D 2013

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO
FMUP FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

HISTONE METHYLASES AND DEMETHYLASES:

THE ROLE OF SMYD3 IN PROSTATE CARCINOGENESIS

ANA FILIPA QUINTELA VIEIRA

TESE DE DOUTORAMENTO APRESENTADA
AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
DA UNIVERSIDADE DO PORTO EM
PATOLOGIA E GENÉTICA MOLECULAR

ANA FILIPA QUINTELA VIEIRA

HISTONE METHYLASES AND DEMETHYLASES: THE ROLE OF SMYD3 IN PROSTATE CARCINOGENESIS

Tese de Candidatura ao grau de Doutor em Patologia e Genética Molecular submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

ORIENTADOR:
PROFESSOR DOUTOR RUI MANUEL FERREIRA HENRIQUE
Professor auxiliar convidado com "Agregação"
Departamento de Patologia e Imunologia Molecular
Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto
Diretor do Serviço de Anatomia Patológica
Investigador sénior do Grupo de Epigenética do Cancro – Centro de Investigação
Instituto Português de Oncologia - Porto

CO-ORIENTADORA
PROFESSORA DOUTORA CARMEN DE LURDES FONSECA JERÓNIMO
Professora associada convidada com "Agregação"
Departamento de Patologia e Imunologia Molecular
Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto
Investigadora auxiliar e coordenadora do Grupo de Epigenética do Cancro – Centro de Investigação
Instituto Português de Oncologia - Porto

Financial Support:

PhD fellowship: SFRH/BD/70564/2010

Fundação para Ciência e Tecnologia (FCT)

LISTA DE PUBLICAÇÕES

No cumprimento do disposto no Decreto-Lei nº 230/2009, declara-se que a autora desta tese participou ativamente na conceção e na execução do trabalho experimental que estiveram na origem dos resultados apresentados, bem como na sua interpretação e na redação dos respetivos manuscritos.

Fazem parte integrante desta tese os seguintes artigos científicos originais já publicados ou submetidos para publicação:

Artigo I – **Vieira FQ**, Costa-Pinheiro P, Ramalho-Carvalho J, Pereira A, Menezes FD, Antunes L, Carneiro I, Oliveira J, Henrique R*, Jerónimo C*. Deregulated expression of selected histone methylases and demethylases in prostate carcinoma. Endocrine-Related Cancer. 2014;21(1):51-61 (*Appendix*) **Co-autores senior*

Artigo II – **Vieira FQ**, Costa-Pinheiro P, Simões-Sousa S, Graça I, Monteiro-Reis S, Almeida-Rios D, Sousa EJ, Baltazar F, Henrique R*, Jerónimo C*
(*Submetido para publicação)* **Co-autores senior*

PREFÁCIO

O cancro da próstata e as suas atuais controvérsias constituem os motivos centrais desta Tese de Doutoramento. Este tipo de neoplasia é a maior causa de mortalidade por cancro em homens nos países desenvolvidos e uma das principais causas de mortalidade e morbilidade em todo o mundo. Nas últimas décadas, a estratégia de rastreio de pacientes com cancro da próstata permitiu a efetiva deteção precoce de tumores, evitando o diagnóstico em fase já avançada e, consequentemente, num estado de incurabilidade. No entanto, esta estratégia assenta fundamentalmente na quantificação do PSA (*prostate specific antigen*) sérico que, à luz dos conhecimentos atuais, apresenta importantes limitações no que respeita à sensibilidade e especificidade. Assim, um valor elevado de PSA sérico não é equivalente a um diagnóstico de cancro da próstata e valores baixos podem ser observados na presença desta neoplasia, incluindo formas clinicamente agressivas. No que diz respeito ao diagnóstico, este é realizado maioritariamente através de biópsia prostática e, sendo esta neoplasia caracterizada por ser multifocal e altamente heterogénea, as limitações inerentes à amostragem colocam problemas quanto à representatividade. Adicionalmente, os parâmetros clínicos e patológicos atualmente utilizados para avaliar a agressividade tumoral apresentam importantes limitações. Assim, um dos maiores desafios atuais no cancro da próstata consiste na redução das elevadas taxas de sobrediagnóstico e, consequente, sobretratamento, responsáveis por morbilidade desnecessária que inclui disfunção eréctil e incontinência urinária. O cancro da próstata é uma neoplasia geralmente caracterizada por crescimento lento, sendo que as formas menos agressivas não afetam a sobrevivência dos pacientes, embora a sua identificação seja, na atualidade, difícil e controversa. Tomando em consideração estes factos, urge encontrar formas de identificar com acuidade os tumores clinicamente agressivos, para os quais o tratamento adequado é fulcral para evitar a morte do paciente e, simultaneamente, discriminá-los dos tumores clinicamente insignificantes, evitando terapêuticas radicais e dessa forma impedindo a diminuição da sua qualidade de vida.

Geneticamente, o carcinoma da próstata caracteriza-se por uma baixa taxa de mutações somáticas. Mesmo as alterações ao nível cromossómico são relativamente infrequentes nas fases iniciais da doença, com a notável exceção dos genes de fusão. Contrariamente, as alterações epigenéticas são bastante mais frequentes e precoces, parecendo desempenhar um papel preponderante na iniciação e progressão neoplásica. O nosso grupo de investigação tem contribuído para a caracterização do metiloma do carcinoma da próstata, o que permitiu o desenvolvimento de biomarcadores com potencial clínico para a deteção precoce da doença. Contudo, o papel desempenhado por

genes codificadores das enzimas envolvidas na remodelação da cromatina, como as metiltransferases e as desmetilases das histonas é, ainda, largamente desconhecido. Atendendo a que a atividade destas enzimas assume particular relevância quer na ativação quer na repressão da transcrição de diversos genes, o seu estudo mais detalhado permitirá aprofundar os conhecimentos atuais quanto aos motivos da desregulação da expressão génica no carcinoma da próstata.

