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THE ROLE OF *MYC* REARRANGEMENTS AND RELATIVE COPY NUMBER GAIN IN PROSTATE CANCER

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

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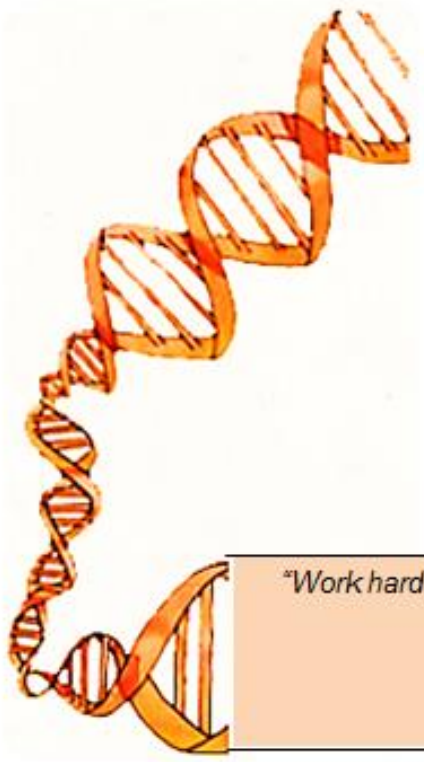

"Work hard and never admire your own science.
It can always be better".

Jacob Hanna

AGRADECIMENTOS

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Expressar por palavras todo o sentimento que carrego neste momento e que se baseia em todas as pessoas que, directa ou indirectamente, contribuíram para eu alcançar mais uma etapa do meu percurso académico, não é de todo fácil.

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porque sem a sua oportunidade e orientação nada disto seria possível.

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provavelmente não estaria hoje comigo – o meu avô.

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SUMMARY

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Prostate cancer (PCa) is the second most frequent malignancy in men worldwide and a common cause of cancer related mortality, constituting a frightening global health problem. The multifocal and highly heterogeneous nature of PCa increases the difficulty to study its progression and to define the most effective prevention strategies or treatment options. The lack of accurate methods able to differentiate between slow-growing tumors of no clinical significance and aggressive carcinomas has reinforced the need of unveiling the underlying molecular genetic alterations and pathways behind prostate cancer initiation and tumorigenesis.

An important breakthrough in the search for novel pathogenic mechanisms in the field of PCa was the finding of chromosomal rearrangements, responsible for de formation gene fusions involving the ETS family of transcription factors. The *TMPRSS2-ERG* is the most frequent type of chromosomal rearrangement present in nearly 50% of localized PCa and in 21% of precursor HGPIN lesions, followed by the *ETV1*, *ETV4*, *ETV5* and *FLI1* genes fused with one of several additional 5´fusion partners. Although ETS fusion genes represent an early event in PCa, secondary copy number changes, such as 8q gain including the *MYC* oncogene, have been shown to be more relevant as prognostic factors. In fact, relative 8q gain is a poor prognostic factor irrespective of *TMPRSS2-ERG* fusion gene status in diagnostic needle biopsy specimens from PCa patients. Moreover, recent integrated analyses of genome and transcriptome led to the discovery of a *C15orf21-MYC* fusion in a single case of hybrid and aggressive case of PCa.

The main aim of this thesis was to explore the role of *MYC* relative copy number increase and structural rearrangements in a set of 50 prostatectomy specimens from patients with clinically localized prostate carcinoma, with available genome-wide microarray expression data. To achieve this goal, a FISH break-apart probe strategy using BAC clones flanking *MYC* and a chromosome 18 centromeric probe, to control of ploidy, was used. Overall, tumor cell populations displaying *MYC* relative copy number increase (*MYC*/CEP18≥1.5) were found in 35% of the prostatectomy specimens and one PCa presented a deletion of the 5´*MYC* region, which may be indicative of *MYC* a rearrangement involving a 5´fusion partner. Additionally, we confirmed the involvement of *MYC* in 4 PCa biopsy specimens with available data indicating a structural rearrangement in 8q24. A probe strategy using BAC clones targeting the 5´ region of *C15orf21* and the 3´region of *MYC* was also used, but *C15orf21* is not the 5' fusion partner in these cases.

In order to identify genes with differential expression among the two FISH subgroups (presence or absence of 8q relative gain), a significance analysis of microarrays (SAM) was used. Gene expression results highlighted three significantly overexpressed genes in the subgroup of patients with 8q relative gain, namely *IKZ2*, *CDON* and *GPRC5A* (FDR=0% and q-value=0%) and all of these are annotated to play a role in cancer, being up-regulated (*IKZF2* and *CDON*) or, in the case of *GPRC5A,* being both up- and down-regulated. According to SAM analysis, *MYC* was not found to be differentially expressed among the two subgroups of patients (*q*-value = 35), but it showed a tendency to present higher expression among those with relative copy number gain (*P* = 0.051). To look for the possible effect of ETS rearrangements status in *MYC* expression, we compared three groups of patients harboring no ETS rearrangements, an *ERG* fusion gene, or other ETS rearrangements (*ETV1*, *ETV4* and *ETV5*) in the presence or absence of 8q gain. A significantly differential expression of *MYC* among the different ETS rearrangements groups of patients was observed only in the patients with no 8q gain (*P*=0.048), and paired comparisons showed that *MYC* expression was significantly higher in the group of patients with ETS rearrangements other than *ERG* (*P*=0.021) compared with ETS negative patients.

RESUMO

RESUMO

O cancro da próstata constitui a segunda neoplasia mais frequente em homens em todo o mundo e é uma causa comum de mortalidade, representando um importante problema de saúde pública. A natureza multifocal e altamente heterogénea desta doença aumenta a dificuldade de estudo da sua progressão, bem como da definição de estratégias mais eficazes, ou melhores formas de tratamento. A falta de métodos mais exactos, capazes de diferenciar entre tumores indolentes e clinicamente insignificantes e carcinomas mais agressivos, reforçam a necessidade de desvendar as alterações genéticas e vias de sinalização subjacentes à carcinogénese prostática.

Uma importante descoberta, na procura de novos mecanismos patológicos na área do cancro da próstata, foi a identificação de rearranjos cromossómicos, responsáveis pela formação de genes de fusão a envolver a família dos factores de transcrição ETS. O *TMPRSS2-ERG* é o rearranjo cromossómico mais frequente, estando presente em quase metade dos tumores de próstata localizados e em 21% das lesões percursoras *HGPIN*, seguido do *ETV1*, *ETV4*, *ETV5* e *FLI1*, geralmente rearranjados com um dos vários parceiros 5´adicionais. Apesar dos genes de fusão ETS representarem um evento precoce em cancro da próstata, alterações secundárias do número de cópias, tal como o ganho do 8q, incluindo o gene *MYC*, foram demonstradas como sendo mais relevantes em termos de factor de prognóstico. De facto, o ganho relativo do 8q é um factor de mau prognóstico, independente do *status* do gene de fusão *TMPRSS2-ERG* em biópsias de pacientes com cancro da próstata. Para além disso, e mais recentemente, análises integradas do genoma e transcriptoma conduziram à descoberta do gene de fusão *C15orf21-MYC* num único caso de cancro da próstata híbrido e agressivo.

O principal objectivo desta foi explorar o papel do aumento relativo do número de cópias do *MYC*, bem como de rearranjos estruturais envolvendo este gene numa série de 50 prostatectomias, de pacientes com cancro da próstata localizado, com dados disponíveis sobre a expressão global do genoma por *microarray*.

Assim, foi feita uma estratégia de análise por *FISH* com uma sonda break-apart incluindo *BAC clones*, que flanqueavam o gene *MYC*, e uma sonda centromérica do cromossoma 18, para controlo da ploidia. No total, as populações de células tumorais que apresentavam aumento relativo do número de cópias do *MYC* foram encontradas em 35% das prostatectomias e uma prostatectomia apresentava uma delecção da região 5´do *MYC,* o que pode ser indicativo de um rearranjo do MYC envolvendo um parceiro de fusão 5´. Confirmámos adicionalmente o envolvimento do gene *MYC* em 4 biópsias de cancro da

próstata com dados disponíveis que indicavam um rearranjo estrutural na região 8q24. Uma estratégia de sondas utilizando *BAC clones*, tendo como alvo a região 5´ do gene *C15orf21* e a região 3´ do gene *MYC* foi usada, mas o gene *C15orf21* não é o parceiro de fusão 5´ nestes casos.

A fim de identificar genes diferencialmente expressos nos dois grupos de FISH (presença ou ausência do ganho relativo do 8q), foi utilizada uma análise de significância do *micoarray* de expressão (SAM). Os resultados do perfil de expressão destacaram 3 genes significativamente sobre-expressos no subgrupo de pacientes com ganho relativo do 8q, nomeadamente *IKZF2*, *CDON* e *GPRC5A* (FDR = 0%, *q*-value = 0%) e todos eles estão descritos como tendo um papel no cancro, estando sobre- expressos (*IKZF2* e *CDON*), ou, no caso do *GPRC5A*, estando tanto sub - como sobre-expresso. De acordo com a análise por SAM, o gene *MYC* não foi encontrado diferencialmente expresso nos dois subgrupos de pacientes (*q* – value = 35), mas demonstrou uma tendência para apresentar uma maior expressão nos pacientes com aumento relativo do número de cópias (*P* = 0.051). Para analisarmos o possível efeito do *status* de rearranjos ETS na expressão do *MYC*, comparamos três grupos de pacientes sem evidência de rearranjos ETS, com um gene de fusão envolvendo o *ERG*, ou tendo outros rearranjos ETS (*ETV1*, *ETV4* e *ETV5*) na presença ou ausência do ganho do 8q. A expressão do *MYC* foi significativamente diferencial entre os diferentes grupos ETS, unicamente no grupo de pacientes com o ganho do 8q (*P*=0.048) e comparações emparelhadas demonstraram que a expressão do *MYC* foi mais elevada no grupo de pacientes com rearranjos de outros ETS, que não o *ERG* (*P*=0.021), quando comparado com os pacientes sem rearranjos ETS.

RELEVANT ABREVIATIONS

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3D-CRT	Three-dimensional conformal radiotherapy
AJCC	American Joint Committee on Cancer
AMACR	α-methylacyl-coA racemase
APC	Adenomatous polyposis coli
AR	Androgen receptor
ARE	Androgen responsive element
AS	Active surveillance
BAC	Bacterial artificial chromosome
BPH	Benign prostatic hyperplasia
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 1, early onset
C15orf21	Chromosome 15 open reading frame 21
CAPB	Cancer prostate and brain
CDK	Ciclin-dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDON	Cell adhesion associated, oncogene regulated
CEP18	Chromosome 18 centromeric probe
CGH	Comparative genomic hybridization
CHEK2	Checkpoint kinase 2
CMV	Cytomegalovirus
CNA	Copy number alteration
Co	Coactivator proteins
COPA	Outlier profile analysis
CRPC	Castration-resistant prostate cancer
DHT	Dihydrotestosterone
DRE	Digital rectal examination
E. coli	Escherichia coli
EAU	European association of urology
ELAC2	elaC homolog 2
ERG	v-ets erythroblastosis virus E26 oncogene homolog
ERK	Extracellular regulated MAP kinase

ERVK-24	Endogenous retrovirus group K, member 24
ESRP1	Epithelial splicing regulatory protein 1
ETS	Erythroblastosis virus E26 transformation-specific
ETV1	ETS variant 1
ETV4	ETS variant 4
ETV5	ETS variant 5
EZH2	Enhancer of zeste homolog 2
FDA	Food and drug administration
FDR	False discovery rate
FFPE	Formalin-fixed paraffin-embeeded
FISH	Fluorescent in situ hybridization
FLI1	Friend leukemia virus integration 1
FNA	Fine-needle aspiration
FOXP3	Forkhead box P3
GPRC5A	Protein-coupled receptor, family C, group 5, member A
GS	Gleason system
GSTP1	Glutathione S-transferase pi 1
GUSB	Glucuronidase beta
GWAS	Genome-wide association study
HGPIN	High-grade prostatic intraepithelial neoplasia
HHV8	Human herpes type 8
HMGN2P46	High mobility group nucleosomal binding domain 2
HNRPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1
HOXB13	Homeobox B13
HPC1	Hereditary prostate cancer, 1
HPC2	Hereditary prostate cancer, 2
HPC20	Hereditary prostate cancer, 20
HPCX	Hereditary prostate cancer, X-linked
HPV	Human papillomavirus
HRPC	Hormone-resistant prostate cancer
HSV2	Human herpes simple virus type 2
IKZF2	Ikaros family zync finger 2
IMRT	Intensity modulated radiotherapy
IUCC	International Union for Cancer Control
LB	Lysogeny broth
LGPIN	Low-grade prostatic intraepithelial neoplasia
LHRH	Luteinizing hormone release hormone

LOH	Loss of heterozygosity
miRNA	Micro RNA
mRNA	Messenger RNA
MSMB	Microseminoprotein, beta
MSR1	Macrophage scavenger receptor 1
MYC	Myc myelocytomatosis viral oncogene homolog
NDRG1	N-myc downstream regulated gene 1
NGS	Next generation sequencing
NKX3.1	NK3 homeobox 1
PANX1	Pannexin 1
PCa	Prostate cancer
PCAP	Predisposing for prostate cancer
PCR	Polymerase chain reaction
PhIP	2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine
PI3K	Phosphatidylinositol 3'-kinase-protein kinase B
PIA	Proliferative inflammatory atrophy
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic
PIM1	Pim-1 oncogene
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homolog
PTGS2	Stress enzyme cyclooxygenase 2
pT stage	Pathological stage
PVT1	Oncogene (non-protein coding)
PZ	Peripheral zone
qRT-PCR	Quantitative reverse-transcription PCR
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
RAS	p21 protein activator (GTPase activating protein) 1
RB1	Retinoblastoma
RHEB	Ras homolog enriched in brain
RICTOR	RPTOR independent companion of MTOR, complex 2
RNASSEL	Ribonuclease L
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse-transcription PCR
SAM	Significance analysis of microarrays
SLC45A3	Solute carrier family 45, member 3

SNP	Single nucleotide polymorphism
SPSS	Statistical Package for Social Sciences
TMEM75	Transmembrane protein 75
TMPRSS2	Transmembrane protease serine II
TNM	Tumor-node-metastasis
TP53	Tumor protein p53
TrERG	Truncated human ERG isoform
TRUS	Transrectal ultrasound-guided biopsy
TSC2	Tuberous sclerosis 2
TSG	Tumor supressor gene
TZ	Transitional zone
UICC	International Union for Cancer Control
WW	Watchfull waiting

INTRODUCTION

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1. Cancer: A Doubtless Major Health Problem

Cancer is a frightening global health problem and it has been quite concerning to see the large increase of this burden. According to GLOBOCAN 2008 estimative, about 12.7 million new cancer cases and 7.6 million deaths have occurred worldwide.

Being a group of diseases, cancer is characterized by uncontrolled growth and spread of abnormal cells. Cancer is the final product of the synergistic or sequential effect of both external (tobacco, infectious organisms, chemicals and radiation), and internal (inherited mutations, hormones, immune conditions) factors.

This human genetic disease is caused by genetic alterations inactivating tumor suppressor genes (TSGs) as well as DNA repair genes, and activating oncogenes (Hanahan and Weinberg, 2000; Porkka and Visakorpi, 2004). Most of the mutations are acquired during cancer progression and are thus considered to be mechanisms of tumorigenesis. On the other hand, some mutations may be inherited, resulting in predisposition to cancer. However, about ten or more years often pass between exposure to external factors and detectable cancer.

The rational for studying the molecular mechanisms behind the development of malignancies is that they may provide means for better diagnostics, prognostics and treatment of cancer. These topics will be discussed in more detail later on.

1.1 Prostate cancer epidemiology

Prostate cancer (PCa) contributes to the overall cancer burden and continues to represent a significant challenge to the clinical community worldwide, being the second most frequently diagnosed non-skin cancer in men worldwide and the sixth deadliest cancer, totalizing 903,500 new cancer cases and 258,400 new cancer-related deaths (Jemal et al., 2011). The low fatality means that many men are alive following a diagnosis of PCa.

Interestingly, both developed and developing countries differ widely in terms of incident and mortality rates, recording higher incidence mainly in Oceania, Western and Northern Europe and North America **(Figure 1)**. Prostate cancer remains relatively rare in Asian population. Incident rates reflect not only differences in risk of disease, but also the extent of diagnosis of latent cancers both by screening of asymptomatic individuals - practice

of prostate-specific antigen (PSA) screening which enables a better and earlier diagnosis – and subsequent biopsy or even by detection of latent cancer in tissue removed during prostatectomy operations or at autopsy (Eble et al., 2004). In contrast, epidemiological data indicates that black males of African descendent in the Caribean and Jamaica regions and African American men have the highest documented PCa mortality rates in the world (Jemal et al., 2011), followed by white people, who in turn have rates considerably higher than Asian populations (including Chinese, Japanese and Korean males) (Eble et al., 2004). Differences in genetic factors (Corder et al., 1995; Devgan et al., 1997; Irvine et al., 1995; Platz et al., 2000; Shook et al., 2007), along with levels of sex hormones (Winters et al., 2001) and growth factors (Platz et al., 1999; Tricoli et al., 1999; Winter et al., 2001) appear therefore to play a major role in explaining the observed racial differences in PCa rates.

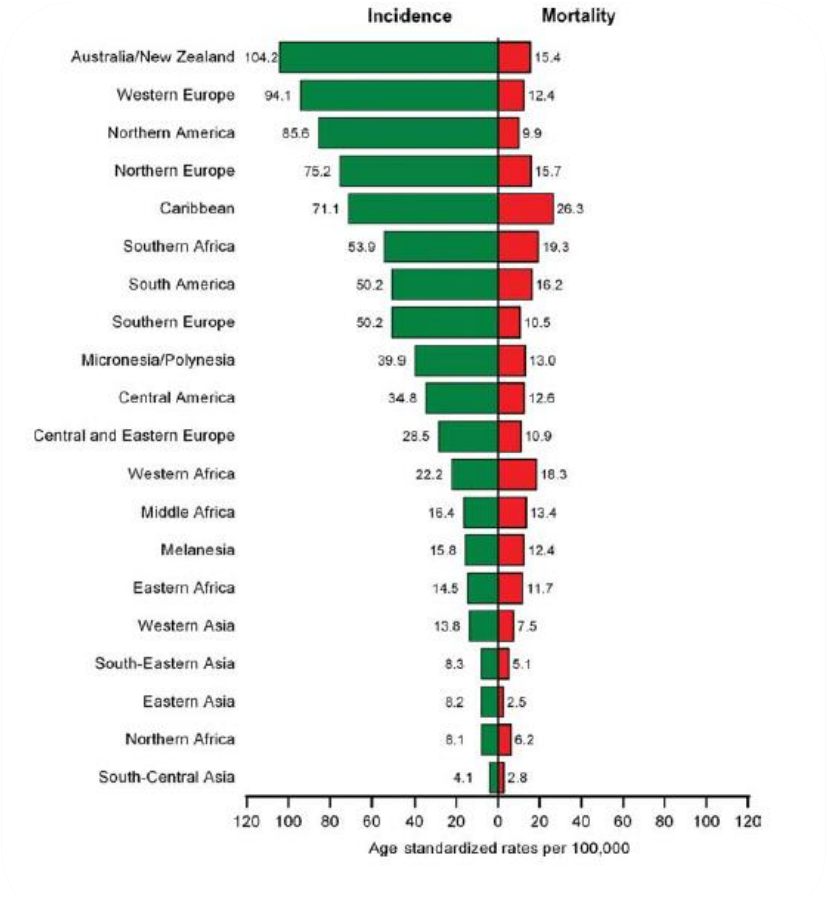


Figure 1 - Age-Standardized prostate cancer incidence and mortality rates by world area. Source: GLOBOCAN 2008 (adapted from (Jemal et al., 2011)).

The number of cases has continuously increased over the past decades, partly due to the higher life expectancy. An additional factor is the western lifestyle, characterized by a highly caloric diet and lack of physical activity.

In Europe, during the year 2008, the estimated number of newly diagnosed cases of PCa was 370,733, accounting for 21.8% of all cancers **(Figure 2)**. In the same year, this cancer was also responsible for 9.4% of the cancer-related deaths (89,629 cases). In Portugal, PCa is the leading cancer among men, **(Figure 2)** with 5,140 estimated incident cases and lies on the third position of cancer-associated deaths (2,021 cases, 13.8%).

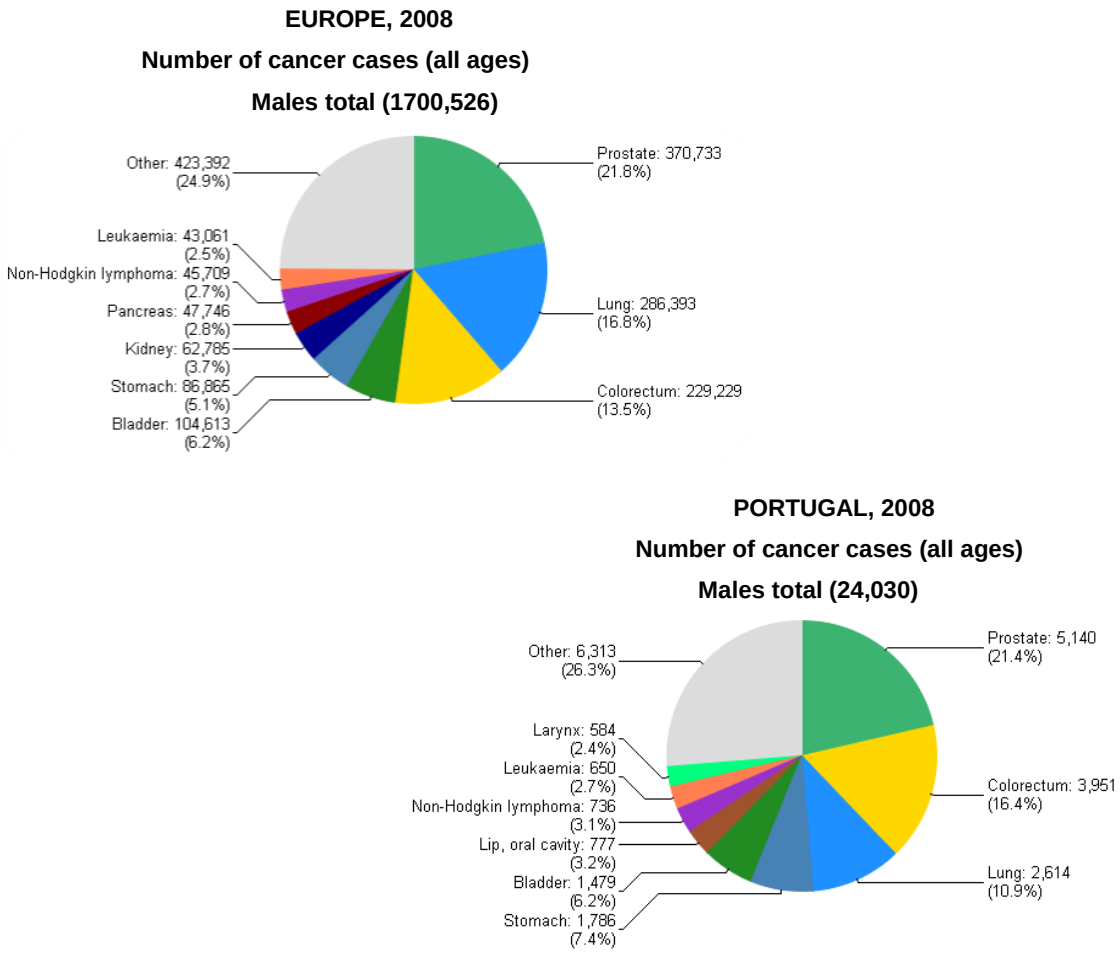


Figure 2 - Estimated cancer incident in Europe and Portugal (number of newly diagnosed cases and proportion of each cancer comparing with all types of cancer). Prostate cancer is the first most common cancer in both Europe and Portugal (adapted from GLOBOCAN, 2008)).

