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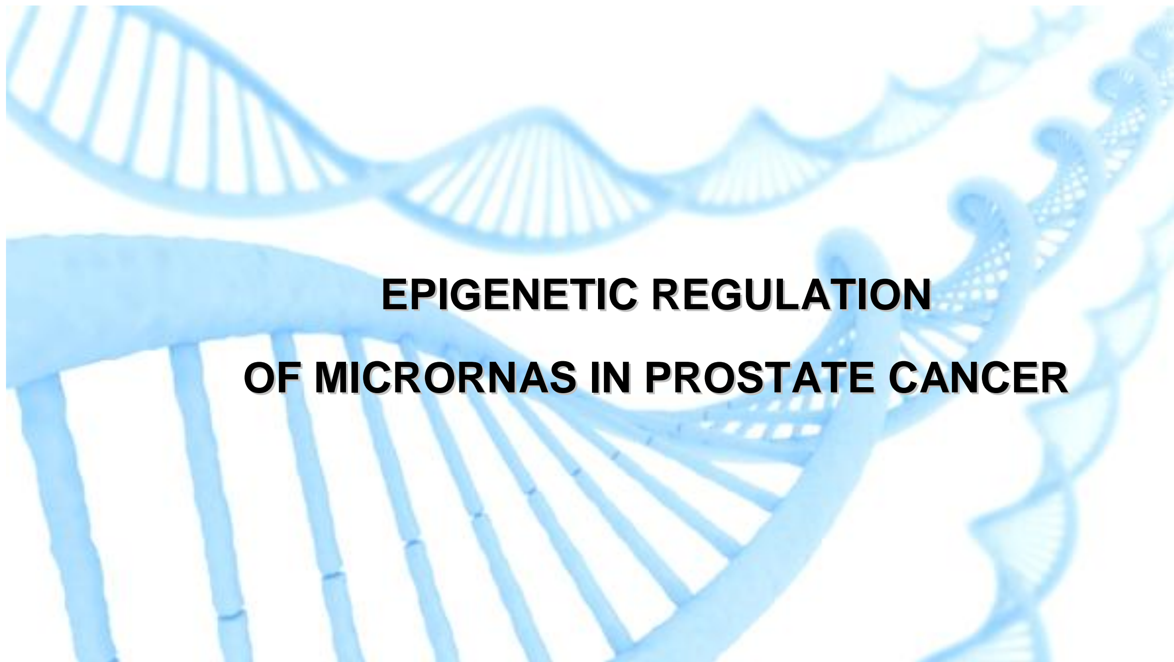

EPIGENETIC REGULATION OF MICRORNAS IN PROSTATE CANCER

by

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EPIGENETIC REGULATION OF MICRORNAS IN PROSTATE CANCER

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RELEVANT ABBREVIATIONS

5-AZA-DC	5-Aza-2'-Deoxycytidine
ACTβ	Actin β
AR	Androgen Receptor
AUC	Area Under the Curve
CpG	Cytosine-phosphate-Guanine
DNA	Deoxyribonucleic Acid
DNMTs	DNA methyltransferases
GS	Gleason's Score
HD	Healthy Donors
HDAC	Histone Deacetylases
HGPIN	High Grade Prostatic Intraepithelial Neoplasia
miRISC	MiRNA-containing RNA-Induced Silencing Complex
MiRNA	MicroRNA
mRNA	Messenger RNA
MSP	Methylation Specific Polymerase Chain Reaction
NPT	Normal Prostatic Tissue
NPV	Negative Predictive Value
PCa	Prostate Cancer
PCR	Polymerase Chain Reaction
PIA	Proliferative Inflammatory Atrophy
PPV	Positive Predictive Value
PSA	Prostate Specific Antigen
qMSP	Real-time Quantitative Methylation-Specific Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse-Transcriptase Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROC	Receiver Operator Characteristics

TGF-β	Transforming Growth Factor β
UTR	Untranslated Region

SUMMARY

BACKGROUND: Prostate cancer (PCa) is one of the most prevalent cancers worldwide, constituting a serious health problem. Clinically localized disease might be successfully treated whereas disseminated disease remains mostly lethal. PCa is thought to be the end product of the interaction of environmental, physiological and molecular/genetic factors. Over the last decade, the role of epigenetic alterations in prostate carcinogenesis has emerged and provided a new framework for the understanding of the mechanisms underlying the disease as well as for the development of novel PCa biomarkers. Although aberrant DNA methylation and post-transcriptional histone modifications have been the main focus of epigenetic-oriented research in PCa, the role of microRNAs (miRNA) deregulation has more recently surfaced. These are small, single-stranded, non-coding, untranslated RNAs that control gene expression at the post-transcriptional level, interacting directly with messenger RNA (mRNA). MiRNAs are globally downregulated in most cancers and although genetic mechanisms have been appointed as the main cause, a role for epigenetic disruption of miRNAs regulation has been recently emphasized.

AIMS: The main aim of this Thesis was to identify new epigenetically downregulated miRNAs in PCa, using an expression profiling based approach, followed by validation in a larger set of clinical samples. In addition, we attempted to identify novel PCa biomarkers suitable for clinical application in early detection, diagnosis and prognosis assessment.

METHODOLOGIES: *In silico* analyses were performed in ten PCa against four morphologically normal prostatic tissues (NPT) based on gene expression profiling data of 740 miRNAs. MiRNAs significantly downregulated in that analysis and re-expressed after treatment with an epigenetic-modulating drug in at least two of three cell lines, were selected for further analysis. Subsequently, candidate miRNAs were surveyed for the presence of a CpG island up to 5000 bp upstream of their mature sequence. Candidate miRNAs fulfilling all these requirements were then validated through DNA methylation analysis in a larger series of tissue samples comprising PCa, NPT and high-grade prostatic intraepithelial neoplasias (HGPIN). Real-time quantitative methylation-specific polymerase chain reaction (qMSP) analysis of 101 PCa, 14 NPT and 56 HGPIN allowed for the determination of promoter methylation levels of the selected miRNAs. Correlation between methylation levels, on the one hand, and expression levels and standard clinicopathologic parameters, on the other hand, was performed. Methylation levels were also used to assess miRNAs performance as PCa biomarkers in tissue samples, and the

best performing miRNAs were then selected for the same type of analysis in urine, using 39 samples from PCa, and 15 samples from donors (HD) as controls. Finally, putative pathways targeted by the epigenetically deregulated miRNAs were also examined to provide a biological rational for their role in prostate carcinogenesis.

RESULTS AND DISCUSSION: Expression profiling identified 173 differently expressed miRNAs out of 740. Of these 173, 47 were considered significantly downregulated and 5 upregulated, comparing PCa to NPT. Concerning response to demethylating treatment, 18 and 120 miRNAs were commonly re-expressed in three and two cell lines, respectively. Combining both datasets and looking for the presence of a CpG island at the promoter region, miR-130a, miR-145 and miR-205 surfaced as the best candidates for further validation. Methylation analyses revealed that their promoter was frequently methylated in PCa tissue compared to NPT, and that alteration was already apparent in HGPIN. However, miR-145 was not shown to be significantly downregulated in most PCa tissue samples, contrarily to the other two miRNAs. Unexpectedly, there was no significant correlation between promoter methylation and expression levels, suggesting that other epigenetic mechanisms might be also involved in altered expression of these miRNAs. Furthermore, expression levels of miR-205 correlated with Gleason’s score and clinical stage, which may be indicative of a potential role as marker of disease aggressiveness. A quantitative assay for promoter methylation of miR-130a and miR-205 displayed high sensitivity and specificity for the discrimination between PCa and NPT (89.11% and 100%, respectively), with an overall accuracy of 90.43% and an area under the curve (AUC) of 0.970. These promising results were further tested in urine samples. Although the sensitivity of the urine assay was modest (25.64%), it was demonstrated for the first time that miRNAs promoter methylation levels may be successfully detected in body fluids of PCa. Because miRNA promoter methylation levels were identified in HGPIN lesions, it is suggested that this epigenetic aberration is an early event in prostate carcinogenesis. The three miRNAs analyzed in this study are predicted to be involved in the regulation of several key cellular pathways including signal transduction, transcription factors, apoptosis and cell adhesion, suggesting a role for miR-130a, miR-145 and miR-205 epigenetic deregulation in prostate carcinogenesis.

CONCLUSIONS: Downregulation of miRNAs due to epigenetic deregulation seems to be an infrequent event in PCa. However, promoter methylation of miR-130a, miR-145 and miR-205, occur early in prostate carcinogenesis and might provide novel biomarkers for PCa detection and diagnosis. Further studies are required to illuminate the biological role of these alterations in PCa initiation and progression.

RESUMO

INTRODUÇÃO: O cancro da próstata é uma das neoplasias mais prevalentes mundialmente, constituindo um grave problema de saúde. A doença clinicamente localizada pode ser tratada com sucesso, enquanto que a doença disseminada é na maioria dos casos fatal. Pensa-se que o cancro da próstata seja o produto final da interação de fatores ambientais, fisiológicos e moleculares/genéticos. Ao longo da última década, o papel das alterações epigenéticas na carcinogénese prostática tem ganho enorme relevância e tem constituído uma fonte sólida de novas bases para entender alguns dos mecanismos relacionados com a doença, e ainda o desenvolvimento de novos biomarcadores. Apesar de alterações como a metilação aberrante do DNA e a modificação pós-transcricional das histonas ter vindo a ser o principal foco dos estudos epigenéticos em cancro da próstata, recentemente a desregulação patológica dos microRNAs tem recebido enorme importância e atenção. Estas pequenas sequencias de RNA de cadeia única, não codificante e não traduzidas controlam a expressão genética pós-transcricional, interagindo directamente com o mRNA. Os microRNAs encontram-se globalmente sub-expressos em muitos cancros e apesar de alterações genéticas terem vindo a ser apontadas como a principal causa, a regulação epigenética dos microRNAs, tem vindo a receber especial atenção.

OBJECTIVOS: O principal objetivo desta Tese foi identificar novos microRNAs sub-expressos em cancro da próstata, devido a mecanismos epigenéticos, recorrendo a uma análise de perfil de expressão, seguida de uma validação num elevado número de amostras clínicas. Tentamos ainda identificar novos biomarcadores para cancro da próstata, passiveis de serem utilizados em deteção e diagnóstico precoce e avaliação de prognóstico.

METODOLOGIAS: Efetuou-se uma análise *in silico*, comparando dez tecidos de adenocarcinoma prostático com quatro tecidos prostáticos morfologicamente normais baseada nos resultados da análise do perfil de expressão de 740 microRNAs. Aqueles significativamente sub-expressos na análise e que demonstraram ser re-expressos após tratamento com um fármaco modulador epigenético em pelo menos duas ou três linhas celulares, foram selecionados para os estudos seguintes. Subsequentemente, os microRNAs candidatos foram examinados acerca da presença de uma ilha CpG até 5000 pares de bases acima da sua sequência madura. Os microRNA candidatos que cumpriam todos estes requisitos foram validados através da análise do estado de

metilação do seu promotor em séries maiores de amostras de tecidos de adenocarcinoma prostático, tecido prostático morfologicamente normal e tecidos de neoplasia intra-epitelial. Uma metodologia de PCR específica para quantificação de metilação foi efetuada em 101 amostras de tecido de adenocarncinoma prostático, 14 tecidos prostáticos morfologicamente normais e 56 tecidos de neoplasia intra-epitelial, permitindo a determinação dos níveis de metilação do promotor dos microRNAs selecionados. Foi também pesquisada uma correlação entre os níveis de metilação, os níveis de expressão e características fisiopatológicas padrão. Os níveis de metilação foram também utilizados para avaliar a performance dos miRNAs selecionados como biomarcadores em amostras de tecido e os mais promissores foram estudados em amostras de urina, utilizando o mesmo tipo de análise, em 39 amostras de adenocarcinoma prostático e 15 amostras de dadores saudáveis como controle. Finalmente, potenciais vias alvo dos microRNAs epigeneticamente regulados foram examinadas, de forma a avaliar o seu impacto na carcinogénese prostática.

RESULTADOS E DISCUSSÃO: A análise do perfil de expressão dos 740 microRNAs identificou 173 expressos diferentemente. Destes 173, 47 foram considerados como significativamente sub-expressos e 5 sobre-expressos, comparando adenocarncinoma prostático e tecidos prostático morfologicamente normal. Relativamente à resposta ao agente desmetilante, 18 e 120 microRNAs foram comummente re-expressos em três e duas linhas celulares, respetivamente. Combinando os resultados e a pesquisa da presença de uma ilha CpG na região do promotor o miR-130a, miR-145 e miR-205 revelaram ser candidatos promissores para posterior validação. A análise de metilação revelou que os seus promotores estão frequentemente metilados em cancro da próstata quando comparado com tecido prostático morfologicamente normal, e que esta alteração era já aparente em neoplasia intra-epitelial. Contudo o miR-145 não demonstrou estar significativamente sub-expresso na maioria das amostras de adenocarcinoma, ao contrário dos outros dois microRNAs. Inesperadamente não foi aparente uma correlação significativa entre a metilação do promotor e os níveis de expressão, sugerindo que outros mecanismos epigenéticos possam também estar envolvidos na alteração da expressão desses microRNAs. Além disso, os níveis de expressão do miR-205 correlacionaram-se com a pontuação de Gleason e com o estadiamento clínico, o que pode ser indicativo de um potencial papel como marcador da agressividade da doença. A análise quantitativa da metilação do promotor do miR-130a e do miR-205 revelou uma elevada sensibilidade e especificidade em discriminar adenocarcinoma prostático e tecido morfologicamente normal (89.11% e 100%, respetivamente), com uma precisão de 90.43% e uma área sobre a curva de 0.970. estes resultados promissores foram então

testados em amostras de urina. Apesar de a sensibilidade demonstrada nos estudos em urina ser modesta (25.64%), demonstrou-se pela primeira vez que os níveis de metilação do promotor de microRNAs podem ser detetados com sucesso em fluidos biológicos de pacientes com cancro da próstata. Uma vez que níveis de metilação do promotor foram identificados como já estando elevados em neoplasia intra-epitelial, sugere que esta alteração epigenética possa constituir um evento precoce na carcinogénese prostática. Os três microRNAs analisados neste estudo parecem estar envolvidos na regulação de diversas vias celulares importantes tais como transdução de sinal, fatores de transcrição, apoptose e adesão celular, sugerindo um papel importante da desregulação epigenética do miR-130a, miR-145 e miR-205 na carcinogénese prostática.

CONCLUSÕES: A sub-expressão de microRNAs devido a alterações epigenéticas parece ser bastante infrequente em cancro da próstata. Contudo, a metilação do promotor do miR-130a, miR-145 e miR-205 parece ocorrer precocemente na carcinogénese prostática e pode fornecer novos biomarcadores para a deteção e diagnóstico da doença. Futuros estudos são necessários para elucidar a função biológica destas alterações na iniciação e progressão desta doença.

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INTRODUCTION

Prostate Cancer

Epidemiology

Nowadays, cancer is one of the most common health problems, with a register of 12.7 million cases and responsible for 7.6 million cancer deaths all around the world in 2008 (Jemal et al., 2011).

Worldwide, prostate cancer (PCa) constitutes one of the three most common cancers among male (Siegel et al., 2012), is the second most commonly diagnosed neoplasia and the sixth leading cause of cancer death in males (Jemal et al., 2011), despite all the recent improvements in diagnosis and treatment. Indeed, in spite of men's long lifetime represent a high risk to develop this disease (about 16–18%), the corresponding risk of death is only about 3% (Fleshner et al., 2012). In fact, international data reveals that PCa accounted for 14% (903,500) of the total new cancer cases and 6% (258,400) of the total cancer deaths in men in 2008 (Jemal et al., 2011). Its incidence rates vary by more than 25-fold worldwide as the develop countries (Oceania, Europe and North America) record highest rates when compared to less develop countries of Africa or the Caribbean region. On the other hand, the highest mortality rates are verified in the less developed countries (Fig. 1 and 2) (Jemal et al., 2011, Center et al., 2012). Indeed, this different global distribution and prognosis may be connected to population's genetic profiles or even to different diagnosis or detection methodologies (Jemal et al., 2011).

Concerning Portugal, the last statistics available revealed PCa as the most incident neoplasia in men, with 5140 cases in 2008, however being the third most lethal cancer (Ferlay et al., 2010) (Fig. 3).

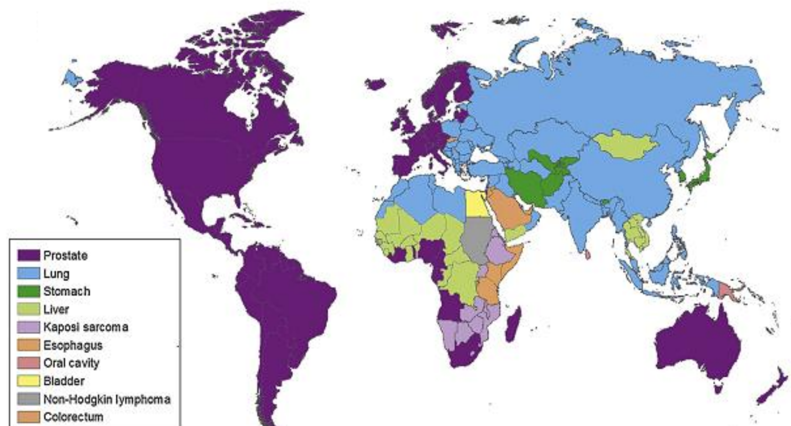


Figure 1 – Most commonly diagnosed cancers among men worldwide. Adapted from (Center et al., 2012)

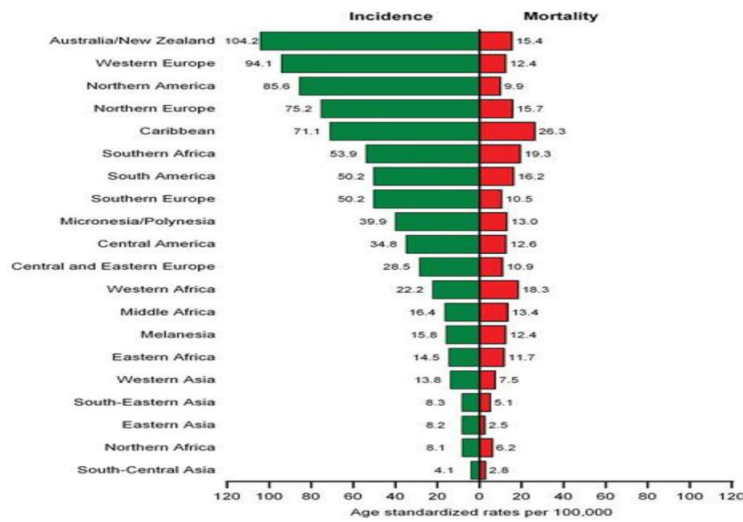
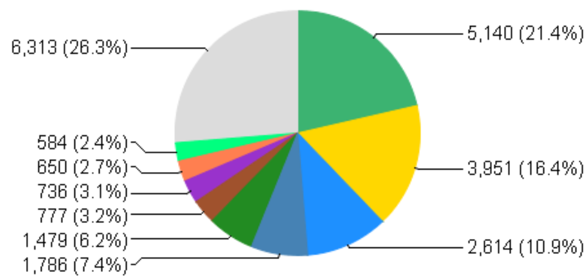


Figure 2 – Age-standardized PCa incidence and mortality rates by geographic area. Adapted from (Jemal et al., 2011)



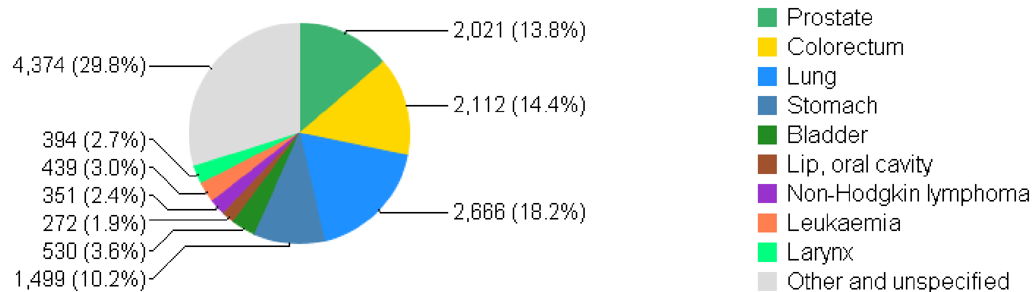


Figure 3 – Incidence and mortality of different types of cancers in Portugal (number of newly diagnosed cancers cases and proportion for each cancer comparing to all types of cancer in both genders). Adapted from (Ferlay et al., 2010)

Clinical Disease and Diagnosis

Prostate is a male exocrine retroperitoneal organ, encircling the neck of the bladder and urethra, which in the normal adult weighs approximately 20 g and is devoid of a distinct capsule (McNeal, 1981). Prostatic parenchyma can be divided into four biologically and anatomically different regions or zones: the peripheral, central and transitional zones and the region of the anterior fibromuscular stroma (McLaughlin et al., 2005) (Fig. 4). Indeed, the central zone surrounding the ejaculatory ducts is the dominant zone for benign hyperplasia development, while the peripheral zone harbors the majority of prostate carcinomas (75%) (McLaughlin et al., 2005, Shen and Abate-Shen, 2010). This organ's main function is to produce and secrete an alkaline fluid, named seminal fluid, which forms part of the ejaculate, aiding spermatozoids motility and nourishment (Dunn and Kazer, 2011).

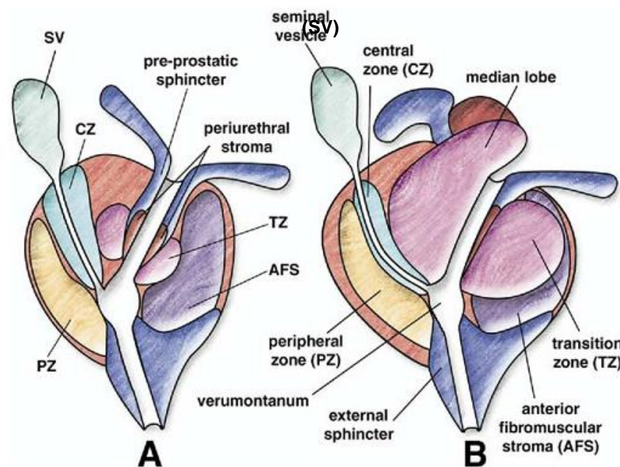


Figure 4 – Zonal anatomy of the normal prostate. (A) Young male with minimal transition zone hypertrophy. (B) Older male with transition zone hypertrophy, which effaces the pre-prostatic sphincter and compresses the peri-ejaculatory duct zone. Abbreviations: AFS - Anterior Fibromuscular Stroma; CZ - Central Zone; PZ - Peripheral Zone; SV - Seminal Vesicle; TZ - Transition Zone. Adapted from (McLaughlin et al., 2005)

Concerning the clinical features of PCa, the localized disease is often asymptomatic, but occasionally it may have some of the same symptoms as the benign hyperplasia, including weak stream, hesitant, urgent and frequent need to urinate, nocturia, incomplete emptying and various degrees of incontinence (Dunn and Kazer, 2011). The clinical condition may also include hematuria, hematospermia, elevated PSA levels, erectile dysfunction (Dunn and Kazer, 2011) and the diagnosis is confirmed by rectal and physical examination and finally needle biopsy. Advanced clinical disease is characterized by bony pain, especially in the hips and pelvis has a cause of metastasis (Dunn and Kazer, 2011).

This malignancy has been recognized as a clinical problem, since ancient Egypt, when it was firstly described, however, effective treatment by surgical procedures (prostatectomy) were only developed in the last century (Capasso, 2005). Concerning PCa diagnosis, the highly accessible blood test for prostate-specific antigen (PSA) constituted the greatest improvement over the past three decades (Shen and Abate-Shen, 2010, Hernandez and Thompson, 2004). This kallikrein-related serine protease is produced in normal prostate secretions, however is released into the blood stream when the normal prostate architecture is disrupted (Lilja et al., 2008). Elevated PSA, which the upper limit that has been considered is 4.0 ng/mL (Hernandez and Thompson, 2004), is usually the primary suspicion criteria for digital rectal examination and undergoing biopsy. In fact, studies like European Randomized Study of Screening for Prostate Cancer where a group of men invited for PCa screening based on PSA was compared to a control group without no active intervention (Schroder et al., 2009), demonstrated that, after a median follow-up of 9 years, men randomized to active surveillance, had a significant reduction in PCa mortality; ratio rate (RR) 0.80 (95% CI 0.65–0.98; adjusted p=0.04) (Schroder et al., 2009). In the same line, other studies revealed the same, like the Göteborg Randomized Population-based Prostate Cancer Screening Trial, in which a group of 20,000 men was divided in half and randomized to a screening program for PSA testing every 2 years against the other half, which was not included in the screening program, serving as a control (Hugosson et al., 2010). In addition, men in the screening group whose PSA concentrations were elevated, were offered additional tests such as digital rectal examination and prostate biopsies (Hugosson et al., 2010). The results of this trial showed that during a median follow up of 14 years, PCa incidence was 12.7% in the screening group and 8.2% in the control group; hazard ratio 1.64 (95% CI 1.50–1.80; p<0.0001) (Hugosson et al., 2010). Also the absolute cumulative risk reduction of death from PCa at 14 years was 0.40% for the control group against the screening group, added to the RR for death from this disease which was 0.56 (95% CI 0.39–0.82; p=0.002) in the screening group compared to the control group (Hugosson et al., 2010). Results from these studies, provide strong evidence that PSA based PCa screening may reduce its mortality. In fact PSA isn't only associated to diagnosis, as it also constitutes a clinical weapon to assay treatment response, once it can be used to evaluate the response to hormonal therapy and to predict disease recurrence, especially after radical prostatectomy (Lilja et al., 2008, Lange et al., 1989).