Neste contexto, esta Tese de Doutoramento teve como principal finalidade a identificação e caracterização de alterações da expressão de metiltransferases e desmetilases das histonas em cancro da próstata, permitindo não apenas avançar no conhecimento da biologia tumoral mas, também, desenvolver novos biomarcadores com relevância no diagnóstico ou prognóstico. Os resultados destes estudos permitirão, ainda, estratificar os pacientes com carcinoma da próstata em subgrupos com diferente agressividade clínica e, eventualmente, identificar alvos terapêuticos preferenciais que resultem no desenvolvimento de terapias personalizadas e mais eficientes com vista ao decréscimo da taxa de mortalidade por cancro da próstata.

O contacto com esta área de estudo surgiu no primeiro ano do meu programa doutoral por sugestão da Prof.ª Regina Silva, que, por conhecer há longos anos a Prof.ª Carmen Jerónimo e o Prof. Rui Henrique e reconhecer o trabalho do seu grupo de investigação, me aconselhou a contactá-los. Apesar de estar a lecionar a tempo inteiro e ter, por isso, limitações de tempo para dedicação ao projeto de doutoramento, penso que consegui convencê-los que daria o meu melhor e que não sairiam desiludidos. No final do meu primeiro ano de trabalho laboratorial (correspondente ao segundo ano do programa doutoral), surgiu a possibilidade de usufruir do estatuto de Equiparação a Bolseiro do Instituto Politécnico do Porto durante dois anos, o que me permitiu, a partir dessa data, dedicar-me a tempo inteiro ao meu projeto de doutoramento.

Começamos por delinear o estudo de forma a caracterizar a expressão das histonas metiltransferases em amostras congeladas de tecidos normais e tumorais de próstata. Apesar de novidade no grupo, utilizamos uma plataforma de *mini-arrays* que nos permitiu avaliar a expressão do transcrito destas enzimas. Mais tarde, e com vista a avaliar o balanço entre estas enzimas e as que catalisam a função inversa, as desmetilases, decidimos aumentar a análise abrangendo os dois grupos de remodeladores da cromatina. Inicialmente, o estudo foi desenhado com uma perspetiva de identificação de biomarcadores de diagnóstico preferencialmente em fluidos biológicos, mas os resultados mais promissores surgiram no campo da caracterização da agressividade dos tumores e predição da recorrência pós-prostatectomia, utilizando amostras de tecido tumoral. Uma vez que, a nível global, não se observou uma desregulação semelhante de enzimas responsáveis pelas mesmas marcas, decidimos

não continuar o estudo por painéis de enzimas mas, antes, explorar o papel biológico das que nos pareceram mais promissoras sob o ponto de vista oncobiológico.

Apesar de não ter sido planeado desde início, dada a minha maior disponibilidade, decidimos ser um pouco mais ambiciosos e alargarmos o projeto no sentido de caracterizar o papel de uma enzima metiltransferase, a SMYD3, que tinha mostrado relevância clínica. Assim, surgiu o subtítulo desta tese, uma vez que uma grande parte do trabalho nela inserido decorreu da caracterização do papel da SMYD3 na carcinogénese prostática. O fato de ter passado a usufruir de uma disponibilidade total para dedicação a este trabalho permitiu-me, num curto espaço de tempo, contactar com diversas metodologias e técnicas, contribuindo para o seu desenvolvimento e implementação no Grupo de Epigenética do Cancro do IPO Porto, o que muito contribuiu para o incremento do meu conhecimento científico nesta área de investigação. É, ainda, de realçar o espírito crítico com que os meus orientadores avaliaram as minhas ideias e projetos de estudo experimental. Contudo, mesmo dentro das restrições orçamentais, sempre me foram proporcionadas todas as condições para o seu desenvolvimento, desde que estas fossem sólidas e bem fundamentadas. Neste contexto, é de salientar o apoio financeiro que decorreu da participação do Grupo de Epigenética do Cancro no consórcio EpiDiaCan, o qual foi selecionado para atribuição de financiamento no âmbito do 7º Quadro Programa (FP7) da União Europeia. Creio que a opção pela área da Epigenética foi, sem dúvida, uma aposta ganha, seja pelo desafio decorrente de ser uma área relativamente recente e fascinante, com muito ainda por explorar, seja pela complexidade mecanística e estrutura que despertou o meu espírito crítico e curioso.

Chego ao fim desta etapa com a noção que o meu projeto está inacabado e incompleto, mas com o sentimento de ter feito o meu melhor, de ter seguido o caminho que queria, desfrutando do imenso prazer que me deu realizá-lo neste grupo de investigação. Sou hoje uma pessoa diferente pelo muito que aprendi, pelas experiências vividas dentro e fora do laboratório e principalmente pelos obstáculos que tive de aprender (constantemente) a ultrapassar. O trabalho de investigação não é fácil mas é aliciante, e os seus resultados nem sempre são proporcionais ao esforço e trabalho investidos. Mas, quando corre bem, é realmente muito gratificante. Nesta etapa tive o prazer de conviver com pessoas fantásticas com as quais muito aprendi e que contribuíram significativamente para que este trabalho chegasse a bom porto. À minha maneira, posso dizer que consegui passar, com sucesso, este gosto pelo que faço (e porque faço) a alguns destes companheiros de viagem pois, no fundo, nunca consegui separar-me inteiramente da faceta de docente e o gosto de ensinar continuará a acompanhar-me.

AGRADECIMENTOS

Esta tese não seria possível sem o contributo pessoal ou profissional de muitas pessoas aos quais quero demonstrar a minha gratidão.

- Aos meus orientadores, o Prof. Rui Henrique e a Prof.ª Carmen Jerónimo pela oportunidade que me deram, por terem sido cruciais no desenho e construção deste projeto e pelo apoio incansável. Ao Prof. Rui por todas as ideias pertinentes que foi dando ao longo da realização deste trabalho, pelo seu rigor e espírito crítico constante e por ser um poço de saber inesgotável. Agradeço ainda por ser o meu ideal de um verdadeiro professor e, por isso, uma inspiração para mim. À Prof.ª Carmen Jerónimo, pelos bons momentos científicos e pessoais vividos, pelo apoio constante no percorrer deste caminho, por ter acreditado no meu valor e ter apostado em mim. Espero sinceramente não a ter desiludido. Além de uma boa líder científica a Prof. Carmen sempre se mostrou muito humana sendo quase uma segunda mãe para todos nós, sempre preocupada connosco e com o nosso futuro.