1.2 Etiology and risk factors

Many factors are thought to contribute to an increased risk of PCa, although consistent evidence is available for only a few of them, such as increasing age, African

ancestry, and a family history of the disease (Bostwick et al., 2004; Gronberg, 2003) **(Table 1)**. Age is one of the well-established risk factors, as the frequency of PCas rises very steeply with age, being exponential in men aged 65 or more (Eble et al., 2004). Ethnic origin seems also to play an important role in prostate carcinogenesis (Jemal et al., 2011). A family history of PCa that includes a first-degree relative is associated with a two-fold increased risk of developing the disease compared to the general population (Carter et al., 1992; Edwards and Eeles, 2004; Steinberg et al., 1990). Furthermore, epidemiologic evidence also supports a major contribution of environmental stresses, dietary factors (increased total fat intake, animal fat intake and red meat) and lifestyle-related factors, including obesity to the makeup of this disease. Further, there is some evidence that occupational exposures of firefighters (toxic combustion products) moderately increase risk (Nelson et al., 2003). The risk of PCa increases whenever Asian individuals immigrate to North America – once more implicating both the environment and lifestyle-related factors in causing PCa in the United States.

Table 1 - Proposed risk factors for Prostate Cancer (adapted from (Gronberg, 2003))

Established	Possible	Uncertain
Age	Lycopene	*AR* polymorphisms
Ethnic origin	Zinc	Vitamin D polymorphisms
Family history	Selenium	Dietary fat

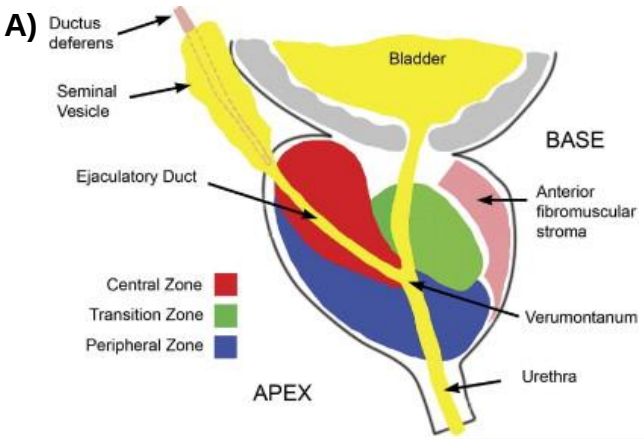
AR indicates androgen receptor.

2. Prostate Cancer Anatomy and Mechanisms of Disease

2.1. Anatomy and histology

Prostate is an accessory gland of the male reproductive system. It is a walnut-sized gland that lies just below the urinary bladder and surrounds the upper part of the urethra. Its primary function is to secrete a slightly alkaline fluid (that forms part of the seminal fluid) into the urethra at the time of ejaculation, which helps to nourish and protect the sperm cells (Marandola et al., 2004). At the histological level, the prostatic pseudostratified epithelium is subdivided in four types of cells: luminal, basal, transient (an intermediate between the previous two) (Isaacs and Coffey, 1989), and neuroendocrine cells (Foster et al., 2002; Hudson, 2004; Peehl, 2005; Shappell et al., 2004; van Leenders and Schalken, 2003). In general terms, the luminal epithelial cells, forming a continuous layer of polarized columnar cells, are responsible for the physiological secretions of the prostatic gland. The basal cells

are much lesser and rest in the basement membrane adjacent to the secretory cells and their absence is a helpful marker of PCa. Finally, neuroendocrine cells are thought to be involved in the regulation of prostatic secretory activity and cell growth (Joshua et al., 2008). The classic work of McNeal (1969, 1981, 1988) defined the human prostate as having a zonal architecture, corresponding to three anatomical zones: the central, the peripheral and the transitional zone (Timms, 2008) (McNeal, 1969, 1981, 1988) **(Figure 3)**. Interestingly the transitional zone (TZ) is more prone to develop benign prostatic hyperplasia (BPH), a nonmalignant condition of older men. On the other hand, most prostate carcinomas occur in the peripheral zone (PZ) **(Figure 3)** (De Marzo et al., 2007; Shen and Abate-Shen, 2010).



B)

	Prostate zone		
	Peripheral	Transition	Central
Focal atrophy			
Acute inflammation			
Chronic inflammation			
Benign prostatic hyperplasia			
High-grade PIN			
Carcinoma			

High prevalence
Medium-high prevalence
Low prevalence
None

Figure 3 - A) Sagital view of prostate gland that specifically illustrates the three major anatomical zones of prostate (adapted from (Cohen et al., 2008)). B) Zonal predisposition to prostate disease. The major part of cancer lesions occur in the peripheral zone, fewer occur in the transitional zone and almost none occur in the central zone (adapted from (De Marzo et al., 2007)).

2.2. Percursor and malignant prostate lesions

The accumulation of multiple somatic genome alterations rather than any individual genetic lesion characterizes the different stages of prostate carcinogenesis **(Figure 5)**. Whereas somatic genome alterations are likely to occur in a stochastic fashion rather than in

a stepwise manner, certain events are known to occur at particular stages of the prostate disease progression (Gonzalgo and Isaacs, 2003).

Following the contemporary model of PCa progression suggested by Gonzalgo and Isaacs (2003) **(Figure 4)**, there are some factors, including genetic predisposition, oxidative damage and inflammatory changes, associated with earliest steps of PCa development.

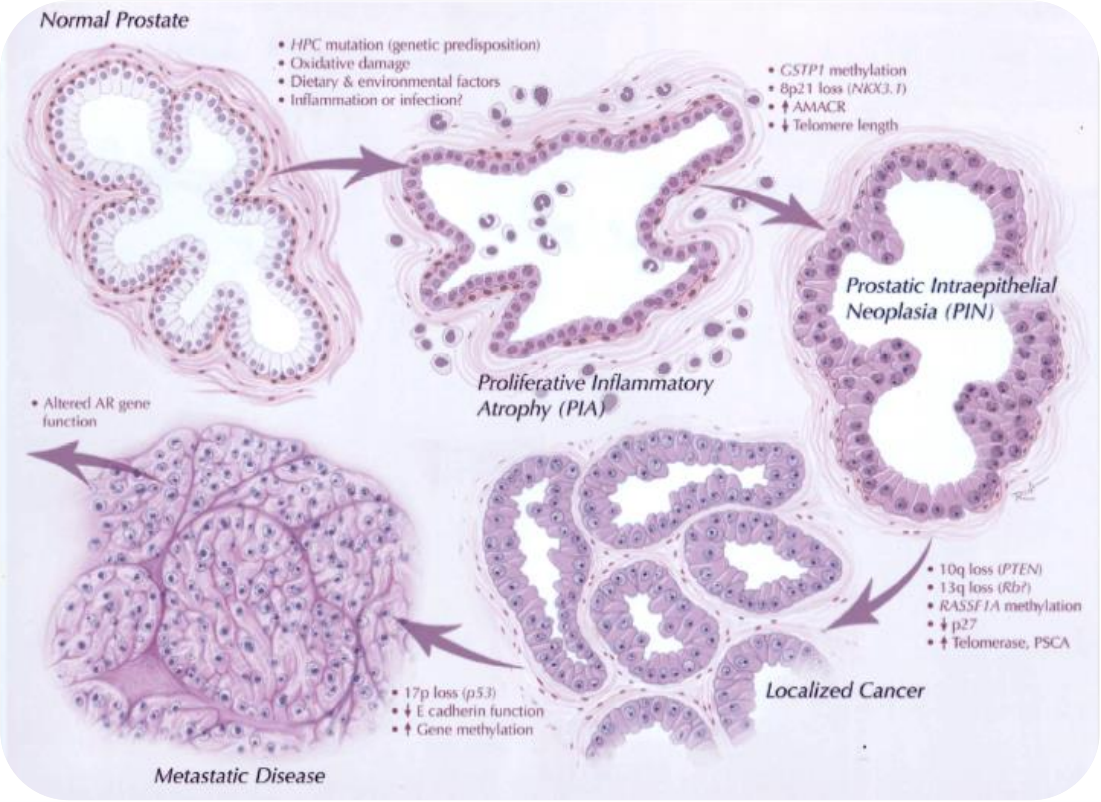


Figure 4 - The Molecular Pathogenesis of Prostate Cancer (adapted from (Gonzalgo and Isaacs, 2003)).

In more than 90% of PCa cases, the absence of Glutathione S-transferase pi 1 (*GSTP1*) in PCa cells, by aberrant promoter hypermethylation of the CpG island sequences, may increase the likelihood for neoplastic transformation (Gonzalgo and Isaacs, 2003; Lin et al., 2001). On the other hand, the same absence of *GSTP1* is also characteristic of prostatic intraepithelial neoplasia (PIN) lesions (Brooks et al., 1998). Although cells carrying inactivated *GSTP1* alleles tend to accumulate during PCa progression, *GSTP1* does not appear to act as a TSG (Lin et al., 2001). Instead, *GSTP1* probably serves as a “caretaker” gene defending prostate cells against genomic damage mediated by carcinogens like 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP), present in well-done or charred meats, or even various oxidants, found at sites of inflammation (Stuart et al., 2000); (Nelson et al., 2001; Shirai et al., 1997). Other alterations like chromosomal loss and telomere shortening may also contribute to genetic instability and progression to invasive disease.

Further methylation changes, loss of TSG function and additional mutational events are associated with metastatic and androgen-independent disease (Gonzalgo and Isaacs, 2003).

Adult males can experience two main types of prostatic disease: BPH and PCa. The former involves the nonmalignant proliferation of epithelial and stromal prostatic cells (Roehrborn, 2008) and is persistently under androgens stimulation (Hayward et al., 1997). BPH progresses in the TZ of prostate gland, around the urethra, and the pressure exerted can lead to urinary tract obstruction, causing consequently lower urinary symptoms. This is the most common urological disease in men, beginning in the third decade of life and affecting over half of the men aged more than 50 years and 90% of men aged more than 80 years (Alcaraz et al., 2009). This pathology is often accompanied by an increase of serum PSA levels, however, in the vast majority of the cases, is not considered a pre-neoplastic lesion.

The most well studied premalignant lesion in the prostate is PIN (Ramon and Denis, 2007). It consists in the development of a variable degree of cytologic atypia in the epithelium lining the ducts and acini, being confined to the glands. At the histologic level, PIN is generally characterized by the appearance of luminal epithelial hyperplasia, reduction in basal cells, enlargement of nuclei and nucleoli, along with cytoplasmic hyperchromasia, and nuclear atypia (Bostwick, 1989; Shappell et al., 2004). Furthermore, two grades of this lesion spectrum (PIN) have been identified: low-grade PIN (LGPIN) and high-grade PIN (HGPIN). The latter is currently considered the only premalignant to PCa on the basis of pathological (Qian et al., 1997), epidemiological (Sakr et al., 1994; Sakr et al., 1993) and cytogenetic (Beheshti et al., 2002) evidence. In addition, HGPIN lesions generally display marked elevation of cellular proliferation markers (Bostwick, 1989; Shappell et al., 2004). Moreover, and based on recent data, the majority of the expression alterations that occur in the progression to PCa, take place in the transition from benign epithelium to HGPIN, rather than from HGPIN to PCa (Tomlins et al., 2007b). HGPIN is characterized by benign prostatic acini and ducts, lined up by cytological atypical cells (Bostwick et al., 2004) and its incidence increases with age (Eble et al., 2004). Bostwick and Quian subdivided HGPIN lesions into at least four different architectural patterns, which have no prognostic significance, ie, tuffed, micropapillary, flat and cribiform (Bostwick et al., 2004). Still, the volume of HGPIN has a positively correlation with tumor stage and Gleason grade, (Qian et al., 1997) as well as the risk of subsequent PCa (Bishara et al., 2004; Kronz et al., 2001). On the other hand, LGPIN alone is not associated with an increased risk for detection of carcinoma.

An alternative, possibly earlier, of PCa is proliferative inflammatory atrophy (PIA), which displays foci of atrophic lesions occurring in association with inflammation. Regions of PIA are also often located in proximity with PIN and adenocarcinoma, and thus PIA has also been proposed to represent a precursor lesion in PCa (De Marzo et al., 1999; De Marzo et

al., 2003). Although linking PIA to PCa is suggestive, this evidence is not as convincing as HGPIN (Joshua et al., 2008).

Finally, prostate adenocarcinoma, which accounts for over 95% of all malignancies of the prostate (DeVita, 2008), is an aged-related disease, initially androgen-dependent, with a highly heterogeneous nature both in terms of pathology and clinical presentation, ranging from indolent, clinically silent, to highly aggressive and lethal tumors (Boyd et al., 2012; Cassidy et al., 2010). Prostate adenocarcinoma is a multifocal and high heterogenic disease, with multiple pathways to the malignant phenotype and, as already described, usually located in the PZ of the prostate, as HGPIN lesions are (De Marzo et al., 2007; DeVita, 2008).

2.3. Natural history of prostate cancer

2.3.1 Latent and clinical cancer

Prostate cancers, being so heterogeneous, display a range of clinical behavior, from slow-growing tumor of no clinical significance to aggressively metastatic and lethal disease **(Figure 5)** (Boyd et al., 2012).

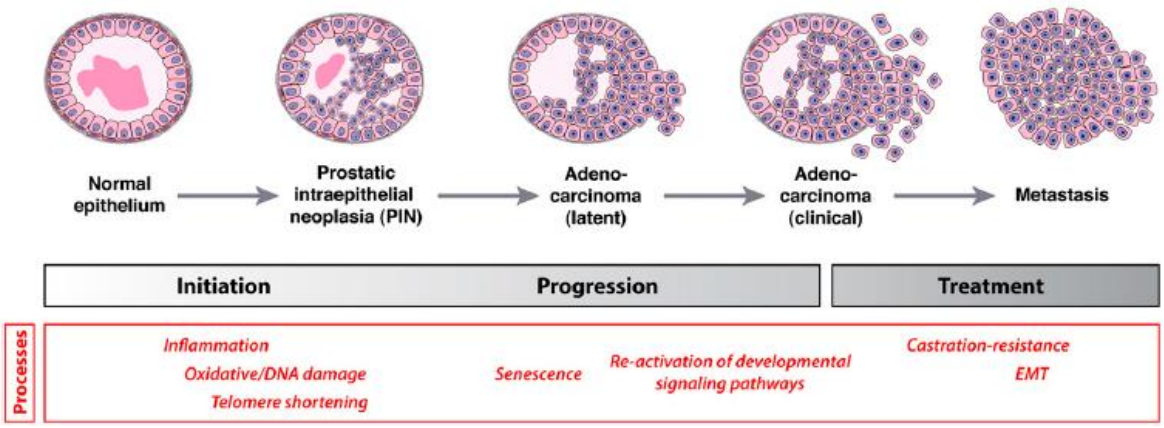


Figure 5 - Human prostate progression pathway. Each stage of disease progression is accompanied by molecular processes that are considered to be significant in each stage (adapted from (Shen and Abate-Shen, 2010)).

Moreover, the adjective “multifocal” is also very adequate to PCa, as primary tumors often contain multiple independent histologic foci of cancer that are often genetically distinct (Aihara et al., 1994; Bostwick et al., 1998; Cheng et al., 1998; Clark et al., 2008; Macintosh et al., 1998; Mehra et al., 2007). On the other hand, and contrarily to what has been stated above, despite the phenotypic heterogeneity of metastatic PCa (Shah et al., 2004), both

molecular and cytogenetic analyses show that metastases in the same patient are clonally related, suggesting that advanced PCa is monoclonal (Liu et al., 2009; Mehra et al., 2008). These evidences indicate that metastatic PCa may arise from the selective advantage of individual clones during cancer progression. However, Shen and Abate-Shen consider that this process of clonal evolution may also result from the androgen-deprivation therapeutic option, which may differentially target cells of varying malignant potential (Shen and Abate-Shen, 2010).

Although PCa is an elderly disease, studies of prostate specimens from healthy men in their 20s and 40s show the presence of multiple histologic foci of cancer (Sakr et al., 1994; Shiraishi et al., 1994; Yatani et al., 1989), conducting to the conclusion that cancer initiation has already taken place at a relatively early age. Thus, the prostate gland is a site of multiple neoplastic transformation events, many of which give rise only to latent PCa and not necessarily progress to clinically detectable disease. Therefore it is suspected that clinical PCa has a different “switch on” program than latent disease, or even may remain under active suppression sufficient to maintain these latent foci in a subclinical state (Shen and Abate-Shen, 2010)

2.4. Molecular subtypes of prostate cancer

Unlike other epithelial tumors, such as breast cancer, PCa lacks distinguishable histopathological subtypes that differ in their prognosis or even treatment response. It is known that the majority of PCas are acinar adenocarcinomas that express the androgen receptor (*AR)*, while other categories of PCa, like ductal adenocarcinoma, mucinous carcinoma, and signet ring carcinoma are extremely rare (Grignon, 2004).

However, a report from Taylor et al support the idea that oncogenomic pathway analyses, which integrate analyses of gene expression, copy number alterations (CNAs), and exon resequencing, may provide a unified approach for distinguishing PCa subtypes and therefore stratifying patient outcome (Taylor et al., 2010). Furthermore, Lapointe et al, crossing genomic data at the level of CNAs obtained from array comparative genomic hybridization (CGH) with gene expression data profiling 55 primary tumors and 9 lymph node metastasis, defined three subtypes of prostate carcinomas. Subtype-1 was mainly characterized by loss of 5q21 and 6q15; subtype-2 by *TMPRSS2-ERG* rearrangements and loss of 8p21 (*NKX3.1*) and subtype-3 by loss of 10q23 (*PTEN*) and gain of 8q24 (*MYC*). This profile was similar to that obtained for lymph node metastasis. Moreover, and regarding the patient outcome, subtype-1 was linked to clinically favorable behavior, and the other two (subtype-2 and -3) were linked with more aggressive disease (Lapointe et al., 2007).

2.5. Metastasis

As already stated, if PCa metastasizes, it undoubtedly goes to bone, giving rise to osteoblastic, rather than osteolytic lesions (Bubendorf et al., 2000; Logothetis and Lin, 2005), although the typical sites of secondary metastasis for PCa are lung, liver and pleura (Shen and Abate-Shen, 2010).

However, the mechanism by which the disseminated tumor cells form metastasis at distant regions of the body remains unclear, as well as the molecular factors that promote metastasis of PCa to the bone (Shen and Abate-Shen, 2010).

3. Processes that Promote Prostate Carcinogenesis

Regarding all the risk factors, already described, for PCa, the single most significant is advanced age. While men who are younger than 40 have 1 in 10.000 chance of developing PCa, this risk increases to 1 in 7 by the age of 60 (American Cancer Society: Cancer Facts and Figures 2012, 2013). Since different populations rate considerably different incidences (Jemal et al., 2011), PCa is not simply a by-product of aging. Therefore, the relationship between PCa and advanced age likely reflects the interplay of environmental, physiological and molecular influences with normal consequences of aging that presumably exacerbate the effects of these influences (Shen and Abate-Shen, 2010). Thus, various studies have described gene expression changes linked with aging, including those involved in inflammation and oxidative stress (Bavik et al., 2006; Begley et al., 2005; Bethel et al., 2009)

3.1. Inflammation

Epidemiological, pathological and molecular evidence have emphasized the idea that chronic inflammation is causally associated with prostate carcinogenesis (Bardia et al., 2009; Haverkamp et al., 2008; Klein and Silverman, 2008). Generally, the cause of prostate inflammation is unclear. However, the initial inciting event could be explained through various potential sources, including direct infection, urine reflux promoting both chemical and physical trauma, dietary factors, estrogens, or a combination of two or more of these factors **(Figure 6)** (Shen and Abate-Shen, 2010). Consequently, any of these factors could easily lead to a break of immune tolerance and the development of an autoimmune response to the prostate (De Marzo et al., 2007).

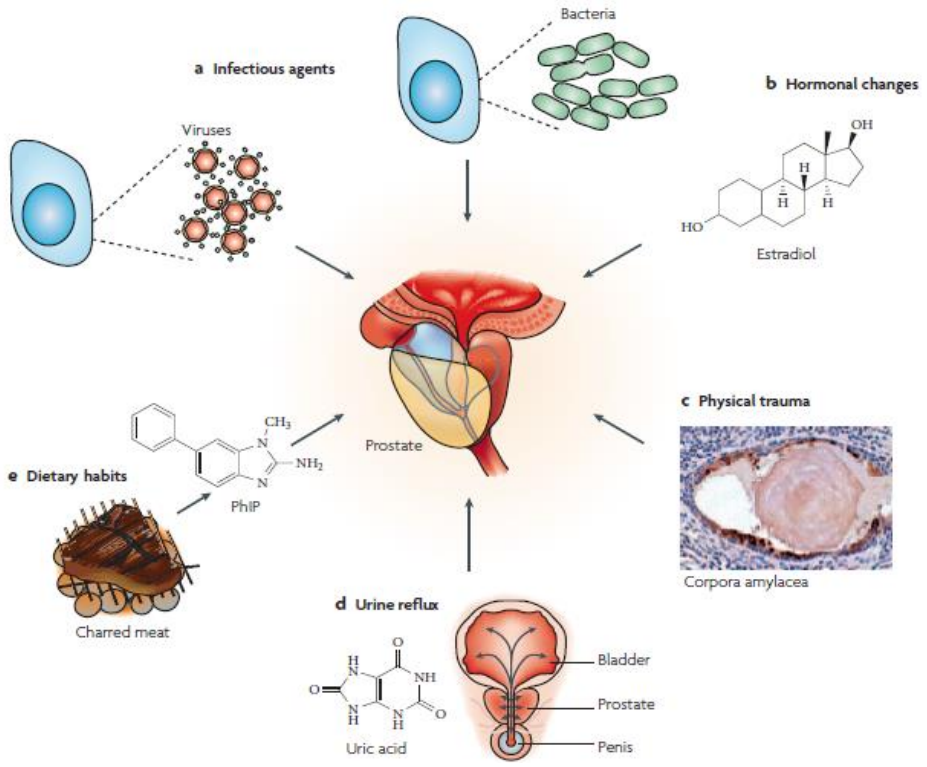


Figure 6 - Possible causes of prostate inflammation. a) Infection. The figure illustrates two cells infected either by bacteria or viruses. b) Hormones. Estrogenic exposures in the prostate. c) Physical trauma. The figure shows a corpora amylacea within a prostatic acinus. d) Urine reflux. e) Dietary habits (adapted from (De Marzo et al., 2007)).