However, screening based on PSA to diagnosis intends, may have some limitations because it may lead to overdiagnosis and overtreatment, due to its lack of sensitivity and specificity (Henrique and Jeronimo, 2004). Interestingly, some problems have been raised by the scientific community as only 1 in 4 men with PSA levels higher

than 4.0 ng/mL would be found to have PCa, while the other 3 here unnecessarily biopsied (Hernandez and Thompson, 2004). Moreover, other PSA values and parameters have emerge in order to overcome PSA lack of sensitivity such as PSA velocity, volume-related PSA, transition zone PSA, PSA density and ratio of free-to-total PSA, however none of those provide a satisfactory sensibility and/or sensitivity (Hernandez and Thompson, 2004, Carter et al., 2007, Bunting, 2002). In addition, adjustments have been proposed, in accordance with age (Gustafsson et al., 1998). Despite all this, PSA is still widely used in the screening of PCa as other clinical biomarkers slow to emerge. In fact, in the last few years, several biomarkers have been suggested in order to promote diagnosis or predict prognosis, such as Human Kallikrein 2, Prostate-specific membrane antigen, presence of fusions genes, between others, however much is still to be known and verified before including them in clinical practice (You et al., 2010).

After biochemical evaluation and physical confirmation, needle biopsy is performed, followed by pathological analysis based on histopathological grading of the tissue. This evaluation is performed by Gleason scoring, which classifies tumors from 2 to 10 concerning tissue architecture, with minimal consideration of tumor cell morphology (Gleason and Mellinger, 1974, Yu and Luo, 2007). This scoring allows two grades by tumor sample and after combination of them, as one reflects dominant architectural pattern and the other a minor architectural pattern, it generates a final Gleason's score. In this way, a tumor sample with combined Gleason's score between 2 – 4 is considered well differentiated, 5 – 6 moderately and 7 – 10 poorly differentiated. In fact, Gleason scoring may constitute a powerful tool in predicting outcome after radical prostatectomy, radiation and hormonal therapy and also a helpful instrument for choosing the best therapeutic approach (Shah, 2009). Indeed, patients with low Gleason score (6 or lower) are often recommended for active surveillance, in the meanwhile, those with a score of 7 are indicated for therapy of any kind, finally those with a score between 8-10 are candidates for adjuvant therapy or radiation treatment (Shah, 2009).

The diagnosis also includes the status of the primary tumor, from organ confined to fully invasive (T1 – 4), with or without lymph node impairment (N0 or 1) and the presence of distant metastasis (M0 and 1) (Ohori et al., 1994).

Treatment

Concerning treatment, localized disease may be effectively suppressed by surgical excision of total organ – radical prostatectomy – or irradiation through external or internal/implanted beam radiation – brachytherapy, however metastatic disease remains

incurable and fatal (Shen and Abate-Shen, 2010, Kumar-Sinha and Chinnaiyan, 2003). In advance disease, the treatment regiments are usually based on androgen deprivation therapy which conducts to apoptosis of malignant tumor cells and reduction of tumor burden and/or circulating PSA levels. Nevertheless this is usually temporary, as in most cases tumor cells became resistant to this therapeutic option, and proliferate independently of androgens, which mechanism is still not fully elucidated (Kumar-Sinha and Chinnaiyan, 2003, Shen and Abate-Shen, 2010, Craft et al., 1999).

Risk Factors

Despite having a high prevalence and mortality, few data is certain about what causes this disease or even the best prevention strategy. In fact, there are several conditions that may compose a risk factor such as age, lifestyle, diet, African American race and familiar history of the disease or even genetic variants (Fowke et al., 2012, Shafique et al., 2012). Concerning age, which is the most significant risk factor, as its occurrence in patients aged below 50 is very low, with around 60% of all cases being registered in men over 70 years old (Macefield et al., 2009). Regarding familiar history, recently a novel HOXB13 G84E variant was associated with a significantly increased risk of hereditary PCa, however, it only accounts for a small fraction of all cases (Ewing et al., 2012). About diet and lifestyle, it seems completely evident that dairy foods constitute one of the most solid predictor for PCa, interestingly at least 7 of 9 cohort studies and 12 of 14 case-control studies observed a positive association between these two variables (Wolk, 2005). Regarding African American men increased odd of developing PCa, it's still a controversy issue as it's not well defined whether it correlates with physiological or socioeconomic factors (Major et al., 2012). Still some authors describe an incidence rate of 233.8/100,000 for African Americans against 149.5/100,000 for Caucasians (Major et al., 2012). In fact, PCa prevention or disease prediction is still a delicate matter, which nowadays has its more solid bases on early detection by PSA screening, with all the controversy already referred. On the mean time, important clinical advances have been reported on prevention of this disease, as recently the Food and Drug Administration concluded that finasteride may reduce the risk of low-grade cancer but doesn't have complete advantages when broad administrated (Theoret et al., 2011). Besides being a controversy issue, studies claim that obesity may constitute a risk factor for developing this disease or at least may influence the grade of PCa, and consequently its aggressiveness and prognosis (Fowke et al., 2012). Other interesting risk factor pointed by several studies is serum cholesterol levels, however it's also still an inconclusive matter

that may be connected with grade and aggressiveness and consequently with mortality (Shafique et al., 2012). Also several chemicals and physic agents have been associated with PCa, such as dioxins, cigarette smoking, some farming pesticides, ultra violet radiation and minerals connected to occupational exposure (Mullins and Loeb, 2012), however none of them as achieved significant and solid arguments yet.

Molecular Pathways of Carcinogenesis

As previously mentioned, PCa is thought to be the end-product of the interplay of environmental, physiological and molecular/genetic factors. Age seems to be the common denominator of all those factors as several associations between gene expression alterations and age progression, including genes related to inflammation, oxidative stress, and cellular senescence, have been pointed (Shen and Abate-Shen, 2010).

Concerning inflammation, its estimated that approximately a fifth of all human cancers including those of the stomach, liver and large intestine arise in a background of chronic inflammation (Haverkamp et al., 2008). Concerning PCa, the lack of solid epidemiologic and histological data connecting it with chronic inflammation, makes this correlation still unclear, although chronic prostatitis may be the origin of proliferative inflammatory atrophy (PIA), which is commonly seen in cancerous prostates and may constitute a precursor state, although this is still a controversial issue (De Marzo et al., 1999). Recently, a study has proposed a link between PCa and sexually transmitted infectious agents like *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Treponema pallidum*, *Human papilloma virus*, *Herpes simplex virus* and *Human herpes virus type 8*, all of which have been detected in prostatic tissue (Wright et al., 2012). That link was suggested by the observation that circumcision before the first sexual intercourse was associated with a 15% reduction in the relative risk of PCa (Wright et al., 2012). Nevertheless, further studies are needed to clarify those findings. Another link between inflammation and PCa derives from the downregulation of a *GSTP1*, which encodes for an enzyme involved in the detoxification of reactive species, which are generated by inflammatory cells (Nakayama et al., 2004). Moreover, oxidative stress and consequent DNA damage may be due to hormonal deregulation, diet and/or epigenetic alterations (Shen and Abate-Shen, 2010, Gupta-Elera et al., 2012, Crawford et al., 2012). Indeed, oxidative stress may play a key role in cancer initiation and progression by regulating DNA function enhancers, cell cycle regulators, transcription factors (Gupta-Elera et al., 2012) or by causing direct DNA damage, which may contribute to telomere shortening (Meeker et al., 2002).

In respect to genomic alterations, chromosomal rearrangements or copy numbers alterations are also involved in prostate carcinogenesis. The most commonly reported are the gains of 8q and losses of 3p, 8p, 10q, 13q and 17p (Dong, 2001, Lapointe et al., 2004). Loss of chromosome 8p is considered a major genetic alteration in PCa initiation as it occurs in about 80% of all PCa and is already present in high grade prostatic intraepithelial neoplasia (HGPIN) lesions, which are putative PCa precursor lesions (Bergerheim et al., 1991). Several molecular pathways have also been linked with to PCa initiation and progression (Fig. 5).

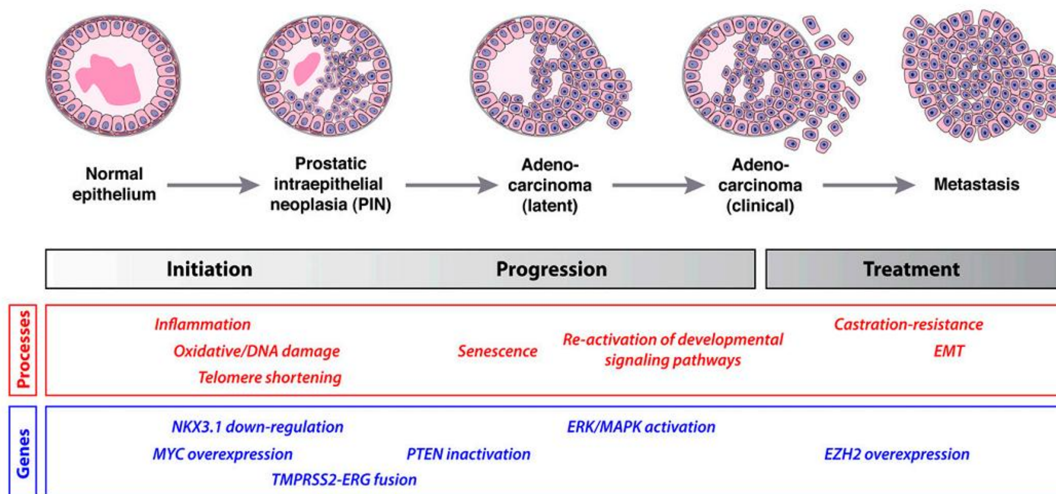


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Figure 5 – Progression pathways for human PCa and its connection to clinical stages. Adapted from (Shen and Abate-Shen, 2010)

An important gene that may be lost during this process is *NKX3.1* which is thought to play a significant role in prostate carcinogenesis (Abate-Shen and Shen, 2000), as it was found to be downregulated in HGPIN lesions (Bethel et al., 2006) as well as in advanced stage disease (Gurel et al., 2010). The function of *NKX3.1* seems to be connected with the regulation of prostate epithelial differentiation and stem cell function (Bhatia-Gaur et al., 1999).

MYC upregulation, usually associated with amplification (at 8q) has been recognized more than one decade ago (Jenkins et al., 1997) and may be present in HGPIN, suggesting a relevant contribution to PCa initiation and progression (Koh et al., 2011). The protein encoded by *MYC* is a transcription factor which is vital in the control of the expression of genes involved in DNA replication, protein synthesis, cell cycle progression, cellular metabolism, chromatin structure, differentiation and stem cell differentiation (Koh et al., 2011).

The tumor suppressor gene *PTEN* is frequently mutated or deleted in PCa (Salmena et al., 2008) and this alteration has been associated with advanced tumor stage, high Gleason grade, presence of lymph node metastasis, hormone refractory

disease, presence of ERG gene fusion and nuclear p53 accumulation (Krohn et al., 2012). The same study also found an association between PTEN's deletion and PSA recurrence (Krohn et al., 2012), suggesting that this genetic alteration may constitute a promising biomarker for PCa diagnosis and/or prognosis.

Several other genes have been reported to be involved in prostate carcinogenesis, including *TP53*, *ZFHX3* , *RB1* and *APC* (Grasso et al., 2012), implicated in several key pathways. Likewise, Akt/mTOR, mitogen-activated protein kinase (MAPK) or EGFR signaling deregulation , have also been linked to this malignancy (Grasso et al., 2012).

A strong enrichment of ETS transcription factor target genes involved in protein synthesis, especially during the transition from benign epithelium to HGPIN lesion has been described (Tomlins et al., 2007). Although this pathway is initially upregulated, it seems to be downregulated during the transition from localized to hormone refractory metastatic PCa (Tomlins et al., 2007), owing to its central role in androgen signaling. Indeed, the same study revealed increased androgen signaling in HGPIN, compared to benign epithelium, but decreased androgen signaling in localized PCa when compared to HGPIN, as well as in high-Gleason grade cancer contrasted with low-grade, achieving the lowest expression levels in hormone refractory disease (Tomlins et al., 2007).

The role of androgens is also pivotal in prostate carcinogenesis. Androgens bind to the human androgen receptor (AR), promoting a cascade of ligand-dependent and protein-protein interactions that may be connected with remodeling of chromatin structure at target promoters, recruitment of basal transcription machinery and RNA polimerase activation (Chmelar et al., 2007, Heinlein and Chang, 2004). However, the precise contribution of AR to prostate carcinogenesis and/or disease progression requires further clarification.

Interestingly, the common fusion genes derived from ETS family members (e.g., ERG and ETV1) and the strong androgen-regulated TMPRSS2 are involved in prostate carcinogenesis owing to androgen induced expression (Hendriksen et al., 2006). The frequency of TMPRSS2-ERG fusion gene is 15% in HGPIN lesions and 50% in localized PCa, suggesting that this genetic alteration may occur after cancer initiation or at early stages of disease progression (Albadine et al., 2009, Mosquera et al., 2008). It may also function as a prognostic marker as some studies have indicated that it may be associated with clinical stage at diagnosis, although no correlation with clinical recurrence or mortality has been found (Pettersson et al., 2012).

Finally, in addition to genetic mechanisms, epigenetic events, including microRNAs (miRNAs) deregulation, have been recognized as critical players in prostate carcinogenesis and their role will be addressed in the following sections (Shen and Abate-Shen, 2010, van der Poel, 2007).

Epigenetics

Epigenetics encompasses DNA or proteins modifications which do not affect DNA sequence but regulate its expression, consequently altering the protein profile (Jeronimo and Henrique, 2011). In fact, the set of changes induced by this particular mechanisms are nowadays gaining significant clinical and pathological magnitude, being recognized as alternatives to gate-keeper genetic mutations or to chromosomal rearrangements (Jeronimo and Henrique, 2011). Whereas these genetic mechanisms are often irreversible, epigenetic mechanisms are dynamic and reversible, occurring as a response to events during embryogenesis or environmental factors and include biochemical modifications of histone proteins, DNA and expression deregulation of noncoding RNAs (Catto et al., 2011).

Indeed, epigenetics may explain why the same genotipes can produce different phenotypes and it constitutes a documented base of some important events that occur in human cells, like the physiologic chromosome X inactivation in females (Heard et al., 2004) or the clinical response to alkylating agents from gliomas depending on the methylation of the promoter region of MGMT (Esteller et al., 2000). Concerning human diseases, epigenetic alterations are increasingly being recognized as deeply implicated, in cancer but also in neurology, cardiovascular and autoimmune disorders (Fernandez et al., 2012). For this purpose, the definition of the whole epigenome constitutes an important contribution, due to the development of powerful new genomic technologies (Fernandez et al., 2012). Regarding the former disease, there are strongly data supporting the role of epigenetic deregulation in malignant transformation and tumor progression, with several clinical applications such as potential biomarkers (Table 1) (Henrique and Jeronimo, 2004, Esteller, 2011, Phe et al., 2010).

Table 1 – Genes that are frequently methylated in PCa and their potential role as a biomarker. Adapted from (Phe et al., 2010)

Gene	Name	Sensitivity (%)	Specificity (%)
GSTP-1	Glutathione S-transferase P1	33–90	84.6–100
CDKN2A	Cyclin-dependent kinase inhibitor 2 A (p1	30–72	
CCND2	Cyclin D2	32	
p14b		9.5	
MGMT	O-6-methylguanine DNA methyltransferase	25–75	
ASC	Apoptosis-associated Speck-like protein containing a CARD	37	
AR	Androgen receptor	8–28	
ESR1	Oestrogen receptor 1	80–95	
ESR2	Oestrogen receptor 2	79–100	
RARβ	Retinoic acid receptor β	68–95	65.4–96.9
EDNRB	Endothelin receptor type B	15–100	11.5
RASSF1A	Ras association domain family protein 1 isoform A	54–96	
MDR1	Multidrug resistance receptor 1	50.8–100	69.2
CDH13	Cadherin 13	45–53.6	
APC	Familial adenomatous polyposis	27–100	50–100
TIMP3	TIMP metallopeptidase inhibitor 3	4	
CDH1	E-cadherin	21–54	
CD44		19–77	
T1G1		41.9–96.3	88.5
LAMA 3	α-3 laminin	44	
LAM B3	Β-3 laminin	18	
LAM C2	γ-3 laminin	41	
SCAV 1	Caveolin 1	90	
PTGS2	Prostaglandin endoperoxide synt	65.4–88	84.6–100
RUNX3	Runt-related transcription factor	44	
WIF1	WNT inhibitory factor 1	28	
COX-2	Cyclo-oxygenase 2		78

DNA Methylation

This epigenetic regulation mechanism is definitely the most studied and relies on the activity of DNA methyltransferases (DNMTs) such as DNMT3L together with either DNMT3A or DNMT3B or by a replicative maintenance procedure that is operated by UHRF1 coupled with DNMT1 (Scholz and Marschalek, 2012). This complex uses S-adenosyl-methionine as the methyl donor to give rise to 5-methylcytosine, by adding

methyl groups to the fifth carbon position of a DNA cytosine after replication (Jeronimo and Henrique, 2011, Goldberg et al., 2007). In fact, nearly all DNA methylation occurs on cytosine residues, located side by side to guanine nucleotides, forming cytosine-phosphate-guanine (CpG) dinucleotides, which usually appear heavily repeated in genomic sequences called CpG islands (Goldberg et al., 2007, Jeronimo and Henrique, 2011). Usually these regions are preferably found in 5' ends, like promoters untranslated regions or exon 1 of human genes and their methylation might prevent gene expression (Jeronimo and Henrique, 2011, Goldberg et al., 2007). There are other genomic regions affected by DNA methylation, located nearby CpG islands, but with less CpG dinucleotides, termed CpG shores, indeed this regions may also regulate gene expression (Dudziec et al., 2011, Jeronimo and Henrique, 2011). Not only abnormal methylation may occur, but also demethylation might take place, promoting inappropriate transcription of genes. This process is mediated by the action of TET family proteins or by GADD45 family members (Scholz and Marschalek, 2012). In fact, some regions, especially those located near the centrosome, transposons, and inserted viral sequences are physiologically methylated, in order to maintain genomic reliability, preventing incorrect recombination events that may lead to genetic errors, such as gene disruption, translocations and chromosomal instability (Jeronimo and Henrique, 2011). DNA methylation may prevent expression either directly through transcriptional activators' obstruction or indirectly by recruitment of methylcytosine-binding proteins (Jeronimo and Henrique, 2011). Indeed, these may promote the enrolment of DNMTs and histone deacetylases (HDAC), which may result in chromatin alterations, repressing transcription (Jeronimo and Henrique, 2011).

Interestingly, in cancer, these methylation patterns are often altered whereas the promoter region of tumor suppressor genes becomes hypermethylated (Fig. 7) or oncogenes undergo hypomethylation (Jeronimo and Henrique, 2011, Lopez-Serra and Esteller, 2008). Hence, these alterations in methylation patterns of gene promoters, whose mechanism and cause are still unknown, might have an impact in expression of several cancer-related genes such as the tumor supressores p16, INK4a, TP53, hMLH1, PTEN and BRCA1 and the oncogenes KRAS, BRAF and PIK3CA (Lopez-Serra and Esteller, 2008, Kwon and Shin, 2011).

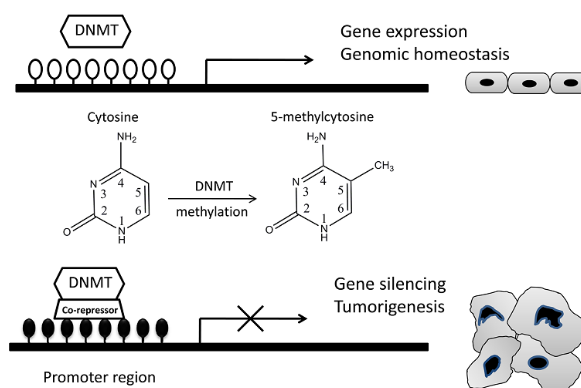

Figure 7 – DNA methylation in normal and cancer cells. A hypomethylated promoter allows normal gene expression while DNA hypermethylation leads to gene silencing. In cancer cells aberrant hypermethylation may promote tumorgenesis. This process is catalyzed by the DNA methyltransferases (DNMTs), by adding a methyl group (CH_3) to the 5-position of the cytosine of the CpG dinucleotides (black circles, methylated sites; white circles, unmethylated sites). Adapted from (Li and Tollefsbol, 2010)

There are several drugs that may hamper DNA methylation, one of the most well studied is 5-Aza-2'-Deoxycytidine (5-AZA-DC) (Fig. 8), which was first synthesized 40 years ago (Christman, 2002). In fact, regardless of having an anti-metabolic activity, it is incorporated in DNA inhibiting DNMTs activity (Christman, 2002). This compound is currently used in in vitro assays in order to verify the role of genes promoter methylation and specific gene´s expression (Christman, 2002).

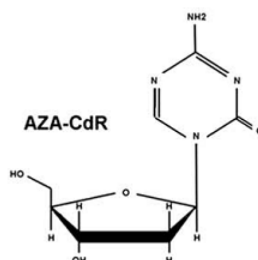

Figure 8 – Chemical structure of 5-Aza-2'-Deoxycytidine. Adapted from (Yang et al., 2012)

Interestingly, in PCa, epigenetic alterations, specifically DNA aberrant methylation, are highly prevalent and occur early in carcinogenesis. Indeed, more than 50 genes involved in key cellular pathways (DNA repair and damage prevention, signal transduction, cell hormone response, cycle control and apoptosis, tumor invasion and architecture, have been commonly found to be hypermethylated (Table 2) (Henrique and Jeronimo, 2004). One of note is *GSTP1*, which may allow the detection of 80-90% of prostate adenocarcinomas with perfect specificity (Henrique and Jeronimo, 2004). Nonetheless, further studies are needed to elucidate how and why this aberrant methylation takes place.

Table 2 – Frequency (%) of hypermethylation in several cancer-related genes in prostate tissue. Adapted from (Henrique and Jeronimo, 2004)

Gene	NPT	HGPIN	PCa
APC	6%	-	27%
AR	0-11%	5%	8-15%
CD44	-	-	32-67%
CDH1	25%	-	0-54%
CDH13	16%	-	31%
CRBP1	0-2.8%	8.6%	47.2%
Cyclin D2	6%	-	32%
DAPK	7-11%	0%	1-36%
EDNRB	83-91%	-	83%
ER (α-A, α-C, β)	0%	-	79-95%
FHIT	0%	-	15%
GSTP1	0-29%	30-70%	36-94%
HIC-1	83-90%	100%	99%
MGMT	0-3%	10%	0-2%
p16	0%	-	3-13%
PR (A, B)	0%	-	0
RARβ2	3-23.3%	20-94%	53-97.5%
RASSF1A	16%	-	53-71%
TIMP-3	6-7%	5%	6%

Abbreviations: NPT- Morphologically Normal Prostate Tissue;
HGPIN- High Grade Prostatic Intraepithelial Neoplasia; PCa- Prostate Cancer

Histone Modifications and Chromatin Remodeling

Chromatin is the higher order of organization of genomic information. Nucleosome constitutes it's basic unit, which is composed by a histone octamic protein core, around which 147 bp of DNA are wrap up (Loizou et al., 2006). These dynamic structures provide not only a physical support to DNA, but also participate in transcriptional regulation, repair and replication (Jeronimo and Henrique, 2011). Indeed, the N-terminal 'tails' of these structures may be target of several enzymes (kinases, acetytransferases or methyltransferases) that using cellular metabolites, such as phosphate, acetyl, or methyl groups promote their phosphorylation, acetylation, and methylation, which may alter gene expression. (Katada et al., 2012, Jeronimo and Henrique, 2011). Globally, these chromatin modifying/remodeling activities constitute normal and physiological mechanisms use by normal cells to process DNA breaks repair and to defend themselves against genomic integrity aggressions (Loizou et al., 2006). In fact, the most well elucidated mechanisms are acetylation which reduces histones affinity for DNA and allow chromatin extension and openness, favoring gene transcription and the other one is histone methylation which may inhibit gene transcription by the opposed mechanism,

depending on which aminoacids are methylated and DNA methylation itself (Fig. 9) (Jeronimo and Henrique, 2011). Concerning PCa, histone modifications, nucleosomal remodeling and chromosomal looping may constitute important epigenetic mechanisms that regulate gene expression. For example the alteration in the repressive histone mark H3K27me3 and the increased expression of H3K27me3 methyltransferase EZH2 conducts to the silencing of tumor suppressor genes (for example GAS2 and ADRB2) (Chen et al., 2010). On the other hand, both the genomic position alteration and protein expression of active histone mark H3K4 methylation may contribute to activation of proto-oncogenes (for example UBE2C) in this neoplasia (Chen et al., 2010). Nucleosome repositioning is not only related to the silencing of tumor suppressor genes, such as MLH1, but is also connected to the activation of genes involved in PCa progression (PSA and TMPRSS2) (Chen et al., 2010). It is also thought that AR gene regulation may have a strong influence of chromatin remodeling or histone modification (Chen et al., 2010).

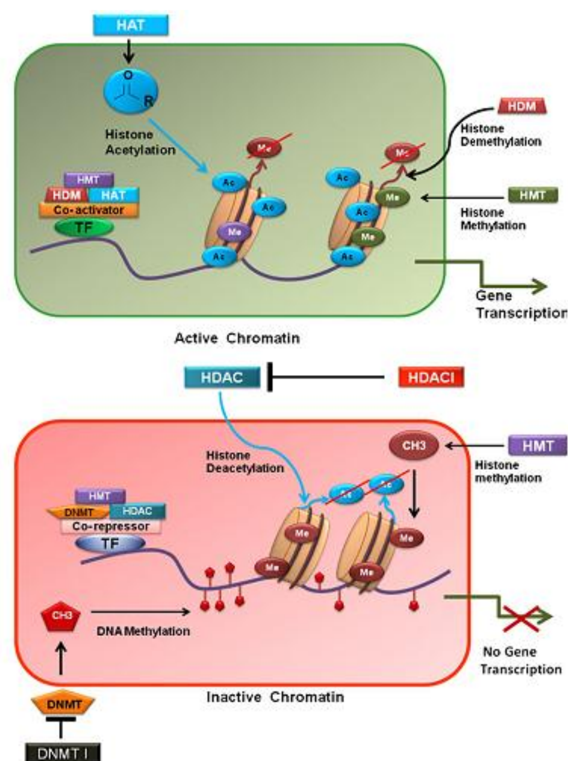

Figure 9 – Active transcription is associated with hyperacetylation of histones by the adding of an acetyl group to specific lysines residing within the N terminal region of histones. Thus, the affinity of histones for DNA is reduced, conducting to and open chromatin conformation which allows transcription factors and RNA polymerase contact with the promoter region of a certain gene. Also, the demethylation of certain lysines on histones (H3K4, H3K36, and H3K79) located on the promoter regions also induces transcription. On the other hand methylation and hypoacetilation of certain other lisines on histones (H3K9, H3K27, and H4K20 residues) may regulate gene expression repression. These modifications are catalyzed by a few chromatin-modifying enzymes such as DNA methyltransferases (DNMTs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs). As a result, inactive genes may be upregulated when the cell is exposed to DNMT-inhibitors and HDAC inhibitors. Adapted from (Mascarenhas et al., 2011)

Non-conding RNAs

Recent evidence indicate that non-coding RNAs may play an important role in controlling multiple genetic and epigenetic phenomena's with a significant impact in normal cellular differentiation and organism development (Goldberg et al., 2007, Mattick and Gagen, 2001). Interestingly in mammals, noncoding RNAs are closely involved in dosage compensation, such as changes in chromatin structure induced by histone modifications (Bernstein and Allis, 2005).