- Ao Prof. Manuel Teixeira, Diretor do Serviço de Genética e do Centro de Investigação do Instituto Português de Oncologia do Porto, por me ter aceite no Centro de Investigação e ter possibilitado que desenvolvesse o meu trabalho com todas as condições necessárias.

- À Prof Fátima Baltazar, do Instituto de Investigação em Ciências da Vida e Saúde em Braga, por ter permitido a realização dos ensaios *in vivo* no grupo que dirige.

- À Prof. Regina por ter estimulado constantemente o meu espírito critico e, principalmente, por ter apoiado a minha dedicação ao doutoramento a tempo integral.

- A todos os coautores dos artigos, pela sua contribuição científica, nomeadamente ao Eng. Luís Antunes e o Dr. Francisco Menezes.

- Ao Pedro, por ter sido um bom companheiro desde o início da viagem. Por toda a ajuda laboratorial e principalmente pelas boas discussões científicas. Valorizo muito a tua opinião e o teu raciocínio lógico, confio no teu trabalho e, por isso, considero-te um membro fulcral no nosso grupo. E não sou a única… Mais importante que isso, obrigada por esta amizade boa que construímos e por todos os abraços que estiveste sempre disposto a dar em momentos menos bons.

- Ao Diogo Rios por toda a ajuda nesta última parte do trabalho, pelas muitas horas (apertadas) na câmara de fluxo, pelas risotas constantes e por tornares qualquer trabalho mais leve e divertido. Pelas conversas deprimentes e/ou parvas que tivemos tantas vezes, pelo ombro amigo e pelos bons conselhos que soubeste dar na hora certa. Espero que continuemos a construir este castelo de legos, como tu já disseste e que,

apesar de termos começado como professora e aluno, possamos agora continuar a ser bons amigos.

- À Inês e à Elsa, companheiras de saídas em casais, pelos muitos bons momentos fora do laboratório, pelos jantares em promoção em que às vezes só se safou mesmo a companhia e pelo companheirismo e amizade que partilhamos. Obrigada por toda a ajuda nas células, por terem partilhado o vosso conhecimento e terem ajudado sempre que foi necessário. À Inês obrigada por teres contribuído no delineamento de muitas experiências e pela ajuda preciosa em muitos momentos de grande aperto. À Elsa por nunca me deixar ficar mal em almoços, lanches ou jantares (pois comes sempre mais que eu) e pela preocupação e disponibilidade constantes na vida laboratorial. Às duas obrigada pelo espírito crítico e pelas boas conversas (científicas ou não) que tivemos.

- À Marcita, minha gémea siamesa, por teres sempre um sorriso na cara e seres a pessoa maravilhosa que és. Porque tens um coração enorme e por quereres o bem de todos muitas vezes à frente do teu. Pela companhia a aprender letras e números (Bokwa babies!) e pelas zumbadas às vezes dessincronizadas. Pela boa disposição constante, pelas risadas que fazem qualquer pessoa sentir-se melhor, pela boa companhia e pelo otimismo que incutes na vida.

- À Joaninha por todas as discussões científicas que tivemos que muito ajudaram a melhorar o meu trabalho, pelo teu espírito crítico, pelos bons conselhos científicos que tanto valorizei, pelas risotas que partilhamos e pela boa companhia que és dentro e fora do laboratório.

- Ao Diogo Silva, por toda a ajuda nas bactérias, pelas boas dicas que deste que tornaram tudo mais fácil, pelo sentido prático que dás à vida e pelas boas conversas que fomos tendo.

- À Andreia e à Isa pela ajuda no laboratório e pela presença sempre bem-disposta a trabalhar.

- Ao Jorge por ter tornado as pausas do almoço mais cultas e mais ricas, sem ti muitos milhões não teriam sido ganhos.

- Ao João, Tiago e Sara por terem contribuído para o planeamento ou realização deste projeto. Ao João pela ajuda na fase inicial deste trabalho, ao Tiago por todas as ajudas tanto nos Westerns como nas técnicas utilizadas na fase final do trabalho (e por, mesmo longe, continuar a ajudar) e à Sara pela ajuda preciosa no ChIP.

- À Marta pelas trocas de experiências aquando da mutagénese e por ser sempre prestável e bem-disposta.

- Ao Serviço de Genética em geral, no qual fui muito bem acolhida e pela cooperação e disponibilidade demonstradas. Um especial agradecimento à Manuela, que

chateei muitas vezes e que sempre se mostrou muito prestável, desenrascando muitas situações complicados.

- À Susana por ter estado sempre disponível nas experiências com os ovos, pelas muitas horas que passamos a fazer (e repetir) experiências e pelo apoio sempre que as coisas correram menos bem.

- À maravilhosa Rute, por seres uma amiga espetacular, por estares sempre presente mesmo quando não comunicamos, pela sinceridade e frontalidade, pelo ombro amigo e pelos bons (e muitos) conselhos. Obrigado Rutiti por seres tão especial.

- Ao Fernando pela amizade e amor, pelo carinho e companheirismo, por não me deixares ir a baixo nos maus momentos e me fazeres crer que tudo iria sempre correr bem. Pela paciência para me aturares (e às vezes não deve ser nada fácil) e, principalmente, por tornares esta vida muito melhor...

- À minha família por me ter apoiado sempre e acreditado que era capaz. Um especial e sentido agradecimento aos meus queridos pais que sempre estiveram do meu lado em todos os momentos bons ou maus. Obrigado pelos bons valores que me incutiram, pela exigência mas também pelo carinho constante que me deram sempre força para fazer mais e melhor.