Regarding the infectious agents, a chronic bacterial prostatitis is a rare recurring infection in which pathogenic bacteria are cultured from prostatic fluid. Furthermore, a potential role for bacterial infection in prostate carcinogenesis has been suggested when multiple bacterial species where identified in most prostatectomy samples examined (Sfanos et al., 2008). Some viruses (like human papillomavirus (HPV), human herpes simplex virus type 2 (HSV2), cytomegalovirus (CMV) and human herpes type 8 (HHV8)), mycobacteria and parasites can also infect and induce an inflammatory response in the prostate. Another factor related with prostate inflammation is urine reflux, ie, urine that travels up back towards the bladder (“retrograde” movement), that can penetrate the ducts and acini of the prostate gland. Crystalline uric acid, a particularly intriguing compound in this regard, directly interacts with a receptor that is part of a molecular pathway within innate immune cells that can potently stimulate inflammation. On the other hand, physical trauma is induced by the trauma of the prostate on a microscopic level by the corpora amylacea **(Figure 6)**. Once corpora amylacea is within a prostatic acinus, its edges appear to be eroding the epithelium, resulting in an increased expression of the stress enzyme cyclooxygenase 2 (PTGS2), as it could be seen in the figure above. Taking into account the dietary habits, like PhIP, present in charred meats, can reach the prostate through the bloodstream or by urine reflux and cause DNA

damage and mutations, thus resulting in an influx of inflammatory cells (Shen and Abate-Shen, 2010). Moreover, this potent heterocyclic amine (PhIP) promotes prostatic hyperplasia and PIN in rodents (Borowsky et al., 2006; Elkahwaji et al., 2009; Elkahwaji et al., 2007; Khalili et al., 2010; Nakai et al., 2007). As abovementioned, one of the well-studied genes responsible for prostate protection against carcinogens such as PhIP is *GSTP1*.

Notably, regions of focal atrophic prostate epithelium can often be identified in older men, frequently associated with an inflammatory response. Furthermore, such regions usually display increased epithelial proliferation, and have been termed PIA (De Marzo et al., 1999). Another potential "promoters" of an inflammatory response in the prostate are hormonal perturbations, including estrogen exposure at crucial development junctures that can result in prostate architectural modifications (De Marzo et al., 2007).

3.2. Oxidative stress and DNA damage

Oxidative stress and its cumulative impact on DNA damage has been suggested as one of the major aging-associated influences on prostate carcinogenesis, by several lines of evidence (DeWeese et al., 2001; Khandrika et al., 2009; Minelli et al., 2009). The constant imbalance of reactive oxygen species (ROS) and detoxifying enzymes, which control cellular levels of ROS, originates the oxidative stress that, in turn, leads to cumulative damage of lipids, proteins and DNA. Evidence linking oxidative stress and PCa initiation include correlative studies showing that major antioxidant enzymes are reduced in human PIN and PCa (Bostwick et al., 2000).Thus prostate gland appears to be very vulnerable to oxidative stress, perhaps as a product of the interplay of inflammation, hormonal deregulation, diet, and/or epigenetic alterations, such *GSTP1* silencing. Furthermore, since the tumor suppressor *NKX3.1* is frequently down-regulated in the onset of the disease, its inactivation may also contribute to the marked vulnerability of prostate to oxidative stress, as well as its damage to DNA, linked with cancer initiation (Shen and Abate-Shen, 2010).

4. Androgens in Prostate Cancer

Circulating androgens are essential for normal prostate development, as well as the onset of PCa through their interactions with the *AR*. Prostate cancer typically initiates as an androgen-sensitive lesion, but frequently develops into an androgen-insensitive status, while progressing to a more advanced stage **(Figure 7)**. As it is illustrated in the figure below, normal prostate gland aging eventually leads to BPH in adult males. On the other hand, malignant development of the prostate may arise from PIN which, in turn, may remain as a

clinically and histologically dormant lesion, progress into organ-confined or locally invasive tumor, or finally evolve into hormone-refractory or metastatic disease (Koochekpour, 2011).

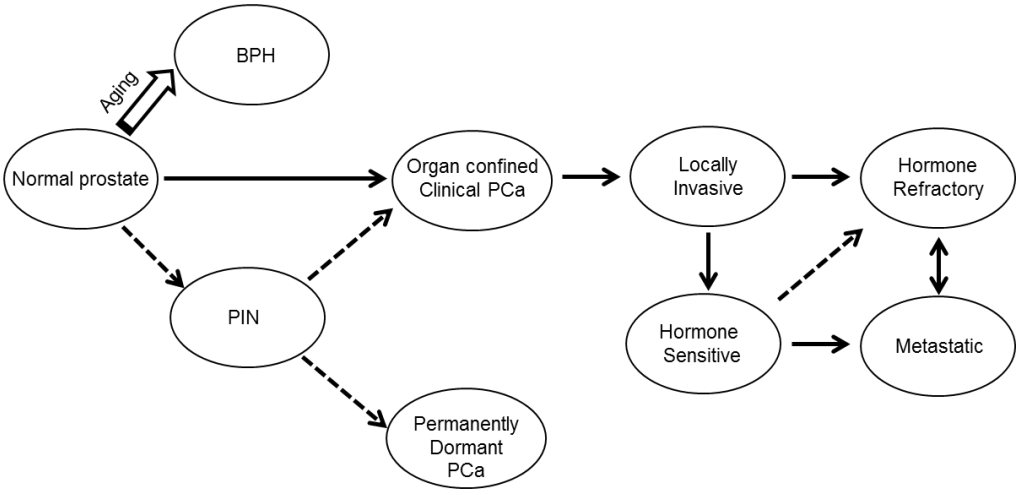


Figure 7 - Prostate cancer development and progression through a multistep fashion (adapted from (Koochekpour, 2011)).

Androgenic hormones are widely believed to regulate proliferation, apoptosis, angiogenesis, metastasis and differentiation (Imamoto et al., 2008). Testosterone and dihydrotestosterone (DHT) are the two main androgens in adult men. While testosterone, synthesized by the testis, is the major male circulating androgen, DHT is the principal androgen in tissues (such as prostate and skin). The latter arises from the conversion of testosterone through the action of the 5α-reductase enzyme and becomes about twice as potent as testosterone at stimulating prostate growth (Wright et al., 1996). It has been clear, since the mid 20th century, that DHT and not testosterone is the primary androgen responsible for the *AR* mediated growth of normal and malignant prostate tissue (Tindall and Rittmaster, 2008). In the unbound state, *AR* is associated, in the cytoplasm, to heat shock proteins **(Figure 8)**. Once bound to the *AR*, DHT causes heat shock protein dissociation, allowing *AR* translocation into the nucleus. Here *AR* binds androgen responsive elements (AREs) as a dimer in the promoter and enhancer regions of target genes. Lastly, the *AR* recruitment of coactivator proteins (Co) to these multiprotein complex enables target gene transcription in a regulated level (Tindall and Rittmaster, 2008).

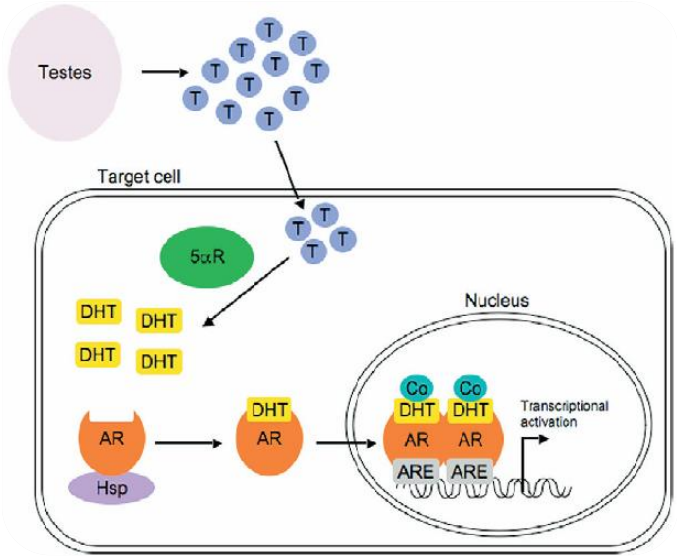


Figure 8 - *AR* signaling pathway in prostate. In androgen responsive target cells, testosterone (T) is converted into DHT by 5αR enzymes. DHT binds to *AR*, causing heat shock protein dissociation, allowing DHT-*AR* complex translocation to the nucleus, where it binds to AREs. Finally, the recruitment of coactivator proteins (Co) enables transcriptional activation of target genes (adapted from (Tindall and Rittmaster, 2008)).

4.1. Progression to an androgen-independent status

Early metastatic PCa is usually treated with androgen ablation, antiandrogens, or a combination of the two, which leads to a significant reduction of androgen-responsive cancer cells (Eisenberger et al., 1998; Koochekpour, 2011; Laufer et al., 2000). Despite an initial and clinically satisfactory response, manifested by rapid cellular apoptosis and evolution to the regressed state, to androgen-deprivation via chemical and surgical castration, progression is inevitable, due to the emergence of androgen-independent cancer cells. The tumor becomes hormone-refractory and more aggressive in the later stages, leading to a poor prognosis, incurable disease, and death (Kruglyak, 1999).

Both PCa and progression to an androgen-independent status are dependent on *AR* expression and function. Its function is quite often heterogeneous, perhaps reflecting an underlying genomic instability (Boyd et al., 2012). Moreover, Zegarra-Moro et al suggested that receptors can drive the proliferation of androgen-independent cells even in the absence of androgens (Zegarra-Moro et al., 2002).

Germline mutations in the *AR* gene are rarely identified in PCa patients, although polymorphisms that alter the response to androgens are frequently observed (Boyd et al., 2012). One example of this is the fact that the length of the human *AR* polymorphic trinucleotide CAG tract, mapping to exon 1 of the *AR* gene, determines androgen sensitivity *in vivo* and short CAG repeat length has been associated with increased *AR* transactivation (Beilin et al., 2000; Mhatre et al., 1993; Simanainen et al., 2011). Regarding the prevalence

of short CAG repeats, it is greater in White men than in Asian men, being the black males the ones that are more prone to carry particularly short repeats (Kumar et al., 2011). It is noteworthy that ethnicity-based differences in mean *AR* CAG tract length correlate with ethnicity-based variations in both incidence and mortality (Buchanan et al., 2001; Hsing et al., 2000). Nevertheless, despite intensive research, the association between CAG repeat length and PCa risk remains unclear (Gu et al., 2012).

5. Prostate Cancer Diagnosis

A major challenge for urologists relates to the initial diagnosis and prognosis of PCa. The main diagnostic tools used to look for evidence of PCa include serum concentration of PSA, digital rectal examination (DRE) and transrectal ultrasound-guided biopsies (TRUS) (Smith et al, 2007). According to the European Association of Urology (EAU) guidelines for the diagnosis of PCa, this disease could be indicated by an abnormal DRE result or elevated serum PSA level (Heidenreich et al., 2011).

5.1. PSA (Prostate Specific Antigen)

Although widely accepted as a prostate tumor marker, PSA has turned out to be organ-specific but not PCa specific (Hessels et al., 2004). PSA is a serine protease produced by epithelial cells lining the prostatic ducts and is secreted directly into the prostatic ductal system **(Figure 9)**.

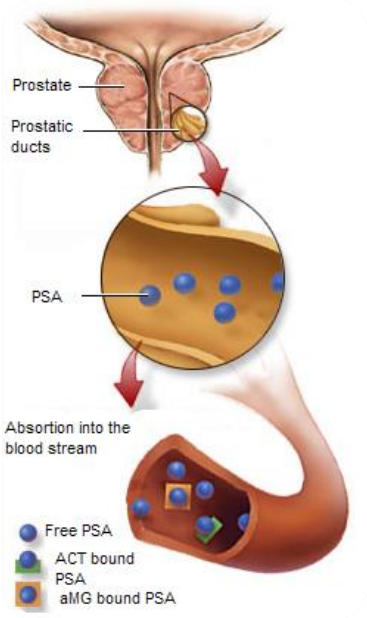


Figure 9 - Schematic illustration of PSA synthesis in epithelial cells and its secretion into prostatic ducts. As PSA is a serine protease its normal mode of existence in the serum is in a complex with -1-anti- chymotrypsin (ACT) and -2-macroglobulin (AMG). Only a small percentage of PSA found in the serum is free (adapted from www.mens-hormonal-health.com).

The main function of PSA is to liquefy the semen (Eble et al., 2004). Generally the normal PSA serum levels are <4ng/mL (Catalona et al., 1991), however it could be “normally” increased in aged and African men. The PSA blood test has changed the landscape of PCa, since its dissemination more than 20 years ago, rising the incidence in a dramatically way and helping to shift the stage of disease to a much earlier and potentially curable stage (Catalona et al., 1991; Cooner et al., 1990). However, testing for the early detection of PCa based on these methods remains a source of uncertainty and controversy (Wolf et al., 2010). The principal aim of screening for PSA was the evidence that elevated PSA levels were associated with asymptomatic/occult PCa.

Still having now a huge lack of definitive answers from randomized trials, the traditional level of 4ng/mL is a reasonable threshold (meaning the higher risk of PCa), but need to suffer further evaluation. The fact that there is no true PSA cutoff point, since PSA level screening produce false positive (lead men without disease to unnecessary additional testing) and false negatives, results suggested that clinicians consider individualized decision making when PSA levels fall in the intermediate range of 2,5ng/mL to 4ng/mL, namely for men at increased risk for PCa based on other risk factors apart from PSA (Wolf et al., 2010).

Any pathological process that leads to a significant increase of prostatic epithelial cells (BPH or PCa) or, in turn, changes the normal organ architecture (prostatitis or PCa), allows an additional amount of PSA to escape the prostate and disseminate to the bloodstream. Any significant increase in PSA serum levels should necessarily indicate a

proper study of the patient, including the prostatic biopsy, if indicated, to confirm the presence of cancer. Concerning now at what age should the early detection start and what is the interval of PSA, this test is not necessary in men >75 years and a baseline PSA ≤ 3ng/mL, because they comprise the very low-risk patients of dying from PCa (Carter et al., 2008).

PSA level is a continuous parameter since the higher the value, the more likely the existence of PCa. However, the results from a US prevention study have shown that many men, even with low levels of serum PSA are prone to develop PCa (Thompson et al., 2004). Several approaches of serum PSA value have been suggested in order to improve PSA specificity in the early detection of PCa, including PSA density; measurement of PSA velocity; age-specific PSA; levels of free/total PSA and PSA molecular forms (Heidenreich et al., 2011). According to the published guidelines by the EAU, a free/total ratio < 20% and a PSA velocity > 0,75ng/mL/yr in combination with high PSA levels are the full criteria to dictate a high-risk man that must be submitted to a prostatic biopsy (Heidenreich et al., 2008). Given the lack of specificity and unclear benefit of PSA testing, alternative markers and methods are required to avoid the overdiagnosis and overtreatment of patients with elevated levels of this prostate-specific antigen (Boyd et al., 2012).

5.2. DRE (Digital Rectal Evaluation)

This was the first and sole ancillary-tool of screening men for PCa before the PSA testing era. Prostate cancer could be detected by a suspect DRE alone, irrespective of the PSA level, in only about 18% of all patients (Carvalhal et al., 1999), but it does not have enough sensitivity to detect the small-volume tumors that are most amenable to cure. About 60% of the PCas detected by this method have already extended beyond the prostatic gland, being therefore at an advanced or even metastatic stage (Epstein, 2010). Taking into account the above reasons, it is important to have an optimized diagnostic strategy using a combination of DRE and serum PSA testing in order to increase the yield of PCa diagnosis.

5.3. (TRUS) Transrectal Ultrasound-Guided Core Biopsy

Apart from other ancillary-tools for the detection of PCa, it is known that the diagnosis of this pathology is based on histological examination (van der Kwast et al., 2003). Therefore, TRUS-guided biopsy has become the recommended method in cases of suspected PCa, since it is the standard way to obtain material to histopathological examination of the prostatic gland (Hara et al., 2008; Takenaka et al., 2008). Recently, studies have shown that protocols used at first biopsy should include be 10 to 12 cores, in

order to increase the PCa detection rate (Eichler et al., 2006; Hara et al., 2008). Moreover, collecting more than 12 cores does not bring significantly more conclusive results (Eichler et al., 2006).

Needle biopsies, unlike fine-needle aspiration (FNA), provide more specific information about tumor extent and sometimes about extra-prostatic extension and seminal vesicles invasion. So, it is of extreme importance that, before any treatment of localized PCa, the diagnosis should be confirmed by core biopsies (Eble et al., 2004). The main disadvantage of needle biopsies is the small size of the tissue available for histological and molecular cytogenetic analysis and the low tumor representativeness (difficulty of identification of few malignant glands among many benign glands) (Epstein, 1995).

5.4. Prognostic factors

5.4.1. Grading

In 1966 Donald F. Gleason created a unique grading system for prostatic adenocarcinoma based on the architectural pattern of tumor growth, as well as on the extent of glandular differentiation (as seen under low-magnification) (Gleason and Mellinger, 1974; Mellinger et al., 1967). The Gleason grading comprises five histological grades of cancer on a scale from 1 (most differentiated) to 5 (poorly differentiated) **(Figure 10; Table 2).**

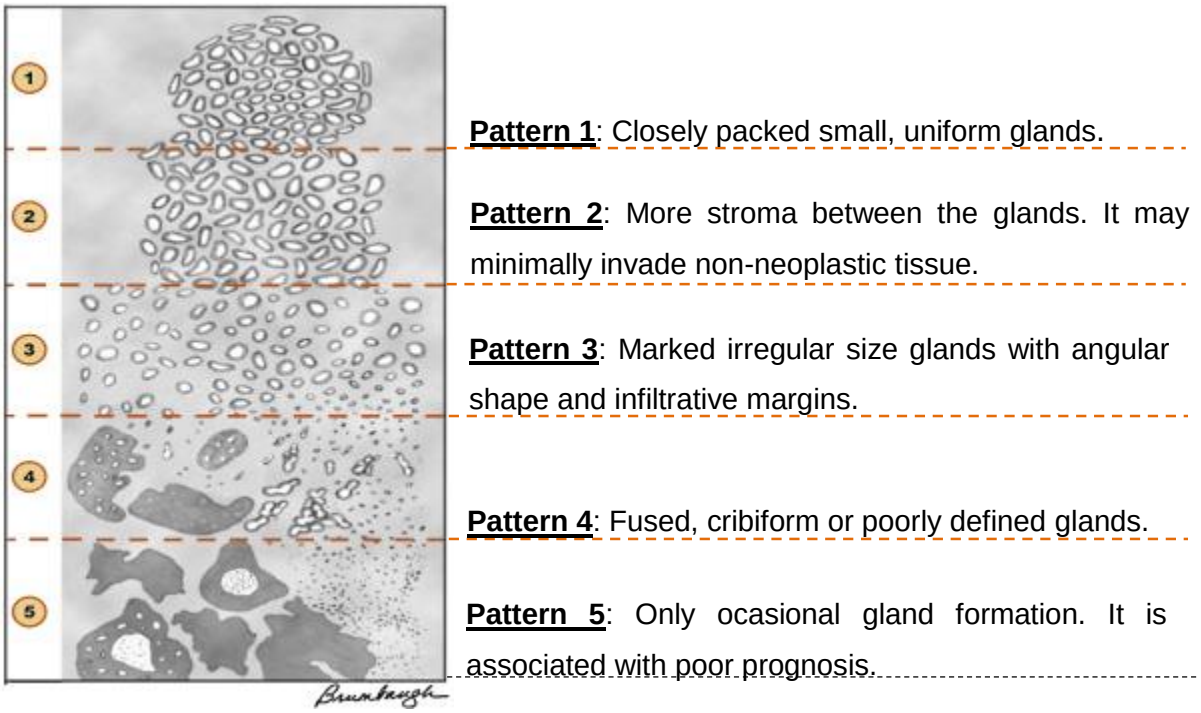


Figure 10 - Updated Gleason score diagram (adapted from (Epstein, 2010))

Grade is one of the strongest predictors of biological behavior (invasiveness and metastatic potential), thus having an important clinical significance to help therapeutic decision making. More than 50% of prostate tumors are characterized by heterogeneity with regard to pathologic development, molecular abnormalities, and clinical outcome (Clark and Cooper, 2009). So, the recognition of this heterogeneity, by Gleason, led to the incorporation of the two dominant and most common grades into his system, establishing the Gleason score (GS). This score reflects better the prognosis of the patient and consists in the addition of both primary (the most predominant) and secondary patterns (the second most frequent). In consequence, the GS system comprises four stages of differentiation that range from 2 to 10 **(Table 2)** (Gleason and Mellinger, 1974). Regarding the prognostic significance **(Table 2)**, a tumor harboring a GS 2 to 6 has better prognosis as it is well to moderately-well differentiated, a Gleason 7 tumor is moderately to poorly differentiated associated with an intermediate prognosis, and a Gleason 8 to 10 tumor is poorly differentiated and is associated with a worse prognosis (Humphrey, 2004). Therefore, the higher the score the more aggressive the tumor is and the higher the chance for the patient to have a poor outcome (Humphrey, 2004).

Table 2 - Relationship between Gleason Score and prognostic significance (adapted from (Humphrey, 2004)).

Gleason score	General terminology
2 - 4 (1+1, 1+2, 2+2)	Well differentiated
5 - 6 (2+3, 3+3)	Intermediately differentiated
7 (3+4, 4+3, 2+5)	Moderately differentiated
8 – 10 (4+4, 4+5, 5+5)	Poorly differentiated

5.4.2. Staging

Staging is the grouping of diseases into broad categories based on the extent of disease, being helpful for the prediction of prognosis, as well as to delineate the more appropriate therapeutic strategy. Current clinical and pathological staging of early PCa relies on the palpability of the tumor by DRE and PSA measurement. Therefore, the most widely used staging system for PCa is the TNM (Tumor-Node-Metastasis) proposed by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) **(Table 3)**. This system takes into account the extent of the primary tumor (T), the involvement of regional lymph node (N) and the presence or absence of metastasis at distant sites of the body (M). Stage could also be reported based on the clinical evidence obtained before the treatment, by using minimally invasive methodologies (clinical stage), or through

pathological findings after surgical remove of the prostate gland (pathological stage). The latter could estimate the prognosis (Edge and Compton, 2010). It is relevant to distinguish intracapsular tumors (stages T1, T2) from extracapsular tumors (T3a, T3b), because it has a strong impact in the therapeutic decision, since only organ confined tumors have a potentially curative treatment (Noldus et al., 2000).