There are two major groups of non-coding RNAs, the small ncRNAs and the long ncRNAs (Hassler and Egger, 2012). Thus, small ncRNAs derive from longer precursors and include transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), microRNAs (miRNAs), piwi interacting RNAs (piRNAs), small nuclear RNAs (snoRNAs) and other less characterized RNAs (Hassler and Egger, 2012). Conversely, long ncRNAs constitute a heterogeneous class of mRNA-like transcripts, yet non-coding, with 200 bp to 100 kb.

There is still much to be elucidated about the mechanisms by which these interference RNAs regulate gene expression and its relation with cancer. These questions will be target of further discussion.

MicroRNAs

MicroRNAs are small (~22 nucleotides), single-stranded, non-coding untranslated RNAs that control gene expression acting post-transcriptionally by destabilization or translational repression of the messenger RNA (mRNA), inhibiting protein synthesis (Ostling et al., 2011, Choudhry and Catto, 2011). Specifically, the 5' end of a miRNA (positions 2–8 nt) binds to a targeting sequence, located at the 3' end of the mRNA- 3' UTR region- depending on the level of complementary between the two sequences (Catto et al., 2011, Betel et al., 2008). Nevertheless, most miRNAs induce a modest reduction (less than two-fold) in their target concentration (Bartel, 2009). In a historical perspective, miRNAs were first described in a work with *Caenorhabditis elegans*, where two regulatory RNA sequences where reported – lin-4 and let-7 – lately these regulatory sequences where also described in other species, including in humans (Bartel, 2009). Currently over 1,223 human miRNAs mature sequences have been reported in the http://www.mirbase.org base catalog (Mestdagh et al., 2012). The miRNAs are expressed from independent transcription units, because they do not contain an open reading frame and are expressed separately from the nearby genes (Lau et al., 2001). Their expression profile varies between species and in each specie during embryogenesis, suggesting that miRNA might be connected to both gene and protein expression and consequently to the regulation of a variety of pathways (Lau et al., 2001).

The Biogenesis of MiRNAs

Presently it is accepted that within the nucleus, miRNAs are transcribed by a polymerase II into a long primary transcripts (pri-miRNAs) which contain both 5'-cap structure (7MGpppG), as well as a 3'-end poly(A) tail, with about 70 nucleotides length (Takada and Asahara, 2012, Iorio and Croce, 2012). Then, miRNAs fold back on themselves to form distinctive hairpin-shaped pre-miRNAs by the action of nuclear RNase III Drosha (Kim, 2005), associated to a double stranded RNA-binding protein DGCR8, known as the microprocessor complex (Iorio and Croce, 2012, Carthew and Sontheimer, 2009). Alternatively, but less frequent, miRNA processing might occur through splicing of pri-miRNA transcripts to release introns which are structurally identical to pre-miRNAs (Carthew and Sontheimer, 2009). Following this nuclear processing, the pre-miRNAs are exported to the cytoplasm, where its maturation and action will take place, this transport is made via one of the nuclear Ran-GTP-dependent transport receptors exportin-5 (Kim, 2005, Iorio and Croce, 2012). Here in, a RNAse III enzyme Dicer, processes pre-miRNAs

into ~22-nucleotide miRNA duplexes (Kim, 2005). Indeed, the PAZ domains of Dicer are crucial to this process, as they interact with the 3' overhang and determines the cleavage site in a ruler-like fashion (Carthew and Sontheimer, 2009). The maturation process is finalized by the cleavage of a precursor miRNA hairpin, into a transitory miRNA/miRNA* duplex, which includes a mature miRNA with a biological activity and a complementary strand (identified by adding a *) usually subject to degradation (Iorio and Croce, 2012), (Bhayani et al., 2012, Griffiths-Jones, 2004).

These mature miRNAs are ready to regulate a variety of pathways, by interfering with the translation process of certain mRNAs. This process requires an incorporation of the miRNA mature sequence into miRNA-containing ribonucleoprotein complex, also called as mirgonaute ou miRISC (miRNA-containing RNA-induced silencing complex) (Kim, 2005), which contains AGO proteins and binds to target mRNA (Iorio and Croce, 2012). The whole miRNAs biogenesis and function process is illustrated on figure 10.

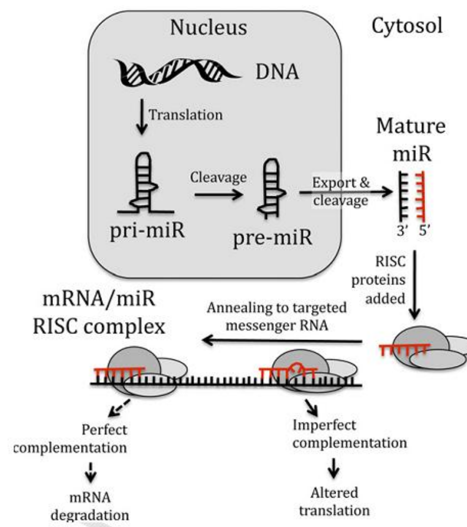

Figure 10 – After synthesis, mature miRNA is incorporated into an RNA-induced silencing complex with Argonaut proteins. This complex targets mRNA through the miRNA seed region, inducing either complete mRNA degradation (by perfect annealing, as seen in plants) or alterations in translation (with imperfect miRNA/mRNA annealing, as seen in mammals). Adapted from (Catto et al., 2011)

Target binding is made by complementarily, into the 3' untranslated regions (UTR) of the target transcripted gene (Iorio and Croce, 2012, Ostling et al., 2011). In fact, as referred, this target interaction does not require complete complementarily between the two sequences, however near perfect base-pairing of the 5' region of the miRNA seems to be determinant in target recognition (Betel et al., 2008). Remarkably, each miRNA might control hundreds of target genes and may modulate up to 60% of all transcripts (Ostling et al., 2011), accounting itself for ~1% of the genome (Kim, 2005). The main target of each miRNA and exactly how its regulation is performed, is still a matter study, however the

reduction of the target gene expression appears to occur by initiation of translation inhibition or by degradation of the target mRNA (Betel et al., 2008). Concerning this issue, there are several databases that provide miRNA target predictions based on complex mathematic algorithms and several criteria such as: sequence complementarily to target sites or calculations of mRNA secondary structure and energetically favorable binding between sequences (Betel et al., 2008).

MiRNAs and Cancer

MiRNAs have also been implicated in cancer, ever since a study revealed that the gene cluster containing the miR-15 and miR-16 was deleted in most patients with chronic lymphocytic leukaemia (Calin et al., 2002). These miRNAs were later described as acting as tumor suppressing genes by targeting the oncogene BCL2, then interfering with cell survival and apoptosis (Cimmino et al., 2005). Moreover, subsequent reports revealed that miRNAs expression are altered in many cancers and have been implicated in tumorigenesis (Catto et al., 2011). MiR-21 constitutes a good example of an oncogenic miRNA that is frequently overexpressed in several tumors, such as breast, colorectal, lung, and pancreatic cancer, as well as, in glioblastomas, neuroblastomas, leukemia and lymphomas (Catto et al., 2011, Kong et al., 2012). Indeed, miRNAs expression constitutes an important mechanism of regulation of several cancer-related genes, relevant for apoptosis avoidance, cell proliferation control, epithelial-to-mesenchymal transition and angiogenic signaling. Furthermore, the potential role of miRNAs as tumor biomarkers is being explored in several cancers.

Mechanisms of MiRNAs Deregulation in Cancer

Interestingly, miRNA may be targeted by several genetic alterations. In fact, nearly 50% of the known miRNAs are located inside or nearby fragile sites and minimal regions of loss of heterozigosity, minimal zones of amplification and common breakpoints which have been already linked to cancer (Kozaki and Inazawa, 2012). Additionally, mutations or polymorphisms on the interference binding site of mRNA coding oncogenes may increasing cancer risk, as described to happen in non-small-cell lung cancer for KRAS (Chin et al., 2008). In addition, several reports verified that most miRNAs have lower expressions in tumors compared to normal tissues, indicating that they may function typically as tumor suppressors (Lu et al., 2005, Agirre et al., 2009, Creighton et al., 2010).

Thus, miRNAs may be targeted by mutations themselves or amplification or methylation events, becoming over or underexpressed (Fig.11).

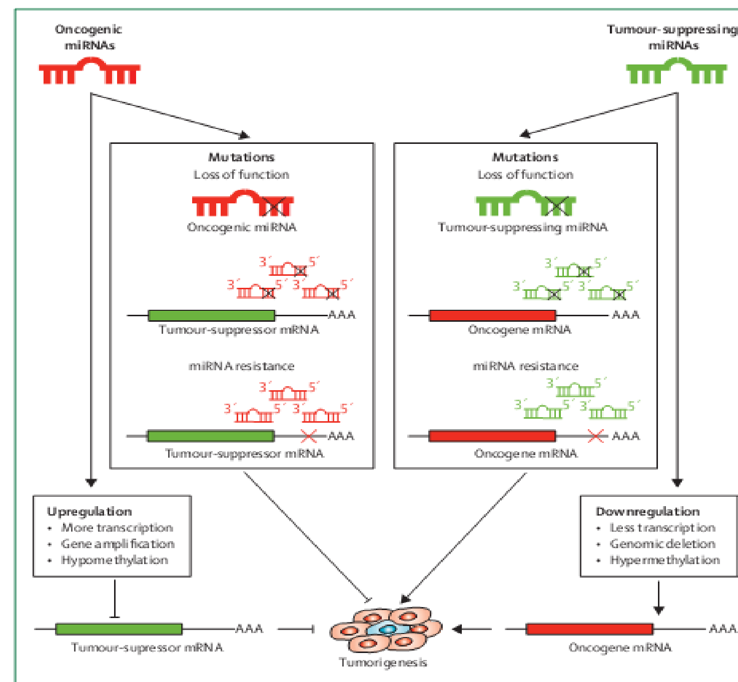

Figure 11 – miRNAs may regulate tumorgenesis at different levels. Oncogenic miRNAs upregulation may reduce expression of tumor-suppressor proteins, contrarily to the downregulation of tumor-suppressing miRNAs, which may increase oncogenic protein levels. Mutations in tumor-suppressing miRNAs and/or on its mRNA binding sites can cause tumorgenesis, on the other hand mutations in oncogenic miRNAs or targets would reduce tumorgenesis. Adapted from (Kong et al., 2012)

Importantly, some studies in recent years correlate miRNAs profile with disease outcome or response to therapy (Calin et al., 2002, Yanaihara et al., 2006). For example, after a median follow-up time of 50 months, miR-96 downregulation was associated with cancer recurrence after surgery (Schaefer et al., 2010). Indeed, miRNA profiles may become a useful tool in assessing clinical stage, as a study using a metastatic versus a non-metastatic PCa xenograft line, found that 140 miRNAs were differently expressed, including miR-16, miR-34a, miR-145 and miR-205 (Watahiki et al., 2011). Owing to the fact that miRNA may also be detected in body fluids, mostly serum and plasma but also in urine samples, they might also serve as biomarkers for early detection, as proposed for miR-141 and miR-375 (Kuner et al., 2012).

Concerning PCa, its first described miRNA profile was reported by Porkka *et al.* (Porkka et al., 2007), in which the authors have identified 51 miRNAs (37 downregulated and 14 upregulated) that were differentially expressed in PCa when compared with benign prostatic lesions. These results were further confirmed by several studies in which a higher frequency of downregulated miRNAs has been reported in PCa, versus to the

lower percentages of miRNAs found to be upregulated in the same malignancy (Schaefer et al., 2010, Catto et al., 2011) (Table 3).

In fact, some miRNAS have already been widely described as being downregulated, specifically miR-145, which has been implicated on apoptosis by regulating *TNFSF10*, a pro-apoptotic gene, as its reconstitution induced cellular death (Zaman et al., 2010) and/or regulating *FSCN1* gene which is related to cell growth, migration and invasion (McLaughlin et al., 2005). Other miRNA that has received wide attention is miR-205, indeed this miRNA seems to be downregulated in PCa and seems to be connected to apoptosis escape by possibly targeting Bcl-w, promoting pharmacologic treatment resistance (Bhatnagar et al., 2010).

Table 3 – A summary of miRNAs with altered expression in PCa, including their targeted mRNAs and pathways. Adapted from (Catto et al., 2011)

MiRNA	Expression	MRNA target	Pathway
miR-20a	Up	*E2F1-3*	Apoptosis
miR-21	Up	*PTEN, AKT, androgen pathway*	Apoptosis, mTOR pathway, androgen independence
miR-24	Up	*FAF1*	Apoptosis
miR-32	Up	*BCL2L11 (Bim)*	Apoptosis
miR-106b	Up	*P21, E2F1*	Cell cycle control/apoptosis and proliferation
miR-125b	Up	*P53, BBC3 (Puma), BAK1*	Apoptosis
miR-148a	Up	*CAND1*	Cell cycle control
miR-221	Up	*p27 (kip1)*	Cell cycle control and androgen independence
miR-222	Up	*p27 (kip1)*	Cell cycle control and androgen independence
miR-521	Up	*Cockayne syndrome protein A*	DNA repair
miR-1	Down	*Exportin-6, tyrosine kinase 9*	Gene expression
miR-7	Down	*ERBB-2 (EGFR, HER2)*	Signal transduction
miR-15a-16 cluster	Down	*CCND1 and WNT3a*	Cell cycle regulation, apoptosis and proliferation
miR-34a	Down	*HuR/Bcl2/SIRT1- >p53/p21/BBC3*	Apoptosis and drug resistance
miR-34c	Down	*E2F3, bcl2*	Apoptosis and proliferation
miR-101	Down	*EZH2*	Gene expression
miR-107	Down	*Granulin*	Proliferation
miR-143	Down	*MYO6, ERK5*	Cell migration, proliferation
miR-145	Down	*MYO6, BNIP3L->AIFM1, CCNA2, TNFSF10*	Cell migration, apoptosis, cell cycle control
miR-146a	Down	*ROCK1*	–
miR-148a	Down	*MSK1*	Proliferation, stress response and drug resistance
miR-205	Down	*IL-24 and IL-32, Cepsilon*	Cell growth and invasion, EMT
miR-331-3P	Down	*ERBB-2, CDCA5, KIF23*	Signal transduction, cell cycle control
miR-449a	Down	*HDAC-1*	Gene expression
miR-1296	Down	*MCM family*	DNA replication
Let-7a	Down	*E2F2 and CCND2*	Cell cycle control and proliferation

Recently, miR-130a, miR-205 and miR-203 have been implicated in androgen receptor and MAPK pathways, however the molecular mechanism causing this downregulation have not, yet, been found (Boll et al., 2012).

Similar to protein coding genes, one reliable explanation for miRNAs' downregulation might be the aberrant methylation of their respective codifying genes. Indeed 13 to 28% of human miRNA genes are located within 3 and 10 kb from a CpG island, respectively (Choudhry and Catto, 2011). In addition, it has been suggested that 81.9% of predicted promoters of intergenic miRNA genes contain at least one CpG island (Wang et al., 2010). On the other hand, concerning intragenetic miRNAs, approximately 13.0% have been reported to be located within 500 bp downstream of a CpG island (Wang et al., 2010). However, due to the fact that the location of the promoter region of miRNAs codifing genes is not fully clarified this is still a controversial issue.

Remarkably, tumor suppressor miRNAs' regulation by hypermethylation, has been suggested since the re-expression of miR-9-1 and miR-127 was achieved after the exposure of human cancer cell lines to 5-AZA-DC (Lehmann et al., 2008) (Saito et al., 2006) (the mechanistic of these studies is elucidated in Figure 12).

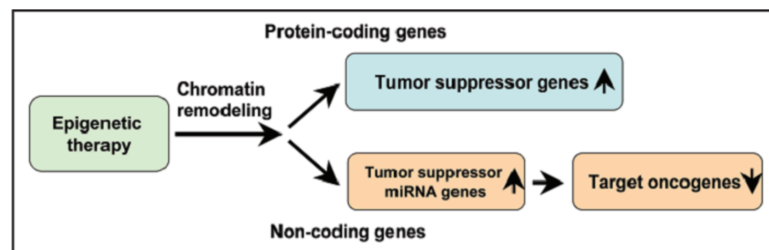

Figure 12 – Activation of coding or non-coding genes that might function as tumor suppressors using an epigenetic therapy with DNMT and/or HDAC inhibitors. The activation of tumor suppressor miRNAs may cause downregulation of target oncogenes. Adapted from (Saito and Jones, 2006)

Concerning PCa, miR-145, miR-205, miR-132, miR-126 and miR-193b have been recently reported to be regulated through methylation (Rauhala et al., 2010, Suh et al., 2011, Saito et al., 2009, Bhatnagar et al., 2010, Formosa et al., 2012). However, most of these studies were only performed in cell lines or in a limited number of primary tumors. Thus, epigenetic regulation of miRNA expression is still a largely unexplored field of research in PCa.

AIMS OF THE STUDY

The key objective of this Master Thesis, performed at the Cancer Epigenetics Group of the Research Center of the Portuguese Oncology Institute – Porto, was to identify new epigenetically downregulated miRNAs in PCa, using an expression profiling based approach. Furthermore, it was our purpose to validate these miRNAs in a larger set of clinical samples, in order to identify a putative tumor biomarker amenable to be used for diagnosis and prognostic assessment of this malignancy.

The specific aims of this project were:

1. Identify miRNAs that are downregulated in PCa compared to normal prostatic tissues (NPT);
2. Identify miRNAs that are upregulated in prostate cancer cell lines exposed to demethylating agents compared to untreated cell lines;
3. Validate the miRNAs putatively regulated by methylation by quantitative methylation-specific PCR in a larger series of tumors;
4. Assess the methylation status of the identified miRNAs in prostatic pre-malignant lesions (HGPIN);
5. Evaluate the performance of the newly identified miRNAs as tumor biomarkers in clinical samples.

METHODOLOGIES

Clinical Samples

Patients and Tissue Sample Collection

A total of 105 men with clinically localized PCa, diagnosed and primarily submitted to radical prostatectomy in I.P.O.F.G – Porto, from 2002 and 2006, were included in this study [stage T1c and T2, according to TNM system (Hermanek et al., 1997)]. Of the total 105 PCa tissues available, 10 were randomly selected to perform the global miRNA expression, while 101 were used for individual validation studies. HGPIN lesions were identified in 56 cases and also collected for further analysis. As sample controls, 14 morphologically NPT specimens were collected from the peripheral zone of prostates that did not harbor PCa obtained from cystoprostatectomy specimens of bladder cancer patients. The 4 NPT used were also randomly chosen from the 14 available patients tissues, to assess the global miRNA expression. All specimens were frozen at -80ºC and then cut with a cryostat for microscopic evaluation and selection of potential areas for analysis. Cut sections were trimmed to maximize target cell content (>70%) and then DNA extraction was performed using phenol-chloroform. From each specimen, parallel fragments were collected, formalin treated and paraffin-embedded for histopathological examination. Gleason's score (Gleason and Mellinger, 1974) and pathological staging (Hermanek et al., 1997) were evaluated by an expert pathologist (Rui Henrique, M.D., PhD). Relevant clinical data was collected from the clinical charts.

Urine Sample Collection and Processing

Morning voided urine samples (one per patient) were collected from 39 patients with PCa diagnosed and treated at the in I.P.O.F.G – Porto, Portugal. 15 male controls were randomly chosen among healthy donors (HD) with no personal or family history of cancer. Patients and controls were enrolled after informed consent. Urine storage and processing conditions were standardized: each sample was immediately centrifuged at 4000 rpm for 10 minutes; the pelleted urine sediment was then washed twice with phosphate-buffered saline, and stored at -80ºC.

These studies were approved by the institutional review board (Comissão de Ética) of Portuguese Oncology Institute - Porto, Portugal.

Cell Culture and Treatment with 5-Aza-2'-Deoxycytidine

In order to identify miRNAs putatively regulated by methylation, representative PCa cell lines were selected and exposed to an epigenetic modulating drug, namely 5-Aza-2'-Deoxycytidine (5-AZA-DC).

PCa cell lines VCaP (positive for the TMPRSS2-ERG fusion gene), PC-3 (androgen independent) and LNCaP (androgen dependent) (American Type Culture Collection, MD, USA) were grown in recommended medium, supplemented with 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA) and antibiotics (100 units/mL of penicillin G and 100 mg/mL of streptomycin; Gibco, Invitrogen, Carlsbad, CA), in a humidified atmosphere of 5% CO_2 at 37Cº, according to instructions. These three cell lines were kindly provided by Prof. Ragnhild A. Lothe from the Department of Cancer Prevention at The Institute for Cancer Research, Oslo, Norway. Cells were grown to 20 to 30% of confluence in 175 cm^3 flasks and then submitted to demethylation treatment with 1μM of 5-AZA-DC (Sigma-Aldrich®, Germany) for 72h. As controls, same cell lines were left untreated for 72 hours, only with medium exchange (Mock Samples). Culture medium and/or the appropriate drug were changed every day, always in the same amount and concentration and all treatments/mocks were made in triplicate. Cells were trypsinized and harvested after period completion and centrifuged. After being washed with PBS 1x, cells were frozen at -80ºC until DNA and RNA extraction, separately. To prepare the stock solution at a concentration of 100 mM of 5-AZA-DC, 10 mg of the compound were dissolved in 438 µL of 50% of acetic acid and stored at -80ºC. Prior to being added to cell culture, 5-AZA-DC solution was diluted in PBS 1x to a final concentration of 10 mM. This solution was stored at -20ºC during the using period.

Gene Expression Analysis

Global microRNA Expression

In order to determine which miRNAs were differentially expressed between NPT against PCa and which of these might be epigenetically regulated by comparing the 5-AZA-DC treated cells against the untreated cell lines, a qRT-PCR miRNA Plates expression profiling was performed. In a total of 20 samples (10 PCa, 4 NPT, and the 3 PCa cell lines mock and exposed to 5-AZA-DC were run in parallel using the miRCURY LNA™ Universal RT microRNA PCR system kit from Exiqon® (Vedbaek, Denmark), which has been previously reported by others (Koo et al., 2012, Jorde et al., 2012).

Using this approach, for each sample, the expression of 740 miRNAs (distributed by two 384 well plates) was measured by the Roche Real-Time PCR System [Light Cycler 480 instrument (Roche, Basel, Switzerland)], following the recommended protocol (Exiqon miRCURY LNA™ Universal RT microRNA PCR, Protocol B – Human and Mouse&Rat microRNA PCR Panels).

Amplification reactions were performed in a 384 well plate containing in each well lyophilised primers specific for the chosen miRNA, and consisted of 10 µL of a mixture containing 20 µL of the cDNA previously synthesized, 1980 µL of deionized nuclease free water, and 2000 µL of SYBR® Green master mix.

Real-time PCR reactions (Fig.13) were carried out at 95°C for 10 minutes, followed by 45 amplification cycles at 95°C for 10 seconds and 60°C for 1 minute with ramp-rate 1.6ºC/s[6] optical read (Fig. 14).

Each plate also contained, 3 interplate calibrators, 2 water blanks, 3 empty wells and 6 reference genes (SNORD38B, U6, SNORD49A, miR-191, miR-423-5p, miR-103) suggested by the kit manufactures and which some were already proposed as universal reference miRNAs like miR-191 and miR-103 (Mestdagh et al., 2009).

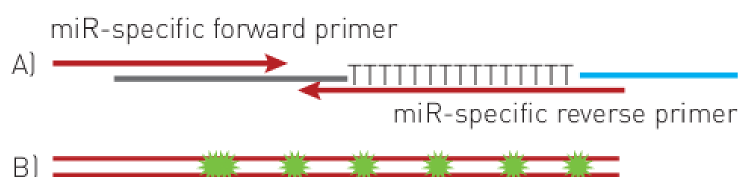

Figure 13 – The cDNA template is then amplified using microRNA-specific and LNA™-enhanced forward and reverse primers (step A). SYBR® Green was used for sequences detection (step B). Adapted from miRCURY LNA™ Universal RT microRNA PCR, Instruction manual Version 4.1 #203300 (August 2011)

The expression for each miRNA is given by the difference between its Ct value and the average Ct value of reference genes, per sample, within a given sample set (Mestdagh et al., 2009). Then, the data was analyzed using the comparative Ct method (Schmittgen and Livak, 2008).

The fold variation of the expression levels were determined by 2-ΔCT to prostate tissues: Prostate tissue ΔCT=CTmiRNA of interest – average CT of reference genes. In cell lines the fold change due to treatment levels were calculated by 2-ΔΔCT (ΔΔCT=ΔCt treated cells - ΔCt mock cells). The final results were generated by a log2 transformation of the real-time PCR data presented as 2-ΔCT or 2-ΔΔCT. Additionally, a fold variation value <-1.0 represents a downregulated miRNA, whereas a fold variation value >1 corresponds to an upregulated miRNA.