RESUMO

O cancro da próstata (CaP) é um dos cancros com maior incidência e prevalência no sexo masculino, sendo uma das principais causas de mortalidade e morbilidade a nível mundial. Dadas as limitações dos parâmetros clínicos e patológicos correntemente utilizados para predição da agressividade do CaP, é de extrema importância a identificação de novos marcadores de diagnóstico ou prognóstico que permitam melhor definir as estratégias de monitorização e terapêutica mais apropriadas. Alterações nos padrões de modificações da cromatina têm sido descritas como resultantes de desregulação da expressão ou atividade de enzimas modificadoras da cromatina, incluindo as metiltransferases (MTHs) e as desmetilases (DMHs) das histonas. A desregulação de algumas destas modificadoras de histonas, como a EZH2 ou LSD1, têm sido associadas com o desenvolvimento e progressão do CaP. No entanto, a importância do papel desempenhado no CaP por outros membros das MTHs ou DMHs, bem como das respetivas marcas de histonas, está ainda pouco esclarecido. Assim, o nosso principal objetivo foi averiguar o papel da alteração da expressão das MTHs e DMHs na carcinogénese prostática e traduzir esses achados em ferramentas com utilidade clínica na abordagem dos pacientes com CaP.

Inicialmente, avaliamos, por RT-qPCR, os níveis de expressão de 37 MTHs e 20 DMHs utilizando tecidos normais e tumorais prostáticos. Numa série de 150 pacientes com CaP clinicamente localizado, submetidos a prostatectomia radical, os níveis de transcrito dos genes *SMYD3*, *SUV39H2*, *PRMT6*, *KDM5A* e *KDM6A* encontravam-se sobreexpressos, enquanto os relativos a *MLL1-5* e *KDM4B* apresentaram níveis inferiores nos tumores quando comparados com os tecidos normais. Notavelmente, a *PRMT6* foi a enzima modificadora de histonas que melhor discriminou tecidos normais de tumorais sugerindo o seu potencial como método auxiliar de diagnóstico no CaP. Adicionalmente, a *SMYD3* apresentou níveis aumentados em tumores mais agressivos (pT3b), tendo estes níveis a capacidade de predizer independentemente o prognóstico nesta série de pacientes. Assim, a expressão da *SMYD3* pode fornecer informações clínicas relevantes no que diz respeito ao potencial de agressividade de um dado carcinoma da próstata.

Seguidamente, foi avaliado o potencial papel oncogénico da SMYD3 em linhas celulares de CaP. O silenciamento da *SMYD3* atenuou o fenótipo maligno das células neoplásicas, tanto *in vitro* (ensaios de viabilidade celular, apoptose, migração e invasão) como *in vivo* [(formação de tumores e angiogénese no ensaio na membrana corioalantoide (CAM)]. De sublinhar que as propriedades oncogénicas da SMYD3 foram associadas à sua atividade como histona metiltransferase. Na investigação de genes alvo da SMYD3 foi identificado o gene *CCND2*, sendo a diminuição da sua expressão

associada com o estabelecimento da marca repressiva H4K20me3 na região promotora, marca essa que é catalisada pela SMYD3.

Concluímos que o perfil de expressão das MTHs e DMHs, especialmente da SMYD3, pode ser de utilidade clínica para avaliação de pacientes com CaP e constituir uma ferramenta auxiliar na seleção da estratégia terapêutica mais apropriada. Adicionalmente, a SMYD3 e, eventualmente, outros modificadores de histonas, poderão constituir um alvo terapêutico interessante em determinados subgrupos de doentes com CaP. No entanto, é necessária uma avaliação mais abrangente dos genes alvo da SMYD3, bem como do impacto na sua desregulação, de forma a melhor compreender o seu papel na carcinogénese da próstata.

SUMMARY

Prostate cancer (PCa) is one of the most incident and prevalent cancers among men, and a leading cause of cancer-related morbidity and mortality, worldwide. Because the currently used parameters to predict the aggressiveness of PCa are rather imperfect, it is extremely important to identify new diagnostic and prognostic markers to better define the most appropriate management and therapeutic strategy. Alteration of chromatin modification patterns have been attributed to altered expression or activity of key chromatin-modifying enzymes, including histone methyltransferases (HMTs) and demethylases (HDMs). Deregulation of some of these histone modifiers, such as EZH2 or LSD1, have been already associated with PCa development and progression. Nevertheless, the importance of other members of HMTs or HDMs and respective histone marks in PCa is currently poorly understood. Therefore, our main goal was to clarify the role of HMTs and HDMs altered expression in prostate carcinogenesis and to translate those findings into clinically useful tools for the management of PCa patients.

Firstly, we assessed the expression levels of 37 HMTs and 20 HDMs in normal and cancerous prostate tissues by RT-qPCR. In a series of 150 PCa patients with clinically localized disease, submitted to radical prostatectomy, *SMYD3*, *SUV39H2*, *PRMT6*, *KDM5A* and *KDM6A* were up-regulated, whereas *MLL1-5* and *KDM4B* were downregulated in PCa compared to normal tissues. Remarkably, *PRMT6* was the histone modifier that best discriminated normal from cancerous tissue samples and might prove useful to assist in PCa diagnosis. Additionally, *SMYD3* presented higher levels in more aggressive tumors (pT3b) and those were able to independently predict prognosis in this patient cohort. Thus, *SMYD3* expression might provide relevant clinical information concerning the potential aggressiveness of a given prostate tumor.

Next, we evaluated the putative oncogenic role of SMYD3 in PCa cells. Silencing of *SMYD3* attenuated the malignant phenotype of PCa cells, both *in vitro* (cell viability, apoptosis, migration and invasion assays) and *in vitro* [tumor formation and angiogenesis in the chorioallantoic membrane (CAM) assay]. Interestingly, we found that SMYD3 oncogenic properties were dependent of its histone methyltransferase activity. In a search for genes targeted by SMYD3, *CCND2* was identified and its downregulation was associated with the establishment of the repressive mark H4K20me3 in the promoter region, which is catalyzed by SMYD3.