Table 3 - Overview of the TNM / pTNM staging system for PCa (adapted from (Edge and Compton, 2010)).

Primary Tumor (T)	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically inapparent tumor not palpable or visible by imaging
T1a	Tumor incidental histologic finding in 5% or less of tissue resected
T1b	Tumor incidental histologic finding in more than 5% of tissue resected
T1c	Tumor identified by needle biopsy (e.g. because of elevated PSA)
T2	Tumor confined within prostate
T2a	Tumor involves one half of one lobe or less
T2b	Tumor involves more than half of one lobe, but not both lobes
T2c	Tumor involves both lobes
T3	Tumor extends beyond the prostate
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicules such as external sphincter , rectum bladder, levator muscles, and/or pelvic wall
Pathological (pT)*	
pT2	Organ confined
pT2a	Unilateral, involving one-half of one lobe or less
pT2b	Unilateral, involving more than one-half of one lobe but not both lobes
pT2c	Bilateral disease
pT3	Extraprostatic extension
pT3a	Extraprostatic extension
pT3b	Seminal vesicle invasion
pT4	Invasion of bladder, rectum
Regional Lymph nodes (N)	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metasatsis
Pahologic (pN)	
pNx	Regional nodes not sampled
pN0	No positive regional nodes
pN1	Metastases in regional node(s)
Distant metastasis (M)	
M0	No distant metástases
M1	Distant metastasis
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

**There is no pathologic T1 classification*

6. Treatment Options for Prostate Cancer

The aim of treatment is to “cure” the cancer or to prolong survival in patients with advanced disease, while preserving the highest possible quality of life in both the long and short term. There are many treatment options depending on age, stage and grade of cancer, PSA level, as well as patient comorbidity and personal preferences (Siegel et al., 2012). According to the evaluation of these features, several therapeutic options are available for PCa patients: active surveillance, radical prostatectomy, radiotherapy, hormone therapy and chemotherapy. Surgery, external beam radiation or brachytherapy may be used to treat early stage disease. More advanced disease is usually treated with androgen deprivation therapy, chemo and radiation therapy or a combination of them (American Cancer Society: Cancer Facts and Figures 2012, 2013).

6.1. Active surveillance

Active surveillance (AS) does not mean the same as watchful waiting (WW) and the two treatment options must be differentiated. The latter is a reasonable and commonly recommended approach, with noncurative intent in patients that are unlikely to benefit from aggressive local therapy (Bhatnagar and Kaplan, 2005). The former must be seen as a suitable therapy for those who also be offered a curative approach (Heidenreich et al., 2011). This treatment option aims to reduce the ratio of overtreatment in patients with clinically localized low-risk PCa based on early data (Albertsen et al., 1998; Chodak et al., 1994) revealing that men with well-differentiated PCa have a survival rate of 80-90%. Some data has recently demonstrated that men with a low-risk PCa and a life expectancy > 10 years are good candidates for AS, whereas only 30% of men will required delayed radical intervention (Klotz et al., 2010).

In conclusion, those men with a clinically localized PCa (T1-T2), a GS ≤ 6; three or fewer biopsies involved with cancer, ≤ 50% of each biopsy involved with cancer and a PSA = 10ng/mL are potential AS patients.

6.2. Radical prostatectomy

Surgical removal of prostatic gland and seminal vesicles is the only potential curative treatment for patients with normal erectile function, clinical localized PCa (Aus et al., 2005). It is recommended in men aged younger than 65 years (about 57%), with a life expectancy ≥ 10 years, low comorbidities, with moderately and poorly differentiated tumors and clinical stage T1 - T2 disease (Aus et al., 2005; Bhatnagar and Kaplan, 2005). The risk of lymph node involvement is low in men with low-risk PCa and <50% positive biopsy cores

(Heidenreich et al., 2011). However, an extended pelvic lymphadenectomy should always be performed in those men with intermediate and high-risk PCa (Briganti et al., 2006).

6.3. Radiotherapy

In Europe, the 1990s saw the introduction of three-dimensional conformal radiotherapy (3D-CRT), the gold standard option for men with advanced localized PCa (Aus et al., 2005) that are no longer candidates to undergo radical prostatectomy. This fact is mainly due to advanced age, thus having a high-risk of not achieving surgical complete clearance or patient preference. At the onset of the third millennium, intensity modulated radiotherapy (IMRT), an optimized and image-guided form of 3D-CRT, is becoming more widely used (Aus et al., 2005; Heidenreich et al., 2011).

Radiotherapy can be effectively delivered by both external beam therapy and brachytherapy. The former uses an external dose-escalated irradiation scheme that offers the same long-term survival results and provides a quality of life at least as good as surgery (Fowler et al, 1996). The latter consists in the interstitial implantation of radioactive seeds in the prostate gland and is offered to patients that have a low-volume and low-grade prostate tumors (Heidenreich et al., 2011; Norderhaug et al., 2003).

6.4. Hormonotherapy

Early metastatic PCa may be controlled by hormone therapy, for long periods, through shrinking the size or limiting the growth of the cancer, thus helping to relieve the pain, since curative intent is no longer an option (American Cancer Society: Cancer Facts and Figures 2012, 2013). Nowadays, in human patients, available options for PCa treatment aim to inactivate the *AR* by androgen deprivation, through surgical (bilateral orchiectomy) or either chemical castration (luteinizing hormone release hormone (LHRH) agonists), blockade with antiandrogens such as flutamide, bicalutamide or even estrogens (Koochekpour, 2011; Shen and Abate-Shen, 2010). In fact, androgen-deprivation is the only reliable treatment approach for advanced PCa, being effective in about 80-90% of men in this stage of the disease (median-free survival of 12 to 33 months) (Denis and Murphy, 1993). As it had been shown by Huggins and colleagues in the 1940s, removal of testicular androgens by surgical or chemical castration will lead to regression of prostate tumors. However, androgen-depletion is usually associated with the recurrence of PCa, as monitored by rising PSA levels, being this recurrent disease termed “castration-resistant” (Huggins and Hodges, 1941; Huggins and Hodges, 2002), which is sensitive to androgens and responsive to a 2º line of hormonal treatment. On the other hand, in hormone-resistant PCa (HRPC) chemotherapy

with docetaxel should be considered (Mottet et al., 2011). More recently, hormonal therapy has been offered as adjuvant therapy in combination with radical prostatectomy or radiotherapy, but only for the latter case it has shown improvement of survival in patients at this advanced stage of PCa.

6.5. Chemotherapy

Chemotherapy in PCa patients, including cytotoxic agents, is an active area of research. The most appropriate indication for chemotherapy is the clinical scenario of symptomatic metastases. In patients with skeletal metastases or a rapid PSA doubling time (DT) < 6 months, primary docetaxel should be considered (Mottet et al., 2011). Because all patients with castration-resistant PCa (CRPC) who received docetaxel-based chemotherapy progresses within 6-8 months, alternative treatments, including vinorelbine, mitoxantrone, or molecular-target therapy are under investigation and might be considered (de Bono et al., 2010; Fizazi et al., 2010; Sternberg et al., 2009)

7. The Prostate Cancer Genetics

7.1. Epigenetic alterations

Being a cause of changes in gene expression, epigenetic perturbations are believed to represent important contributing factors in prostate carcinogenesis, and may provide useful biomarkers for disease progression (Li et al., 2005; Nelson et al., 2009; Nelson et al., 2007). These epigenetic mechanisms occur with advancing age in the prostate (Kwabi-Addo et al., 2007), early in prostate carcinogenesis (Yegnasubramanian et al., 2004), coordinately (Florl et al., 2004) and influence crucial processes in tumor formation. Although the cause of these epigenetic events remains unclear, some evidences suggest that these epigenetic regulatory mechanisms appear sensitive to external factors, including diet and oxidative stress, and consequently may act as interpreters of the effect of these environmental effects in prostate carcinogenesis (Aitchison et al., 2007; Herceg, 2007).

There are three main interacting epigenetic phenomena: DNA methylation, histone modification and micro-RNAs (miRNAs). Regarding the first epigenetic phenomena, focal hypermethylation of critical genes has attracted most interest on deducing the pathogenesis of PCa (Joshua et al., 2008). DNA methylation has been implicated in silencing genes involved in diverse tumor processes, namely signal transduction, hormone response, cell

cycle control, and oxidative damage response, such as *GSTP1* (Joshua et al., 2008; Li et al., 2005; Shen and Abate-Shen, 2010).

Histone modifications, such as acetylation and methylation, are other relevant epigenetic alterations. It is known that prostate tumors display global changes in chromatin modification coincident with cancer progression (Ke et al., 2009; Kondo et al., 2008). One prominent and key modification associated with prostate carcinogenesis is trimethylation of lysine 27 of histone H3 (H3K27-me3), mediated by the histone methyltransferase *EZH2.* This polycomb group gene *EZH2* **(Figure 5)** (Shen and Abate-Shen, 2010) is a key oncogenic driver, being up-regulated in advanced disease, in some cases through amplification, and metastases, thus being associated with aggressive tumors (Bachmann et al., 2006) and worse prognosis (Saramaki et al., 2006; Varambally et al., 2002; Yu et al., 2007). Moreover, global changes in histone modifications are also associated with cellular senescence (Funayama and Ishikawa, 2007).

Short (~22bp) non-coding RNAs (miRNAs) are known to regulate both normal processes of growth and development and pathogenic processes associated with cancer. Furthermore, several authors have performed expression profiling studies of human prostate tumors and xenografts, suggesting that the expression patterns of miRNAs may distinguish indolent from aggressive tumors (Ambs et al., 2008; Coppola et al., 2010; DeVere White et al., 2009; Ozen et al., 2008; Porkka et al., 2007), as well as have implicated specific miRNAs in CRPC (Shi et al., 2007; Sun et al., 2009).

7.2. Germline mutations and polymorphisms

Several studies have shown a familial aggregation of PCa and this could be explained by the inheritance of gene mutations that cause this disease. Some PCa susceptibility loci were recently identified by microsatellite-based linkage studies **(Table 4)**, including the hereditary prostate cancer 1 (*HPC1)* locus on 1q23-25 (harboring the *RNASEL* gene) (Carpten et al., 2002; Smith et al., 1996; Xu, 2000); the predisposing for cancer prostate (*PCAP*) locus on 1q42-43 (Berthon et al., 1998); the cancer prostate and brain (*CAPB*) locus on 1p36 (Gibbs et al., 1999); the *HPC2* locus on 17p (which *ELAC2* is the candidate allele) (Tavtigian et al., 2001); the *HPC20* locus on 20q13 (Berry et al., 2000) and the *HPCX* locus on Xq27-28 (Xu et al., 1998). Last but not least, arises the identification of a new susceptibility region at 17q21 (harboring the *BRCA1* gene) (Gillanders et al., 2004), discovered when the results of several linkage studies (Lange et al., 2003; Schleutker et al., 2003; Wiklund et al., 2003; Xu et al., 2003; Xu et al., 2001) were reanalyzed as a combined dataset based on 426 families with hereditary PCa (Gillanders et al., 2004). Single nucleotide

polymorphism (SNP) based linkage analyses confirmed that chromosome 2, chromosome 6, region 8p22-23, and chromosome 12 harbor PCa susceptibility loci. A new candidate gene was identified by Xu *et al* – pannexin 1 (*PANX1*, also known as *MSR1)* mapping to 8p22-23 (Xu et al., 2001).

Recently, a new susceptibility gene, homeobox B13 *HOXB13*, for the locus 17q21-22 was discovered by Ewing (Ewing et al., 2012), which harbors a rare mutation (G84E) that is associated with an increased risk of familial PCa (Boyd et al., 2012). *HOXB13* is a homeobox transcription factor with a crucial role in normal prostate development. However, its function on prostate carcinogenesis is still controversial, since it has been implicated as both a TSG and an oncogene (Kim et al., 2010a; Kim et al., 2010b).

Table 4 - Prostate cancer susceptibility loci discovered by linkage analysis. Nevertheless, some of these linkage loci HPC1, PCAP, and CAPB (chromosome 1) and the HPC2 (chromosome 2) revealed difficulty to replicated with confidence (adapted from (Boyd et al., 2012)).

Chromosomal position	Susceptibility locus	Associated gene
1p36	*CAPB*	-
1q23-25	*HPC1*	*RNASEL*
1q42-43	*PCAP*	-
2q23	-	-
4q21	-	-
5q31	-	-
6p22	-	-
7p21	-	-
7q32.1	-	-
8p22-23	-	*MSR1*
8q24	-	-
9q34	-	-
12p13-14	-	-
16q23	-	-
17p	*HPC2*	*ELAC2*
17q21-22	-	*HOXB13*
17q21	-	*BRCA1*
19q12-13.11	-	-
20q13	*HPC20*	-
22q12.3-13.1	-	*APOL3*
Xq27-28	*HPCX*	-

With the development of SNP array technology and in order to identify common variants, at multiple loci, that have a moderate effect on PCa risk, a powerful tool has emerged - genome-wide association study (GWAS) (Eeles et al., 2009; Gudmundsson et al.,

2009; Gudmundsson et al., 2007; Gudmundsson et al., 2008; Kader et al., 2009; Takata et al., 2010; Thomas et al., 2008). Study data from the first two PCa GWASs highlighted a 3.8 Mb region on the 8q24 chromosome band, comprising the oncogene *MYC* (Amundadottir et al., 2006; Freedman et al., 2006). Consequently, at least nine SNP loci were shown to be independently associated with PCa risk, mapping to this chromosome region, in addition to 29 predisposition SNP loci in other chromosomes (Ahn et al., 2011; Al Olama et al., 2009; Amundadottir et al., 2006; Gudmundsson et al., 2007; Kote-Jarai et al., 2011). Taking into account the later GWAS performed by Kote-Jarai and colleagues, that have identified seven new PCa susceptibility loci at 2p11, 3q23, 3q26, 5p12, 6p21, 12q13 and Xq12 (Kote-Jarai et al., 2011), in total nearly 50 PCa susceptibility loci have now been identified (Gudmundsson et al., 2008; Kote-Jarai et al., 2011; Thomas et al., 2008).

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7.3. Somatic genetic alterations

A central goal in cancer research is to identify genes that play a key role in cancer progression, either by being mutationally inactivated or downregulated, such as TSGs or caretakers/gatekeepers, or by being activated, such as oncogenes **(Figure 11) (Table 5)**.

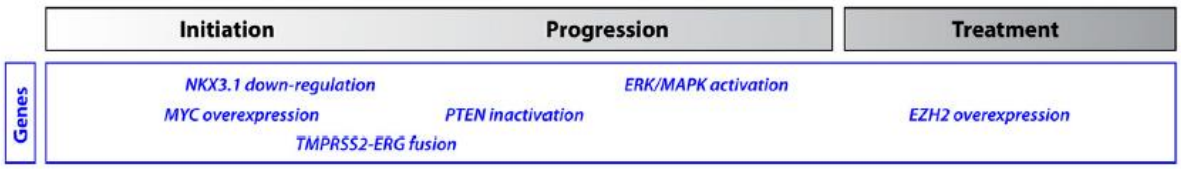

Figure 11 - Human prostate cancer progression pathway. Each stage of disease progression is accompanied by gene/pathways that are considered to be important in each stage (adapted from (Shen and Abate-Shen, 2010)).

7.3.1 Negative regulators of carcinogenesis: tumor suppressor genes

Among all the well-established TSGs in other neoplasias, only tumor protein p53 (*TP53)* and phosphatase and tensin homologue (*PTEN)* have been shown to play a role in prostate carcinogenesis, where losses of 17p13 and 10q23, respectively, are indeed observed in advanced carcinomas. Mutations in the *TP53* are present in a minority of primary tumors (10-20%) and may undergo clonal selection during the process of progression to metastatic PCa (20-30%) of advanced localized prostate tumors (Brooks et al., 1996; Grignon et al., 1997; Quinn et al., 2000; Stapleton et al., 1997).

PTEN encodes a phosphatase active against both proteins and lipid substrates. The mechanism by which *PTEN* might act as a TSG in the prostate may involve the inhibition of the phosphatidylinositol 3´-kinase-protein kinase B (PI3K-AKT) signaling pathway that is

essential for cell-cycle progression and cell survival, allowing normal cell death (Furnari et al., 1998; Li and Sun, 1998; Ramaswamy et al., 1999; Sun et al., 1999).

Furthermore, recent studies have investigated *PTEN* copy number, mutational *status*, and/or protein expression in primary and castration-resistant tumors using multiple experimental approaches (Schmitz et al., 2007; Sircar et al., 2009; Taylor et al., 2010; Verhagen et al., 2006). These studies, in consensus with previous reports, have concluded that *PTEN* undergoes copy number loss as an early event in prostate carcinogenesis **(Figure 11)**, and is correlated with progression to aggressive, castration-resistant disease (Shen and Abate-Shen, 2010). Moreover, mutations on *PTEN* gene are more common (30-60%) in metastatic samples than they are in primary tumors (5-27%) (Karan et al., 2003; Suzuki et al., 1998), being correlated with a high grade (GS) and stage (McMenamin et al., 1999). Low levels of *PTEN* activity may be retained in PCa – an observation that parallels the NK3 homeobox 1 (*NKX3.1*) haploinsuficiency and the p27 cell regulator (Abate-Shen et al., 2008; Gao et al., 2004).

Regarding the *in vivo* models, germline loss of *PTEN* in heterozygous mutants or conditional deletion in the prostate epithelium results in PIN and/or adenocarcinoma (Di Cristofano et al., 1998; Podsypanina et al., 1999; Trotman et al., 2003; Wang et al., 2003). Moreover, the inactivation of *PTEN* has been shown to cooperate with loss of function of the *NKX3.1* homeobox gene, up regulation of *MYC*, or the *TMPRSS2-ERG* fusion (Carver et al., 2009; Kim et al., 2009; Kim et al., 2002; King et al., 2009).

On the other hand, further investigations of *PTEN* loss – together with perturbations of cell cycle regulators such as p27, p18 ^{ink4c}, and p14 ^{arf} (Bai et al., 2006; Chen et al., 2009; Di Cristofano et al., 2001) or components of key signaling pathways like *RHEB*, *TSC2* and *RICTOR* (Guertin et al., 2009; Ma et al., 2005; Nardella et al., 2008) – have emphasized the relevance of haploinsuficiency in PCa context (Shen and Abate-Shen, 2010). Notably, *PTEN* reduction or loss in PCa actually predisposes to the emergence of CRPC (Mulholland et al., 2006; Shen and Abate-Shen, 2007). While this may reflect the ability of *PTEN* to interact with androgen receptor (*AR*), the mechanistic details by which *PTEN* loss promotes castration-resistance remains incompletely understood (Karan et al., 2003; Shen and Abate-Shen, 2010).

Another well-studied candidate in recurrently deleted genomic region is *NKX3.1*. No “gatekeeper” genes for the development of PCa, analogous to the adenomatous polyposis coli (APC) gene in colorectal cancer, have been identified (Kinzler and Vogelstein, 1997). *NKX3.1*, which maps to the 8p21 locus, encodes a homeobox gene that is likely to be essential for prostate development and is therefore a candidate gatekeeper gene (Bieberich et al., 1996; Sciavolino et al., 1997). Moreover, its relevance to prostatic carcinogenesis was originally described on the basis of loss of heterozygozity (LOH) of chromosome 8p21 in up

to 85% of HGPIN lesions and adenocarcinomas (Bethel et al., 2006; Emmert-Buck et al., 1995; Haggman et al., 1997; Swalwell et al., 2002; Vocke et al., 1996). Notwithstanding, LOH of 8p21 progressively increases in frequency with cancer grade **(Figure 11)**, but the remaining allele of *NKX3.*1 remains unmutated (Bethel et al., 2006; Ornstein et al., 2001; Vocke et al., 1996; Voeller et al., 1997). Furthermore, one study has reported *NKX3.1* deletion or loss of function in 20% of PIN lesions, 34% of androgen-independent PCas and in 78% of PCa metastases, justifying why it has been correlated with disease progression (Bowen et al., 2000). Therefore, it has been concluded that there is an association between 8p deletions and *NKX3.1* expression in more advanced PCa, suggesting that genetic deletions may be more important in the progression of invasive disease whilst decrease *NKX3.1* expression is more important in initial stages of the disease (Joshua et al., 2008). Once more regarding the expression of this gatekeeper gene in PCa, although early studies had suggested that *NKX3.1* expression is completely lost in advanced cancers (Bowen et al., 2000), recent analyses, using a highly sensitive antibody, indicate that low levels of *NKX3.1* expression can be demonstrated in nearly all PCas and metastases examined (Gurel et al., 2010). Therefore, it appears to be a selection for reduction, but not loss, of *NKX3.1* expression during PCa progression.

Moving to the functional studies, analyses of *NKX3.1* in human tumor cells and genetically engineered mice have provided insights into its potential role in cancer initiation. When *NKX3.1* was inactivated in mice, it resulted in a defective response to oxidative damage, while its expression in human PCa cell lines protects against DNA damage and is regulated by inflammation (Bowen and Gelmann, 2010; Markowski et al., 2008; Ouyang et al., 2005). *NKX3.1* represents a haploinsuficiency TSG that behaves has a gatekeeper gene in prostate carcinogenesis initiation (Gelmann, 2003; Kim et al., 2002; Magee et al., 2003).

Other TSGs with known somatic mutations in PCa are identified in the table below **(Table 5)** and particularly *RB1*, *CDKN2A* and *TP53* are inactivated in hormone-refractory and metastatic tumors, suggesting a role of these genes in PCa progression.

7.3.2 Elevated expression and gain of function: oncogenes

Regarding the somatic “gain-of-function” alterations identified in PCa, the most frequent oncogenic changes belong to the category of amplifications, such as the 8q24 and Xq11 loci. These chromosome regions encompass putative target genes, such as *MYC* and *AR*, respectively, both well-known proto-oncogenes.

The *MYC* (avian v-myc myelocytomatosis viral oncogene homolog) gene is a member of the basic Helix-loop-Helix Leucine Zipper (b-HLH-LZ) family of transcription factors that plays a role in cell-cycle progression, apoptosis and cellular transformation (Grandori et al.,

2000; Lavigne et al., 1998). It is known that both overexpression and amplification of *MYC* are more prone to occur in recurrent and metastatic lesions than in primary tumors. In order to corroborate this fact, Jenkins et al, by using a Fluorescence *In Situ* (FISH) approach to detect *MYC* amplification and chromosomal abnormalities, have stated that amplification was identified in 21% of metastatic and in only 8% of carcinoma foci, but not in PIN foci (Jenkins et al., 1997). Similarly, Visakorpi and colleagues (1995) found gains of 8q more frequently in locally recurrent cancer, rather than in primary cancer (Visakorpi et al., 1995) and amplification of 8q was observed in 75% of lymph node metastases (Van Den Berg et al., 1995). Moreover, Nupponen et al have demonstrated that the 8q24 gain was present in up to 90% of advanced tumors (Nupponen et al., 1998), thus correlating with high histological grade (Jenkins et al., 1997). Later on, Ribeiro et al, have shown that 8q24 gain is an independent predictor of poor survival in PCa patients (Ribeiro et al., 2007). However, Gurel *et al* (2008) have recently suggested a role for *MYC* overexpression in cancer initiation, as nuclear *MYC* protein is up-regulated in many PIN lesions and the majority of carcinomas in the absence of gene amplification (Gurel et al., 2008).