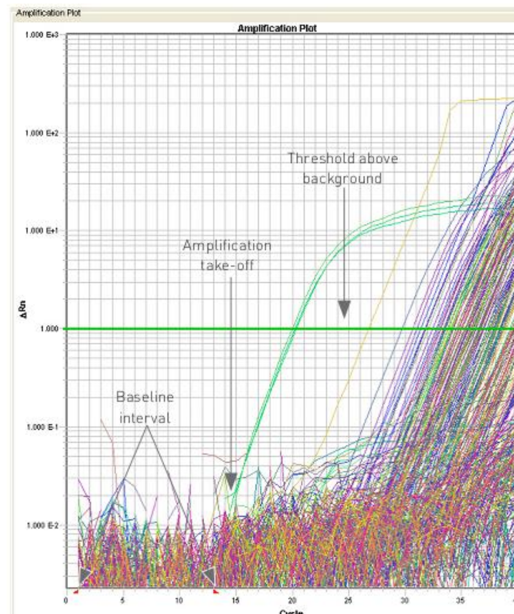

Figure 14 – Illustrative qRT-PCR amplification, with evidence to the baseline interval (happens before the amplification takes off) and the threshold above background. Adapted from miRCURY LNA™ Universal RT microRNA PCR, Instruction manual Version 4.1 #203300 (August 2011)

MiRNAs upregulated in more than 1.0-fold after 5-AZA-DC treatment in at least two of three PCa cell lines and simultaneously downregulated in tumor samples compared with normal tissue were considered to be potential targets for further methylation studies. Finally, all selected miRNAs were confirmed to be expressed in Prostatic tissue using a publically available database (www.microrna.org).

Quantitative Gene Expression Validation

The miRNAs identified by miRCURY LNA™ Universal RT microRNA PCR system kit were further confirmed by quantitative reverse-transcriptase (qRT-PCR) expression analysis.

RNA Extraction

Total RNA was extracted from all 105 PCa samples, three cell lines treated and untreated and from the 14 NPT samples, using PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. RNA concentrations were determined using a ND-1000 Nanodrop (NanoDrop Technologies, Wilmington, USA) and the RNA quality was verified by an electrophoresis in agarose gel.

cDNA Synthesis

Total cDNA was obtained from total RNA. In brief, the RNA was tailed with a poly(A) sequence at its 3'end and then reverse transcribed to cDNA using a universal poly(T) primer with a 3'end degenerate anchor and a 5'end universal tag (Fig.15).

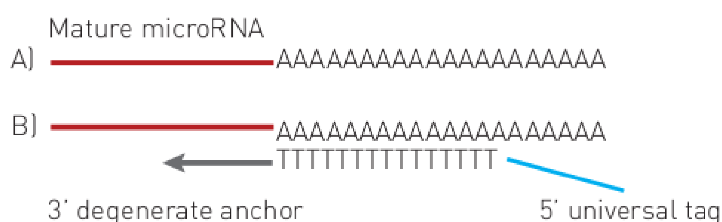

Figure 15 – A poly-A tail is added to the mature microRNA template (step A). cDNA is synthesized using a poly-T primer with a 3' degenerate anchor and a 5' universal tag (step B). Adapted from miRCURY LNA™ Universal RT microRNA PCR, Instruction manual Version 4.1 #203300 (August 2011)

For each sample, 20 ng of total RNA was reverse transcribed into cDNA using the standard protocol of miRCURY LNA™ Universal RT microRNA PCR (Exiqon®, Vedbaek, Denmark). The following components were added to an RNAse-free PCR tube on ice: 4 µL of a previously diluted at 5 ng/µL RNA solution, 4 µL of a 5x Reaction buffer (includes universal reverse transcription primers), 9 µL of nuclease-free water, 2 µL of enzyme mix and 1 µL of RNA spike in, previously re-suspended. Final mix reaction was incubated for 60 minutes at 42ºC, followed by 5 minutes at 95% for reverse transcriptase inhibition, using a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Final products were stored at -20ºC according to manufactures' instructions.

Quantitative Reverse-Transcriptase PCR (qRT-PCR)

Thus, for individual assays, the cDNA from the 101 tissue samples which was previously synthesized, was freshly diluted 80x (395 µl to each 5 µl of cDNA) in nuclease free water (MP Biomedicals, LLC, OH, USA) in low-nucleic acid binding tubes and then 4 µl of this solution was added in a 96 well plate, to a previously prepared solution containing 0.2 µL of a passive ROX reference dye (Invitrogen, Carlsbad, CA, USA), 1 µL of miRNAs primers, specific to a certain genomic sequence (Exiqon®, Vedbaek, Denmark) (Table 4) and 5 µL of SYBR® Green Master mix, according with manufactures' instructions. Primers were received lyophilized and re-suspended with nuclease free water (MP Biomedicals, LLC, OH, USA). Amplification reactions were carried out in triplicates on a 7500 Sequence Detection System (Applied Biosystems, USA), at 95°C for 10 minutes, followed by 45 amplification cycles at 95°C for 10 seconds and 60°C for 1 minute with

ramp-rate 1.6˚C/s[6] optical read. Each plate also contained two negative template controls and serial dilutions of a positive control, consisted of total human prostate RNA (Ambion, Austin, Texas, USA) previously converted to cDNA (dilution factor of 10x), which were used to construct a calibration curve for each plate to quantify the amount of transcript in each reaction.

A run was considered valid when the further requisites were achieved: a) Slopes of each calibration curve above -3.20 corresponding to a PCR efficiency near 100%; b) R^2 of at least three relevant data points ≥ 0.99; c) No template controls not amplified; d) Threshold cycle value for each gene ≤ 40;

For each sample, the triplicate with a standard deviation greater than 0.38, was removed. Also, for quality control, all amplification curves were visualized and scored without information of the clinical data. Finally, the ratio obtained by the former mathematic operation, which constitutes an index of the percentage of input copies of DNA that exist in that sample, was multiplied by 1000 for easier tabulation (expression levels = target gene/ average of the two reference genes x1000). The two reference genes used for normalization were miR-191 and miR-423-5p as these obtained the lowest coefficient of variation in Global microRNA Expression profile, between samples.

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Table 4 - Oligonucleotide target sequences to be studied and amplified by qRT-PCR in this work.

Target miRNA	Mature Sequence to be amplified
miR-191	CAACGGAAUCCCAAAAGCAGCUG
miR-130a	CAGUGCAAUGUUAAAAGGGCAU
miR-205	UCCUUCAUUCCACCGGAGUCUG
miR-145	GUCCAGUUUUCCCAGGAAUCCCU
miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU

DNA Methylation Analysis

In Silico Screening for CpG Islands

In order to confirm that the genes codifying for the miRNAs previously identified were putatively regulated by methylation, the presence of CpG islands at their promoter region was assessed *in silico* by Methyl Primer Express v 1.0 CpG as described by Costa *et al.* (Costa et al., 2010). The criteria used to define a CpG island were the existence of a

minimum stretch of 100 bp with at least 50% CG content and a ratio observed/expected CpG of at least 0.6. The minimal distance to differentiate two different adjacent CpG islands was 100 bp. For that, sequences of the candidate miRNA genes were obtained from the human genome database (http://www.ensembl.org). CpG islands were searched in a region up to 5000 bp 5'-upstream from the mature miRNA. The miRNAs that did not contain any CpG island were excluded from further analyses. The methylation status of the miRNAs' genes harboring a CpG was further assessed.

DNA Extraction

DNA from all clinical samples was extracted by the phenol-chloroform, according to standard protocol, as described by Pearson *et al.* (Pearson and Stirling, 2003). Thus, tissue digestion was achieved by adding 2700 µL of SE solution (75mM NaCl; 25 mM EDTA) 300 µL of 10% SDS (Sodium Dodecyl Sulfate) and 25 µL of proteinase K (20 mg/mL) (Sigma-Aldrich®, Germany) to each tube, followed by incubation overnight in a water-bath at 55ºC until total digestion was achieved. When necessary, the incubation went for 2 or 3 days, and proteinase K was added twice a day, during the incubation period. After digestion, extraction was completed with phenol/chloroform [Sigma, Germany]/ [Merck, Germany] in Phase Lock GelTM tubes. After centrifugation (20 minutes at 4000 rpm), the upper aqueous phase was transferred to a new tube. The DNA precipitation followed through with 1000 µL of 100% cold ethanol and 165 µL of ammonium acetate 7.5 M ammonium acetate (Sigma-Aldrich®, Germany), mixed and incubated overnight at -20ºC in order to promote DNA precipitation. The samples were washed in 70% ethanol solution and the pellets were air dried and eluted in 30 µL of sterile distilled water (B. Braun, Melsungen, Germany). DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, USA) and stored at - 20ºC until used.

Sodium Bisulfite Treatment of DNA

The basic principle of sodium bisulfite modification of DNA is that all unmethylated cytosines are deaminated, sulphonated and then converted to thymines, whereas methylated cytosines remain unaltered in the presence of NaOH and sodium bisulfite (Esteller, 2009, Tost, 2009). Consequently, the sequence of treated DNA will differ depending on whether the DNA is originally methylated or not (Fig.16).

Bisulfite modification of DNA

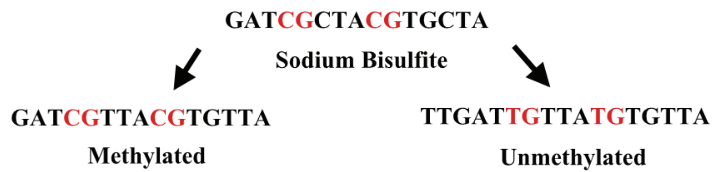

Figure 16 – Diagram of bisulfate modification of Methylated and Unmethylated DNA. Adapted from (Esteller, 2009)

In detail, the genomic DNA fragments are denatured to single stranded DNA, for a more effective bisulfite action, secondly cytosines form adducts, across the 5-6 double bond with an oxidant reagent such as bisulfate ion, which promotes deamination of the cytosine to give a uracil-bisulfite derivate. Thus, a subsequent alkali treatment will form a uracil by the removal of the sulphonate group (Fig. 17) (Tost, 2009, Clark et al., 1994). This reaction is highly specific and is controlled by pH, bisulfate concentration, temperature and the length of the genetic material (Clark et al., 1994). After this reaction the strands of DNA are no longer complementary, allowing its analyses by PCR methods (Tost, 2009).

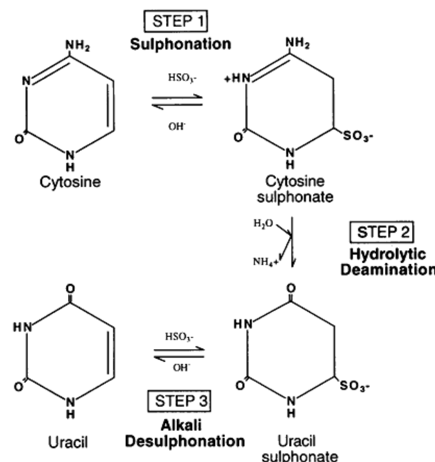

Figure 17 – Schematic diagram of bisulfite conversion reaction. Adapted from (Clark et al., 1994)

Genomic DNAs from tissues and urine were modified by sodium bisulfite, using EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA, USA) and this procedure was performed in accordance to manufacturer's guidelines. Briefly, in a PCR tube,1 µg of DNA in a total volume of 20 µL in sterile distilled water (B. Braun, Melsungen, Germany) was added to 130 µL of the CT conversion Reagent of the above mentioned commercial kit and incubated in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA,USA) for 10 minutes at 98ºC, and 180 minutes at 64ºC, in order to complete the cited chemical reaction. Following, the DNA was recovered in 600 µL using M-Binding buffer placed in a Zymo-Spin IC™ column and centrifuged for 30 seconds at 10,000 rpm, followed by a

cleanup step with M-Wash Buffer in the column. After eliminating the M-Wash Buffer from the column, 200 µL of M-Desulphonation Buffer were added and the DNA was submitted to desulphonation by this buffer for 20 minutes at room temperature. After a centrifugation to remove M-Desulphonation Buffer from the Column, DNA was washed twice with M-Wash Buffer. Finally, the column was placed in a new 1.5 mL-tube and DNA was eluted by incubation with 30 µL of sterile bidistilled water (B.Braun, Melsungen, Germany) for 5 minutes at room temperature followed by a centrifugation at 12,000 rpm for 30 seconds. This last step was performed again to obtain a total volume of 60 µL. CpGenome™ Universal Methylated DNA (Millipore, CA, USA) and CpGenome™ Universal Unmethylated DNA (Millipore, CA, USA) were also modified. CpGenome™ Universal Methylated and Unmethylated DNA (Millipore, CA, USA) were eluted in a final volume of 30 µL. Finally, bisulfite modified DNA was stored at -50ºC until further use.

Methylation Specific Polymerase Chain Reaction (MSP)

MSP constitutes both a sensitive and specific methodology for evaluating promoter hypermethylation of CpG islands (Herman et al., 1996, Tost, 2009). Hence, after bisulfite modification, the amplification is possible using specific primers that can distinguish methylated from unmethylated DNA. To insure specificity they must include at least two CG residues, with at least one of them located near the 3' region and also include non-CpG cytosines to amplify only modified DNA (Tost, 2009). The primers sequences, chosen from regions containing frequent cytosines, were designed using Methyl Primer Express v 1.0, for miR-130a, while miR-205 (Bhatnagar et al., 2010) and miR-145 (Suh et al., 2011) have been already published elsewhere (Table 5). In the present study, MSP was used to assess primers' specificity for the methylated sequence, to further be analyzed by real-time quantitative methylation-specific PCR (qMSP). For that, CpGenome™ Universal Mehylated DNA (Millipore, CA, USA), previously modified, was used as a positive control for methylation, while negative controls were modified from CpGenome™ Universal Unmehylated DNA (Millipore, CA, USA). Water blanks were also included in each assay. Thus, bisulfite modified DNA (2 uL) was amplified by PCR, using a primer pair for methylated and unmethylated CpG sequences, in a total volume of 20 uL, each. The amplification mix contained 0.4 µL of 10 mM of dNTPs mix (Fermentas, Ontario, Canada), 1 µL of each pair (forward and reverse) of methylated or nonmethylated primers at 10 µM, 2 µL of 10x DyNAzyme™ II Hot Start Reaction Buffer (Finnzymes, Finland), 0.24 µL of DyNAzyme II Hot Start (2 U/µL) (Finnzymes, Finland) and 14.36 µL of sterile distilled water (B. Braun, Melsungen, Germany), according with manufactures' instructions. In each assay negative and positive controls were tested simultaneously for

each pair of methylated primers, as well as, a water-blank to assess possible contaminations. The amplification conditions were performed as indicated by DyNAzyme™ II Hot Start manufacturer's conditions, at 94°C for 10 minutes, followed by 35 cycles at 94°C for 30 seconds, for each pair of primers an optimal annealing temperature (60ºC for miR-130a, 64ºC for miR-145, and 59ºC for miR-205) was performed for 30 seconds and 72°C for 30 seconds, followed by a final extension for 5 minutes at 72°C. The amplification products were loaded on a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

Table 5 – Oligonucleotide primers used for MSP and promoter methylation levels quantification by qMSP.

Target miRNA	MSP primers
miR-130a	Forward Methylated - ATAAATTTTGTCGGGGAGAGC
	Reverse Methylated - AATACCCCGATCAACGAAAA
miR-145	Forward Methylated - GGGTTTTCGGTATTTTTTAGGGTAATTGAAGTTTC
	Reverse Methylated - TAAAATACCACACGTCGCCG
miR-205	Forward Methylated - GAGTTTAAGTTGCGTATGGAAGC
	Reverse Methylated - AAAACAAATATTTCTTTTATAATCCGAA

Real-time Quantitative MSP (qMSP)

QMSP was performed for the same miRNAs in order to quantify the levels of CpGs promoter methylation, of each identified miRNAs. To date, most of the studies detecting miRNAs hypermethylation have used conventional MSP, a sensitive but not quantitative assay. Thus, using the same primers previously used for MSP fluorescence based, qMSP were performed using Power SYBR® Green PCR Master Mix (Applied Biosystems, USA), as performed by Savva-Bordalo *et al.* (Savva-Bordalo et al., 2010). Briefly, 2 μL of modified DNA from tissue and urine samples were amplified in a final reaction volume of 20 μL, which consisted of: 10 μL of Master Mix, 1 μL of forward and reverse methylated primers at 10μM and 7 μL of sterile deionized nuclease free water (MP Biomedicals, LLC, OH, USA). Analyses was performed in a 96 well plate in a 7500 Sequence Detection System (Applied Biosystems, USA), using the following amplification conditions: 50°C for 2 minutes, followed by 95°C for 10 minutes, then 45 cycles at 95°C for 15 seconds and specific primer annealing temperature [miR-130a, miR-145 and ACTB (used as reference gene) at 60ºC, and miR-205 at 59ºC) for 1 minute (Savva-Bordalo et al., 2010). After all cycles were completed, a dissociation-curve analysis was performed by the following

conditions: 95°C for 15 seconds, 60°C for 20 seconds and 95°C for 15 seconds. Samples were run in triplicate and multiple water blanks were used as control for contamination (negative control). To build the standard curve, five dilutions (dilution factor of 5x) of the same stock of bisulfite modified CpGenome™ Universal Methylated DNA (Millipore, CA, USA) were run in each plate. A run was considered valid when the further requisites were achieved: a) Slopes of each calibration curve above -3.60 corresponding to a PCR efficiency near 100%; b) R^2 of at least three relevant data points ≥ 0.99; c) no template controls not amplified; d) The positive methylation control had to supply a significant methylation signal; e) Threshold cycle value for each gene ≤ 40; f) No negative template controls were amplified.

For each sample, the triplicate with a standard deviation greater than 0.38, was removed. Also for quality control, all amplification curves were visualized and scored without knowledge of the clinical data. Finally, the ratio generated by the former mathematic operation, which constitutes an index of the percentage of input copies of DNA that are completely methylated at the specific primer site was multiplied by 1000 for easier tabulation (methylation levels = target gene/reference gene × 1000) (Savva-Bordalo et al., 2010). To classify samples as methylated or unmethylated, an empirical cutoff value was selected based on the highest methylation ratio value obtained for NPT or HD samples, ensuring an absolute specificity of the assay.

Identification of Prostate Cancer Cellular Pathways Targeted by Epigenetically Deregulated MiRNAs

After the identification and validation of 3 epigenetically regulated miRNAs, we investigated their putative target genes and forecast its implication to prostatic carcinogenesis.

Bioinformatics' Uncovering of MiRNAs Targets

The prediction of miRNA-mRNA interactions remains a challenging task due to the interactions complexity and still a limited knowledge of the entire process. There are numerous target prediction algorithms to find and speculate numerous targets that exploit different approaches and methods to predict such interactions. The current available algorithms can be divided in two categories based on the use or non-use of conservation comparison. The algorithms based mostly on conservation criteria are for example miRanda, PicTar, TargetScan and DIANA-microT while PITA and rna22 belong group of

those who use other parameters such as free energy of binding or secondary structures of 3'UTRs that can promote or prevent miRNA binding (Witkos et al., 2011). There is no consensus regarding the best algorithm, since all have advantages and disadvantages. Therefore, the algorithm that we have used was DIANA-microT, which has been already widely used in several studies (Albertini et al., 2011, Maragkakis et al., 2009, Formosa et al., 2012).

This algorithm uses a 38 nt-long frame that is moved along the 3'UTR, and the minimum energy of potential miRNA binding is measured and compared with the energy of 100 per cent complementary sequence bound to the 3'UTR region (Witkos et al., 2011). Additionally, this database searches for sites with canonical central bulge, requiring 7-9 nt-long complementary in 5' region of target miRNA (Witkos et al., 2011).

This database considers mainly conservative alignment for scoring but also non-conservative sites and it provides a probability of existence for each result depending on its pairing and conservation profile (Witkos et al., 2011). Therefore, for the identification of the pathways that are targeted by the identified miRNA, a higher and specific threshold score of 0.9 was used according to previously reported (Vlachos et al., 2012).

All the targets predicted by this algorithm will be properly validated in future studies.

Statistical Analysis

The Wilcoxon Signed Rank non-parametric test was performed for two-groups comparison of gene expression (Khan, 2004) and all miRNAs which showed a significant differential expression ($p<0.05$) were further considered for analysis.

The frequency of methylated cases, as well as the median and interquartile range of miR-130a, miR-145 and miR-205 promoter methylation was determined in PCa tissues and NPT. Also, the frequency of methylated cases as well as the median and interquartile range of miR-130a and miR-205 promoter methylation levels were determined in PCa patients and HD urine samples. To classify each sample as methylated or unmethylated an empirical cutoff value was established based on the higher methylation level observed in NPT tissues or HD urines, respectively. Differences in quantitative levels between NPT, HGPIN and PCa tissues or PCa and HD urines were assessed by the nonparametric Kruskal-Wallis test, followed by Bonferroni-adjusted Mann-Whitney U test for pairwise comparisons, when appropriate.

For statistical analysis purposes, PCa samples were divided into three Gleason's Score (GS) categories (GS $<$ 7, GS $=$ 7, and GS $>$ 7). Clinical stage at diagnosis

comprised two categories (stage II and III). The relationship between methylation or expression levels and other clinicopathological variables such as serum PSA levels at the time of diagnosis, GS and pathological stage were evaluated using the Kruskal-Wallis or the Mann-Whitney U tests, as appropriate. The Spearman non parametric correlation test was used to correlate methylation levels with age and also to correlate methylation levels with expression levels of each miRNA in the 101 PCa cases selected for both analyses.

For the purpose of examining the biomarker potential, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of miR-130a, miR-145 and miR-205, in tissue samples, and of miR-130a and miR-205, in urine samples, alone or in association, were calculated. In addition, multivariate logistic regression was used to examine associations between miRNAs with biomarker potential. For multivariate logistic regression the backward stepwise (Wald) selection method was used. Then, a receiver operator characteristics (ROC) curve was performed by plotting the true-positive rate (sensitivity) against the false-positive rate (1-specificity) and the area under the curve (AUC) was also calculated individually and for combination of the selected miRNAs.

Two sided p-values were considered significant when inferior to 0.05 for all tests, with Bonferroni's correction, when appropriate. Statistical analyses were performed using SPSS version 20.0.

RESULTS

Clinical and Pathological Characteristics

Tissue samples from 105 PCa, 56 HGPIN lesions, and 14 NPT samples were tested. The clinical and pathological characteristics of the patients are illustrated in Table 6. No significant differences were found for the median age of NPT, HGPIN and PCa patients, using the Kruskal-wallis test. The clinical characteristics of the patient who provided urine samples are illustrated in Table 7. However significant differences were found in urine samples, for the median age of HD and PCa patients (p<0.001), using the Mann–Whitney U test.

Table 6 – Clinical and pathological characteristics of patients from which tissue samples were obtained.

Clinicopatholgical Features	***PCa***	***HGPIN***	***NPT***
Patients, n	101	*56*	*14*
Median age, years*	64 (49 - 74)	65 (53 - 75)	65 (45 - 80)
PSA (ng/mL), median (range)*	7.6 (3.4 - 35.5)	*n.a.*	*n.a.*
Pathological Stage, n (%)			
pT2	56 (55.4)	*n.a.*	*n.a.*
pT3	45 (44.6)	*n.a.*	*n.a.*
Gleason Score, n (%)			
< 7	30 (29.7)	*n.a.*	*n.a.*
= 7	56 (55.4)	*n.a.*	*n.a.*
> 7	15 (14.9)	*n.a.*	*n.a.*

Abbreviations: NPT- Morphologically Normal Prostate Tissue; HGPIN- High Grade Prostatic Intraepithelial Neoplasia; PCa- Prostate Cancer and n.a.- Not Applicable

Table 7 – Gender and age distribution of patients which provided urine samples for this study.

	HD	**PCa**
Patients, n	15	39
Gender, n (%)		
Male	15 (100%)	39 (100%)
Female	0 (0%)	0 (0%)
Median age, yrs (range)	52 (43-64)	65 (52-88)

Abbreviations: PCa- Prostate Cancer; HD- Healthy Donors

Identification of Epigenetically Regulated MiRNAs

After comparing the expression values between PCa tissues and NPT, only 173 miRNAs, out of the 740 analyzed, shown to be differently expressed with a $p<0.05$ by the Signed Rank non-parametric test. After applying the previously mentioned fold variation cut-off, 47 miRNAs were significantly downregulated, whereas 5 miRNAs were found to be upregulated (Fig. 18). From the downregulated miRNAs, the highest fold variation values were displayed by miR-187 (~3.0 fold) and miR-224, miR-31*, miR-548b-3p, miR-193b*, miR-205 and miR-221*(~2.0 fold) (Table 8). Conversely, miR-449a and miR-32 were upregulated with a fold variation of 4.0 and 3.0, respectively.

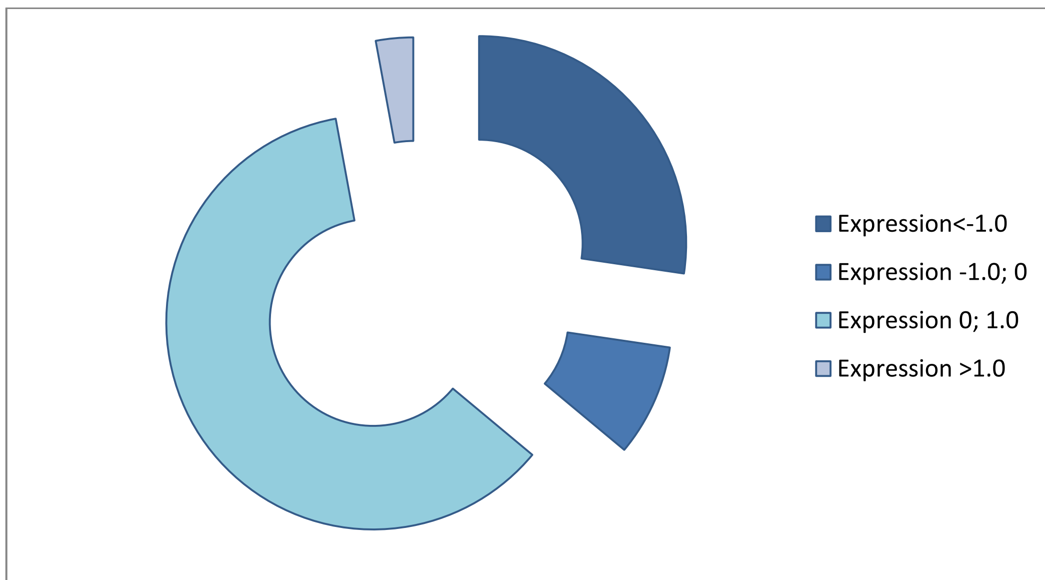

Figure 18 – Number of differentially expressed miRNAs in PCa tissues concerning respective fold variations.