We concluded that expression profiling of HMTs and HDMs, especially *SMYD3*, might be of clinical usefulness for assessment of PCa patients and assist in pre-therapeutic decision-making. In addition, SMYD3, and eventually other histone modifiers, might constitute an attractive therapeutic target in defined subsets of PCa patients.

Nevertheless, a more comprehensive identification of target genes and how SMYD3 deregulation impacts on its expression is required to fully understand its role in prostate carcinogenesis.

TABLE OF CONTENTS

FIGURES INDEX

CHAPTER I

CHAPTER III

CHAPTER IV

TABLES INDEX

CHAPTER I

CHAPTER III

CHAPTER IV

LIST OF ABBREVIATIONS AND ACRONYMS

15-LOX-1	15-Lipoxygenase-1
ADT	Androgen Deprivation Therapy
AJCC	American Joint Committee on Cancer
AR	Androgen Receptor
AS	Active Surveillance
BPH	Benign Prostatic Hyperplasia
CAM	Chick Chorioallantoic Membrane
CCND2	Cyclin D2
c-met	met proto-oncogene
CRPC	Castration-Resistant Prostate Cancer
DFS	Disease-Free Survival
DRE	Digital Rectal Examination
EAU	European Association of Urology
ER	Estrogen Receptor
EZH2	Enhancer of Zeste Homolog 2
GS	Gleason Score
GUSB	Glucuronidase, Beta
H3R2	Arginine 2 of histone 3
H3K4	Lysine 4 of histone 3
H3K9	Lysine 9 of Histone 3
H3K27	Lysine 27 of histone 3
H4R3	Arginine 3 of histone 4
H4K5	Lysine 5 of histone 4
H4K20	Lysine 20 of histone 4
HDM	histone demethylase
HE	hematoxylin and eosin
HMT	Histone Methyltransferase or Histone Methylase
ISUP	International Society of Urological Pathology
JMJC	Jumonji C
KDM	Lysine-Specific demethylase
KMT	lysine histone methyltransferase or lysine histone methylase
LSD	Lysine specific demethylase

me1	Monomethylation
me2	Dimethylation
me3	Trimethylation
miRNAs	MicroRNAs
MLL	Mixed-Lineage Leukemia
MRI	Magnetic Resonance Imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
ncRNAs	Non-Coding RNAs
Nkx2.8	NK2 homeobox 8
NPT	Normal Prostate Tissue
NSD1	Nuclear Receptor Binding SET Domain Protein 1
PCa	Prostate cancer
pRB	Retinoblastoma Protein
PRDM2	PR Domain Containing 2 With ZNF Domain
PRMT	Protein Arginine Methyltransferase
PSA	Prostate Specific Antigen
PTMs	Post-Translational Modifications
RIZ1	*(Same as PRMD2)*
RP	Radical Prostatectomy
SBE	Specific DNA Sequences
SMYD3	SET And MYND Domain Containing 3
SUV39H2	Suppressor Of Variegation 3-9 Homolog 2
TFRC	Transferrin Receptor
TRUS	Transrectal Ultrasound
TSS	Transcriptional Start-Site
UICC	Union for International Cancer Control
VEGFR1	Vascular Endothelial Growth Factor Receptor 1
WNT10B	Wingless-Type MMTV Integration Site Family Member 10B

Chapter I

General Introduction

PROSTATE CANCER

EPIDEMIOLOGY

Prostate cancer (PCa) is the second most frequently diagnosed cancer in men and fourth most incident cancer overall (Figure 1). However, as developed countries presented almost three-quarters of the registered cases, this cancer emerged as the most frequent amongst men of those regions [1, 2]. The same trend is observed in Europe, where PCa has emerged as the most frequent cancer amongst men with an estimated 416.7 per 100,000 of new cancer cases diagnosed in 2012 [3]. Worldwide, incident rates can vary by more than 25-fold, with the highest rates recorded in Australia/New Zealand, Northern America, Western and Northern Europe and the lowest rates observed in Asia [2]. Regarding incidence there was a rising trend mainly due to increased detection of latent disease after the widespread of prostate specific antigen (PSA) testing in the late-1980s and its subsequent and rapid uptake by urologists as a screening test [3]. A slight decline in the past few years was noted most likely owed to the depletion of the pool of detectable cases [4].

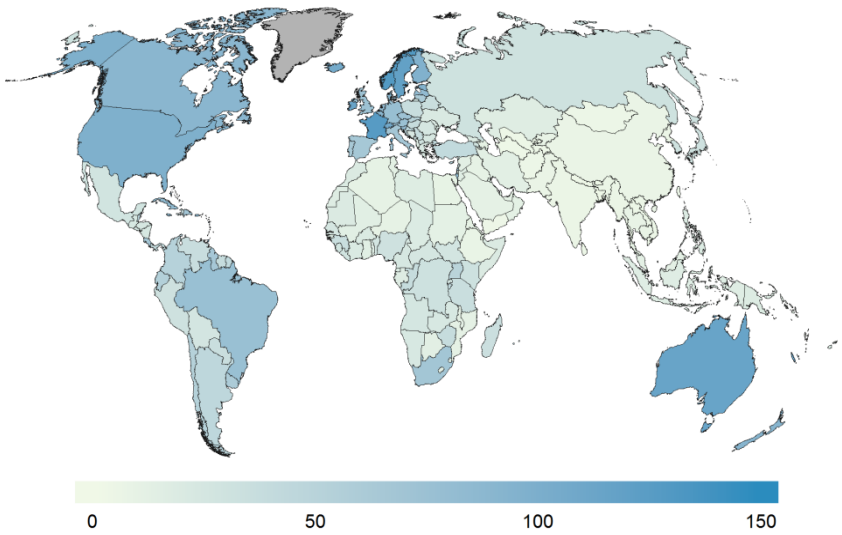

Figure 1 – Estimated age-standardized incidence rate for PCa per 100,000 worldwide. *Adapted from [2].*

PCa is the fifth leading cause of death from cancer in men with an estimated 307,000 deaths in 2012 (Figure 2). In contrast with high variable incident rates associated with prevalence of PSA testing and subsequent biopsy, mortality rates worldwide reflect a much lower variation between countries, with a similar number of deaths in developed or developing regions. Overall, mortality rates are generally higher in predominantly black populations (Caribbean and sub-Saharan Africa), very low in Asia and intermediate in Europe and Oceania. Although decreasing mortality trends have been observed in several

European countries and United States in the middle 1990s, relative impact of the introduction of curative treatment versus early detection by PSA is still subject to much debate [2, 5].