These results may be consistent with the identification of a major susceptibility locus at 8q24 in several large-scale GWASs of PCa, as well as other epithelial cancers (Al Olama et al., 2009; Amundadottir et al., 2006; Freedman et al., 2006; Gudmundsson et al., 2009; Gudmundsson et al., 2007), but detailed analyses have not yet revealed any correlation between risk alleles and *MYC* RNA expression levels in prostate tumors samples, or even the presence of any non- protein-coding genes such as miRNAs (Pomerantz et al., 2009). Nonetheless, long-range regulatory elements for *MYC* have been recently identified in this region, arising de possibility that the risk alleles may disturb the regulation of *MYC* (Jia et al., 2009; Sotelo et al., 2010). Regarding *MYC* regulation/ repression, another study conducted by Wang and colleagues has showed that the X-linked forkhead box P3 gene (*FOXP3*) encodes a winged helix transcription factor that represses *MYC* expression, being itself mutated in PCa (Wang et al., 2009).

At the functional dimension, it was proved by Ellwood-Yen et al that transgenic mice expressing human *MYC* display rapid development of PIN, followed by progression to invasive adenocarcinoma despite having rare metastases (Ellwood-Yen et al., 2003). Interestingly, bioinformatic analyses have identified an expression signature characterized by down-regulation of *NKX3.1* and up-regulation of *PIM1*, which is an oncogene known to collaborate with *MYC* in lymphomas (Ellwood-Yen et al., 2003). In consistence with these data, lentiviral coexpression of human *MYC* with mouse *PIM1* in tissue recombinants results in cooperative formation of carcinomas with neuroendocrine differentiation (Wang et al., 2010). Taking into account all these reports, it is suggested that both overexpression and

amplification of the *MYC* gene may play a role in the progression and evolution of prostate carcinoma.

Another “gain-of-function” gene implicated in recurrence of PCa is *AR*, located in the Xq11 locus. Although somatic *AR* mutations are rarely detected in early stage disease, mutation or amplification is significantly increased in advanced androgen-independent tumors, thus suggesting a role of *AR* mutations in tumor progression (Feldman and Feldman, 2001; Koivisto et al., 1997). About 20-30% of the castration-resistant PCas are *AR* mutated or amplified (Boyd et al., 2012).

One question that persists is how can *AR* gene continue to drive the growth of androgen-depleted cells? Several mechanisms have been proposed and one possible explanation for this question is the occurrence of mutations in the receptor that cause its constitutional activation or, in turn, enable activation by other alternative ligands like steroids (Feldman and Feldman, 2001; Gregory et al., 2001; Linja et al., 2001). Over 70 different somatic missense *AR* mutations have been described, but only a few have been functionally studied (Gottlieb et al., 2004).

Table 5 - Common somatic genetic changes in prostate cancer (adapted from (De Marzo et al., 2007; Nelson et al., 2003)

Gene and gene type	Location	Biochemical function	Cellular function
Tumor suppressor genes			
CDKN1B	12p12-13	CDK inhibitor	Cell cycle
PTEN	10q23.31	Protein/lipid phosphatase	Signaling
TP53	17p13.1	Transcription factor	Growth arrest/apoptosis
Oncogenes			
MYC	8q24	Transcription factor	Cell proliferation
AR	Xq11-12	Androgen receptor	Signaling
Caretaker genes			
GSTP1	11q13	Glutatione transferase	Detoxification metabolism
Gatekeeper genes			
NKX3.1	8p21.2	Transcription factor	Cell proliferation and differentiation
Other somatic changes			
PTGS2*, *APC*, *RB1*, *BRAF*, *PIK3CA*, *CHEK2	Various		

Abbreviations: CDK, cyclin-dependent kinase.

7.3.3. Chromosomal copy number changes in prostate cancer

Advances in molecular cytogenetics and genomics facilitated the characterization of common genomic alterations in prostate cancer and have been established as reliable sources of diagnostic and prognostic information (Rose et al., 2004; Squire et al., 2011). Prostatic tumors contain somatic mutations, including gene deletions, amplifications and chromosomal rearrangements (Nelson et al., 2003). In 2006, Ribeiro et al analyzed by comparative genomic hybridization (CGH) the genetic profile of a consecutive series of prostate needle biopsies obtained prospectively from 100 PCa suspects (Ribeiro et al., 2006a). By screening the whole genome at the same time, the advent of CGH provided the breakthrough in the field by having the capacity of identifying chromosomal regions affected by genomic imbalances (Kallioniemi et al., 1994). The pattern of copy number changes present in prostate tumors found by Ribeiro et al (2006) is depicted in **Figure 12**. Briefly, the most common copy number losses are 8p, 13q, 6q, 16q, 5q and 10q. Regarding the recurrent copy number gains, they were seen at 8q, 7q, 3q, 7p, 1q and 5p **(Figure 12)** (Ribeiro et al., 2006a). Therefore, these chromosomal regions are more prone to harbor oncogenes. Other more complex patterns, as well as an accumulation in the number of genomic gains and amplifications (Xq11.2-q12, *AR*), emerge in more advanced disease (Squire et al., 2011).

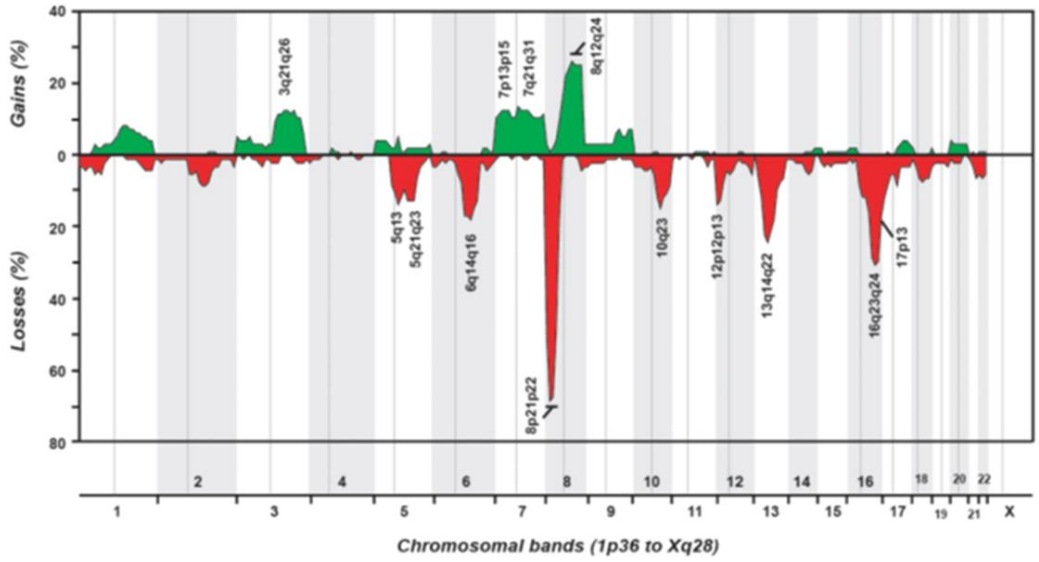

Figure 12 - Genomic findings in 61 prostate carcinomas in needle biopsies done in 100 prostate cancer suspects. Gains and losses of genetic material are depicted along all chromosomes (X axis), with the most frequently altered bands being indicated (adapted from (Ribeiro et al., 2006b)).

Moreover, Ribeiro *et al* (2006) has proposed a genetic pathway of PCa with two distinct initiating events, namely, 8p and 13q losses. These primary imbalances are then preferentially followed by 8q gain and 6q, 16q and 18q losses **(Figure 13)** which in turn are

followed by a set of late events that make recurrent and metastatic PCas genetically more complex (Ribeiro et al., 2006a).

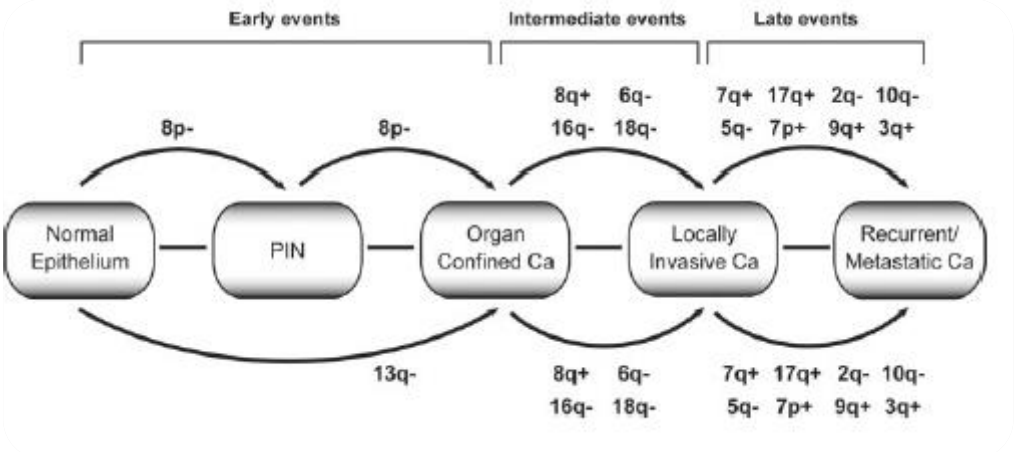

Figure 13 - Genetic model of prostate cancer progression based on genomic imbalances detected by comparative genomic hybridization. DNA copy number changes detected in abnormal prostate cancer samples were categorized as early, intermediate, or late events, according to time of occurrence and principal component analysis. Two potential pathways of genetic progression are proposed, one starting with 8p loss and the other starting with 13q loss (adapted from (Ribeiro et al., 2006a)

The fact that several of these genomic alterations have also been identified in both PIN and PIA lesions indicates the precursor relationship of these lesions to PCa (Shen and Abate-Shen, 2010).

8. Chromosomal Rearrangements and Gene Fusions

An important breakthrough in the search for novel pathogenic mechanisms in PCa was the finding of fusion oncogenes (Tomlins et al., 2005). The discovery that 50% of prostate cancers harbor recurrent gene rearrangements may enable molecular subtyping and identification of patients with aggressive disease (Boyd et al., 2012; Rubin et al., 2011). Often, these oncogenic fusions usually juxtapose a hormone-specific promoter that acts as an “on” switch for the oncogene, resulting in the deregulated gene expression, altered levels of expression or expression of chimeric proteins with transforming properties (Cooper and Fletcher, 2002; Rubin et al., 2011) The genes involved in these fusions are transcription factors, which in their altered form constitutively activate or inactivate specific target genes causing cellular transformation (Cooper and Fletcher, 2002). Thus, the discovery of the Erythroblastosis virus E26 transformation-specific (ETS) family transcription factor gene fusions by Tomlins et al (2005) dramatically changed the field of solid tumor biology.

8.1. ETS *family of transcription factors*

Twenty-nine human ETS transcription factor family members have been identified, subdivided into 5 subfamilies. All share a conserved 80-amino-acid DNA binding domain that recognizes the core DNA sequence 5´- GGA (A/T) - 3´ (Clark and Cooper, 2009). These transcription factors have the capacity to alter the expression of proteins involved in a range of pathways including stem cell development, cell senescence, proliferation, migration, apoptosis and tumorigenesis (Clark and Cooper, 2009). Although ETS gene translocations represent an early event in PCa, they seem to be insufficient on their own to induce cancer formation (Clark and Cooper, 2009).

8.2. TMPRSS2 – ERG fusion

Recent advances in the power of genomic profiling and bioinformatics have bypassed the technical limitations of cytogenetic analysis of solid malignancies. For example, Cancer Outlier Profile Analysis (COPA) has revealed recurrent chromosomal translocations between the ETS transcription factor family and the androgen-regulated transmembrane protease serine II *TMPRSS2* gene (Tomlins et al., 2005). Further, these genomic rearrangements leading to the formation of the *TMPRSS2*-ETS gene fusions are the most frequent alterations observed in PCa (Tomlins et al., 2005). Rearrangement of the *TMPRSS2* with ETS-related gene (*ERG*) has been recurrently found in about 50% of localized PCa (Albadine et al., 2009; Clark et al., 2008; Mao et al., 2010; Mosquera et al., 2008; Perner et al., 2006; Tomlins et al., 2005), and in 21% of precursor HGPIN lesions (Cerveira et al., 2006), becoming the principal genomic alteration and a characteristic signature of prostatic malignancies (Tomlins et al., 2009). Furthermore, this rearrangement either occurs after cancer initiation or, alternatively, corresponds to an early event at the transition between benign and PIN epithelium **(Figure 5)** (Perner et al., 2007). Although *TMPRSS2-ERG* fusions are detected less in PIN lesions than in tumor lesions, they are frequently detected in PIN lesions that are adjacent to fusion-positive tumors (Carver et al., 2009; Perner et al., 2007).

This *TMPRSS2-ERG* fusion (*TMPRSS2* exon 1 fused to *ERG* exon 4) results in overexpression of a 5´ truncated form of the *ERG* transcription factor under the control of the androgen-responsive promoter of *TMPRSS2* **(Figure 14)** (Clark et al., 2007; Clark and Cooper, 2009; Iljin et al., 2006; Perner et al., 2006; Tomlins et al., 2005; Wang et al., 2006).

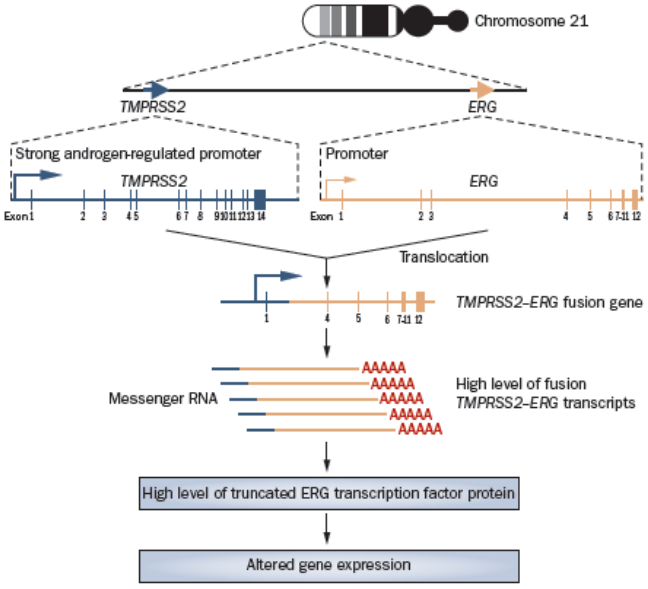

Figure 14 - Formation and consequences of *TMPRSS2-ERG* fusions. High level of truncated *ERG* transcript factor proteins is believed to cause alterations in expression of target genes (adapted from (Clark and Cooper, 2009)

Kezovitch et al have proposed that up-regulation of *ERG* in human prostate cancer activates cell invasion programs that subsequently displace prostate basal epithelium by the luminal cells and the development of PIN (Klezovitch et al., 2008).

Wang et al characterized in detail the expression of *TMPRSS2-ERG* fusion mRNAs and correlated the isoforms expressed and expression levels with clinical outcome in cancers from men undergoing radical prostatectomy. There are several possible transcripts of this fusion gene. Expression of an isoform, in which the native ATG in exon 2 of the *TMPRSS2* gene is in frame with exon 4 of the *ERG* gene, was associated with clinical and pathologic variables of aggressive disease. Expression of other isoforms, in which the native *ERG* ATG in exon 3 was the first in-frame ATG, was associated with seminal vesicle invasion, which is correlated with poor outcome following radical prostatectomy. Thus, both the isoforms of *TMPRSS2-ERG* fusions expressed and expression level may affect prostate cancer progression (Wang et al., 2006). The mechanism behind this rearrangement is either an interstitial deletion in 21q22.2-3 (Iljin et al., 2006; Mertz et al., 2007; Perner et al., 2006), where *TMPRSS2* and *ERG* are located ~3Mbp apart, or an insertion of the sequences between the two fusion partners into another chromosome (Bott et al., 2005; Teixeira, 2008). However, a translocation mechanism could also be possible **(Figure 15)** (Liu et al., 2007; Teixeira, 2008). The close proximity of these two genes explains why this common rearrangement had not previously been detected in conventional karyotypic analyses (Tomlins et al., 2005).

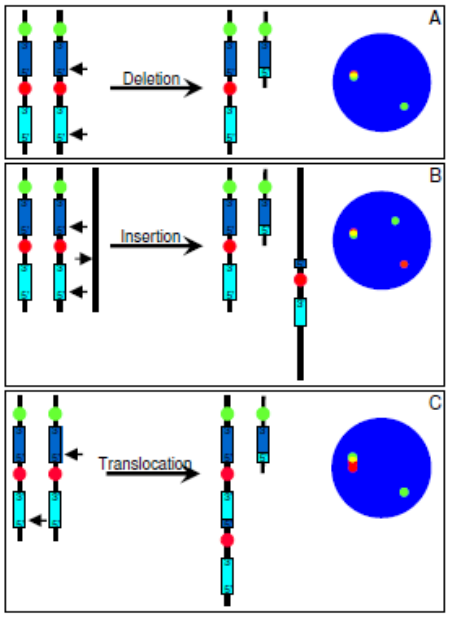

Figure 15 - Chromosome mechanisms giving rise to the TMPRSS2-ERG fusion oncogene (adapted from (Teixeira, 2008)).

Moreover, formation of these chromosomal rearrangements may be an indirect consequence of *AR* function, since studies in androgen-responsive LNCaP cells have shown that *AR* binding induces chromosomal proximity between the *TMPRSS2* and *ERG* loci that can lead to formation of *TMPRSS2-ERG* fusions following DNA damage (Lin et al., 2009; Mani et al., 2009). Additionally, and even in the absence of genotoxic stress, androgen signaling can recruit topoisomerase II to *AR*-binding sites and consequently lead to induction of double stranded breaks (Haffner et al., 2010). At the functional level, despite the prevalence of these genomic rearrangements, the functional significance of *TMPRSS2-ERG* fusion and other ETS rearrangements in PCa remains not fully resolved (Shen and Abate-Shen, 2010).

Recently, Yu et al performed whole-genome chromatin immunoprecipitation analyses and concluded that *ERG* has the ability to bind *AR* downstream target genes and disrupts *AR* signaling in PCa cells through epigenetic silencing, which is consistent with a role in inhibiting prostate epithelial differentiation (Yu et al., 2010). Furthermore, some authors performed analyses of ETS activation in cell culture assays as well as transgenic mice and suggested that this ETS activation promotes epithelium-mesenchyme transitions (EMT) and confers tumor-invasive properties (Klezovitch et al., 2008; Tomlins et al., 2008; Tomlins et al., 2007b; Wang et al., 2008), although the effects are relatively moderate.

With only a year of interval, both Tomlins and Klezovich reported that in transgenic mice, expression of truncated human *ERG* isoform (TrERG) resulted in a minimal or weak PIN phenotype after 5-6 months of age, but progression to PCa was not observed, suggesting that additional transforming events are required (Klezovitch et al., 2008; Tomlins

et al., 2007b). However, transgenic *ERG* overexpression in *PTEN* heterozygous mice (PTEN+/-) leads to rapid progression of HGPIN lesions to invasive carcinoma (Carver et al., 2009). On the other hand, King et al found that the same mice background only leads to the development of PIN lesions and a third molecular event would be needed to promote the progression to prostate carcinoma (King et al., 2009). Furthermore, these authors also report that transgenic expression of *ERG* in prostate epithelium is sufficient to induce PIN lesions, refuting what has been previously described. Additionally, Zong et al (2009) corroborate the results published by Craver et al (2009), further stating that overexpression of *MYC* in the prostate glands of TrERG mice, suggesting the oncogene *MYC* as one of the downstream effectors of *ERG*-mediated oncogenesis (Zong et al., 2009). Moreover, and corroborating this interpretation, Sun et al (2008) showed that overexpression of *ERG,* as a result of *TMPRSS2-ERG* fusion, in VCaP cells contributes to the neoplastic process upregulating *C-MYC* oncogene and by abrogating the differentiation of prostate epithelium as indicated by prostate epithelial markers, such as *PSA*, *SLC45A3* and *MSMB* **(Figure 16)** (Sun et al., 2008).

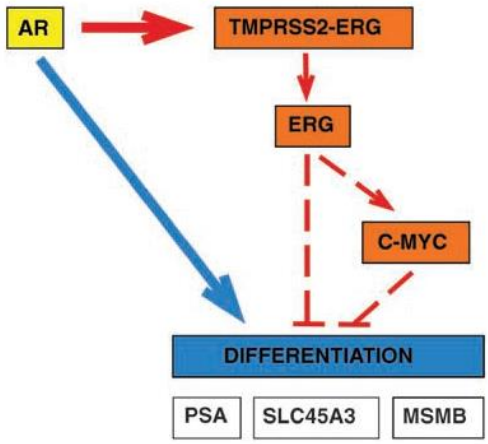

Figure 16 - Proposed model for *ERG* functions in prostate cancer. *ERG* upregulates C-*MYC* and interferes with differentiation of prostate cancer epithelial cells (adapted from (Sun et al., 2008)).

Another recent finding stated that *TMPRSS2-ERG* positive tumors are also correlated with the deletion of a small genomic region mapping to 3p14, suggestive of another cooperative interaction in tumorigenesis (Taylor et al., 2010). In the same year, using a high-resolution SNP array genomic copy number analysis, Mao et al have shown a difference in the frequency of the *TMPRSS2-ERG* fusion gene between Chinese and UK-based populations (Mao et al., 2010). Moreover, a difference in fusion gene frequency between Western and Asian countries has since been supported by studies involving Korean and Japanese populations (Lee et al., 2010; Magi-Galluzzi et al., 2011; Miyagi et al., 2010).

Finally, it can be concluded that ETS rearrangements are selected primarily for their ability to disrupt differentiation and/or to promote cancer progression along with the cooperation of other transforming events (Shen and Abate-Shen, 2010).

8.3. Other ETS *implicated in gene fusions*

Apart from *ERG,* which is the most common 3´partner, three other ETS family genes, namely *ETV1*, *ETV4* and *ETV5,* have also been found fused with *TMPRSS2* or with other 5´partners, although less frequently (Prensner and Chinnaiyan, 2009). Furthermore, Paulo et al (2012) has recently reported *FLI1* gene as the fifth ETS transcription factor involved in fusion genes in PCa. By using FISH probes flanking *FLI1* gene, RT-PCR and sequencing analyses, they could show that the 5´partner was *SLC45A3* (exon 1). So, *FLI1* is as a novel ETS transcription factor involved in gene fusions in PCa. Intratumor genetic heterogeneity of ETS rearrangements was occasionally found in index primary tumors (Paulo et al., 2012a).