Table 8 – Downregulated miRNAs with a fold variation lower than -1.0.

Ranking	miRNA	Mean Fold Variation (Log 2)	Chromosome localization	P value*
1	miR-187	-3.07	**18q122**	P=0.002
2	miR-224	-2.72	**Xq28**	P=0.013
3	miR-31*	-2.47	**9p213**	P=0.027
4	miR-548b-3p	-2.43	**6q2231**	P=0.013
5	miR-193b*	-2.18	**16p1312**	P=0.002
6	miR-205	-2.13	**1q322**	P=0.004
7	miR-221*	-2.02	**Xp113**	P=0.014
8	miR-27b*	-1.91	**9q2232**	P=0.019
9	miR-204	-1.84	**9q2112**	P=0.002
10	miR-624*	-1.81	**14q12**	P=0.002
11	miR-628-3p	-1.79	**15q213**	P=0.036
12	miR-502-3p	-1.78	**Xp1123**	P=0.024
13	miR-214	-1.73	**1q243**	P=0.036
14	miR-221	-1.72	**Xp113**	P=0.008
15	miR-555	-1.71	**1q22**	P=0.024
16	miR-139-5p	-1.67	**11q134**	P=0.014
17	miR-100	-1.67	**11q241**	P=0.024
18	miR-505*	-1.64	**Xq271**	P=0.006
19	miR-338-5p	-1.60	**17q253**	P=0.013
20	miR-125b-2*	-1.54	**21q211**	P=0.008
21	miR-145*	-1.54	**5q32**	P=0.024
22	miR-328	-1.51	**16q221**	P=0.019
23	miR-1271	-1.49	**5q35**	P=0.002
24	miR-224*	-1.49	**Xq28**	P=0.024
25	miR-145	-1.48	**5q32**	P=0.006
26	miR-455-3p	-1.42	**9q32**	P=0.004
27	miR-30a*	-1.42	**6q13**	P=0.024
28	miR-222	-1.40	**Xp113**	P=0.004
29	miR-193b	-1.37	**16p1312**	P=0.028
30	miR-133b	-1.35	**6p122**	P=0.036
31	miR-300	-1.29	**14q3231**	P=0.036
32	miR-143*	-1.29	**5q32**	P=0.024
33	miR-1468	-1.29	**Xq112**	P=0.024
34	miR-296-5p	-1.28	**20q1332**	P=0.036
35	miR-509-3p	-1.27	**Xq273**	P=0.014
36	miR-29b-1*	-1.19	**7q323**	P=0.024
37	miR-320a	-1.17	**8p213**	P=0.014
38	miR-23b*	-1.16	**9q2232**	P=0.004
39	miR-181a-2*	-1.12	**9q333**	P=0.028
40	miR-675b	-1.12	**11p155**	P=0.028
41	miR-193a-5p	-1.10	**17q112**	P=0.014
42	miR-23b	-1.09	**9q2232**	P=0.014
43	miR-138	-1.03	**16q13**	P=0.046
44	miR-130a	-1.03	**11q121**	P=0.001
45	miR-378	-1.03	**5q32**	P=0.036
46	miR-1181	-1.03	**19p132**	P=0.036
47	miR-99b	-0.99	**19q1341**	P=0.014

** Wilcoxon Signed Rank non-parametric test*

Additionally, the relative expression fold variation of the same 740 miRNAs was assessed for the three PCa cell lines (LNCaP, VCaP and PC3) (treated with 1 µM 5-AZA-DC *vs* untreated). The results for each cell line are depicted in Figure 19. In PC3 cell line, 227 miRNAs were re-expressed after treatment, whereas there were 202 miRNAs for LNCaP and 218 miRNAs for VCaP. Interestingly, only 18 miRNAs were commonly re-expressed in the three treated cell lines, while 120 miRNAs were re-expressed in two out of the three treated cell lines. Of these, only miR-130a, miR-145, miR-205, miR-509-3p, miR-23b*, miR-455-3p and miR-1181 were significantly downregulated in PCa, and thus, identified as putatively regulated by gene promoter methylation.

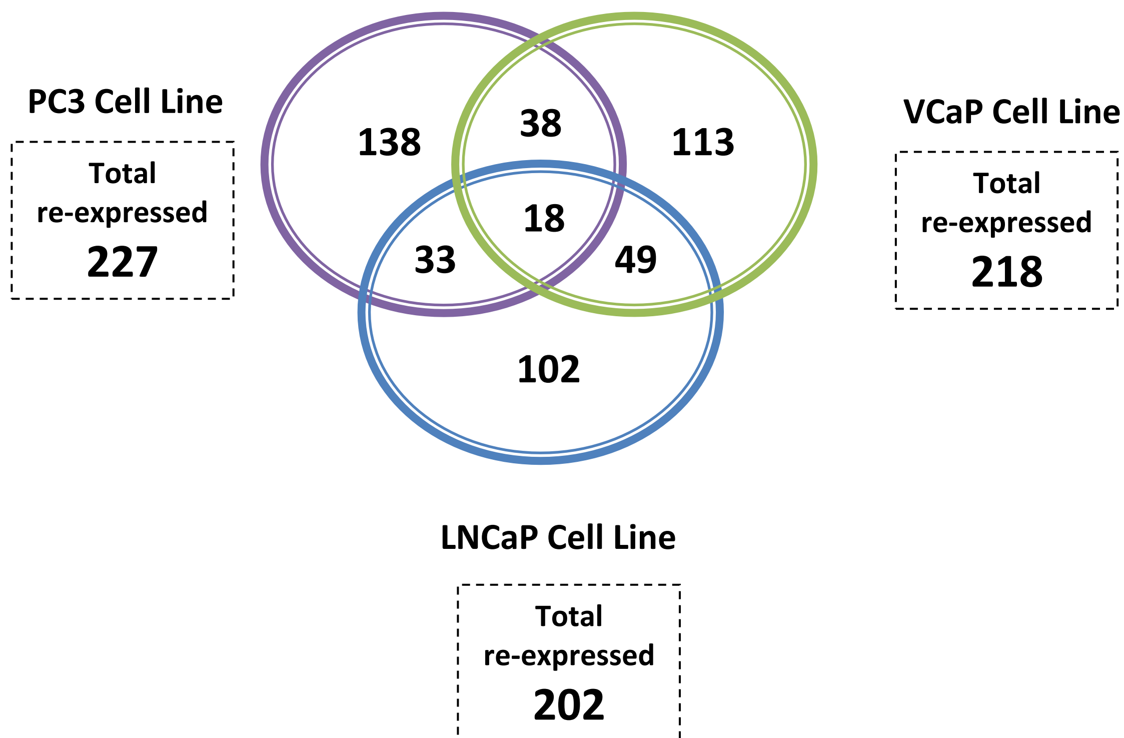

Figure 19 – The Venn diagram shows the number of miRNAs re-expressed with a fold variation higher than 1.0 in LNCaP, PC-3 and VCaP after treatment with 5-AZA-DC, in comparison with the non treated.

Finally, the expression status of these miRNAs was verified in normal prostate through their search in www.microrna.org database. Hence, miR-509-3p and miR-1181 miR-23b* were excluded of further analysis, since no information was found, concerning its expression in normal prostatic tissue. Concerning miR-455-3p it had no CpG island locate 5000 bp upstream.

Importantly, concerning the 3 identified miRNAs, we describe a novel miRNA, miR-130a, which has never been reported to be epigenetically regulated, and miR-145, miR-205 both already described to be regulated by methylation in PCa cell lines and tissues.

A flow-chart of the strategy used for the identification of putatively epigenetic regulated miRNAs is depicted in Figure 20.

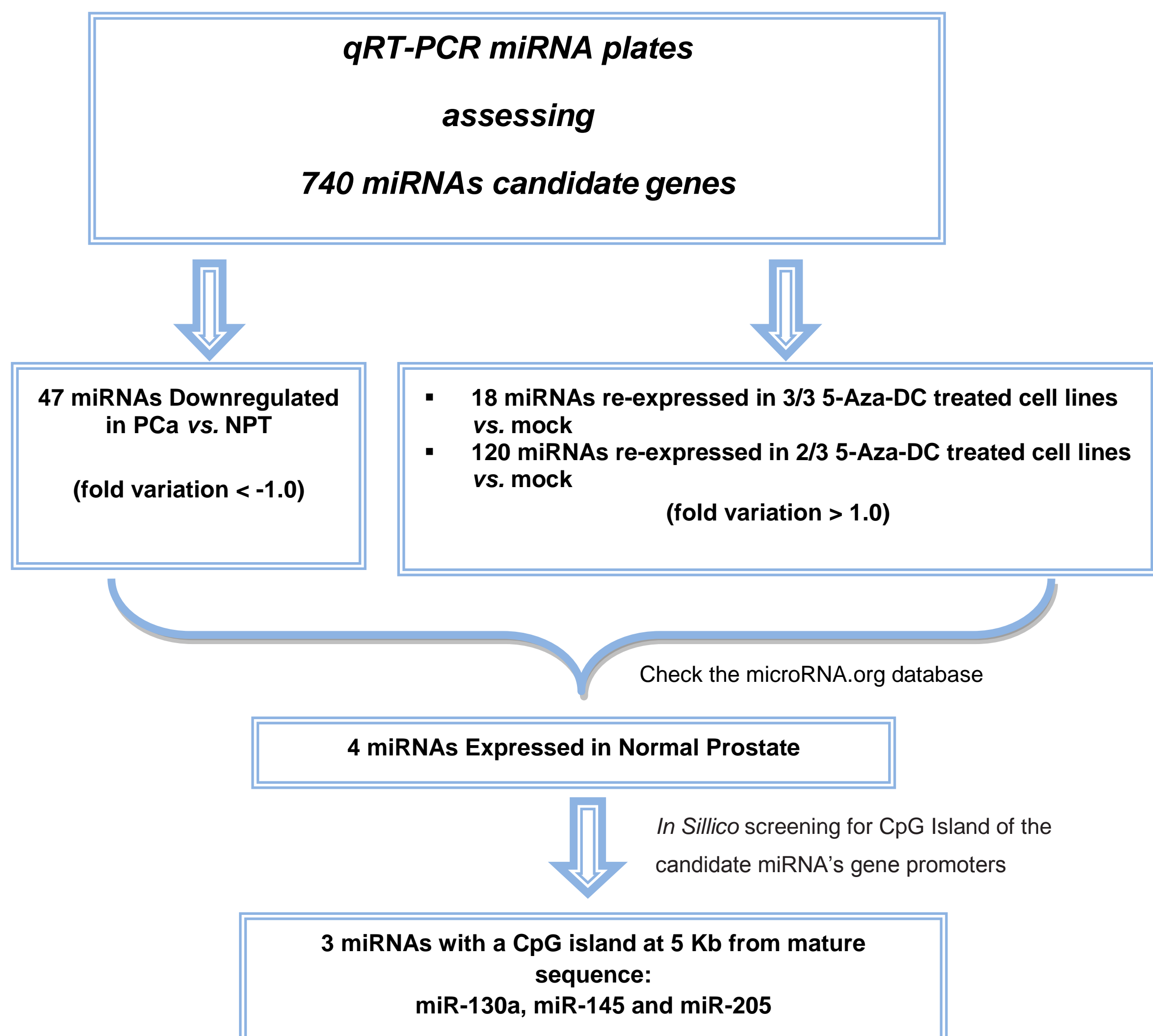

Figure 20 – Overview of the screening approach performed in this study. A combination of *in silico* analyses, molecular studies in human PCa cell lines, and analysis of primary PCa and health prostate tissue specimens used for the identification of miRNAs genes affected by differential methylation in human PCa.

Analysis of MiRNAs Promoter Methylation in Tissues

After testing the primers for each miRNA by conventional MSP, and verifying their specificity (data not shown), qMSP analysis was performed to determine the methylation levels of the promoter regions of miR-130a, miR-145 and miR-205 in tissue samples.

An empirical cutoff value (highest methylation level observed in NPT) was used to categorize cases as methylated *vs.* unmethylated. Thus, miR-130a showed the highest frequency of methylation in PCa (82.2%), followed by miR-205 (35.6%) and miR-145 (26.73%). Median and interquartiles values for the methylation levels are represented on Table 9. Differences among the three groups of tissues for the methylation frequencies and methylation levels were statistically significant for the 3 tested miRNAs (Kruskall-Wallis test, p<0.0001 for both).

Table 9 – Frequency and distribution of the methylation levels for each miRNA in PCa tissue samples

	PCa		***HGPIN***		***NPT***		
Gene	*n (%)*	*Median (IQR)*	*n (%)*	*Median (IQR)*	*n (%)*	*Median (IQR)*	*P Value**
miR-130a	*83 (82.17%)*	*200.91 (144.86- 283.26)*	*23 (41.07%)*	*87.06 (29.36- 192.93)*	*0 (0%)*	*27.78 (13.18- 56.03)*	*<0.0001*
miR-145	*27 (26.73%)*	*346.11 (216.04- 471.39)*	*23 (41.07%)*	*442.83 (313.80- 558.57)*	*0 (0%)*	*117.98 (75.28- 253.26)*	*<0.0001*
miR-205	*36 (35.64%)*	*345.30 (204.52- 640.41)*	*24 (42.86%)*	*266.40 (183.81- 376.68)*	*0 (0%)*	*58.09 (17.62- 177.22)*	*<0.0001*

*Abbreviations: IQR- Interquartile Range; NPT- Morphologically Normal Prostate Tissue; HGPIN- High Grade Prostatic Intraepithelial Neoplasia; PCa- Prostate Cancer; * Kruskall-Wallis test*

Indeed, pair-wise comparisons showed that in PCa the methylation levels of the 3 miRNAs were significantly higher than those of NPT (Bonferroni-adjusted Mann-Whitney U test, p<0.0001) (Fig. 21, 22 and 23). The same trend was verified concerning the comparison between PCa and HGPINs, in which for miR-130a (Fig. 21) (Bonferroni-adjusted Mann-Whitney U test, p<0.0001) and for miR-205 (Fig. 23) (Bonferroni-adjusted Mann-Whitney U test, p=0.004) the highest levels were found in the tumor samples. Contrarily, for miR-145 significantly higher methylation levels were observed in HGPIN (Fig. 22) (Bonferroni-adjusted Mann-Whitney U test, p=0.011). Moreover, the methylation levels displayed by HGPIN lesions were also significantly higher than by NPT [miR-130a (Bonferroni-adjusted Mann-Whitney U test, p=0.003), miR-205 (Bonferroni-adjusted

Mann-Whitney U test, p<0.0001) and miR-145 (Bonferroni-adjusted Mann-Whitney U test, p<0.0001)] (Fig. 21, 22 and 23).

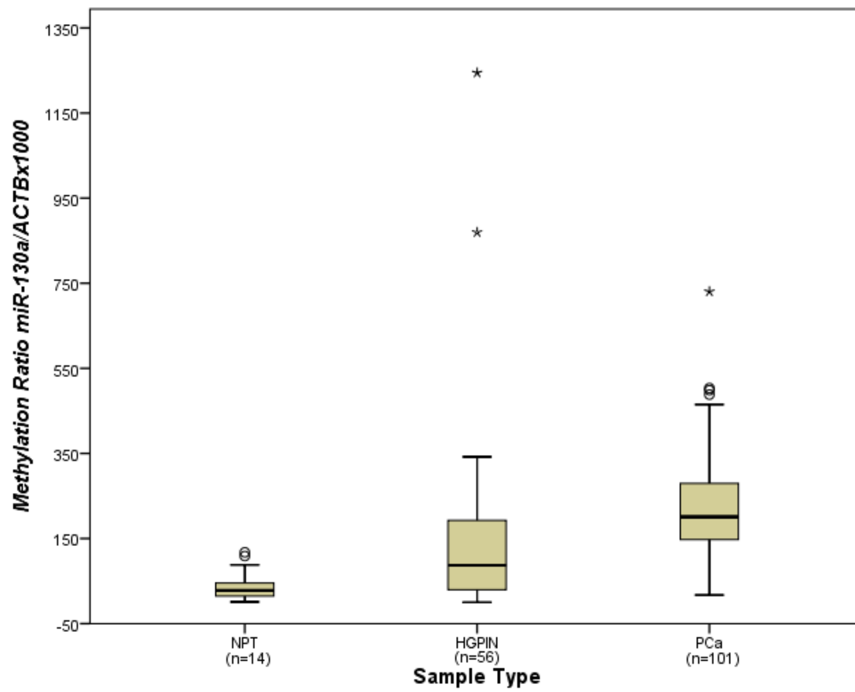

Figure 21 – Distribution of miR-130a promoter methylation levels in prostatic tissue samples. Abbreviations: NPT- Morphologically Normal Prostate Tissue; HGPIN- High Grade Prostatic Intraepithelial Neoplasia; PCa- Prostate Cancer

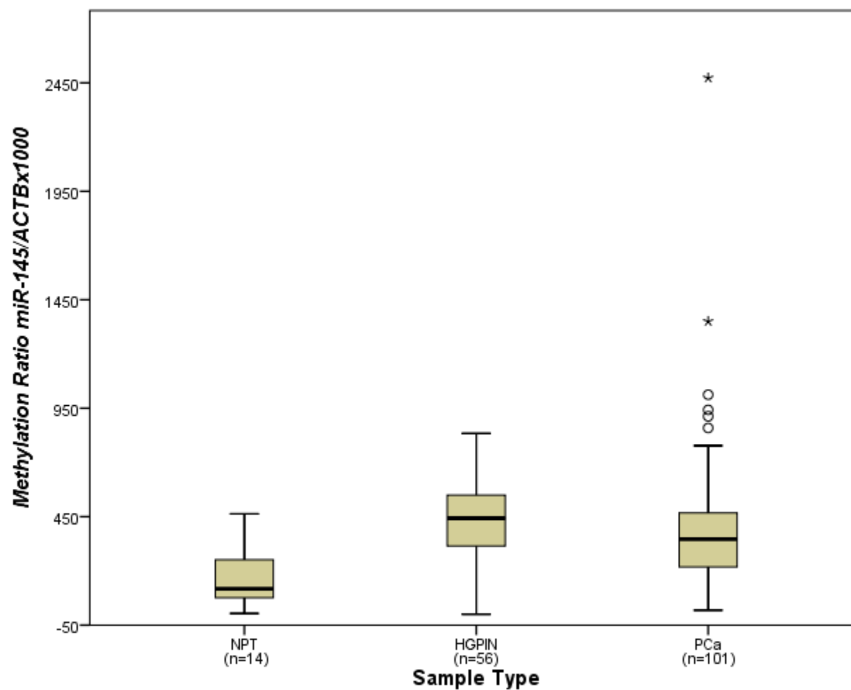

Figure 22 – Distribution of miR-145 promoter methylation levels in prostatic tissue samples. Abbreviations: NPT- Morphologically Normal Prostate Tissue; HGPIN- High Grade Prostatic Intraepithelial Neoplasia; PCa- Prostate Cancer

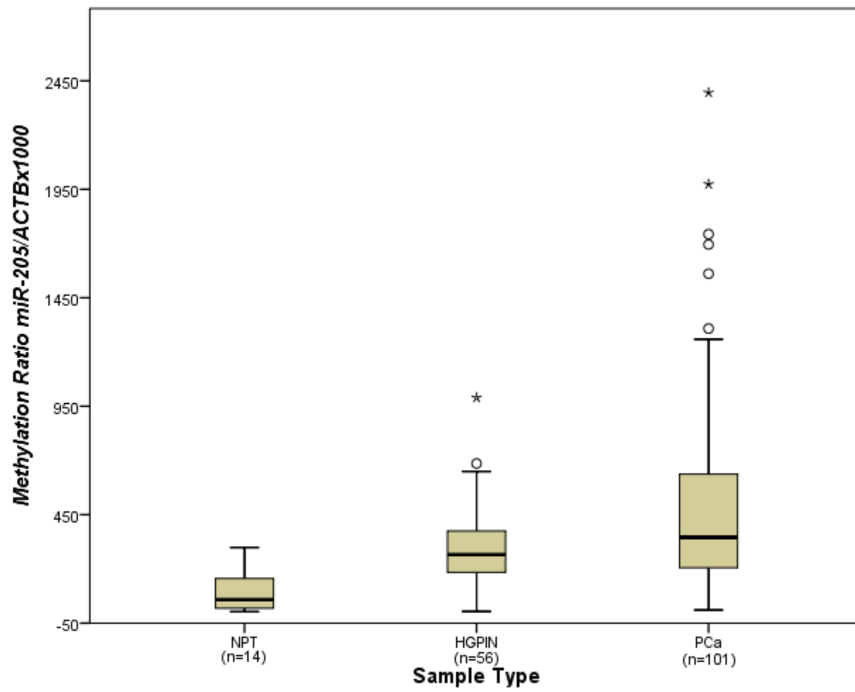

Figure 23 – Distribution of miR-205 methylation levels in prostatic tissue samples. Abbreviations: NPT- Morphologically Normal Prostate Tissue; HGPIN- High Grade Prostatic Intraepithelial Neoplasia; PCa- Prostate Cancer

No significant correlations were found between methylation levels of the target genes and any of the clinical-pathological variables (age, pre-operative serum PSA, Gleason score or pathological stage).

mRNA Relative Expression Levels in Tissue

The expression levels of the three miRNAs, miR-130a, miR-145 and miR-205, were also determined in the same series of tissue samples. Indeed, miR-130a (Mann-Whitney U test, P=0.004) and miR-205 (Mann-Whitney U test, P<0.0001) were significantly downregulated in PCa, as shown in Figures 24 and 26.

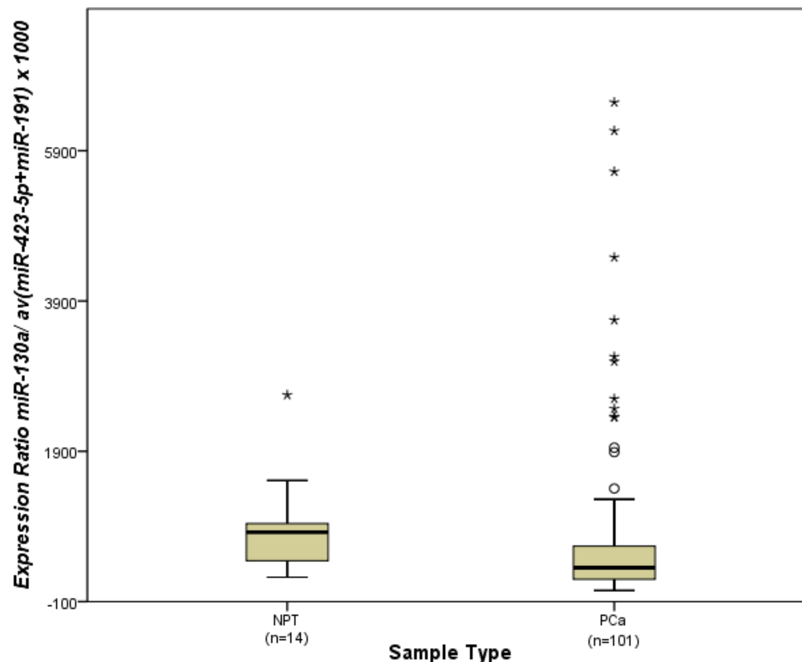

Figure 24 – Distribution of miR-130a transcript expression levels in prostatic tissues. Abbreviations: NPT-Morphologically Normal Prostate Tissue; PCa- Prostate Cancer; Av- Average

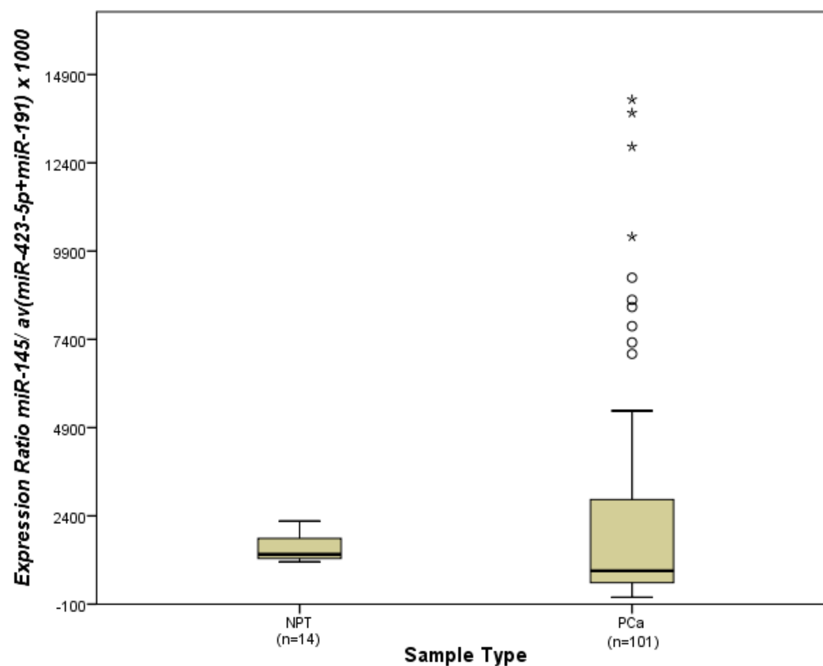

Figure 25 – Distribution of miR-145 transcript expression levels in prostatic tissue. Abbreviations: NPT-Morphologically Normal Prostate Tissue; PCa- Prostate Cancer; Av- Average

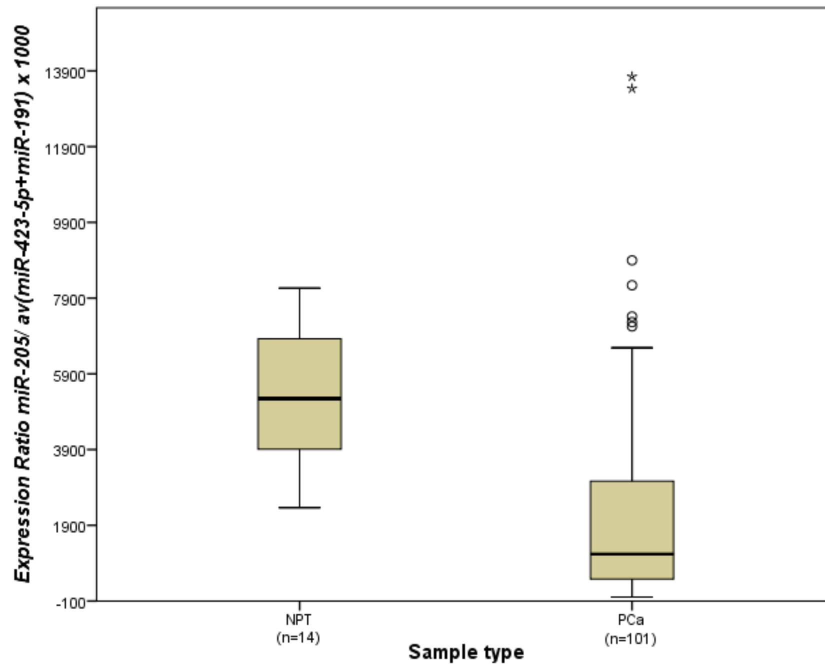

Figure 26 – Distribution of miR-205 transcript expression levels in prostatic tissue. Abbreviations: NPT- Morphologically Normal Prostate Tissue; PCa- Prostate Cancer; Av- Average

Concerning miR-145, although the same trend has been observed, no statistical significance was attained (Fig. 25).