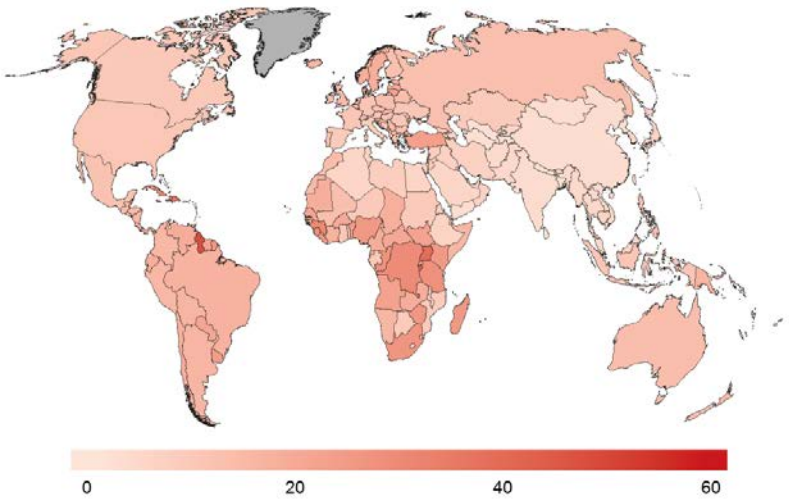

Figure 2 – Estimated age-standardized mortality rate for PCa per 100,000 worldwide. *Adapted from [2].*

In Portugal, PCa is the most frequent cancer in men and third leading cause of death from cancer, with 6,622 new cases registered and 1,582 deaths by PCa in 2012 (Figure 3). Worldwide, it is expected that PCa will reach more than 2 million new cases and 633 000 new deaths by 2035, largely because of the growth and aging of global population which turns prostate cancer into a major health concern [2].

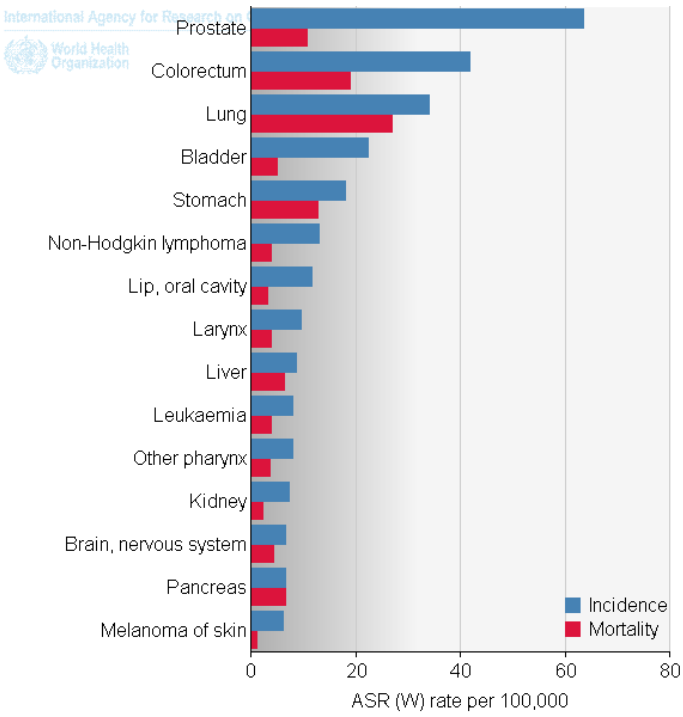

Figure 3 – Estimated age-standardized incidence and mortality rates in men in Portugal. *Adapted from [2].*

RISK FACTORS

Currently, the only risk factors fully established for PCa are age, ethnicity and family history [6].

PCa incidence rises strongly with age with approximately 85% of cases occurring in males over 65 and only 0.1% diagnosed under the age of 50 [7, 8]. Mainly due to its latency, a considerable percentage of PCa's are undetectable before progressing to clinically significant disease, but it is predicted that all man would develop this neoplasia if they live more than 100 years old [8].

African American men have the highest rates of PCa in the world and are frequently diagnosed earlier and at a more advanced stage than white American men [9, 10]. In contrast, Asians/Pacific Islanders display the lowest incident and mortality rates. Nevertheless, studies based on migration patterns show distinct changes in the incidence of this malignancy that might reflect the influence of exogenous factors such as differences in diet and in access to care and screening programs.

Men with family history of PCa have an increased risk of developing this disease, suggesting inheritance of genes involved in prostate carcinogenesis. Even though, familial aggregation of PCa might also be explained by similarities in environmental factors which ultimately lead to development of this disease [7, 11].

Additionally, these different incidence rates among populations might also be due to genetic differences, once several reports showed differences in microsatellites allelic frequencies at the androgen receptor (AR) locus as well as polymorphic variation [12].

PROSTATE ADENOCARCINOMA

Prostate gland is a walnut-sized organ surrounding the urethra at the base of the bladder whose main function is to produce important components of the seminal fluid [13]. McNeal model for prostate structure, widely adopted by clinicians, mainly divides prostate gland in four distinct zones: peripheral, central, transition zones and anterior fibromuscular stroma [14, 15]. Benign prostatic hyperplasia (BPH), a common non-malignant condition, is found in the transition zone, whereas almost all prostate neoplasias arise from the peripheral zone [13, 16]. Adenocarcinoma comprises 95% of the malignant neoplasms of the prostate and is presented as a heterogeneous and multifocal disease [14]. Regarding cellular content, normal and mature prostatic epithelium-basal comprises basal, secretory luminal and neuroendocrine cells. The luminal or glandular cells constitute the exocrine compartment of the prostate producing PSA and prostatic acid phosphatase into the glandular lumina. Those cells express high levels of AR and depend on androgens for

their survival. Some authors defend that luminal cells are responsible for the origin of prostate neoplasia, although this concept is still controversial [17].