In addition, a number of 5´ partners, including *SLC45A3, ERVK-24* (also known as *HERVK_22*), *HNRPA2B1, C15orf21, NDRG1*, *HERPUD1*, *ACSL3*, *HERVK17*, *CANT1*, *DDX5*, *KLK2*, *FOXP1*, *EST14*, and the chromosomal region 14q13.3-14q21.1 have been identified in *ETS* gene fusions (Clark and Cooper, 2009; Lapointe et al., 2007; Maher et al., 2009; Pflueger et al., 2009; Tomlins et al., 2006). By using a paired-end RNA sequencing approach, Dorothee and colleagues (2009) discovered a fusion involving the already mentioned androgen-inducible tumor suppressor *NDRG1* (N-myc downstream regulated gene 1) and *ERG* in two out of 101 PCa cases of men with localized and locally advanced disease (Pflueger et al., 2009). Furthermore, this fusion is predicted to encode a chimeric protein (Pflueger et al., 2009). Taking into account these evidences, PCa seems to be prone to recurrent gene fusions involving androgen-responsive genes (like *TMPRSS2*, *SLC45A3* and *NDRG1*) and ETS transcription factors (such as *ERG*, *ETV1* and *ETV4*).

Recently, Wu et al (2012) where able to identify a novel form of hybrid and aggressive PCa, involving the oncogene *MYC*, using genome and transcriptome sequencing. One individual was diagnosed with conventional but aggressive PCa and both primary and metastatic tissues were collected before hormone therapy. The transcriptome analyses revealed signatures of both luminal and neuroendocrine cell types. Remarkably, the repertoire of expressed but apparently private gene fusions, including *C15orf21* (also known as *HMGN2P46*) and *MYC*, recapitulated this biology. This luminal-neuroendocrine tumor appears to represent a novel and highly aggressive case of PCa with propensity for rapid progression to castrate-resistance (Wu et al., 2012). Moreover, Grandori and colleagues suggest that the ability of overexpressed *MYC* to facilitate proliferation and inhibit the final

differentiation fits well the fact that tumors of diverse origins contain genetic rearrangements involving *MYC* family genes (Grandori et al., 2000).

Next generation sequencing (NGS) technology has discovered novel gene fusions in PCa, including several non-ETS fusions such as *SLC45A3-BRAF* and *ESRP1-RAF1* (Maher et al., 2009; Palanisamy et al., 2010; Pflueger et al., 2009). These gene fusions are known to drive tumorigenesis, despite occurring at a low frequency (<5%) compared with *TMPRSS2-ETS* (Boyd et al., 2012). However, Palanisamy *et al* (2010) demonstrated that it is possible to treat cancers expressing these fusion genes with RAF or RAS/RAF/ERK pathway inhibitors (Palanisamy et al., 2010).

Upon analyzing genome-wide copy number change data from 77 PCa tumors, Boyd et al (2012), identified a higher frequency of known TSGs – including p53, *PTEN*, *BRCA1*, and *BRCA2* – than oncogenes at recurrent chromosome breakpoint, but have yet to determine whether any of these affected genes are fusion gene partners (Boyd et al., 2012; Mao et al., 2011).

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9. Genetic Prognostic Factors in Prostate Cancer

An ETS fusion gene is arguably the initial genetic event in a large subset of PCa. However, chromosomal instability, describing the cellular processes that increases the rate at which large portions of chromosomes are gained, lost or rearranged in tumors, appears to be more important in PCa (Squire et al., 2011). Thus, chromosome copy number changes, such as 8q gain (Barros-Silva et al., 2011) and 10q loss (involving *PTEN*) (Yoshimoto et al., 2008), may be more relevant as prognostic factors.

The strongest target oncogene for 8q gain is *MYC*, at cytoband 8q24 (Nupponen et al., 1998). Up to 90% of advanced tumors show 8q gain, when compared to 5% of organ confined tumors (Nupponen et al., 1998). It has been recently shown, using CGH, that 8q gain is an independent predictor of poor survival to PCa patients as patients whose tumors displayed this alteration are more likely to have an adverse outcome (Ribeiro et al., 2006a; Ribeiro et al., 2007). Given that CGH may be difficult to implement in the clinical practice, Ribeiro et al chose to test the feasibility of using a three-color fluorescent assay to access 8q status in diagnostic paraffin-embedded biopsy samples from PCa patients (Ribeiro et al., 2007).

Ribeiro et al (2007) used a standard FISH protocol with a dual-color probe flanking the gene *MYC* at 8q24 and a centromeric probe for chromosome 18 (CEP18) used as ploidy control. Relative 8q24 gain was assessed by a ratio between *MYC* and CEP18 signals within each nucleus of a representative cancer cell population. Cases categorized as having

relative 8q gain (whenever *MYC*/CEP18 ≥1.5) presented a significantly higher risk of dying from this malignance (Ribeiro et al., 2007). On the other hand, Barros-Silva et al (2011) evaluated the prognostic value of the *TMPRSS2-ERG* fusion gene combined with chromosome arm 8q relative gain in a consecutive series of diagnostic needle from patients with PCa. They were able to conclude, using FISH, that relative 8q gain around *MYC* was associated with poor disease-specific survival irrespective of *TMPRSS2-ERG* fusion gene status (Barros-Silva et al., 2011). Furthermore, Paulo et al, made correlation analyses with the clinico-pathological data and were able to show an association of *ERG* rearrangements with localized advanced disease and *MYC* overexpression and also an association of *ETV1* rearrangements with *PTEN* downregulation. *MYC* expression was higher and *PTEN* expression was lower in PCa with ETS rearrangements than in those without. Looking at *ERG* and *ETV1* rearrangements separately, *MYC* was significantly upregulated only in PCa harboring *ERG* rearrangement, while *PTEN* was downregulated only in PCa containing *ETV1* rearrangement (Paulo et al., 2012a). This strong positive correlation found by Paulo and colleagues between *ERG* and *MYC* expression in PCa with *ERG* rearrangement is in agreement with earlier reports showing that *MYC* is a downstream target of the overexpressed *ERG* transcription factor (Sun et al., 2008).

In the present year, the aim of the study by Fromont et al (2013) was to analyze, in a large cohort of PCa tissues, the amplification status of 8q24 at the *MYC* locus, together with the protein expression of *MYC* and other candidate genes located at 8q24, in order to correlate genomic amplification and protein expression status with disease stage, aggressiveness and recurrence after treatment. Fromont and colleagues reached the conclusion that 8q24 amplification at the *MYC* locus (found in 29% of the cases) correlated with *MYC* protein expression and was associated with disease progression, GS and cancer cell proliferation. Moreover, the amplification status, but not *MYC* protein expression was strongly predictive of biochemical recurrence after radical prostatectomy, independently from known prognostic factors, including TNM stage and GS, and could therefore be useful to better predict the outcome for both intermediate-risk (pT2 and Gleason 7) and high-risk Pca (Fromont et al., 2013).

AIMS

In order to better understand the role of *MYC* in prostate carcinogenesis, the specific aims of this study were:

- To characterize the relative copy number of the *MYC* oncogene in a series of prostatectomy specimens from PCa patients with available genome-wide expression microarray data and ETS rearrangement status.

- To evaluate the involvement of *MYC* in structural rearrangements in prostate cancer. In cases with *MYC* rearrangements, the involvement of the only known *MYC* 5´fusion partner, *C15orf21*, will be looked for.

- To identify differentially expressed genes between prostate carcinomas with and without *MYC* relative copy number gain.

- To look for associations between *MYC* structural rearrangements and copy number changes and clinico-pathological data in prostate cancer patients.

MATERIAL AND METHODS

MATERIAL AND METHODS

1. Sample Selection and Clinical Data

A collection of **200** formalin-fixed paraffin-embedded (FFPE) radical prostatectomy specimens from patients with clinically localized prostate carcinoma treated at the Portuguese Oncology Institute - Porto (IPO-Porto) has been previously studied by our group for *ETS* rearrangements and *MYC* mRNA expression (Paulo et al., 2012a).

Of these 200 patients, global gene-expression data has been previously obtained for 50 by our group using the Affymetrix GeneChip Human Exon 1.0 ST arrays. Expression Console v1.1 software was used to obtain exon-level robust multi-array average (RMA) – normalized expression values for the core probe sets only (Paulo et al., 2012b). These 50 cases were therefore selected for the evaluation of the *MYC* relative copy number and structural rearrangements by FISH. Relevant clinical data at diagnosis were obtained from medical records. Patient age at diagnosis ranged from 49 to 74 (mean 62, median 64, and median PSA value was 7.59 ng/mL (range 2.66 - 18.6). At diagnosis, 4.2% of the patients had the disease classified as pathological stage (pT Stage) 2a, 47.9% as pT Stage 2b, 37.5% as pT Stage 3a and 10.4% as pT Stage 3b.

An independent series of **4** diagnostic paraffin-embedded biopsy samples from PCa patients, also from IPO-Porto, for which we had earlier data indicating a 8q24 structural rearrangement, (Barros-Silva et al., 2011; Ribeiro et al., 2007) was also studied to evaluate the involvement of *MYC* and its known 5´fusion partner, *C15orf21*.

2. FISH with BAC Clones

2.1. BAC Clones Selection

A break-apart probe strategy using BAC clones flanking *MYC* was used **(Table 6; Figure 17)** to evaluate the relative copy number gain and structural rearrangements of this gene in the **50** radical prostatectomy specimens and to evaluate whether the **4** prostate biopsy specimens with 8q24 structural changes harbored a rearrangement involving *MYC.*

Additionally, to investigate the involvement of the known 5´fusion partner in gene fusions involving *MYC*, a probe strategy flanking the 5´ region of *C15orf21* and the 3´region of *MYC* was also used. Bacterial artificial chromosome (BAC) clones targeting the 5´and 3´ regions of *MYC* and the 5´region of *C15orf21* were selected using the University of

California, Santa Cruz (UCSC) Human Genome Browser and obtained from BACPAC Resources Center (Oakland, California, USA) **(Table 6; Figure 17).**

Table 6 - BAC probes used to access *MYC* relative copy number and structural rearrangements, as well as its known 5´ fusion partner, *C15orf21*.

Gene Symbol	5´Probe (SpectrumRed)	3´Probe (SpectrumGreen)
MYC	CTD – 2313L9	RP11 – 946L14
C15orf21	RP11 – 42E1	NA

NA indicates not applicable

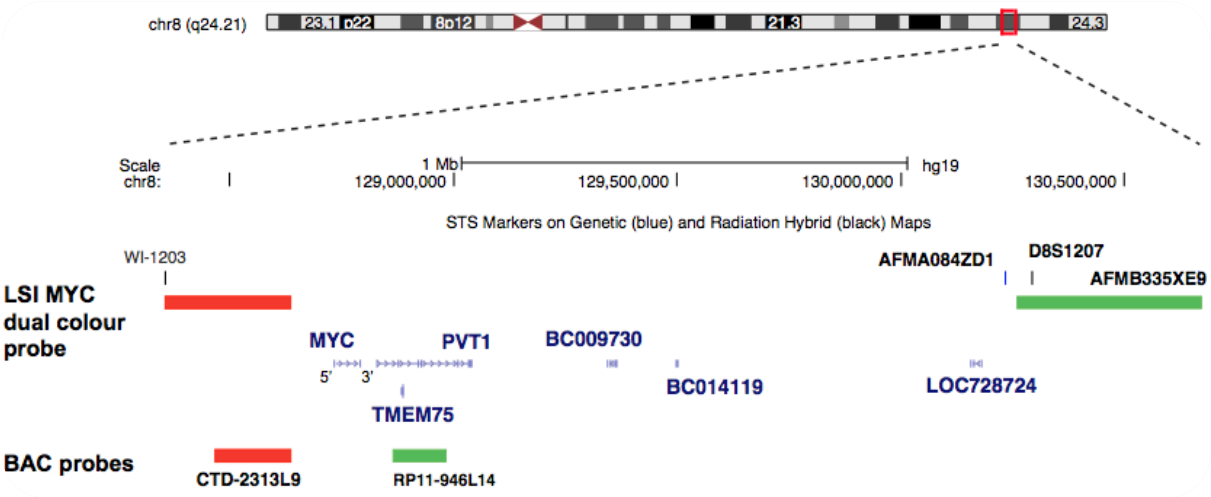

Figure 17 - Representative scheme of the both FISH probe strategies flanking *MYC* in order to identify a possible rearrangement involving this gene. A) A commercial dual-color probe flanking the *MYC* gene. The 400-kb probe labelled with SpectrumGreen starts 1.5Mb of the 3´ end of *MYC*. B) A BAC dual-color break-apart probe flanking *MYC*. The RP11-946L14 SpectrumGreen labeled probe starts 0.06Mb of the 3´end of *MYC*.

2.2. BAC Clones Growth

BAC clones were grown in 50 mL Falcon tubes, containing 10 mL of lysogeny broth (LB) medium supplemented with 12.5 µg/mL of chloramphenicol, during 16 hours, overnight, in an orbital shaking incubator **(Figure 18)**. This 16h period corresponds to the transition from logarithmic into stationary phase of the bacterial growth. At this time, the DNA is not yet degraded due to overaging of the culture, as in the later stationary phase.

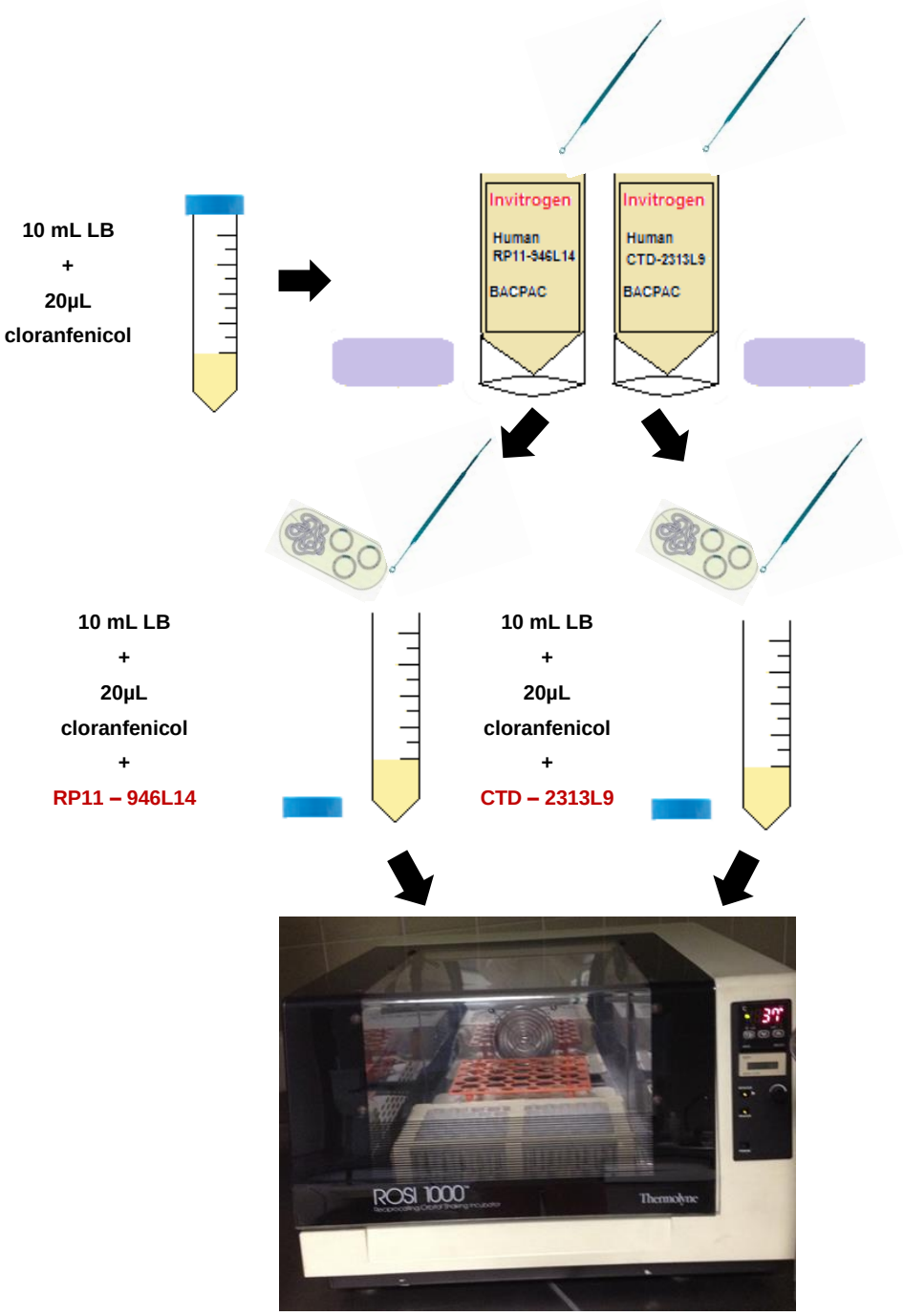

Figure 18 - Schematic representation of the growth process of both BAC clones.

Competent bacteria were harvested by centrifugation at 4000 rpm, during 30 minutes, at room temperature (RT) **(Figure 19)**. The supernatant was then discarded and the pellet, was dried, upside down, for at least 15 minutes.

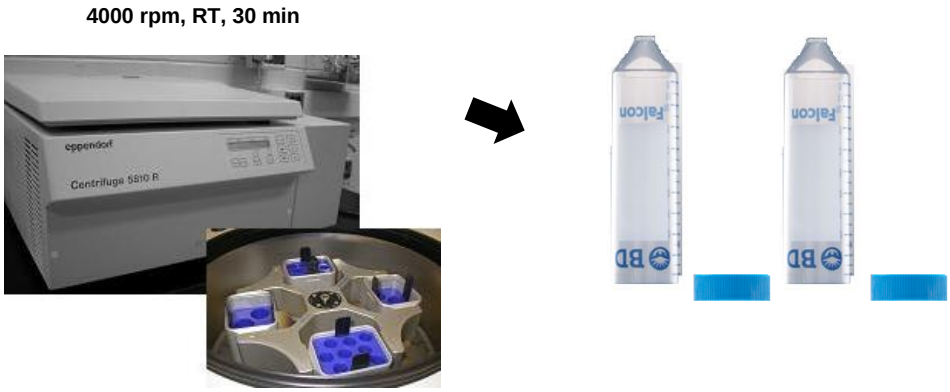

Figure 19 - Centrifugation and drying of each BAc´s pellet.

2.3. Plasmidic BAC DNA Extraction

BAC DNA was extracted using the NucleoSpin^{R} Plasmid Quick Pure Kit (MACHEREY - NAGEL, Düren, Germany) according to the manufacturer´s instructions. Briefly, the pelleted bacteria were resuspended in A1 resuspension buffer (supplemented with RNAse), and plasmid DNA was liberated from the *Escherichia coli* (E. coli) host cells by SDS/alkaline lysis (buffer A2). Buffer A3 was then added to neutralize the resulting lysate creating appropriate conditions for binding of plasmid DNA to the silica membrane of the NucleoSpin^{R} Plasmid Column. Precipitated protein, genomic DNA, and cell debris were then pelleted by a room temperature centrifugation for 10 min, at 11,000 x *g*. The supernatant was loaded onto a NucleoSpin^{R} Plasmid Column and after a 1 min, at 11, 000 x *g* centrifugation. To wash the column, ethanolic buffer A4 was used. Pure plasmid DNA was finally eluted with a slightly alkaline buffer (elution buffer) (5 mM Tris/HCl, pH 8.5), being then incubated during 2 min at room temperature, before a centrifugation for 1 min, at 11,000 x *g*.

Purity and DNA concentration were measured in a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, USA).

2.4. Amplification

Extracted DNA was diluted to a concentration of 10ng/µL for further plasmidic DNA amplification. The amplification was performed by using the GenomiPhi V2 DNA amplification kit (WGA kit, GE, Healthcare, UK) according to the manufacturer´s instructions. In this reaction, 1 µL (10ng) plasmidic DNA was added to 9 µL of sample buffer. The plasmidic DNA was briefly heat-denaturated at 95ºC, during 3 minutes and then cooled to 4ºC in sample buffer containing random primers that non-specifically bind to the DNA. Then, a master mix,

consisting in 9 µL of reaction buffer and 1 µL of Enzyme Mix, was prepared. This master mix contains all the components required for DNA amplification, including DNA polymerase, additional random primers, dNTPs salts and buffers, and amplification proceeded during 16 cycles, at 30ºC, for 16 hours. After this, the enzyme was heat-inactivated during 10 minutes incubation at 65ºC.

2.5. Nick Translation

Before hybridization, plasmidic DNA was labeled with SpectrumGreen or SpectrumRed (Abbott Laboratories, UK) conjugated nucleotides in nick translation reactions using the Nick Translation DNA Labelling System (Cat. NO. ENZ – 42910; Enzo^{R} Life Sciences, USA). This reaction consisted in two cycles in which the DNA was incubated at 15ºC, during 60 minutes, followed by an increase in temperature to 65ºC, during 5 minutes. About 5,5 µL of labeled BAC probe was then mixed with 5 µL of unlabeled Cot-1 DNA (Life Technologies, Rockville, MD), ethanol precipitated, dried and dissolved in hybridization buffer (Abbott Laboratories). Adequate mapping and probe specificity of all BAC clones were confirmed by hybridization onto normal human metaphases.

2.6. FISH in tissue sections

In this technique a labeled DNA probe is hybridized to cytological targets such as metaphase chromosomes, interphase nuclei, extended chromatin fibers or, more recently, DNA microarrays (Speicher and Carter, 2005). Such hybridization allows the identification of gain, loss or rearrangement of a specific gene or a set of genes. FISH in FFPE enables the analysis of cells maintaining tissue organization, allows observation of eventual heterogeneity present in the tumor and can be applied to samples not subjected to cell culture, obtained from fixed cells for karyotyping, paraffin blocks, aspirative cytology, etc. However, and like any other technique, FISH also carries some drawbacks, including probe´s lower penetration, tissue autofluorescence, overlapping nuclei, no intact nuclei, low efficiency of hybridization due to reduced or, in turn, very prolongated fixation times.