No correlation was found between transcript expression levels of any of the analyzed miRs and respective gene promoter methylation levels. Moreover, no significant correlations were found between the transcript levels of miR-130a and miR-145 and clinical-pathological variables (age, pre-operative serum PSA, Gleason's score or pathological stage). However, concerning miR-205, significant correlation was found between transcript levels and both Gleason score and pathological stage. Interestingly, miR-205 expression levels were lower in high Gleason's scores tumors (Kruskall-Wallis test, p=0.001). Indeed, significant differences were found between GS<7 and GS=7 (p=0.018), between GS=7 and GS>7 (p=0.026) and between GS>7 and GS<7 (p=0.001) by the Mann-Whitney U test (Fig. 27). Furthermore, miR-205 lower expression levels inversely correlated with pathological stage by the Mann-Whitney U test (p=0.006) (Fig. 28).

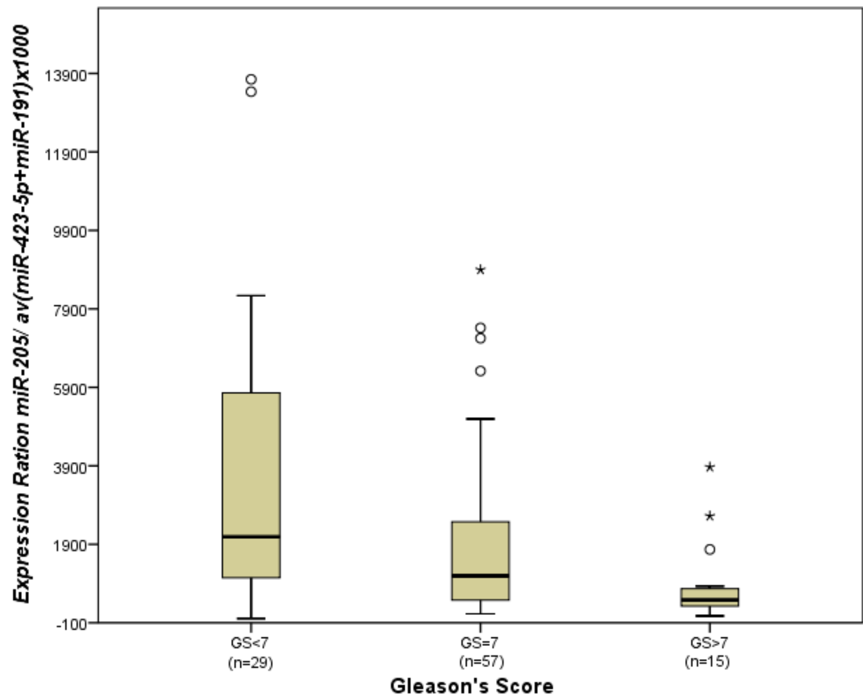

Figure 27 – Expression levels of miR-205 in samples of 101 PCa prostatectomies according to Gleason's Score. Abbreviations: GS- Gleason's score; Av- Average

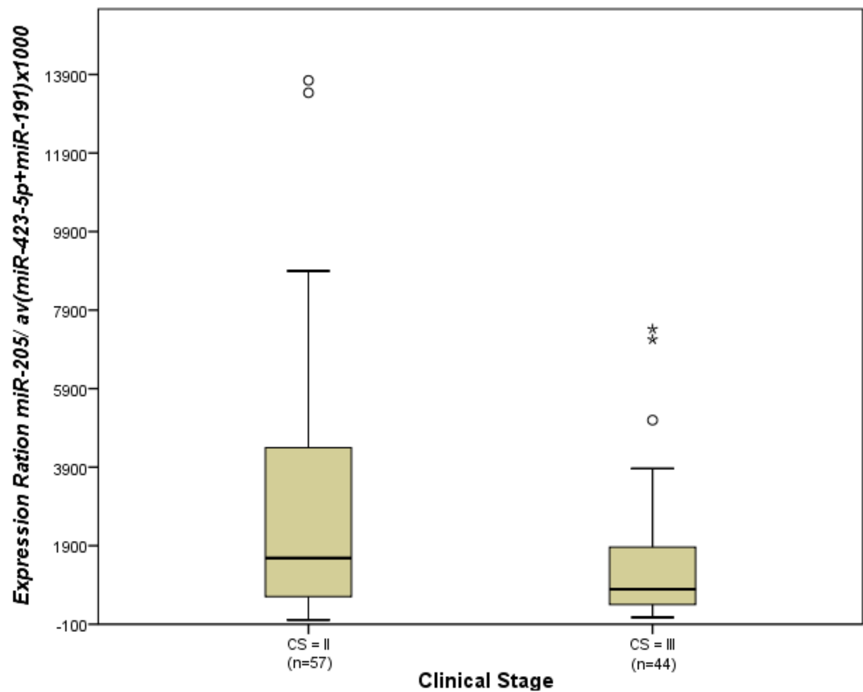

Figure 28 – Expression levels of miR-205 in samples of 101 PCa prostatectomies according to clinical stage. Abbreviations: CS- clinical stage; Av- Average

Evaluation of the Biomarkers Diagnostic Potential Using Tissue and Urine Samples

Performance of MiR-130a, MiR-205 and MiR-145 Methylation as Tumor Markers in Tissue

The diagnostic performance of the three miRNAs was assessed using the cutoff values of methylation levels previously referred and determined for each of these gene promoters (117.54 for miR-130a, 463.15 for miR-145 and 298.39 for miR-205). Validity and information estimates for each miRNA or the best combination of genes are displayed in Table 10.

Table 10 – Diagnostic performance of selected miRNAs methylation as a PCa biomarker, alone or in association.

	Parameter - Value (%)				
Gene	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**
miR-130a	82.18	100	100	43.75	84.35
miR-145	26.73	100	100	15.91	35.65
miR-205	64.36	100	100	28.00	68.70
miR-130a and *miR-205*	89.11	100	100	56.00	90.43
miR-130a, miR-145 and miR-205	91.09	100	100	60.87	92.17

Abbreviations: PPV - Positive Predictive Value; NPV Negative Predictive Value (NPV)

Although, a specificity of 100% was apparent for all tested miRNAs, the sensitivity has ranged from 27% to 82%, for each miRNA alone. The best sensitivity was achieved combining in the same panel the methylation analysis of two miRNAs (miR-130a and miR-205). According to the model of logistic regression applied, the inclusion of miR-145 in the panel, did no increment significantly the performance of the two miRNAs-panel. ROC curve analysis allowed for the determination of the AUC (95% CI) for each miRNAs gene: 0.956 (0.917- 0.996) for miR-130a, 0.828 (0.710- 0.946) for miR-145 and 0.907 (0.832-0.982) for miR-205 (Fig. 29). The ROC curve based on the above mentioned panel of two

markers (miR-130a and miR-205) resulted in an area under the curve (AUC) of 0.970 (0.941-0.998), at a significance of P<0.0001, by the multivariate logistic regression Wald test (Fig. 30).

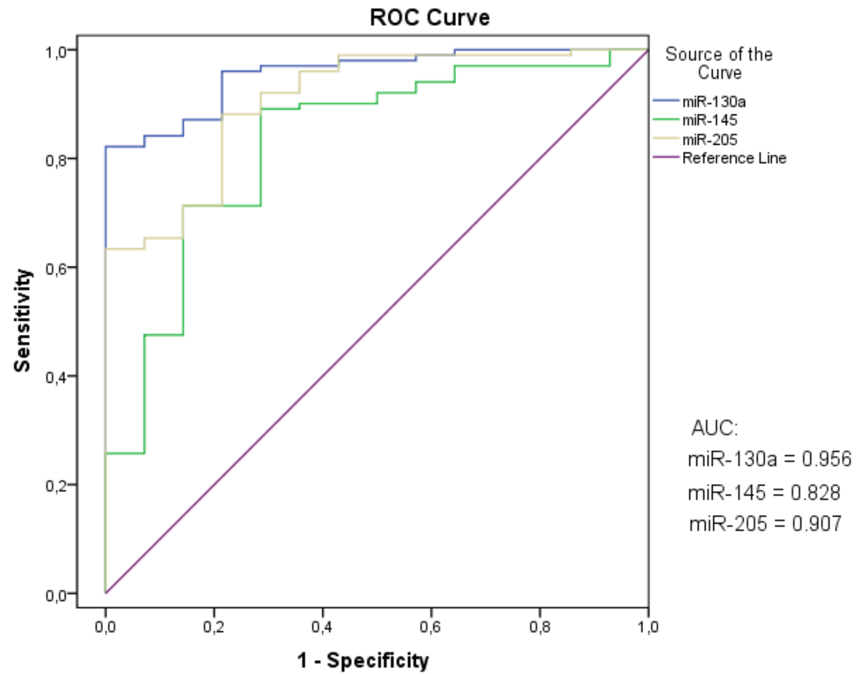

Figure 29 – Receiver operating characteristic curve in PCa tissue for each individual gene (miR-130a, miR-205 and miR-145).

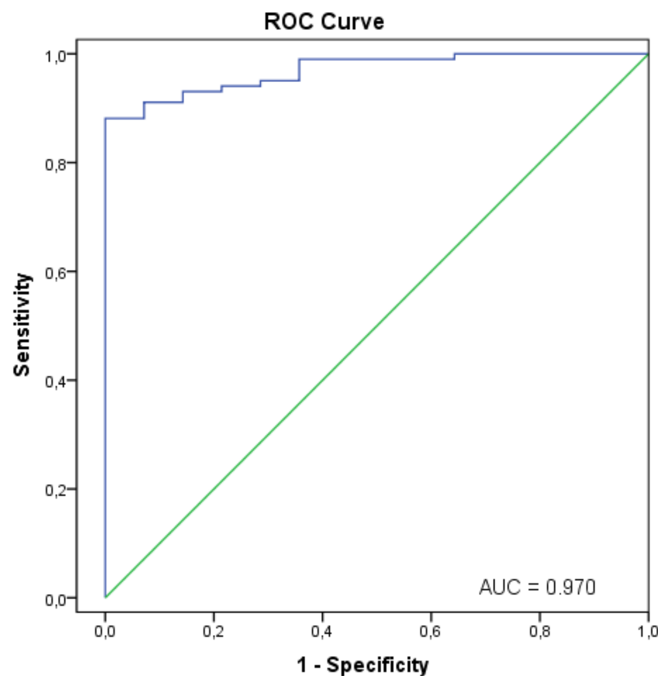

Figure 30 - Receiver operating characteristic curve in PCa tissue for the best combination of two genes (miR-130a and miR-205).

Performance of MiR-130a and MiR-205 Methylation as Tumor Markers in Urine Samples

We have additionally investigated the methylation status of the promoter region of the miRNAs panel which have shown the best performance in terms of sensitivity, specificity and ROC curve (thus miR-145 was not tested), in distinguishing PCa patients from controls in tissue samples, in urine samples. Nevertheless, due to time constrains, we have only performed a preliminary study in a few number of urine sediments: 39 PCa urine patients and 15 urine samples obtained from HD.

Interestingly, the Bonferroni-adjusted Mann-Whitney U test revealed that miR-130a methylation levels were significantly higher in urine samples from PCa than from healthy donors (p=0.015) (Fig. 31), however the same was not demonstrated for miR-205.

The possible diagnostic performance of the miR-130a alone was assessed using the cutoff values of methylation levels of 114.05. Validity and information estimates are displayed in Table 11.

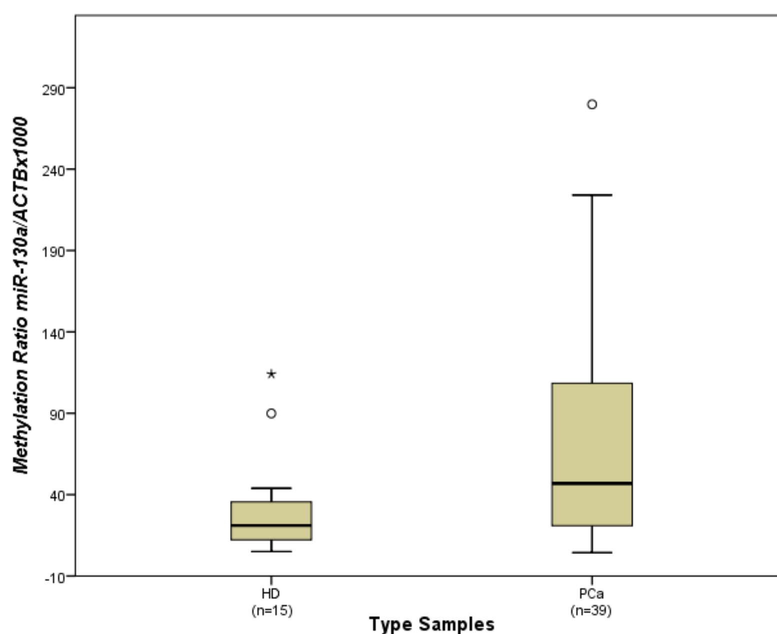

Figure 31 – Distribution of the methylation levels of miR-130a in urine samples. Abbreviations: HD- Healthy Donors; PCa- Prostate Cancer

Table 11 – Diagnostic performance of miR-130a methylation in urine samples.

Parameter	Value (%)
Sensitivity	25.64
Specificity	100
PPV	100
NPV	34.09
Accuracy	46.30

Abbreviations: PPV - Positive Predictive Value; NPV Negative Predictive Value (NPV)

MiRNAs Potential Targets

In order to investigate possible target genes and respective signaling pathways of the three methylation regulated miRNAs previously identified, a well know database DIANA-microT was surveyed, using the described criteria in the material and methods section. Possible target pathways of miR-130a, miR-145 and miR-205 are listed in Table 12, Table 13 and Table 14, respectively. Globally, several critical pathways involved in tumor progression seem to be targeted by the three miRNAs herein identified as epigenetically deregulated in PCa.

Table 12 – Possible pathways targeted by miR-130a.

Putative Target Pathways	Gene Name	-ln(value)
TGF-beta signaling pathway	*LTBP1, ROCK1, ZFYVE9, SMAD5, ACVR1, SKP1A, NOG, INHBB, GDF6, PPP2R1B, ACVR2B, TGFBR2, EP300*	13.84
Calcium signaling pathway	*PDE1C, PLN, PDGFRA, LHCGR, ADCY1, ERBB3, ATP2A2, SLC8A1, ITPR1, ADCY2, SPHK2, GRIN2A, PLCB1, ERBB4*	7.01
Wnt signaling pathway	*WNT2B, NFATC2, ROCK1, SKP1A, DAAM1, WNT1, FBXW11, PPP2R1B, NLK, PLCB1, EP300, FZD6*	4.46
Ubiquitin mediated proteolysis	*UBE2D1, UBE2D2, HERC3, BIRC6, CUL3, UBE2W, SKP1A, FBXW11, UBE4B, ANAPC5, CUL5*	4.35
Gap junction	*PDGFRA, GJA1, ADCY1, ITPR1, PRKG1, ADCY2, SOS1, PLCB1*	3.15
Cell cycle	*E2F3, YWHAB, CDKN1A, GADD45A, E2F2, CDC14A, SKP1A, ANAPC5, EP300*	3.07
mTOR signaling pathway	*TSC1, PRKAA1, ULK2, IGF1*	2.94
Phosphatidylinositol signaling system	*SYNJ1, ITPR1, PTEN,PTENP1, ITPK1, PLCB1*	2.28
MAPK signaling pathway	*NFATC2, PDGFRA, GADD45A, MAP3K4, PPM1B, SOS1, DUSP16, RPS6KA5, NLK, HSPA8, TGFBR2, RASA1, MAP3K12, MAX*	2.22
Adherens junction	*PTPRM, MET, WASL, NLK, TGFBR2, EP300*	2.21
Focal adhesion	*PDGFRA, MET, ROCK1, CAV2, ITGB8, SOS1, PTEN,PTENP1, PAK6, ITGA11, COL2A1, IGF1, ITGA5*	2.09
ABC transporters	*ABCA1, ABCC5, ABCD3, ABCB7*	1.86
Ether lipid metabolism	*ENPP6, LYCAT, PAFAH1B1*	1.37
ErbB signaling pathway	*CDKN1A, ERBB3, SOS1, PAK6, ERBB4, EREG*	1.34
Methionine metabolism	*DNMT1, MAT2B*	0.93

Table 13 – Possible pathways targeted by miR-145.

Possible Pathways	Gene Name	-ln(value)
Adherens junction	*ACTB, IGF1R, PTPRF, YES1, NLK, TGFBR2, ACTG1, SMAD4, SMAD3*	21.3
TGF-beta signaling pathway	*ZFYVE9, SMAD5, SKP1A, INHBB, TGFBR2, SMAD4, SMAD3*	8.67
Wnt signaling pathway	*FZD7, CTNNBIP1, SKP1A, PPP3CA, NLK, CCND2, SENP2, SMAD4, SMAD3*	8.04
Tight junction	*ACTB, MAGI2, IGSF5, VAPA, YES1, MPP5, ACTG1, EPB41L3*	6.7
Focal adhesion	*ACTB, FN1, IGF1R, FLNB, ITGB8, CCND2, ACTG1, PAK7*	3.45
Cell adhesion molecules	*MPZL1, CD40, CDH2, PTPRF, ITGB8, HLA-DRB5*	3.38
p53 signaling pathway	*CDK6, CCND2, BAX, BBC3*	3.02
MAPK signaling pathway	*EVI1, FLNB, MAP4K2, RASA2, PPP3CA, NLK, TGFBR2, DUSP6, RASA1*	2.75
Cell cycle	*CDK6, SKP1A, CCND2, SMAD4, SMAD3*	2.35
Calcium signaling pathway	*ADRB3, PTGFR, ERBB3, PPP3CA, NOS1, ERBB4*	1.87

Table 14 – Possible pathways targeted by miR-205.

Possible Pathways	Gene Name	-ln(value)
Tight junction	*MAGI3, MAGI2, PRKCA, YES1, CLDN11, PTEN,PTENP1, PARD6B, EPB41, MYH1*	6.82
Adherens junction	*PTPRM, YES1, SORBS1, FGFR1, INSR, SMAD4*	6.17
Ubiquitin mediated proteolysis	*UBE2G1, UBE2NL, UBE1, MAP3K1, UBE2N, SIAH1, ANAPC5*	3.49
Notch signaling pathway	*APH1A, NOTCH2, PCAF*	2.35
ABC transporters – General	*ABCC9, ABCD1, ABCB7*	2.17
Phosphatidylinositol signaling	*PRKCA, INPPL1, PTEN,PTENP1, PLCB1*	2.03
mTOR signaling pathway	*VEGFA, RPS6KA3, EIF4E*	1.79
Inositol phosphate metabolism	*INPPL1, PTEN,PTENP1, PLCB1*	1.79
Wnt signaling pathway	*PRKCA, NKD1, SIAH1, PLCB1, NFAT5, SMAD4*	1.61
Cell cycle	*CDKN2B, CHEK2, CDC25B, ANAPC5, SMAD4*	1.57
TGF-beta signaling pathway	*INHBA, CDKN2B, SMAD1, SMAD4*	1.23
JAK-STAT signaling pathway	*TPO*	1.23
Cell adhesion molecules	*PTPRM, NRCAM, CLDN11, NRXN1, PTPRC*	1.22
Gap junction	*PRKCA, NPR2, PLCB1, GNAQ*	1.09

DISCUSSION

The mechanisms involved in PCa initiation and progression are not fully understood at present, demanding the search for yet unidentified molecular alterations which underlie tumor heterogeneity. Moreover, the growing concerns about PCa overtreatment provide an opportunity for the discovery of novel biomarkers which not only are accurately able to detect PCa but are also capable of identifying the aggressive forms of the disease. For more than a decade, our research team has been involved in the identification of epigenetic-based markers for PCa. The initial research efforts were devoted to the characterization of the methylome but the fast evolution of Epigenetics has now made clear that other epigenetic alterations, such as histone onco-modifications and miRNAs deregulation, might play a critical role in prostate carcinogenesis. Because epigenetic mechanisms are closely inter-related, we aimed to identify miRNA genes deregulated by promoter methylation in PCa, in an attempt to further illuminate the biological mechanisms underlying PCa and, eventually, provide new PCa biomarkers or therapeutic targets. Thus, comprehensive *in silico* analyses were performed to identify miRNAs downregulated in PCa and simultaneously re-expressed in response to 5-AZA-DC exposure in PCa cell lines. Subsequently, the candidate miRNA genes were validated in a large number of clinical samples through a qMSP assay and their putative role as cancer biomarkers was assessed. In brief, we showed, for the first time, that miR-130a downregulation in PCa is due to an epigenetic mechanism, namely aberrant DNA promoter methylation, and that this is an early event in prostate carcinogenesis. We have also found associations between miR-205 expression and clinical-pathological variables. Concerning miR-145, we found that, contrarily to previous reports, it is not significantly downregulated in PCa, although it may still be regulated by epigenetic mechanisms. Finally, we demonstrated that selected miRNAs promoter methylation may provide useful biomarkers for accurate identification of PCa in tissue and urine samples.

Expression profiling analysis identified several miRNAs differentially expressed in PCa samples compared to NPT and most were downregulated (47 vs. 5), a finding which is in line with previous reports (Porkka et al., 2007). These results are also in accordance with the more comprehensive observation that miRNAs are globally downregulated in most human cancers, probably reflecting the lower differentiation of malignant cells (Lu et al., 2005). The validity of this first approach is provided by the fact that several miRNAs which we found to be downregulated in PCa, including miR-130a, miR-145, miR-221, miR-100, miR-99b miR-224 and miR-205, have been already reported by other

researchers (Porkka et al., 2007, Szczyrba et al., 2010, Boll et al., 2012, Sun et al., 2011). Genomic deletions have been generally considered the cause of miRNA dowregulation (Calin et al., 2004), and, indeed, we found that some miRNAs (miR-548b-3p and miR-30a* at 6q16-22, and miR-328 at 16q) located at frequently deleted regions in PCa (Lu and Hano, 2008, Carter et al., 1990) were downregulated in our series. However, epigenetic alterations have recently emerged as an alternative mechanism (Bandres et al., 2009), and these were the main focus of this study.

We hypothesized that among epigenetic alterations involved in miRNA deregulation, aberrant promoter methylation would be an obvious mechanism, in similarity with protein coding genes. Among the relatively large number of candidate miRNA genes fulfilling strict selection criteria (re-expressed in at least two cell lines, dowregulated in PCa, expressed in normal prostate tissues, and having at the least one CpG island 5000 bp upstream its mature sequence), only three miRNA genes - miR-130a, miR-145 and miR-205 – emerged as candidates for deregulation by promoter methylation. Thus, it is likely that the re-expression of a proportion of miRNAs in cell lines may be due to cell death induced by 5-AZA-DC, which has cytotoxic properties, as previously suggested (Christman, 2002). Indeed, miR-520g and miR-497, which we found to be re-expressed in this study, have previously been found to be upregulated in response to treatment with 5-AZA-DC in bladder cancer cell lines (Yoshitomi et al., 2011). Recent studies have also found a few miRNAs regulated by methylation in PCa in cell lines treated with 3 μM of 5-AZA-DC and later compared to clinical samples (Formosa et al., 2012). The results of this study are not fully in line with ours but it must be emphasized that different technologies were used for miRNA profiling, as well as higher drug concentrations, a 10 kb upstream limit for CpG searching and lower cut-off values considered for downregulation (Formosa et al., 2012). These data not only demonstrate that miRNA deregulation in PCa is probably a relatively uncommon phenomenon but also that different methodologies are likely to yield quite dissimilar results.