SCREENING AND DIAGNOSIS OF PROSTATE CANCER

PCa is frequently asymptomatic in its early stages and, although most of cases are indolent with good prognosis, others are extremely aggressive leading to patients' death. Considering that PCa is a slow-growth disease, 70% of deaths due to PCa occur after 75 years of age [18]. Nowadays, PSA serum concentration and digital rectal examination (DRE) are the only available screening tools for PCa; nevertheless, their impact on prostate cancer mortality remains unclear [19]. PSA is not a PCa specific marker as several other conditions can also lead to an increase of its production, namely prostatitis, BPH, medications or urologic manipulations [20]. Currently, for diagnosis purposes, European Association of Urology (EAU) guidelines consider transrectal ultrasound (TRUS)-guided systemic biopsy in men with abnormal DRE and/or a PSA value higher than 4.0ng/ml, but clinicians should also be aware of patient's age, potential comorbidities, and therapeutic consequences. Importantly, it should be taken in consideration that there are PCa cases with normal DRE and/or lower levels of PSA [19, 21-23].

Presently, PCa screening is responsible for overdiagnosis and overtreatment due to identification of indolent tumors that would not threaten men's life. US Preventive Services Task Force even do recommend against PCa screening in all men, regardless of age or risk, without even considering a discussion of the risks and benefits of screening [18]. Nevertheless, other studies still defend that DRE and PSA together as early detection tools have an impact in PCa mortality reduction as they have allowed the identification of early-stage tumors, thus increasing chances of cure [13, 24].

PROSTATE CANCER GRADING

The Gleason score (GS) is the universal standard for grading PCa and is based on the histologic evaluation of glandular epithelial architecture pattern. It consists in classifying the two most prevalent patterns assigning a value from 1 to 5: 1 corresponds to a well-differentiated pattern and 5 to a poorly differentiated pattern. The GS is obtained by adding the classification of the two most common patterns in order to reflect the PCa morphological heterogeneity, therefore ranging from 2 to 10 [13]. In 2005, this classification was updated by International Society of Urological Pathology (ISUP) (Figure 4), which agreed that GS in a prostate biopsy should contemplate the Gleason grade of the most extensive carcinoma component and the highest grade, regardless of its extent.

When needle biopsy comprises different cores with different grades, it is recommended to report the grades of each core separately and the highest one should be considered to determine treatment [25]. In radical prostatectomy (RP) specimens, both the primary and the secondary Gleason grade should be reported with a comment on the tertiary pattern [19]. The predominant pattern has an important role in prognostic evaluation, namely in GS 7 in which the predominance of pattern 4 (Gleason 4+3) carries more than a three-fold higher risk of PCa mortality than Gleason pattern 3+4 [26].

Remarkably, decades after its implementation, Gleason grading system remains one of the most powerful prognostic tools for PCa, alongside with pathological stage, being crucial in predicting PCa natural history and assessment of the risk of recurrence after RP or radiotherapy [21]. Inter-observer reproducibility and number of sampling biopsies are still the major concern when using GS, although the updated system has proved to increase in 20% agreement between observers [27, 28].

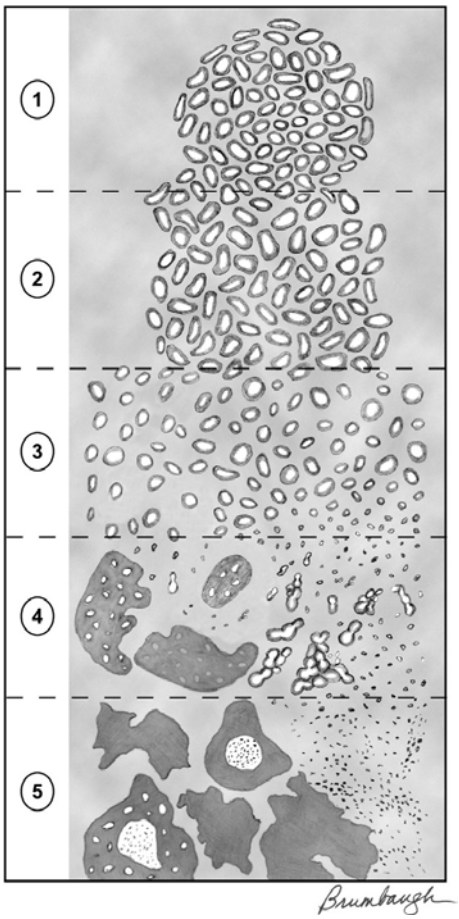

Figure 4 – Updated Gleason Grading System. Pattern 1 – Closely-packed, uniform, rounded to oval glands; Pattern 2 – more loosely arranged glands with smooth ends that minimally invade non neoplastic tissue; Pattern 3 – Irregular size and shape glands with more infiltrative margins; Pattern 4 – Fused, cribriform or ill-defined glands; Pattern 5 – almost no glandular differentiation. *Adapted from [25].*

CLINICAL AND PATHOLOGICAL STAGING

Cancer staging systems are important tools to assess the extent of a cancer and thus decisive in the choice of treatment options and in prognostic evaluation [16, 29]. Among clinicians, the most commonly used staging system is TNM system, proposed by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) [30]. The system is based on the extension of primary tumor (T), presence and extension of involved lymph nodes (N) and distant metastases (M) [31] (Table 1). There are two types of TNM stages determined by AJCC and UICC: pre-treatment or clinical stage and postsurgical or pathological stage [30, 32].