Four- µm-thick sections from paraffin-embedded tumor blocks of 50 prostatectomy specimens and 4 biopsy specimens were performed onto Superfrost Plus Adhesion slides (Menzel–Glaser, Braunschweig, Germany). Slides were then deparaffinized in two passages through xylol, followed by other two passages through 100% ethanol, 10 minutes each. For slides pre-treatment, they were incubated in 2x SSC for 3 minutes, followed by the incubation with NaSCN 1M at 80ºC for 12 minutes and then rinsed in 2xSSC for 3 minutes. The

enzymatic digestion was made through incubation of a pepsin solution (4mg/mL) with each slide at 37ºC for 10 minutes in the Hybrite (Vysis) for prostatectomy and 8 minutes for biopsy specimens. In order to finish the digestion, the slides were placed two times in a 2xSSC solution for 2 minutes each, followed by an increasing series of ethanols, 70%, 85% and 100%, for 3 minutes each. After the dehidratation, the specific probe sets were applied onto each sample. In the 50 prostatectomy series, 5´ and 3´*MYC* probes combined with a centromeric probe for chromosome 18 (CEP18, SpectrumAqua, Vysis) were used. For the 4 biopsy specimens, 5´*C15orf21* and 3´*MYC* probes were used **(Table 6)**. Slides were placed in a Hybrite denaturation/hybridization system (Vysis) and co-denaturated at 80ºC for 8 minutes, followed by hybridization for 18 hours at 37ºC. The slides were then washed in a 2xSSC/0.5% IGEPAL (Sigma Aldrich) solution for 5 minutes and 2XSSC/0.1% IGEPAL at RT for 3 minutes. Slides were then counterstained with 4´, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, California, USA). Finally, the slides were analyzed and fluorescent images corresponding to DAPI, Spectrum Green, Spectrum Orange, and Spectrum Aqua were sequentially captured in a Zeiss Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany) coupled with a Cohu 4900 CCD camera and a CytoVision system version 3.9 (Applied Imaging, Santa Clara, California, USA) **(Figure 20)**.

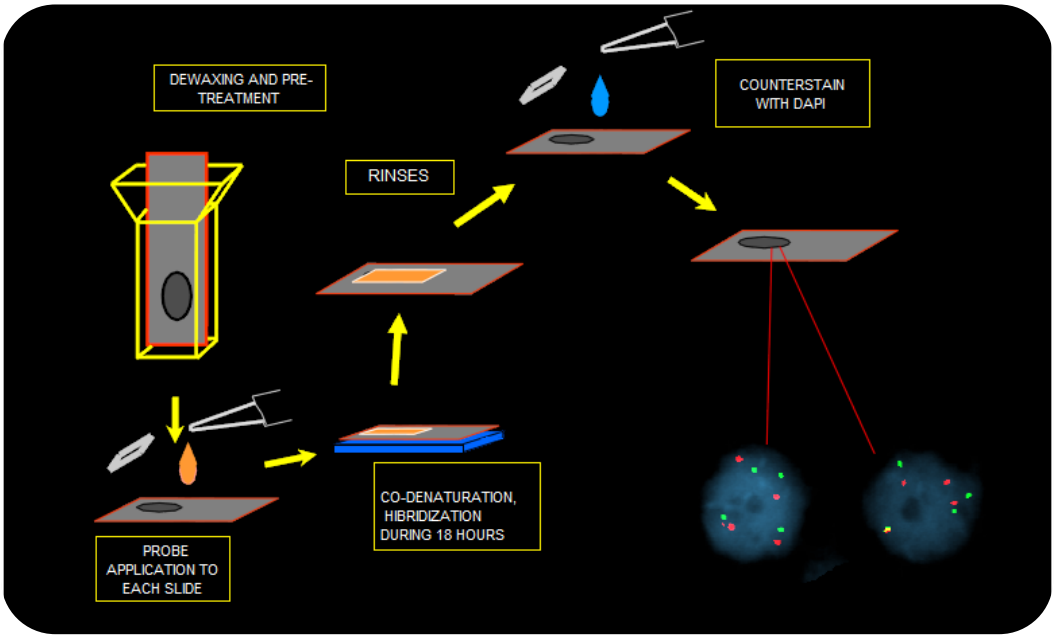

Figure 20 - Schematic representation of FISH methodology in paraffin-embedded tisues.

Relative 8q24 gain was assessed as previously described (Ribeiro et al., 2007; Ribeiro et al., 2006b). A ratio between *MYC* and CEP18 signals within each nucleus of a representative cancer cell population was computed for each sample. Cases were categorized as negative for relative 8q gain whenever *MYC*/CEP18 < 1.5 and as having relative 8q gain when *MYC*/CEP18 ≥1.5 (Ribeiro et al., 2007). Additionally, cases with *MYC*/CEP18 ≥ 2 ratio were deemed amplified.

According to the break-apart pattern of the chosen probes for the detection of *MYC* relative copy number gain **(Figure 17)**, a prostatectomy was deemed normal when two co-localized FISH signals were found. On the other hand, an abnormal signal pattern was considered representative when present in a minimum of 50 morphologically intact, non-overlapping nuclei.

3. Expression Microarrays

DNA microarrays contain oligonucleotide or cDNA probes for measuring the expression of thousands of genes, to identify changes in expression between different biologic states (Tusher et al., 2001). However, methods are needed to determine the significance of these genes, so Tusher and colleagues described a statistical method, Significant Analysis of Microarray (SAM) that assigns a score to each gene on the basis of its change in expression relative to the standard deviation of repeated measurements for that gene. Genes with scores greater than a threshold are deemed potentially significant, and the percentage of such genes identified by chance is the false discovery rate (FDR). The threshold can be adjusted to identify smaller or larger set of genes, and FDRs are calculated for each set. Concerning the *q*-value, it is the lowest false discovery rate at which that gene is called significant based on the work of John Storey (Storey, 2002). It is like the commonly used *P*-value, but adapted to the analysis of a large number of genes

Regarding SAM analysis, normalized, log-transformed and median-centered array results for all the prostatectomy samples, previously obtained by our group (Paulo et al., 2012b) were thereby submitted to two-class unpaired t-statistic method to determine the panel of genes with differential expression among the two FISH subgroups (presence or absence of 8q relative gain).

4. Statistical Analyses

For comparison of categorical data in the correlation analysis with clinico-pathological parameters, the Pearson Chi-Square test was used. Student´s *t* test was used for parametric data, when comparing two means. For non-parametric data both the Mann-Whitney *U* and the Kruskal-Walis test were used. The former was used to compare both PSA values and gene expression levels of *MYC* across the two FISH subgroups (presence or absence of 8q relative gain) and among the subgroup of patients with absence of 8q relative gain and according with ETS rearrangement status. The latter was applied when comparing the expression of *MYC* among the three subgroups of patients having 8q relative gain and

different ETS rearrangement status. A *P* value smaller than 0.05 was considered statistically significant. Statistical analyses were performed using the Statistical Package for Social Sciences software, version 20.0 (SPSS Inc., Chicago, IL).

RESULTS

RESULTS

1. Relative *MYC* Copy Number

The starting point of this study was a subset of **50** prostate carcinomas with available global gene-expression data using the Affymetrix GeneChip Human Exon 1.0 ST arrays, (Paulo et al., 2012b). The relative copy number of *MYC* in this cohort was assessed by FISH. Of these 50 cases, 2 were considered not analyzable. Overall, tumor cell populations with *MYC* copy number increase were found in **20** PCas. Of these, **3** PCas had a *MYC*/CEP18 ratio lower than 1.5, being therefore considered negative for relative 8q gain **(Table 1 on appendix)**. Of the **17** PCas with relative 8q gain (*MYC*/CEP18≥1.5), **6** displayed *MYC* amplification (*MYC*/CEP18 ≥ 2) **(Figure 21)**.

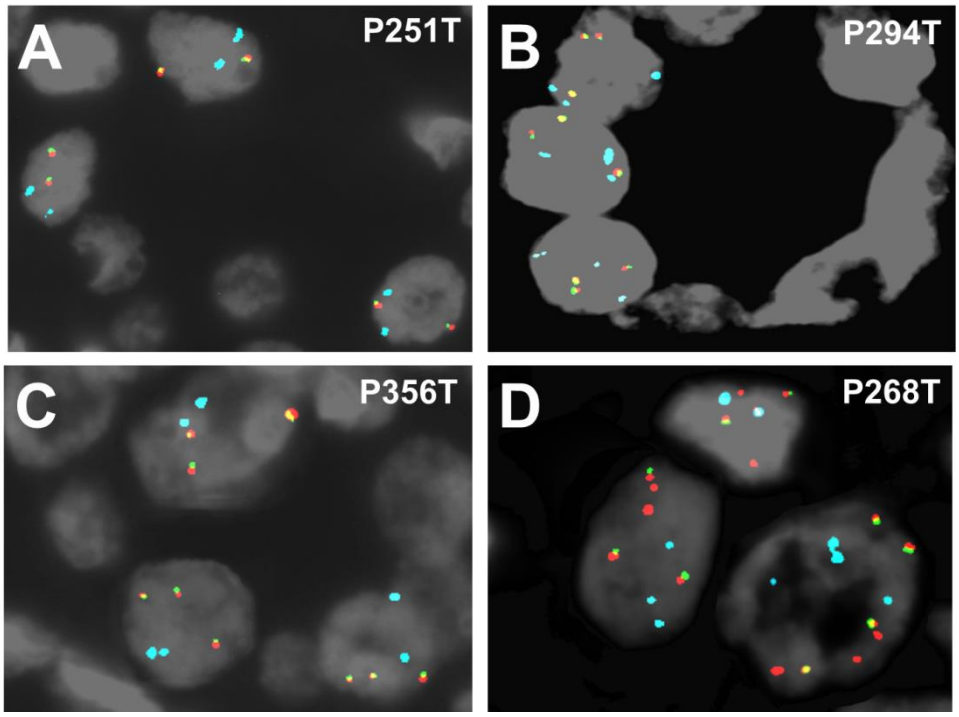

Figure 21 - Representative FISH images from selected prostatectomy specimens analyzed with BAC clones targeting 5´ and 3´*MYC*, labeled red and green respectively, and with chromosome 18 centromeric probe (aqua). A) Nuclei with two co-localized (red and green) signals of *MYC*, and two centromeric signals (aqua) representing a normal result. B) Nuclei showing three co-localized signals of *MYC* and three centromeric signals of chromosome 18, representing no relative copy number gain of *MYC*. C) Nuclei presenting relative copy number gain of *MYC*, illustrated by three co-localized signals of *MYC* and two centromeric signals of chromosome 18 (MYC/CEP18=1.5). D) Nuclei showing two to four co-localized signals of *MYC*, two to three additional red signals and two to four centromeric signals of chromosome 18 (MYC/CEP18>1.5).

2. *MYC* Structural Rearrangements

Deletion of 3´*MYC* was found in two prostatectomy specimens and a 5´*MYC* deletion was found in one additional case **(Table 7)**. Whereas both indicate a structural rearrangement of the *MYC* gene, the latter is compatible with a *MYC* rearrangement involving a 5´ fusion partner **(Figure 22)**.

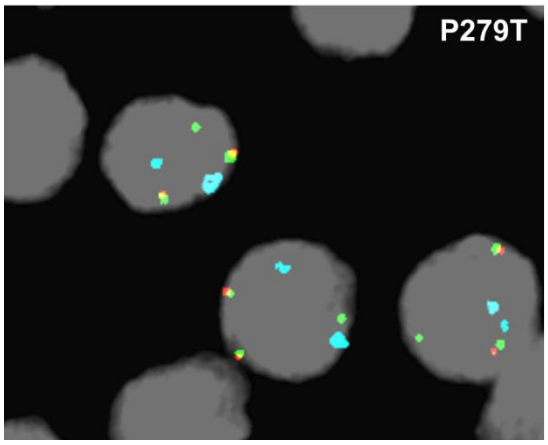

Figure 22 - Representative FISH image from a selected prostatectomy specimen, P279T, analyzed with BAC clones targeting 5´ and 3´*MYC*, labeled red and green respectively, and with chromosome 18 centromeric probe. This figure illustrates nuclei with two co-localized signals of *MYC*, one additional green signal, indicating a *MYC* structural rearrangement, and two centromeric signals of chromosome 18.

To search for additional cases with possible gene fusions involving *MYC*, we studied an independent set of **4** biopsy specimens in which we had earlier evidence of 8q24 structural rearrangements using commercially available probes relatively far apart from *MYC* (Barros-Silva et al., 2011; Ribeiro et al., 2007). In the current study, we used BAC probes with the particularity of closely flanking *MYC*, therefore allowing to evaluate if this gene is involved in structural rearrangements **(Figure 17)**. The results are summarized in **Table 7** and illustrated by **Figure 23**. Although results represented on Figure 22 are illustrative of the most frequent tumor cell populations, more than one single genetic alteration was observed in the biopsy specimens, a normal phenomenon due to the high heterogeneity present in prostate tumors.

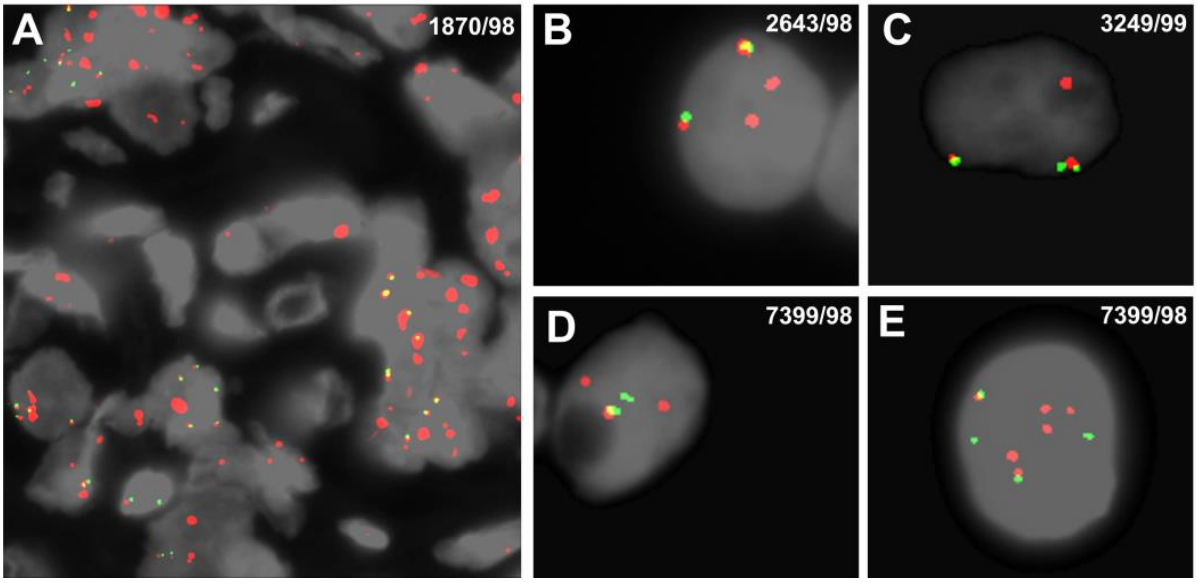

Figure 23 - Representative FISH images from 4 selected biopsy speciments analyzed with BAC clones targeting 5´ and 3´*MYC*, labeled red and green, respectively. A) Nuclei with amplification of 5´*MYC* region. B) Nucleus showing two co-localized signals of *MYC* and two additional red signals. C) Nucleus presenting two co-localized signals of *MYC* and one green and one red signal far apart. In this particular case, the green signal is localized relatively near from the two co-localized signals of *MYC*. D) Nucleus showing one co-localized signal of *MYC*, one green signal and two isolated red signals. E) Nucleus with two co-localized signals of *MYC*, two green signals and four red signals. Both D) and E) images represent tumor cells from the same biopsy specimen.

Table 7 - Experimental findings of a rearrangement involving *MYC* in the three prostatectomy specimens and in the 4 biopsy specimens.

Sample	FISH
P279T	2F, 1G, 2Aq
P268T	(1-4)F, (2-4)R, (1-4)Aq
P499T	(3-8)F, (1-2)R, (2-6)Aq
A - 1870/98	Amp R
B - 2643/98	(1-3)F, (1-2)R
C - 3249/99	(2-3)F, 1R, 1G
D and E - 7399/98	1F, 2R, 1G/ 2F, 4R, 2G

F indicates fusion (co-localization); G, spectrum green, R, spectrum red.

We then looked for the involvement of the only known *MYC* 5´fusion partner, *C15orf21*. The analysis of these prostate biopsy specimens did not indicate a chromosomal rearrangement typical of a fusion gene involving the *C15orf21* gene, as no co-localization of the probes flanking *C15orf21* and *MYC* was found **(Figure 24)**.

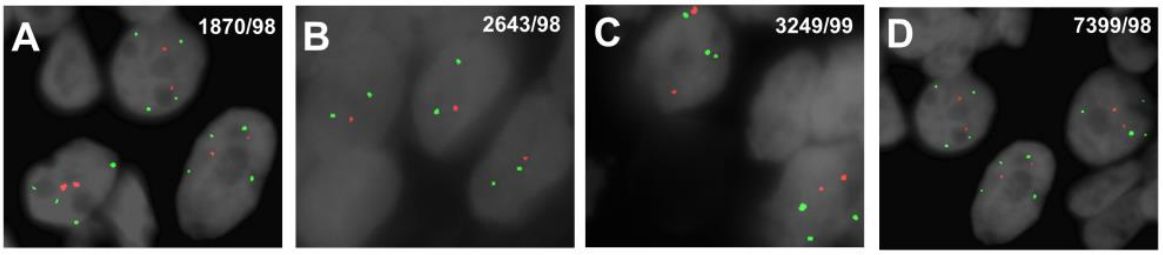

Figure 24 - **Representative FISH images from 4 selected biopsy speciments analyzed with BAC clones targeting 5´*C15orf21* and 3´*MYC*, labeled red and green, respectively.** A) Nuclei presenting two aditional green signals and two red signals. B) Nuclei with two green signals and a 5´ *C15orf21* deletion, marked by the absence of one red signal. C) Nuclei presenting three green signals of *MYC* and two *C15orf21* red signals. D) Nuclei showing two additional green signals and two red signals.

3. Differentially Expressed Genes According to 8q Status

The application of Significance Analysis of Microarrays (SAM) in our data allowed the identification of three significantly overexpressed genes in the subgroup with 8q relative gain, namely *IKZF2*, *CDON*, and *GPRC5A* **(Figure 25)**, with a FDR = 0% and a *q*-value = 0. According with this analysis, *MYC* was not found to be differentially expressed among the two subgroups of patients (*q*-value = 35). Apart from *MYC*, *ERG* and *ETV1* are the two ETS genes most commonly involved in gene fusions in PCa and were therefore included in the figure.

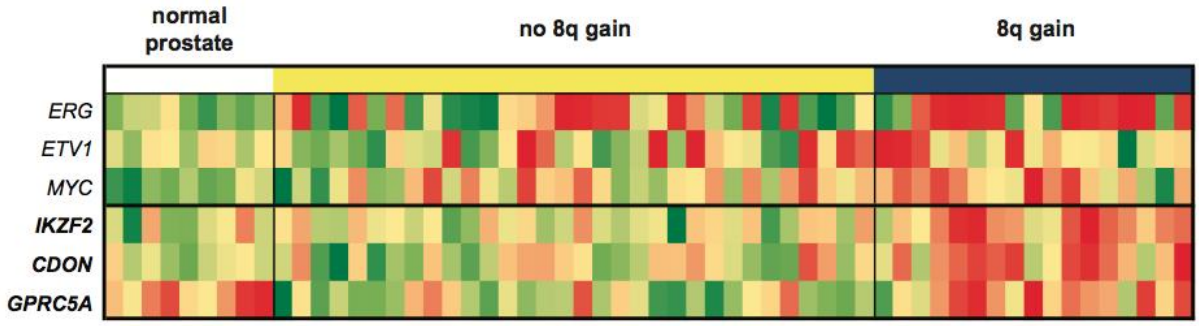

Figure 25 - Differentially expressed genes among sample subgroups: no 8q gain versus 8q gain discovered by SAM. Genes overexpressed in the 8q gain subgroup are highlighted in bold (*IKZF2*, *CDON, GPRC5*). For illustrative purpose, expression of *ERG*, *ETV1*, and *MYC* are also displayed in the figure. Each cell represents a sample and its expression was defined by different colors. Red represents the samples overexpressed and dark green corresponds to the samples with lower expression.

3.1. *MYC as a target gene of 8q24 copy number gain*

As stated above, SAM analysis excluded *MYC* as a significantly overexpressed gene in PCa with 8q gain. However, given that it is commonly stated in the literature the association of *MYC* and 8q gain (Fromont et al., 2013; Jenkins et al., 1997; Nupponen et al., 1998), we decided to look at it using conventional statistical approaches, including the Mann-Whitney *U* test.

Although there is indeed a higher expression of *MYC* in the 8q gain subgroup, the difference is only borderline for statistical significance (*P*=0.051) **(Figure 26)**, which is compatible with the SAM analysis.

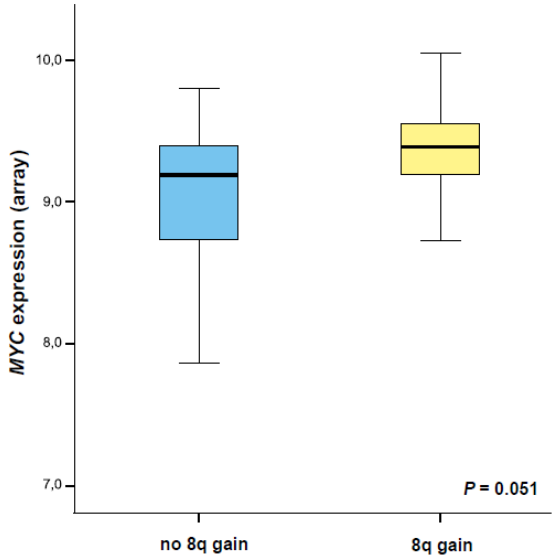

Figure 26 – Differential expression of *MYC* across the two patient subgroups (no 8q gain and 8q gain) previously stratified by the FISH analysis of the 48 prostate carcinomas.

3.2. *Correlation of 8q24 and* ETS *rearrangement status*

To look for the possible effect of ETS rearrangements status in *MYC* expression, we compared three groups of patients, harboring no ETS rearrangements, an *ERG* rearrangement, or other ETS rearrangements (*ETV1*, *ETV4* and *ETV5*) in the presence **(Figure 27)** or absence **(Figure 28)** of 8q gain.

Considering patients with 8q gain, no differences in *MYC* expression were found among the three ETS subgroups (*P*= 0.852).

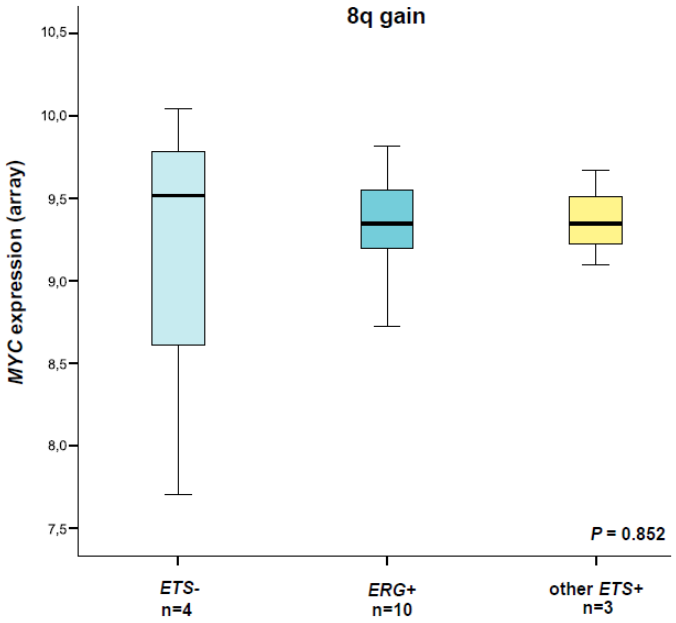

Figure 27 - Differential expression of *MYC* across the three subgroups stratified by both 8q relative gain and ETS rearrangement status.