Whereas miR-145 and miR-205 have already been reported as deregulated by promoter methylation in PCa, either in cell lines or clinical samples (Hulf et al., 2012, Bhatnagar et al., 2010, Hulf et al., 2011), as well as in other tumor models (Wiklund et al., 2011, Tellez et al., 2011), the main novelty of this study is the identification of miR-130a as an epigenetically-regulated miRNA. This finding has been indirectly corroborated by the recent demonstration that miR-130a was downregulated in PCa by other researchers, although the underlying mechanism was not identified (Boll et al., 2012). Concerning miR-145, although it was previously found to be downregulated in PCa (Suh et al., 2011), we verified that the reduction of expression levels in primary tissues was less dramatic than expected. Although methylation is implicated in miR-145 dowregulation, it is likely that

other mechanisms are also involved, including monoallelic methylation and histone deacetylation. Indeed, PCa cell lines treated with demethylation agent plus a potent histone deacetylase inhibitor, display higher re-expression levels of miR-145 than cell lines treated with demethylation agent only (Zaman et al., 2010, Ke et al., 2009). The same mechanism might explain the lack of correlation between methylation and expression levels found not only for miR-145, but also for miR-130a and miR-205. Remarkably, miR-205 seems also to be regulated by histone acetylation at lysine 9 of histone 3 in PCa cell lines (Hulf et al., 2011). This might explain the inverse correlation between miR-205 expression levels with Gleason score and clinical stage and the lack of it with promoter methylation levels, in accordance with previous studies which found lower miR-205 expression in advanced PCa (Schaefer et al., 2010, Boll et al., 2012).

This study is the first to demonstrate that promoter methylation of miR-130a, miR-205 and miR-145 genes precedes the development of invasive PCa as they occur in HGPIN lesions, which are generally considered PCa precursors. Interestingly, methylation levels in HGPIN are intermediate between those of NPT and PCa, suggesting that this epigenetic alteration initially affects only a small subset of morphologically normal epithelial cells, which might benefit from a growth / survival advantage. This may foster the neoplastic transformation into HGPIN cells and further progression to an invasive phenotype, as previously suggested for other genes (Henrique et al., 2006), with which these alterations might act in concert. This trend is more obvious for miR-130a and miR-205, although the higher miR-145 gene promoter methylation levels found in HGPIN vs. PCa might be due to epigenetic heterogeneity as suggested for other epigenetic regulated genes in prostate carcinogenesis (Henrique et al., 2006).

A major aim of this study was to assess the biomarker capabilities of epigenetically deregulated miRNAs for PCa detection. We found that a panel comprising miR-130a and miR-205 promoter methylation is able to accurately discriminate PCa from NPT in tissue samples and may, thus, constitute an interesting ancillary tool for histopathological assessment of diagnostically challenging prostate lesions. It may also be used in patients with clinical suspicion of cancer, abnormal digital rectal examination and persistent elevation of PSA, who are to be submitted to a second biopsy. The inclusion of miR-145 in the gene panel would only increment sensitivity in 2%, and, thus, we considered that no relevant gain was obtained from extending the molecular analysis, especially in minute tissue samples. Remarkably, the analysis of miR-130a and miR-205 promoter methylation in tissue samples compares well with quantitative promoter methylation analysis of protein coding genes for PCa discrimination such as GSTP1 and APC (Henrique et al., 2006). However, the performance of miR-130a promoter methylation for PCa detection in urine has been disappointing owing to the low sensitivity and NPV (25.64% and 34.09%,

respectively), notwithstanding perfect specificity and PPV, providing an overall accuracy of 46.30%. The performance of this biomarker is undoubtedly inferior to that reported for other epigenetic biomarkers developed for PCa detection in urine samples, such as *GSTP1* (Woodson et al., 2008, Phe et al., 2010). Nevertheless, the feasibility of detecting miRNA promoter methylation in prostate clinical samples (urine, in the present study) has been clearly demonstrated for the first time and technical refinement must be sought to improve the performance of miR-130a promoter methylation.

The biological impact of the epigenetic deregulation of miR-130a, miR-145 and miR-205 was investigated using a bioinformatic tool. Interestingly, all considered miRNAs are linked with several cellular pathways such as signal transduction, transcription factors, apoptosis and cell adhesion, which are involved in tumor initiation and progression. Common pathways targeted by those miRNAs include TGF-beta, MAPK, Wnt, cell cycle regulation and adhesion. Remarkably, some of these pathways have been found to be upregulated in PCa and potentially enhancing tumorigenicity or invasion, such as TGF-beta (Steiner et al., 1994), Wnt signaling (Chen et al., 2004) and some cell cycle regulators such as SMADs and CDKs (Aaltomaa et al., 1999, Lu et al., 2007). Thus, it is tempting to speculate whether these alterations are due to downregulation of miR-130a, miR-145 and miR-205 through promoter methylation, and eventually other epigenetic mechanisms. Recent reports emphasize the role of these miRNAs in cancer. Concerning miR-130a deregulation, it has been associated with authophagy and DICER complex (Kovaleva et al., 2012), angiogenesis (Chen and Gorski, 2008), as well as to androgen receptors co-regulators and MAPK signaling (Boll et al., 2012). Moreover, miR-205 has been associated with invasion and cell motility as a part of epithelial-to-mesenchymal transition (Gandellini et al., 2009) and apoptosis (Bhatnagar et al., 2010), whereas miR-145 has been linked with cell growth, migration (Fuse et al., 2011) and angiogenesis (Xu et al., 2012). These reports are in line with our findings. For instance, androgen signaling activity is considered a key molecular change in the transition from normal prostate epithelium to HGPIN as well as in PCa progression (Tomlins et al., 2007). Clearly, functional studies are now required to validate these interesting findings.

CONCLUSIONS AND FUTURE PERSPECTIVES

- Although epigenetic aberration affecting protein coding genes have been widely characterized in PCa, available data concerning epigenetic deregulation of miRNAs is rather scarce. We found that miR-130a, miR-145 and miR-205 gene promoters are frequently methylated in PCa and HGPIN lesions, sustaining an important role for these alterations in PCa initiation and progression.

- We found that a panel comprising miR-130a and miR-205 promoter methylation is able to accurately discriminate PCa from non-cancerous prostate tissues and may constitute a new ancillary tool for PCa diagnosis. However, the performance of these markers in urine samples, intended for PCa detection, is suboptimal and requires further improvement. Future studies should seek for meaningful correlations between miRNAs promoter methylation and patients's survival, providing a basis for the use of this alteration as prognostic biomarkers, which may identify the most aggressive forms of disease.

- Concerning target prediction, miR-130a, miR-205 and miR-145 seem to target relavant oncogenes implicated in prostate carcinogenesis. However, additional databases (e.g., PicTar, TargetScan or miRanda) need to be consulted to corroborate these preliminary findings. Moreover, microarray analysis should be performed using the selected target genes using PCa tissue against NPT. Finally, gene reporter assays need to be performed to validate putative miRNA-mRNA interactions, including luciferase reporter assays in PCa cellular cultures transfected with the selected miRNA, followed by proteome analysis.

All these findings constitute the basis of further studies aimed to elucidate the role of epigenetically deregulated miRNAs in prostate carcinogenesis, improve biomarker performance and identify new therapeutic targets.

BIBLIOGRAPHY

AALTOMAA, S., ESKELINEN, M. & LIPPONEN, P. 1999. Expression of cyclin A and D proteins in prostate cancer and their relation to clinopathological variables and patient survival. *Prostate,* 38**,** 175-82.

ABATE-SHEN, C. & SHEN, M. M. 2000. Molecular genetics of prostate cancer. *Genes Dev,* 14**,** 2410-34.

AGIRRE, X., VILAS-ZORNOZA, A., JIMENEZ-VELASCO, A., MARTIN-SUBERO, J. I., CORDEU, L., GARATE, L., SAN JOSE-ENERIZ, E., ABIZANDA, G., RODRIGUEZ-OTERO, P., FORTES, P., RIFON, J., BANDRES, E., CALASANZ, M. J., MARTIN, V., HEINIGER, A., TORRES, A., SIEBERT, R., ROMAN-GOMEZ, J. & PROSPER, F. 2009. Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia. *Cancer Res,* 69**,** 4443-53.

ALBADINE, R., LATOUR, M., TOUBAJI, A., HAFFNER, M., ISAACS, W. B., E, A. P., MEEKER, A. K., DEMARZO, A. M., EPSTEIN, J. I. & NETTO, G. J. 2009. TMPRSS2-ERG gene fusion status in minute (minimal) prostatic adenocarcinoma. *Mod Pathol,* 22**,** 1415-22.

ALBERTINI, M. C., OLIVIERI, F., LAZZARINI, R., PILOLLI, F., GALLI, F., SPADA, G., ACCORSI, A., RIPPO, M. R. & PROCOPIO, A. D. 2011. Predicting microRNA modulation in human prostate cancer using a simple String IDentifier (SID1.0). *J Biomed Inform,* 44**,** 615-20.

BANDRES, E., AGIRRE, X., BITARTE, N., RAMIREZ, N., ZARATE, R., ROMAN-GOMEZ, J., PROSPER, F. & GARCIA-FONCILLAS, J. 2009. Epigenetic regulation of microRNA expression in colorectal cancer. *Int J Cancer,* 125**,** 2737-43.

BARTEL, D. P. 2009. MicroRNAs: target recognition and regulatory functions. *Cell,* 136**,** 215-33.

BERGERHEIM, U. S., KUNIMI, K., COLLINS, V. P. & EKMAN, P. 1991. Deletion mapping of chromosomes 8, 10, and 16 in human prostatic carcinoma. *Genes Chromosomes Cancer,* 3**,** 215-20.

BERNSTEIN, E. & ALLIS, C. D. 2005. RNA meets chromatin. *Genes Dev,* 19**,** 1635-55.

BETEL, D., WILSON, M., GABOW, A., MARKS, D. S. & SANDER, C. 2008. The microRNA.org resource: targets and expression. *Nucleic Acids Res,* 36**,** D149-53.

BETHEL, C. R., FAITH, D., LI, X., GUAN, B., HICKS, J. L., LAN, F., JENKINS, R. B., BIEBERICH, C. J. & DE MARZO, A. M. 2006. Decreased NKX3.1 protein expression in focal prostatic atrophy, prostatic intraepithelial neoplasia, and adenocarcinoma: association with gleason score and chromosome 8p deletion. *Cancer Res,* 66**,** 10683-90.

BHATIA-GAUR, R., DONJACOUR, A. A., SCIAVOLINO, P. J., KIM, M., DESAI, N., YOUNG, P., NORTON, C. R., GRIDLEY, T., CARDIFF, R. D., CUNHA, G. R., ABATE-SHEN, C. & SHEN, M. M. 1999. Roles for Nkx3.1 in prostate development and cancer. *Genes Dev,* 13**,** 966-77.

BHATNAGAR, N., LI, X., PADI, S. K., ZHANG, Q., TANG, M. S. & GUO, B. 2010. Downregulation of miR-205 and miR-31 confers resistance to chemotherapy-induced apoptosis in prostate cancer cells. *Cell Death Dis,* 1**,** e105.

BHAYANI, M. K., CALIN, G. A. & LAI, S. Y. 2012. Functional relevance of miRNA sequences in human disease. *Mutat Res,* 731**,** 14-9.

BOLL, K., REICHE, K., KASACK, K., MORBT, N., KRETZSCHMAR, A. K., TOMM, J. M., VERHAEGH, G., SCHALKEN, J., VON BERGEN, M., HORN, F. & HACKERMULLER, J. 2012. MiR-130a, miR-203 and miR-205 jointly repress key oncogenic pathways and are downregulated in prostate carcinoma. *Oncogene*.

BUNTING, P. S. 2002. Screening for prostate cancer with prostate-specific antigen: beware the biases. *Clin Chim Acta,* 315**,** 71-97.

CALIN, G. A., DUMITRU, C. D., SHIMIZU, M., BICHI, R., ZUPO, S., NOCH, E., ALDLER, H., RATTAN, S., KEATING, M., RAI, K., RASSENTI, L., KIPPS, T., NEGRINI, M., BULLRICH, F. & CROCE, C. M.

2002. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A,* 99**,** 15524-9.

CALIN, G. A., SEVIGNANI, C., DUMITRU, C. D., HYSLOP, T., NOCH, E., YENDAMURI, S., SHIMIZU, M., RATTAN, S., BULLRICH, F., NEGRINI, M. & CROCE, C. M. 2004. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A,* 101**,** 2999-3004.

CAPASSO, L. L. 2005. Antiquity of cancer. *Int J Cancer,* 113**,** 2-13.

CARTER, B. S., EWING, C. M., WARD, W. S., TREIGER, B. F., AALDERS, T. W., SCHALKEN, J. A., EPSTEIN, J. I. & ISAACS, W. B. 1990. Allelic loss of chromosomes 16q and 10q in human prostate cancer. *Proc Natl Acad Sci U S A,* 87**,** 8751-5.

CARTER, H. B., KETTERMANN, A., FERRUCCI, L., LANDIS, P. & METTER, E. J. 2007. Prostate-specific antigen velocity risk count assessment: a new concept for detection of life-threatening prostate cancer during window of curability. *Urology,* 70**,** 685-90.

CARTHEW, R. W. & SONTHEIMER, E. J. 2009. Origins and Mechanisms of miRNAs and siRNAs. *Cell,* 136**,** 642-55.

CATTO, J. W., ALCARAZ, A., BJARTELL, A. S., DE VERE WHITE, R., EVANS, C. P., FUSSEL, S., HAMDY, F. C., KALLIONIEMI, O., MENGUAL, L., SCHLOMM, T. & VISAKORPI, T. 2011. MicroRNA in prostate, bladder, and kidney cancer: a systematic review. *Eur Urol,* 59**,** 671-81.

CENTER, M. M., JEMAL, A., LORTET-TIEULENT, J., WARD, E., FERLAY, J., BRAWLEY, O. & BRAY, F. 2012. International variation in prostate cancer incidence and mortality rates. *Eur Urol,* 61**,** 1079-92.

CHEN, G., SHUKEIR, N., POTTI, A., SIRCAR, K., APRIKIAN, A., GOLTZMAN, D. & RABBANI, S. A. 2004. Up-regulation of Wnt-1 and beta-catenin production in patients with advanced metastatic prostate carcinoma: potential pathogenetic and prognostic implications. *Cancer,* 101**,** 1345-56.

CHEN, Y. & GORSKI, D. H. 2008. Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. *Blood,* 111**,** 1217-26.

CHEN, Z., WANG, L., WANG, Q. & LI, W. 2010. Histone modifications and chromatin organization in prostate cancer. *Epigenomics,* 2**,** 551-60.

CHIN, L. J., RATNER, E., LENG, S., ZHAI, R., NALLUR, S., BABAR, I., MULLER, R. U., STRAKA, E., SU, L., BURKI, E. A., CROWELL, R. E., PATEL, R., KULKARNI, T., HOMER, R., ZELTERMAN, D., KIDD, K. K., ZHU, Y., CHRISTIANI, D. C., BELINSKY, S. A., SLACK, F. J. & WEIDHAAS, J. B. 2008. A SNP in a let-7 microRNA complementary site in the KRAS 3' untranslated region increases non-small cell lung cancer risk. *Cancer Res,* 68**,** 8535-40.

CHMELAR, R., BUCHANAN, G., NEED, E. F., TILLEY, W. & GREENBERG, N. M. 2007. Androgen receptor coregulators and their involvement in the development and progression of prostate cancer. *International Journal of Cancer,* 120**,** 719-733.

CHOUDHRY, H. & CATTO, J. W. 2011. Epigenetic regulation of microRNA expression in cancer. *Methods Mol Biol,* 676**,** 165-84.

CHRISTMAN, J. K. 2002. 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene,* 21**,** 5483-95.

CIMMINO, A., CALIN, G. A., FABBRI, M., IORIO, M. V., FERRACIN, M., SHIMIZU, M., WOJCIK, S. E., AQEILAN, R. I., ZUPO, S., DONO, M., RASSENTI, L., ALDER, H., VOLINIA, S., LIU, C. G., KIPPS, T. J., NEGRINI, M. & CROCE, C. M. 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A,* 102**,** 13944-9.

CLARK, S. J., HARRISON, J., PAUL, C. L. & FROMMER, M. 1994. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res,* 22**,** 2990-7.

COSTA, V. L., HENRIQUE, R., DANIELSEN, S. A., DUARTE-PEREIRA, S., EKNAES, M., SKOTHEIM, R. I., RODRIGUES, A., MAGALHAES, J. S., OLIVEIRA, J., LOTHE, R. A., TEIXEIRA, M. R., JERONIMO, C. & LIND, G. E. 2010. Three epigenetic biomarkers, GDF15, TMEFF2, and VIM, accurately

predict bladder cancer from DNA-based analyses of urine samples. *Clin Cancer Res,* 16**,** 5842-51.

CRAFT, N., CHHOR, C., TRAN, C., BELLDEGRUN, A., DEKERNION, J., WITTE, O. N., SAID, J., REITER, R. E. & SAWYERS, C. L. 1999. Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process. *Cancer Res,* 59**,** 5030-6.

CRAWFORD, A., FASSETT, R. G., GERAGHTY, D. P., KUNDE, D. A., BALL, M. J., ROBERTSON, I. K. & COOMBES, J. S. 2012. Relationships between single nucleotide polymorphisms of antioxidant enzymes and disease. *Gene,* 501**,** 89-103.

CREIGHTON, C. J., FOUNTAIN, M. D., YU, Z., NAGARAJA, A. K., ZHU, H., KHAN, M., OLOKPA, E., ZARIFF, A., GUNARATNE, P. H., MATZUK, M. M. & ANDERSON, M. L. 2010. Molecular profiling uncovers a p53-associated role for microRNA-31 in inhibiting the proliferation of serous ovarian carcinomas and other cancers. *Cancer Res,* 70**,** 1906-15.

DE MARZO, A. M., MARCHI, V. L., EPSTEIN, J. I. & NELSON, W. G. 1999. Proliferative inflammatory atrophy of the prostate: implications for prostatic carcinogenesis. *Am J Pathol,* 155**,** 1985-92.

DONG, J. T. 2001. Chromosomal deletions and tumor suppressor genes in prostate cancer. *Cancer Metastasis Rev,* 20**,** 173-93.

DUDZIEC, E., MIAH, S., CHOUDHRY, H. M., OWEN, H. C., BLIZARD, S., GLOVER, M., HAMDY, F. C. & CATTO, J. W. 2011. Hypermethylation of CpG islands and shores around specific microRNAs and mirtrons is associated with the phenotype and presence of bladder cancer. *Clin Cancer Res,* 17**,** 1287-96.

DUNN, M. W. & KAZER, M. W. 2011. Prostate cancer overview. *Semin Oncol Nurs,* 27**,** 241-50.

ESTELLER, M. 2009. *Epigenetics in biology and medicine,* Boca Raton, CRC Press.

ESTELLER, M. 2011. Cancer Epigenetics for the 21st Century: What's Next? *Genes Cancer,* 2**,** 604-6.

ESTELLER, M., GARCIA-FONCILLAS, J., ANDION, E., GOODMAN, S. N., HIDALGO, O. F., VANACLOCHA, V., BAYLIN, S. B. & HERMAN, J. G. 2000. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med,* 343**,** 1350-4.

EWING, C. M., RAY, A. M., LANGE, E. M., ZUHLKE, K. A., ROBBINS, C. M., TEMBE, W. D., WILEY, K. E., ISAACS, S. D., JOHNG, D., WANG, Y., BIZON, C., YAN, G., GIELZAK, M., PARTIN, A. W., SHANMUGAM, V., IZATT, T., SINARI, S., CRAIG, D. W., ZHENG, S. L., WALSH, P. C., MONTIE, J. E., XU, J., CARPTEN, J. D., ISAACS, W. B. & COONEY, K. A. 2012. Germline mutations in HOXB13 and prostate-cancer risk. *N Engl J Med,* 366**,** 141-9.

FERLAY, J., SHIN, H. R., BRAY, F., FORMAN, D., MATHERS, C. & PARKIN, D. M. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer,* 127**,** 2893-917.

FERNANDEZ, A. F., ASSENOV, Y., MARTIN-SUBERO, J. I., BALINT, B., SIEBERT, R., TANIGUCHI, H., YAMAMOTO, H., HIDALGO, M., TAN, A. C., GALM, O., FERRER, I., SANCHEZ-CESPEDES, M., VILLANUEVA, A., CARMONA, J., SANCHEZ-MUT, J. V., BERDASCO, M., MORENO, V., CAPELLA, G., MONK, D., BALLESTAR, E., ROPERO, S., MARTINEZ, R., SANCHEZ-CARBAYO, M., PROSPER, F., AGIRRE, X., FRAGA, M. F., GRANA, O., PEREZ-JURADO, L., MORA, J., PUIG, S., PRAT, J., BADIMON, L., PUCA, A. A., MELTZER, S. J., LENGAUER, T., BRIDGEWATER, J., BOCK, C. & ESTELLER, M. 2012. A DNA methylation fingerprint of 1628 human samples. *Genome Res,* 22**,** 407-19.

FLESHNER, N. E., LUCIA, M. S., EGERDIE, B., AARON, L., EURE, G., NANDY, I., BLACK, L. & RITTMASTER, R. S. 2012. Dutasteride in localised prostate cancer management: the REDEEM randomised, double-blind, placebo-controlled trial. *Lancet,* 379**,** 1103-11.

FORMOSA, A., LENA, A. M., MARKERT, E. K., CORTELLI, S., MIANO, R., MAURIELLO, A., CROCE, N., VANDESOMPELE, J., MESTDAGH, P., FINAZZI-AGRO, E., LEVINE, A. J., MELINO, G., BERNARDINI, S. & CANDI, E. 2012. DNA methylation silences miR-132 in prostate cancer. *Oncogene*.

FOWKE, J. H., MOTLEY, S. S., CONCEPCION, R. S., PENSON, D. F. & BAROCAS, D. A. 2012. Obesity, body composition, and prostate cancer. *BMC Cancer,* 12**,** 23.

FUSE, M., NOHATA, N., KOJIMA, S., SAKAMOTO, S., CHIYOMARU, T., KAWAKAMI, K., ENOKIDA, H., NAKAGAWA, M., NAYA, Y., ICHIKAWA, T. & SEKI, N. 2011. Restoration of miR-145 expression suppresses cell proliferation, migration and invasion in prostate cancer by targeting FSCN1. *Int J Oncol,* 38**,** 1093-101.

GANDELLINI, P., FOLINI, M., LONGONI, N., PENNATI, M., BINDA, M., COLECCHIA, M., SALVIONI, R., SUPINO, R., MORETTI, R., LIMONTA, P., VALDAGNI, R., DAIDONE, M. G. & ZAFFARONI, N. 2009. miR-205 Exerts tumor-suppressive functions in human prostate through down-regulation of protein kinase Cepsilon. *Cancer Res,* 69**,** 2287-95.

GLEASON, D. F. & MELLINGER, G. T. 1974. Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. *J Urol,* 111**,** 58-64.

GOLDBERG, A. D., ALLIS, C. D. & BERNSTEIN, E. 2007. Epigenetics: a landscape takes shape. *Cell,* 128**,** 635-8.

GRASSO, C. S., WU, Y. M., ROBINSON, D. R., CAO, X., DHANASEKARAN, S. M., KHAN, A. P., QUIST, M. J., JING, X., LONIGRO, R. J., BRENNER, J. C., ASANGANI, I. A., ATEEQ, B., CHUN, S. Y., SIDDIQUI, J., SAM, L., ANSTETT, M., MEHRA, R., PRENSNER, J. R., PALANISAMY, N., RYSLIK, G. A., VANDIN, F., RAPHAEL, B. J., KUNJU, L. P., RHODES, D. R., PIENTA, K. J., CHINNAIYAN, A. M. & TOMLINS, S. A. 2012. The mutational landscape of lethal castration-resistant prostate cancer. *Nature,* 487**,** 239-43.

GRIFFITHS-JONES, S. 2004. The microRNA Registry. *Nucleic Acids Res,* 32**,** D109-11.

GUPTA-ELERA, G., GARRETT, A. R., ROBISON, R. A. & O'NEILL, K. L. 2012. The role of oxidative stress in prostate cancer. *Eur J Cancer Prev,* 21**,** 155-62.

GUREL, B., ALI, T. Z., MONTGOMERY, E. A., BEGUM, S., HICKS, J., GOGGINS, M., EBERHART, C. G., CLARK, D. P., BIEBERICH, C. J., EPSTEIN, J. I. & DE MARZO, A. M. 2010. NKX3.1 as a marker of prostatic origin in metastatic tumors. *Am J Surg Pathol,* 34**,** 1097-105.

GUSTAFSSON, O., MANSOUR, E., NORMING, U., CARLSSON, A., TORNBLOM, M. & NYMAN, C. R. 1998. Prostate-specific antigen (PSA), PSA density and age-adjusted PSA reference values in screening for prostate cancer--a study of a randomly selected population of 2,400 men. *Scand J Urol Nephrol,* 32**,** 373-7.

HASSLER, M. R. & EGGER, G. 2012. Epigenomics of cancer - emerging new concepts. *Biochimie*.

HAVERKAMP, J., CHARBONNEAU, B. & RATLIFF, T. L. 2008. Prostate inflammation and its potential impact on prostate cancer: a current review. *J Cell Biochem,* 103**,** 1344-53.

HEARD, E., CHAUMEIL, J., MASUI, O. & OKAMOTO, I. 2004. Mammalian X-chromosome inactivation: an epigenetics paradigm. *Cold Spring Harb Symp Quant Biol,* 69**,** 89-102.

HEINLEIN, C. A. & CHANG, C. 2004. Androgen receptor in prostate cancer. *Endocr Rev,* 25**,** 276-308.

HENDRIKSEN, P. J., DITS, N. F., KOKAME, K., VELDHOVEN, A., VAN WEERDEN, W. M., BANGMA, C. H., TRAPMAN, J. & JENSTER, G. 2006. Evolution of the androgen receptor pathway during progression of prostate cancer. *Cancer Res,* 66**,** 5012-20.

HENRIQUE, R. & JERONIMO, C. 2004. Molecular detection of prostate cancer: a role for GSTP1 hypermethylation. *Eur Urol,* 46**,** 660-9; discussion 669.

HENRIQUE, R., JERONIMO, C., TEIXEIRA, M. R., HOQUE, M. O., CARVALHO, A. L., PAIS, I., RIBEIRO, F. R., OLIVEIRA, J., LOPES, C. & SIDRANSKY, D. 2006. Epigenetic heterogeneity of high-grade prostatic intraepithelial neoplasia: clues for clonal progression in prostate carcinogenesis. *Mol Cancer Res,* 4**,** 1-8.

HERMAN, J. G., GRAFF, J. R., MYOHANEN, S., NELKIN, B. D. & BAYLIN, S. B. 1996. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A,* 93**,** 9821-6.