On the other hand, in patients with no 8q gain, differential *MYC* expression was observed among the different ETS groups (*P*=0.048). Paired comparisons showed that *MYC* expression was significantly higher in patients with ETS rearrangements other than *ERG* (*P*=0.021) compared to ETS- patients. Although patients with *ERG* rearrangements had higher *MYC* expression than ETS- patients, the difference was not statistically different (*P*= 0.118).

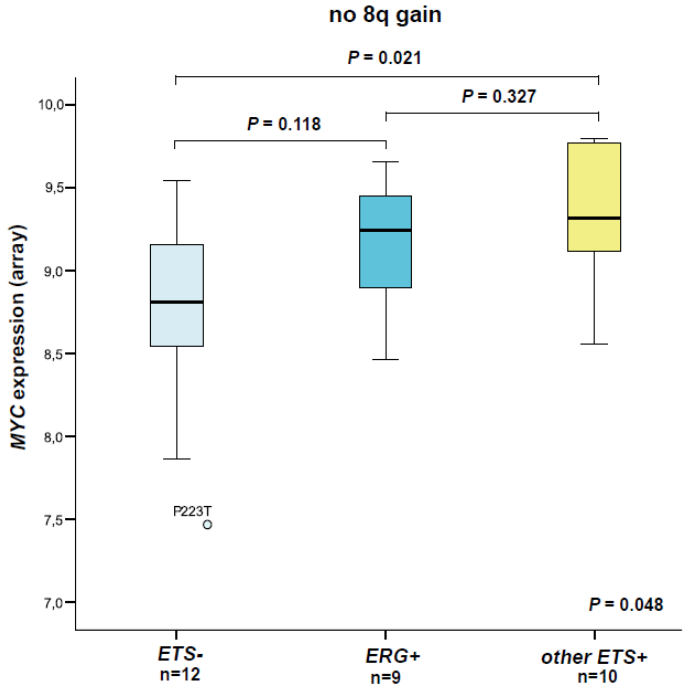

Figure 28 - Differential expression of *MYC* across the three subgroups stratified by both no 8q relative gain and ETS rearrangement status.

4. Relative 8q Gain and Clinico-Pathological Associations

Statistical comparisons with clinico-pathological data showed no association of 8q relative gain with age (*P*=0.757), PSA level at diagnosis (*P*=0.271), GS (*P*=0.303), and pT stage (*P*=0.483). All the clinico-pathological parameters are summarized in Table 8.

Table 8 – Correlation analysis of the FISH results for the presence or absence of the 8q relative gain with clinico-pathological parameters.

	8q relative gain		
Clinical parameters	**-**	**+**	***P***
Age (±std)	62.20 ± 6.75	62.67 ± 5.85	0.757
PSA at diagnosis (median)	7.45	7.80	0.271
Gleason score			
GS<7	11 (73.3%)	4 (26.7%)	0.303
GS=7	20 (62.5%)	12 (37.5%)	
GS>7	0 (0.0%)	1 (100.0%)	
pT Stage			
2a	2 (100.0%)	0 (0.0%)	0.483
2b	15 (65.2%)	8 (34.8%)	
3a	12 (66.7%)	6 (33.3%)	
3b	2 (40.0%)	3 (60.0%)	

DISCUSSION

DISCUSSION

Gene fusions involving the ETS transcription factor family of genes are a recurrent feature of PCa. These gene aberrations, caused by chromosomal structural abnormalities, originate fusion transcripts that lead to overexpression of N-truncated ETS proteins, or, more rarely, to full-length ETS proteins or chimeric fusion proteins. In 2005, Tomlins reported the fusion of the prostate-specific, androgen-regulated promoter region of *TMPRSS2* gene with *ERG*, the most common ETS rearrangement, being present in 50% of the localized PCas, and in 21% of precursor HGPIN neoplasia lesions (Albadine et al., 2009; Cerveira et al., 2006; Clark et al., 2007; Liu et al., 2007; Mao et al., 2010; Mosquera et al., 2008; Perner et al., 2006; Soller et al., 2006; Tomlins et al., 2005). Other rarer fusion events can occur involving the *ETV1* (Attard et al., 2008; Hermans et al., 2008; Tomlins et al., 2007a; Tomlins et al., 2005), *ETV4* (Tomlins et al., 2006), *ETV5* (Helgeson et al., 2008), and *FLI1* genes (Paulo et al., 2012a). Besides the *TMPRSS2*, several other 5´ ETS fusion partners have also been described, namely *HERPUD1*, *HERVK17, SLC45A3*, *ERVK-24* (also known as *HERVK_22q*), *HNRPA2B1, C15orf21*, *NDRG1*, *CANT1*, *DDX5*, *KLK2*, *FOXP1*, *EST14*, *ACSL3*, and the chromosomal region 14q13.3-14q21.1 (Lapointe et al., 2007; Pflueger et al., 2009; Tomlins et al., 2006). Although *TMPRSS2-ERG* is suggested to be an early event, presumably occurring at the transition between benign and PIN epithelium (Perner et al., 2007), secondary chromosome changes, such as 8q gain comprising *MYC*, have been shown to be one of the best candidate genetic prognostic factors. It is remarkable that 8q gain detected in diagnostic needle biopsies of PCa suspects, by either CGH or FISH, is significantly associated with death from disease (Ribeiro et al., 2006b). Additionally, it has been shown that 8q gain predicts poor disease-specific survival independently of the presence of *TMPRSS2-ERG* fusion gene (Barros-Silva et al., 2011). The recent discovery of a novel and highly aggressive hybrid case of PCa, harboring the novel *C15orf21-MYC f*usion gene, highlights the potential involvement of the *MYC* oncogene in prostate carcinogenesis by several mechanisms. However, the role of *MYC* in prostatic carcinogenesis is still poorly understood.

To increase our knowledge on the involvement of the *MYC* oncogene in prostate carcinogenesis, we evaluated in this study *MYC* relative copy number and structural rearrangements in 50 prostatectomy specimens of patients with available genome-wide microarray expression data and clinic-pathological parameters. In addition, we evaluated further the involvement of *MYC* in 4 PCa biopsy specimens with available data indicating a

structural rearrangement in 8q24. In case of validation of *MYC* rearrangements, the involvement of the only known *MYC* 5´fusion partner, *C15orf21*, will be checked.

1. *MYC* Relative Copy Number Gain

MYC relative copy number was evaluated in 50 prostatectomy specimens using FISH. The break-apart probe strategy using BAC clones closely flanking *MYC* (8q24) is expected to identify both prostate carcinomas with 8q gain and structural rearrangements involving *MYC*, and the dual-color labeling enables the scoring of copy number changes and structural rearrangements in archival prostatectomy specimens. To control for ploidy in each case, we chose a chromosome 18 probe, because the centromeric region of this chromosome is rarely affected in PCa, as opposed to other commercial available SpectrumAqua probes (chromosomes 8, 10, and 17) (Ribeiro et al., 2006b).

Overall, tumor cell populations with *MYC* relative copy number increase (*MYC*/CEP18≥1.5) were found in 35% of the prostate carcinomas, and of these 6 cases displayed *MYC* amplification (*MYC*/CEP18>2). Jenkins et al (1997) and Fromont et al (2013) reported similar results as ours. The former studied twenty-five prostatectomy and pelvic lymphadenectomy specimens and analyzed, by FISH, the relative extra *MYC* copy number. These authors used DNA probes for the centromere region of chromosome 8 (CEP8) and for the 8q24 (*MYC*) region and found relative *MYC* copy number increase (*MYC*/CEP8≥1.5) in 11% of the patients (Jenkins et al., 1997). On the other hand, the cohort studied by Fromont and colleagues (2013) was composed by 202 prostatectomy specimens, and they also evaluated by FISH the relative *MYC* copy number increase by using a commercial dual-color probe (*MYC*/CEN8) that consists in a mixture of an orange fluorochrome direct-labeled probe specific for the alpha satellite centromeric region of chromosome 8 (D8Z2) and a green fluorochrome direct labeled SPEC *MYC* probe specific for the *MYC* gene at 8q24. These authors observed that 29% of the tumors tested showed increase of 8q24 at the *MYC* locus (MYC/CEN8≥1.5).

2. *MYC* Structural Rearrangements

The same probe strategy used to evaluate *MYC* copy number was able to identify structural rearrangements involving this gene. In fact, two cases with a 3´*MYC* deletion and

one case with a 5´*MYC* deletion were found in the series of 50 prostatectomy specimens evaluated for *MYC* copy number changes, with the latter being the best candidate for a *MYC* rearrangement involving a 5´ fusion partner.

Additionally, our group has analyzed by FISH a retrospective series of paraffin-embedded biopsies from 60 PCa patients for relative 8q24 gain (Ribeiro et al., 2007). For each of the sixty biopsy samples, a commercial dual-color probe flanking the *MYC* gene at 8q24 and a CEP18 probe labeled with SpectrumAqua were used in each sample. The *MYC* probe set consists of a ~277kb SpectrumOrange labeled probe and a ~400kb probe labeled with SpectrumGreen **(Figure 17)**. The dual-color labeling of that probe allowed the detection of rearrangements presumably involving *MYC* in 4 biopsy specimens that include gain or loss of the individual 3´*MYC* and 5´*MYC* probes (Ribeiro et al., 2007). However, other genes, such as plasmocytoma variant translocation 1 gene (*PVT1*) and transmembrane protein 75 (*TMEM75*) gene, reside in this locus and therefore might be possible candidates to be involved in the breakpoint. Due to the long distance between the dual-color flanking probes, it was not clear where the breakpoint had occurred. Our strategy in this study was therefore the choice of a break-apart probe using BAC clones closely flanking *MYC* that could provide a more precise location of the breakpoint region. The *MYC* BAC probe set consists of a ~166kb SpectrumRed labeled probe and a ~172kb probe labeled with SpectrumGreen **(Figure 17)**. Our results corroborated the initial suspiction of Ribeiro and colleagues (2007) and the mentioned gain or loss of the individual 3´*MYC* and 5´*MYC* probes was demonstrated by a split between the green and red signals (3´*MYC* and 5´*MYC* regions, respectively), reflected either by a deletion of the 3´*MYC* **(Figure 23D)** or by the increase copy number of the 5´*MYC* region **(Figure 23A and E)**.

4. Evaluation of a Possible 5´ Fusion Partner of *MYC*

Gene fusions involving the ETS transcription factor family of genes are a recurrent feature in PCa and the 5´fusion partner *C15orf21* gene has been reported to be involved in these ETS gene fusions, namely with *ETV1* (Barros-Silva et al., 2013; Lapointe et al., 2007; Pflueger et al., 2009; Tomlins et al., 2006). However, *C15orf21* has recently been reported to be also involved in gene fusions with *MYC* (Wu et al., 2012). These authors, using genome and transcriptome sequencing, identified a novel form of hybrid and aggressive PCa, involving the oncogene *MYC*. The transcriptome analyses revealed signatures of both luminal and neuroendocrine cell types. Remarkably, the repertoire of expressed but apparently private gene fusions, including *C15orf21* and *MYC*, recapitulated this biology. This hybrid luminal-neuroendocrine tumor appears to represent a novel and highly

aggressive case of PCa with propensity for rapid progression to castrate-resistance. After confirming the structural rearrangement involving *MYC* in the 4 biopsy specimens, we then looked for the involvement of the only known 5´fusion partner of *MYC*, *C15orf21*. However, our results did not confirm that *C15orf21* was the 5´fusion partner of *MYC*, as no co-localization of the probes flanking *C15orf21* and *MYC* was found. The presumed 5´fusion partner of *MYC* in these cases remains therefore unknown.

5. Overexpressed Genes Associated with Relative *MYC* Gain

The SAM analysis identified three significantly overexpressed genes, namely *IKZF2*, *CDON*, and *GPRC5A*, in the *MYC* relative gain subgroup of patients **(Figure 25)**. *IKZF2*, ikaros family zinc finger 2, also known as helios, is an hematopoietic-specific transcription factor involved in the regulation of lymphocyte development. It is also known that this protein forms homo and hetero-dimers with other ikaros family members, and is thought to function predominantly in early hematopoietic development. Ikaros was found as a marker of T-cell activation and proliferation (Akimova et al., 2011) and a short isoform of this gene has been recently reported to be overexpressed in a patient with T-cell acute lymphoblastic leukemia (Nakase et al., 2002). On the other hand, ikaros has been found to be involved in a gene fusion involving B-cell CLL/lymphoma 11B (zinc finger protein) (*BCL11B*) gene in an adult T-cell leukemia patient (Fujimoto et al., 2012). Further, this transcription factor has been related with lymphoid malignancies (Rebollo and Schmitt, 2003) and with both non-Hodgking and Hodgkin lymphoma (Antica et al., 2008).

Cell-adhesion associated, oncogene regulated (*CDON*) encodes a cell surface receptor that is a member of the immunoglobulin superfamily. The encoded protein, CDO, being a member of a cell surface receptor complex, mediates cell-cell interactions between muscle precursor cells and positively regulates myogenesis. A recent study published by Hayashi and colleagues (2011) showed, by using quantitative RT-PCR analysis, overexpression of this gene in about 83% of PCa tissues, and its expression is involved in tumor cell growth and invasion. Moreover, knockdown of *CDON* in DU145 cells induced *5* –fluorouracil-induced apoptosis and inhibited invasion ability, suggesting that this gene has a high potential as a therapeutic target for PCa (Hayashi et al., 2011) However, they did not test the association of *CDON* with *MYC* copy number

Lastly, the protein-coupled receptor, family C, group 5, member A (*GPRC5A*) gene, being a member of the type 3G protein-coupling receptor family, may be involved in the interaction between retinoic acid an G protein signaling pathways. Retinoic acid plays a

critical role in development, cellular growth and differentiation and this gene may also play a role in both embryonic development and epithelial cell differentiation. Jörissen and colleagues (2009) conducted a systematic analysis of *GPRC5A* (also known as *RAI3*) expression in normal and cancerous human breast tissue at both mRNA and protein levels and reported, based on cDNA dot blot and immunohistochemistry experiments, that both *RAI3* mRNA and *RAI3* protein were abundantly expressed in human breast carcinoma (Jorissen et al., 2009). However, this retinoic acid-inducible gene was identified by Tao et al (2007) as a new lung tumor suppressor gene (Tao et al., 2007). Moreover, *GPRC5A* tumor suppressor loss of expression was found conserved, prevalent and associated with survival in human lung adenocarcinoma (Kadara et al., 2010). It is, however, important to note that *GPRC5A* expression was also high in normal prostate tissue.

4.1. MYC as a target gene of 8q24 copy number gain

SAM analysis revealed that *MYC* was not differentially expressed among the two subgroups previously stratified by FISH (presence or absence of 8q relative gain), (FDR = 0%; *q*-value = 35). Given that some authors have associated 8q gain with *MYC* overexpression in a subset of prostate adenocarcinoma cases (Fromont et al., 2013; Jenkins et al., 1997; Nupponen et al., 1998; Nupponen et al., 1999), we also evaluated the expression of *MYC* among the same two subgroups of patients, and indeed there was a tendency (*P* = 0.051) for an association between *MYC* copy number gain and higher expression of this gene. Although the method of obtaining the *MYC* gene expression levels was different from ours, Gurel et al (2008) compared *MYC* protein levels in PCa by semiquantitative image analysis of immunohistochemistry stained specimens, and did not find a correlation between gain of 8q24 by FISH and *MYC* expression level (Gurel et al., 2008). Furthermore, gain of 8q24 is rare in PIN lesions and localized low-grade prostate adenocarcinomas, yet *MYC* overexpression is common on these lesions. Moreover, other genes, including *TRPS1*, *EIF3S3*, *RAD21*, *KIAA0916*, and *PSCA*, reside in or near this locus and have been put forth as potential targets of 8q amplification (Nupponen et al., 1999; Porkka et al., 2004; Reiter et al., 2000; Saramaki et al., 2001; Tsuchiya et al., 2002; van Duin et al., 2005).

Sun et al (2008) reported that in VCaP cells the overexpressed *ERG*, as a result of harboring a *TMPRSS2-ERG* fusion, upregulates C-*MYC* oncogene (Sun et al., 2008). In order to look for the possible effect of ETS rearrangements status in *MYC* expression, we compared three groups of patients, harboring no ETS rearrangements, harboring an *ERG* fusion gene, or other ETS rearrangements (*ETV1*, *ETV4* and *ETV5*) in the presence or absence of 8q gain. Regarding the patients with 8q gain, our results did not show a

significant differential expression of *MYC* across the three ETS subgroups (*P* = 0.852). Concerning the patients with no 8q relative gain, a differential *MYC* expression was observed among the different ETS subgroups (*P* = 0.048), and its expression was higher in the other ETS+ subgroup of patients. We then compared the subgroup of patients harboring other ETS+ rearrangements with the other two subgroups (harboring no ETS rearrangements, and harboring only *ERG* rearrangements), and our results showed that, in the absence of 8q relative gain, the other ETS genes, but not *ERG*, were associated with increased expression of *MYC* (*P* = 0.021). Despite the apparently overlapping oncogenic potential of *ERG* and *ETV1* gene fusions, it has not been established whether different ETS transcription factors have specific or shared downstream targets. In order to address this issue, Paulo and colleagues (2012), by using exon-level expression arrays in a series of 50 PCa presenting different ETS rearrangements (21 samples with *ERG* rearrangements, 13 samples presenting *ETV1* rearrangements, 2 sample each harboring *ETV4* and *ETV5* rearrangements, and 14 samples without ETS rearrangements), concluded that both *ERG* and *ETV1* regulate specific and shared target genes (Paulo et al., 2012b). Moreover, when the expression profiles of the two PCa with *ETV4* and *ETV5* rearrangements where included in the hierarchical clustering, they clustered among the *ETV1*-positive PCa samples, suggesting that *ETV4* and *ETV5* might be, at least in part, shared by *ETV1*, something that was expected since these genes belong to the same *PEA3* family of transcription factors (Hollenhorst et al., 2007; Paulo et al., 2012b).

5. Clinico-Pathologic Associations with Relative *MYC* Gain

We did not find a statistically significant association of the clinico-pathologic parameters with 8q relative gain. It is however interesting to note that the frequency of 8q gains increased with the degree of indifferentiation (ie, higher Gleason scores). Barros-Silva et al (2011) have previously reported that relative 8q gain was less frequent in low GS prostate tumors (Barros-Silva et al., 2011). Regarding the disease progression (pT Stage), none of the pT2a prostatectomy specimens had 8q gain, rising to about 30% in pT2b and pT3a, and finally to 60% of pT3b patients. Our findings are in agreement with the already stated by Ribeiro et al (2006), that 8q gain appears at an intermediate event, in the transition of organ confined to locally invasive PCa (Ribeiro et al., 2006a).

CONCLUSIONS AND FUTURE PERSPECTIVES

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- Relative copy number gain of *MYC* was found in 35% of the prostatectomy specimens, and involvement of this gene in a structural rearrangement with a unknown 5´fusion partner was only found in one prostatectomy specimen. In order to search for possible 5´ *MYC* fusion partners, we could perform 5´rapid amplification of cDNA ends (5´RACE) as we already have extracted RNA for this prostatectomy specimen.

- Although we confirmed the involvement of *MYC* in the 4 PCa biopsy specimens with available data indicating a structural rearrangement in 8q24, the only known *MYC* 5' fusion partner, *C15orf21,* was excluded as the 5' fusion partner in this case.

- The SAM analysis highlighted three significantly overexpressed genes in subgroup with relative *MYC* copy number gain, namely *IKZF2*, *CDON* and *GPRC5A3*. Since *CDON* has already been reported to be overexpressed in PCa tissues, it will be interesting to confirm if *CDON* overexpression can also be detectable at the protein level by immunohistochemistry.

- Considering patients with 8q gain, no differences in *MYC* expression were found among different ETS molecular subtypes. On the other hand, in patients with no 8q gain, differential *MYC* expression was observed among the three ETS molecular subtypes, being *MYC* expression higher in the group of patients harboring ETS rearrangements other than *ERG*.

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APPENDIX

APPENDIX

Table 1 - Summary of the experimental findings in 48 prostate tumors

Sample	MYC expression (array)	FISH
P279T	10,04590	**2F, 1G, 2Aq**
P291T	9,81674	**3F, 2Aq**
P272T	9,79870	**2F, 2Aq**
P488T	9,79230	**2F, 2Aq**
P298T	9,77782	**3F, 1Aq**
P261T	9,77219	**2F, 2Aq**
P456T	9,67331	**3F, 2Aq**
P288T	9,65540	**2F, 2Aq**
P300T	9,54934	**3F, 2Aq**
P263T	9,54788	**2F, 2Aq**
P281T	9,51871	**3F, 1Aq**
P356T	9,51362	**3F, 2Aq**
P467T	9,50377	**2F, 2Aq**
P451T	9,45699	**2F, 2Aq**
P487T	9,45084	**2F, 2Aq**
P268T	9,39545	**(1-4)F, (2-4)R, (1-4)Aq**
P209T	9,38808	**(3F, 2Aq); 3F, 1Aq**
P499T	9,34923	**(3-8)F, (1-2)R, (2-6)Aq**
P257T	9,34543	**2F, 2Aq**
P542T	9,34401	**2F, 2Aq**
P276T	9,32716	**2F, 2Aq**
P238T	9,30989	**3F, 1Aq**
P522T	9,29374	**2F, 2Aq**
P274T	9,27316	**2F, 2Aq**
P301T	9,25564	**4F, 2Aq**
P289T	9,24508	**2F, 2Aq**
P307T	9,22398	**3F, 3Aq**
P303T	9,19531	**3F, 2Aq**
P351T	9,18999	**2F, 2Aq**
P264T	9,12502	**2F, 2Aq**
P525T	9,11501	**2F, 2Aq**
P229T	9,10989	**2F, 2Aq**
P344T	9,10117	**3F;2Aq**
P241T	9,03349	**3F, 2Aq**
P262T	8,92137	**2F, 2Aq**
P227T	8,89570	**2F; 2Aq**
P297T	8,84844	**2F; 2Aq**

P265T	8,77253	**2F, 2Aq**
P230T	8,72831	**3F, 2Aq**
P470T	8,69278	**2F, 2Aq**
P461T	8,61359	**2F, 2Aq**
P251T	8,59438	**2F,2Aq**
P305T	8,56142	**3F, 3Aq**
P242T	8,48194	**2F, 2Aq**
P294T	8,46684	**3F, 3Aq**
P228T	7,86252	**2F, 2Aq**
P245T	7,70471	**(7-15)F, (2-4)Aq**
P223T	7,45986	**2F, 2Aq**

CEP 18 indicates centromeric probe for chromosome 18; F, fusion; Aq, spectrum aqua; G, spectrum green; R, spectrum red, n.a., not analyzable.