HERMANEK, P., WITTEKIND, C. & SEIB, H. J. 1997. [Current TNM aspects. 5. TNM classification and stage grouping]. *Zentralbl Chir,* 122**,** 934-7.

HERNANDEZ, J. & THOMPSON, I. M. 2004. Prostate-specific antigen: a review of the validation of the most commonly used cancer biomarker. *Cancer,* 101**,** 894-904.

HUGOSSON, J., CARLSSON, S., AUS, G., BERGDAHL, S., KHATAMI, A., LODDING, P., PIHL, C. G., STRANNE, J., HOLMBERG, E. & LILJA, H. 2010. Mortality results from the Goteborg randomised population-based prostate-cancer screening trial. *Lancet Oncol,* 11**,** 725-32.

HULF, T., SIBBRITT, T., WIKLUND, E. D., BERT, S., STRBENAC, D., STATHAM, A. L., ROBINSON, M. D. & CLARK, S. J. 2011. Discovery pipeline for epigenetically deregulated miRNAs in cancer: integration of primary miRNA transcription. *BMC Genomics,* 12**,** 54.

HULF, T., SIBBRITT, T., WIKLUND, E. D., PATTERSON, K., SONG, J. Z., STIRZAKER, C., QU, W., NAIR, S., HORVATH, L. G., ARMSTRONG, N. J., KENCH, J. G., SUTHERLAND, R. L. & CLARK, S. J. 2012. Epigenetic-induced repression of microRNA-205 is associated with MED1 activation and a poorer prognosis in localized prostate cancer. *Oncogene*.

IORIO, M. V. & CROCE, C. M. 2012. microRNA involvement in human cancer. *Carcinogenesis*.

JEMAL, A., BRAY, F., CENTER, M. M., FERLAY, J., WARD, E. & FORMAN, D. 2011. Global cancer statistics. *CA Cancer J Clin,* 61**,** 69-90.

JENKINS, R. B., QIAN, J., LIEBER, M. M. & BOSTWICK, D. G. 1997. Detection of c-myc oncogene amplification and chromosomal anomalies in metastatic prostatic carcinoma by fluorescence in situ hybridization. *Cancer Res,* 57**,** 524-31.

JERONIMO, C. & HENRIQUE, R. 2011. Epigenetic biomarkers in urological tumors: A systematic review. *Cancer Lett*.

JORDE, R., SVARTBERG, J., JOAKIMSEN, R. M. & COUCHERON, D. H. 2012. Plasma profile of microRNA after supplementation with high doses of vitamin D3 for 12 months. *BMC Res Notes,* 5**,** 245.

KATADA, S., IMHOF, A. & SASSONE-CORSI, P. 2012. Connecting threads: epigenetics and metabolism. *Cell,* 148**,** 24-8.

KE, X. S., QU, Y., ROSTAD, K., LI, W. C., LIN, B., HALVORSEN, O. J., HAUKAAS, S. A., JONASSEN, I., PETERSEN, K., GOLDFINGER, N., ROTTER, V., AKSLEN, L. A., OYAN, A. M. & KALLAND, K. H. 2009. Genome-wide profiling of histone h3 lysine 4 and lysine 27 trimethylation reveals an epigenetic signature in prostate carcinogenesis. *PLoS One,* 4**,** e4687.

KHAN, H. A. 2004. ArraySolver: an algorithm for colour-coded graphical display and Wilcoxon signed-rank statistics for comparing microarray gene expression data. *Comp Funct Genomics,* 5**,** 39-47.

KIM, V. N. 2005. MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol,* 6**,** 376-85.

KOH, C. M., IWATA, T., ZHENG, Q., BETHEL, C., YEGNASUBRAMANIAN, S. & DE MARZO, A. M. 2011. Myc enforces overexpression of EZH2 in early prostatic neoplasia via transcriptional and post-transcriptional mechanisms. *Oncotarget,* 2**,** 669-83.

KONG, Y. W., FERLAND-MCCOLLOUGH, D., JACKSON, T. J. & BUSHELL, M. 2012. microRNAs in cancer management. *Lancet Oncol,* 13**,** e249-58.

KOO, S., MARTIN, G. S., SCHULZ, K. J., RONCK, M. & TOUSSAINT, L. G. 2012. Serial selection for invasiveness increases expression of miR-143/miR-145 in glioblastoma cell lines. *BMC Cancer,* 12**,** 143.

KOVALEVA, V., MORA, R., PARK, Y. J., PLASS, C., CHIRAMEL, A. I., BARTENSCHLAGER, R., DOHNER, H., STILGENBAUER, S., PSCHERER, A., LICHTER, P. & SEIFFERT, M. 2012. miRNA-130a targets ATG2B and DICER1 to inhibit autophagy and trigger killing of chronic lymphocytic leukemia cells. *Cancer Res,* 72**,** 1763-72.

KOZAKI, K. & INAZAWA, J. 2012. Tumor-suppressive microRNA silenced by tumor-specific DNA hypermethylation in cancer cells. *Cancer Sci,* 103**,** 837-45.

KROHN, A., DIEDLER, T., BURKHARDT, L., MAYER, P. S., DE SILVA, C., MEYER-KORNBLUM, M., KOTSCHAU, D., TENNSTEDT, P., HUANG, J., GERHAUSER, C., MADER, M., KURTZ, S., SIRMA, H., SAAD, F., STEUBER, T., GRAEFEN, M., PLASS, C., SAUTER, G., SIMON, R., MINNER, S. & SCHLOMM, T. 2012. Genomic Deletion of PTEN Is Associated with Tumor Progression and

Early PSA Recurrence in ERG Fusion-Positive and Fusion-Negative Prostate Cancer. *Am J Pathol*.
KUMAR-SINHA, C. & CHINNAIYAN, A. M. 2003. Molecular markers to identify patients at risk for recurrence after primary treatment for prostate cancer. *Urology,* 62 Suppl 1**,** 19-35.
KUNER, R., BRASE, J. C., SULTMANN, H. & WUTTIG, D. 2012. microRNA biomarkers in body fluids of prostate cancer patients. *Methods*.
KWON, M. J. & SHIN, Y. K. 2011. Epigenetic regulation of cancer-associated genes in ovarian cancer. *Int J Mol Sci,* 12**,** 983-1008.
LANGE, P. H., ERCOLE, C. J., LIGHTNER, D. J., FRALEY, E. E. & VESSELLA, R. 1989. The value of serum prostate specific antigen determinations before and after radical prostatectomy. *J Urol,* 141**,** 873-9.
LAPOINTE, J., LI, C., HIGGINS, J. P., VAN DE RIJN, M., BAIR, E., MONTGOMERY, K., FERRARI, M., EGEVAD, L., RAYFORD, W., BERGERHEIM, U., EKMAN, P., DEMARZO, A. M., TIBSHIRANI, R., BOTSTEIN, D., BROWN, P. O., BROOKS, J. D. & POLLACK, J. R. 2004. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *Proc Natl Acad Sci U S A,* 101**,** 811-6.
LAU, N. C., LIM, L. P., WEINSTEIN, E. G. & BARTEL, D. P. 2001. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science,* 294**,** 858-62.
LEHMANN, U., HASEMEIER, B., CHRISTGEN, M., MULLER, M., ROMERMANN, D., LANGER, F. & KREIPE, H. 2008. Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer. *J Pathol,* 214**,** 17-24.
LI, Y. & TOLLEFSBOL, T. O. 2010. Impact on DNA methylation in cancer prevention and therapy by bioactive dietary components. *Curr Med Chem,* 17**,** 2141-51.
LILJA, H., ULMERT, D. & VICKERS, A. J. 2008. Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. *Nat Rev Cancer,* 8**,** 268-78.
LOIZOU, J. I., MURR, R., FINKBEINER, M. G., SAWAN, C., WANG, Z. Q. & HERCEG, Z. 2006. Epigenetic information in chromatin: the code of entry for DNA repair. *Cell Cycle,* 5**,** 696-701.
LOPEZ-SERRA, L. & ESTELLER, M. 2008. Proteins that bind methylated DNA and human cancer: reading the wrong words. *Br J Cancer,* 98**,** 1881-5.
LU, J., GETZ, G., MISKA, E. A., ALVAREZ-SAAVEDRA, E., LAMB, J., PECK, D., SWEET-CORDERO, A., EBERT, B. L., MAK, R. H., FERRANDO, A. A., DOWNING, J. R., JACKS, T., HORVITZ, H. R. & GOLUB, T. R. 2005. MicroRNA expression profiles classify human cancers. *Nature,* 435**,** 834-8.
LU, S., LEE, J., REVELO, M., WANG, X. & DONG, Z. 2007. Smad3 is overexpressed in advanced human prostate cancer and necessary for progressive growth of prostate cancer cells in nude mice. *Clin Cancer Res,* 13**,** 5692-702.
LU, T. & HANO, H. 2008. Deletion at chromosome arms 6q16-22 and 10q22.3-23.1 associated with initiation of prostate cancer. *Prostate Cancer Prostatic Dis,* 11**,** 357-61.
MACEFIELD, R. C., LANE, J. A., METCALFE, C., DOWN, L., NEAL, D. E., HAMDY, F. C. & DONOVAN, J. L. 2009. Do the risk factors of age, family history of prostate cancer or a higher prostate specific antigen level raise anxiety at prostate biopsy? *Eur J Cancer,* 45**,** 2569-73.
MAJOR, J. M., NORMAN OLIVER, M., DOUBENI, C. A., HOLLENBECK, A. R., GRAUBARD, B. I. & SINHA, R. 2012. Socioeconomic status, healthcare density, and risk of prostate cancer among African American and Caucasian men in a large prospective study. *Cancer Causes Control,* 23**,** 1185-91.
MARAGKAKIS, M., ALEXIOU, P., PAPADOPOULOS, G. L., RECZKO, M., DALAMAGAS, T., GIANNOPOULOS, G., GOUMAS, G., KOUKIS, E., KOURTIS, K., SIMOSSIS, V. A., SETHUPATHY, P., VERGOULIS, T., KOZIRIS, N., SELLIS, T., TSANAKAS, P. & HATZIGEORGIOU, A. G. 2009. Accurate microRNA target prediction correlates with protein repression levels. *BMC Bioinformatics,* 10**,** 295.

MASCARENHAS, J., ROPER, N., CHAURASIA, P. & HOFFMAN, R. 2011. Epigenetic abnormalities in myeloproliferative neoplasms: a target for novel therapeutic strategies. *Clin Epigenetics,* 2**,** 197-212.

MATTICK, J. S. & GAGEN, M. J. 2001. The evolution of controlled multitasked gene networks: the role of introns and other noncoding RNAs in the development of complex organisms. *Mol Biol Evol,* 18**,** 1611-30.

MCLAUGHLIN, P. W., TROYER, S., BERRI, S., NARAYANA, V., MEIROWITZ, A., ROBERSON, P. L. & MONTIE, J. 2005. Functional anatomy of the prostate: implications for treatment planning. *Int J Radiat Oncol Biol Phys,* 63**,** 479-91.

MCNEAL, J. E. 1981. Normal and pathologic anatomy of prostate. *Urology,* 17**,** 11-6.

MEEKER, A. K., HICKS, J. L., PLATZ, E. A., MARCH, G. E., BENNETT, C. J., DELANNOY, M. J. & DE MARZO, A. M. 2002. Telomere shortening is an early somatic DNA alteration in human prostate tumorigenesis. *Cancer Res,* 62**,** 6405-9.

MESTDAGH, P., DERVEAUX, S. & VANDESOMPELE, J. 2012. Whole-genome RT-qPCR microRNA expression profiling. *Methods Mol Biol,* 815**,** 121-30.

MESTDAGH, P., VAN VLIERBERGHE, P., DE WEER, A., MUTH, D., WESTERMANN, F., SPELEMAN, F. & VANDESOMPELE, J. 2009. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol,* 10**,** R64.

miRCURY LNA™ Universal RT microRNA PCR, Instruction manual Version 4.1 #203300 (August 2011)

MOSQUERA, J. M., PERNER, S., GENEGA, E. M., SANDA, M., HOFER, M. D., MERTZ, K. D., PARIS, P. L., SIMKO, J., BISMAR, T. A., AYALA, G., SHAH, R. B., LODA, M. & RUBIN, M. A. 2008. Characterization of TMPRSS2-ERG fusion high-grade prostatic intraepithelial neoplasia and potential clinical implications. *Clin Cancer Res,* 14**,** 3380-5.

MULLINS, J. K. & LOEB, S. 2012. Environmental exposures and prostate cancer. *Urol Oncol,* 30**,** 216-9.

NAKAYAMA, M., GONZALGO, M. L., YEGNASUBRAMANIAN, S., LIN, X., DE MARZO, A. M. & NELSON, W. G. 2004. GSTP1 CpG island hypermethylation as a molecular biomarker for prostate cancer. *J Cell Biochem,* 91**,** 540-52.

OHORI, M., WHEELER, T. M. & SCARDINO, P. T. 1994. The New American Joint Committee on Cancer and International Union Against Cancer TNM classification of prostate cancer. Clinicopathologic correlations. *Cancer,* 74**,** 104-14.

OSTLING, P., LEIVONEN, S. K., AAKULA, A., KOHONEN, P., MAKELA, R., HAGMAN, Z., EDSJO, A., KANGASPESKA, S., EDGREN, H., NICORICI, D., BJARTELL, A., CEDER, Y., PERALA, M. & KALLIONIEMI, O. 2011. Systematic analysis of microRNAs targeting the androgen receptor in prostate cancer cells. *Cancer Res,* 71**,** 1956-67.

PEARSON, H. & STIRLING, D. 2003. DNA extraction from tissue. *Methods Mol Biol,* 226**,** 33-4.

PETTERSSON, A., GRAFF, R. E., BAUER, S. R., PITT, M. J., LIS, R. T., STACK, E. C., MARTIN, N. E., KUNZ, L., PENNEY, K. L., LIGON, A., SUPPAN, C., FLAVIN, R., SESSO, H., RIDER, J. R., SWEENEY, C. S., STAMPFER, M. J., FIORENTINO, M., KANTOFF, P. W., SANDA, M. G., GIOVANNUCCI, E., DING, E. L., LODA, M. & MUCCI, L. A. 2012. The TMPRSS2:ERG Rearrangement, ERG Expression, and Prostate Cancer Outcomes: a Cohort Study and Meta-analysis. *Cancer Epidemiol Biomarkers Prev*.

PHE, V., CUSSENOT, O. & ROUPRET, M. 2010. Methylated genes as potential biomarkers in prostate cancer. *BJU Int,* 105**,** 1364-70.

PORKKA, K. P., PFEIFFER, M. J., WALTERING, K. K., VESSELLA, R. L., TAMMELA, T. L. & VISAKORPI, T. 2007. MicroRNA expression profiling in prostate cancer. *Cancer Res,* 67**,** 6130-5.

RAUHALA, H. E., JALAVA, S. E., ISOTALO, J., BRACKEN, H., LEHMUSVAARA, S., TAMMELA, T. L., OJA, H. & VISAKORPI, T. 2010. miR-193b is an epigenetically regulated putative tumor suppressor in prostate cancer. *Int J Cancer,* 127**,** 1363-72.

SAITO, Y., FRIEDMAN, J. M., CHIHARA, Y., EGGER, G., CHUANG, J. C. & LIANG, G. 2009. Epigenetic therapy upregulates the tumor suppressor microRNA-126 and its host gene EGFL7 in human cancer cells. *Biochem Biophys Res Commun,* 379**,** 726-31.

SAITO, Y. & JONES, P. A. 2006. Epigenetic activation of tumor suppressor microRNAs in human cancer cells. *Cell Cycle,* 5**,** 2220-2.

SAITO, Y., LIANG, G., EGGER, G., FRIEDMAN, J. M., CHUANG, J. C., COETZEE, G. A. & JONES, P. A. 2006. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell,* 9**,** 435-43.

SALMENA, L., CARRACEDO, A. & PANDOLFI, P. P. 2008. Tenets of PTEN tumor suppression. *Cell,* 133**,** 403-14.

SAVVA-BORDALO, J., RAMALHO-CARVALHO, J., PINHEIRO, M., COSTA, V. L., RODRIGUES, A., DIAS, P. C., VEIGA, I., MACHADO, M., TEIXEIRA, M. R., HENRIQUE, R. & JERONIMO, C. 2010. Promoter methylation and large intragenic rearrangements of DPYD are not implicated in severe toxicity to 5-fluorouracil-based chemotherapy in gastrointestinal cancer patients. *BMC Cancer,* 10**,** 470.

SCHAEFER, A., JUNG, M., MOLLENKOPF, H. J., WAGNER, I., STEPHAN, C., JENTZMIK, F., MILLER, K., LEIN, M., KRISTIANSEN, G. & JUNG, K. 2010. Diagnostic and prognostic implications of microRNA profiling in prostate carcinoma. *Int J Cancer,* 126**,** 1166-76.

SCHMITTGEN, T. D. & LIVAK, K. J. 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc,* 3**,** 1101-8.

SCHOLZ, B. & MARSCHALEK, R. 2012. Epigenetics and blood disorders. *Br J Haematol,* 158**,** 307-22.

SCHRODER, F. H., HUGOSSON, J., ROOBOL, M. J., TAMMELA, T. L., CIATTO, S., NELEN, V., KWIATKOWSKI, M., LUJAN, M., LILJA, H., ZAPPA, M., DENIS, L. J., RECKER, F., BERENGUER, A., MAATTANEN, L., BANGMA, C. H., AUS, G., VILLERS, A., REBILLARD, X., VAN DER KWAST, T., BLIJENBERG, B. G., MOSS, S. M., DE KONING, H. J. & AUVINEN, A. 2009. Screening and prostate-cancer mortality in a randomized European study. *N Engl J Med,* 360**,** 1320-8.

SHAFIQUE, K., MCLOONE, P., QURESHI, K., LEUNG, H., HART, C. & MORRISON, D. S. 2012. Cholesterol and the risk of grade-specific prostate cancer incidence: evidence from two large prospective cohort studies with up to 37 years' follow up. *BMC Cancer,* 12**,** 25.

SHAH, R. B. 2009. Current perspectives on the Gleason grading of prostate cancer. *Arch Pathol Lab Med,* 133**,** 1810-6.

SHEN, M. M. & ABATE-SHEN, C. 2010. Molecular genetics of prostate cancer: new prospects for old challenges. *Genes Dev,* 24**,** 1967-2000.

SIEGEL, R., DESANTIS, C., VIRGO, K., STEIN, K., MARIOTTO, A., SMITH, T., COOPER, D., GANSLER, T., LERRO, C., FEDEWA, S., LIN, C., LEACH, C., CANNADY, R. S., CHO, H., SCOPPA, S., HACHEY, M., KIRCH, R., JEMAL, A. & WARD, E. 2012. Cancer treatment and survivorship statistics, 2012. *CA Cancer J Clin*.

STEINER, M. S., ZHOU, Z. Z., TONB, D. C. & BARRACK, E. R. 1994. Expression of transforming growth factor-beta 1 in prostate cancer. *Endocrinology,* 135**,** 2240-7.

SUH, S. O., CHEN, Y., ZAMAN, M. S., HIRATA, H., YAMAMURA, S., SHAHRYARI, V., LIU, J., TABATABAI, Z. L., KAKAR, S., DENG, G., TANAKA, Y. & DAHIYA, R. 2011. MicroRNA-145 is regulated by DNA methylation and p53 gene mutation in prostate cancer. *Carcinogenesis,* 32**,** 772-8.

SUN, D., LEE, Y. S., MALHOTRA, A., KIM, H. K., MATECIC, M., EVANS, C., JENSEN, R. V., MOSKALUK, C. A. & DUTTA, A. 2011. miR-99 family of MicroRNAs suppresses the expression of prostate-specific antigen and prostate cancer cell proliferation. *Cancer Res,* 71**,** 1313-24.

SZCZYRBA, J., LOPRICH, E., WACH, S., JUNG, V., UNTEREGGER, G., BARTH, S., GROBHOLZ, R., WIELAND, W., STOHR, R., HARTMANN, A., WULLICH, B. & GRASSER, F. 2010. The microRNA profile of prostate carcinoma obtained by deep sequencing. *Mol Cancer Res,* 8**,** 529-38.

TAKADA, S. & ASAHARA, H. 2012. Current strategies for microRNA research. *Mod Rheumatol*.

TELLEZ, C. S., JURI, D. E., DO, K., BERNAUER, A. M., THOMAS, C. L., DAMIANI, L. A., TESSEMA, M., LENG, S. & BELINSKY, S. A. 2011. EMT and stem cell-like properties associated with miR-205 and miR-200 epigenetic silencing are early manifestations during carcinogen-induced transformation of human lung epithelial cells. *Cancer Res,* 71**,** 3087-97.

THEORET, M. R., NING, Y. M., ZHANG, J. J., JUSTICE, R., KEEGAN, P. & PAZDUR, R. 2011. The risks and benefits of 5alpha-reductase inhibitors for prostate-cancer prevention. *N Engl J Med,* 365**,** 97-9.

TOMLINS, S. A., MEHRA, R., RHODES, D. R., CAO, X., WANG, L., DHANASEKARAN, S. M., KALYANA-SUNDARAM, S., WEI, J. T., RUBIN, M. A., PIENTA, K. J., SHAH, R. B. & CHINNAIYAN, A. M. 2007. Integrative molecular concept modeling of prostate cancer progression. *Nat Genet,* 39**,** 41-51.

TOST, J. 2009. *DNA methylation : methods and protocols,* New York, Humana Press.

VAN DER POEL, H. G. 2007. Molecular markers in the diagnosis of prostate cancer. *Crit Rev Oncol Hematol,* 61**,** 104-39.

VLACHOS, I. S., KOSTOULAS, N., VERGOULIS, T., GEORGAKILAS, G., RECZKO, M., MARAGKAKIS, M., PARASKEVOPOULOU, M. D., PRIONIDIS, K., DALAMAGAS, T. & HATZIGEORGIOU, A. G. 2012. DIANA miRPath v.2.0: investigating the combinatorial effect of microRNAs in pathways. *Nucleic Acids Res,* 40**,** W498-W504.

WANG, G., WANG, Y., SHEN, C., HUANG, Y. W., HUANG, K., HUANG, T. H., NEPHEW, K. P., LI, L. & LIU, Y. 2010. RNA polymerase II binding patterns reveal genomic regions involved in microRNA gene regulation. *PLoS One,* 5**,** e13798.

WATAHIKI, A., WANG, Y., MORRIS, J., DENNIS, K., O'DWYER, H. M., GLEAVE, M. & GOUT, P. W. 2011. MicroRNAs associated with metastatic prostate cancer. *PLoS One,* 6**,** e24950.

WIKLUND, E. D., BRAMSEN, J. B., HULF, T., DYRSKJOT, L., RAMANATHAN, R., HANSEN, T. B., VILLADSEN, S. B., GAO, S., OSTENFELD, M. S., BORRE, M., PETER, M. E., ORNTOFT, T. F., KJEMS, J. & CLARK, S. J. 2011. Coordinated epigenetic repression of the miR-200 family and miR-205 in invasive bladder cancer. *Int J Cancer,* 128**,** 1327-34.

WITKOS, T. M., KOSCIANSKA, E. & KRZYZOSIAK, W. J. 2011. Practical Aspects of microRNA Target Prediction. *Curr Mol Med,* 11**,** 93-109.

WOLK, A. 2005. Diet, lifestyle and risk of prostate cancer. *Acta Oncol,* 44**,** 277-81.

WOODSON, K., O'REILLY, K. J., HANSON, J. C., NELSON, D., WALK, E. L. & TANGREA, J. A. 2008. The usefulness of the detection of GSTP1 methylation in urine as a biomarker in the diagnosis of prostate cancer. *J Urol,* 179**,** 508-11; discussion 511-2.

WRIGHT, J. L., LIN, D. W. & STANFORD, J. L. 2012. Circumcision and the risk of prostate cancer. *Cancer*.

XU, Q., LIU, L. Z., QIAN, X., CHEN, Q., JIANG, Y., LI, D., LAI, L. & JIANG, B. H. 2012. MiR-145 directly targets p70S6K1 in cancer cells to inhibit tumor growth and angiogenesis. *Nucleic Acids Res,* 40**,** 761-74.

YANAIHARA, N., CAPLEN, N., BOWMAN, E., SEIKE, M., KUMAMOTO, K., YI, M., STEPHENS, R. M., OKAMOTO, A., YOKOTA, J., TANAKA, T., CALIN, G. A., LIU, C. G., CROCE, C. M. & HARRIS, C. C. 2006. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell,* 9**,** 189-98.

YANG, X., NOUSHMEHR, H., HAN, H., ANDREU-VIEYRA, C., LIANG, G. & JONES, P. A. 2012. Gene reactivation by 5-aza-2'-deoxycytidine-induced demethylation requires SRCAP-mediated H2A.Z insertion to establish nucleosome depleted regions. *PLoS Genet,* 8**,** e1002604.

YOSHITOMI, T., KAWAKAMI, K., ENOKIDA, H., CHIYOMARU, T., KAGARA, I., TATARANO, S., YOSHINO, H., ARIMURA, H., NISHIYAMA, K., SEKI, N. & NAKAGAWA, M. 2011. Restoration of miR-517a expression induces cell apoptosis in bladder cancer cell lines. *Oncol Rep,* 25**,** 1661-8.

YOU, J., COZZI, P., WALSH, B., WILLCOX, M., KEARSLEY, J., RUSSELL, P. & LI, Y. 2010. Innovative biomarkers for prostate cancer early diagnosis and progression. *Crit Rev Oncol Hematol,* 73**,** 10-22.

YU, Y. P. & LUO, J. H. 2007. Pathological factors evaluating prostate cancer. *Histol Histopathol,* 22**,** 1291-300.

ZAMAN, M. S., CHEN, Y., DENG, G., SHAHRYARI, V., SUH, S. O., SAINI, S., MAJID, S., LIU, J., KHATRI, G., TANAKA, Y. & DAHIYA, R. 2010. The functional significance of microRNA-145 in prostate cancer. *Br J Cancer,* 103**,** 256-64.