Clinical staging is established prior to first treatment and is achieved accounting data from DRE, TRUS or magnetic resonance imaging (MRI), and serum PSA levels [30, 33]. This classification does not change after surgical resection and pathological stage determination.

Pathological staging requires histological evaluation; hence the tumors extent within the prostate and in surrounding tissues can only be determined after RP, with micro and macroscopic analysis of prostatectomy specimen and the dissected regional lymph nodes. Distant lymph nodes involvement outside the pelvic region, are considered metastatic disease, notwithstanding its lymphatic nature. PCa spreads preferentially to bone, therefore in advanced stages bone metastasis are frequently observed [34].

Moreover, as PCa is frequently multifocal and clinical stage truly fails in evaluation and prediction of extraprostatic extension or seminal vesical invasion. Conversely, pathological stage is widely accepted as a more accurate tool to predict recurrence being also used as a prognostic factor [29, 35].

The combination of several tumor features, namely prostate capsule invasion, preoperative serum PSA levels, GS for the RP specimen, positive surgical margins, lymph node metastases, seminal vesicles involvement and distant metastases are considered for establishing clinical nomograms, which have proved to be useful to assess prognosis and risk for PCa in clinical practice [13, 29, 36].

Table 1 – The 2010 American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) TNM staging classification for prostate cancer. *Adapted from [32]*

PRIMARY TUMOR (T)	
CLINICAL	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically unapparent tumor neither palpable nor visible by imaging
T1a	Tumor incidental histologic finding in 5% or less of tumor resected
T1b	Tumor incidental histologic finding in more than 5% of tumor resected
T1c	Tumor identified by needle biopsy
T2	Tumor confined within prostate gland
T2a	Tumor involves one half of one side or less
T2b	Tumor involves more than one half of one lobe but not both lobes
T2c	Tumor involves both lobes
T3	Tumor extends through prostate capsule
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles, such as: external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
PATHOLOGIC (PT)*	
pT2	Organ confined
pT2a	Unilateral, one half of one side or less
pT2b	Unilateral, involving more than one half of one lobe but not both lobes
pT2c	Bilateral disease
pT3	Extraprostatic extension
pT3a	Extraprostatic extension or microscopic invasion of bladder neck
pT3b	Seminal vesicle invasion
pT4	Invasion of rectum, levator muscles and/or pelvic wall
REGIONAL LYMPH NODES (N)	
CLINICAL	
Nx	Regional lymph nodes were not assessed
N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node(s)
PATHOLOGIC (PN)	
pNx	Regional nodes not sampled
pN0	No positive regional nodes
pN1	Metastasis in regional node(s)
DISTANT METASTASIS (M)	
M0	No distant metastasis
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 pT1 classification

PROSTATE CANCER TREATMENT

Even though the great majority of PCa cases have slow growth rates, there are some aggressive cases that must be promptly identified. The likelihood of cure is greater for non metastatic disease, requiring different treatment options. In this regard, stage of disease, PSA levels, GS, life expectancy of the patient and their possible side effects are taken into account [31, 37]. The challenge is to adequate treatment strategies avoiding patients' overtreatment, but always for curative purposes [13].

Regarding clinically localized PCa, active surveillance (AS), surgery with RP, external-beam radiation therapy, and interstitial radiation therapy (brachytherapy) are the current treatment options.

AS is considered for patients with low risk of developing an aggressive disease. These patients have clinically confined PCa (T1-T2), GS ≤ 6 and PSA level < 10 ng/mL [19] and are subject to periodic PSA measurement and prostatic biopsies in order to assess disease progression.

RP with or without extended pelvic lymphadenectomy is a surgical option advocated for high-risk locally advanced PCa [37]. Patients that are eligible for surgery include those with a biopsy GS ≤ 8, PSA level < 20 ng/ml, tumor ≤ cT3a and life expectancy of 10 or more years [19]. Recently, focal cryotherapy has emerged as a less morbid option and an alternative option for low-risk patients [19].

Radiotherapy is considered the second major therapeutic modality for localized high-risk PCa, after RP. This procedure comprises external-beam radiotherapy, brachytherapy or, more recently, proton beam therapy and has fewer side effects in urinary disorders and erectile functions, with similar survival rates in low-risk PCa when comparing to RP [37, 38]. Different radiotherapies can be used as monotherapy, in combination with other radiotherapies or as an adjuvant post-prostatectomy [13].

When the disease is considered clinically advanced, hormonal therapy is the most recommended treatment option. Because surgical removal of the prostate and radiotherapy both have side effects, such as incontinence and erectile dysfunction, they are not indicated in patients with metastatic disease [37].

Since prostate cells are androgen-dependent, the activation of AR, even without androgen stimulation, is the driven force of prostate growth. Androgen deprivation therapy (ADT) either by chemical or surgical castration is recommended in locally advanced and metastatic disease. Nevertheless, this strategy is also used in combination with surgical or radiation therapy [19, 39, 40].

Notwithstanding, ADT leads to 70-80% symptoms reduction, almost all tumors will progress and become resistant to androgen suppression, a status named castration-

resistant PCa (CRPC) [41, 42]. Albeit its low efficiency, chemotherapy with docetaxel and prednisone, is the first-line treatment in metastatic CRPC. Moreover, when this strategy fails, all subsequent treatments are only used as palliative support [42-44].

EPIGENETICS

Currently, epigenetics is accepted as a set of processes which have long-term effects on gene expression programs but no interference within DNA sequence [45]. These mechanisms consist in several heritable changes that are early established during embryonic development, responsible for initiation and maintenance of cellular differentiation, even within cell replication and division, which might explain different cell phenotypes for identical genetic information [46, 47].

Epigenetics machinery is critical to chromatin structure and gene transcriptional activity, comprising three major mechanisms: DNA methylation, covalent modifications of histones and non-coding RNAs (Figure 5) [48]. Although part of cellular natural and normal physiology, epigenetic patterns might be modified in response to intrinsic and extrinsic stimuli that can lead to gene expression deregulation and ultimately to disease onset [45, 49].

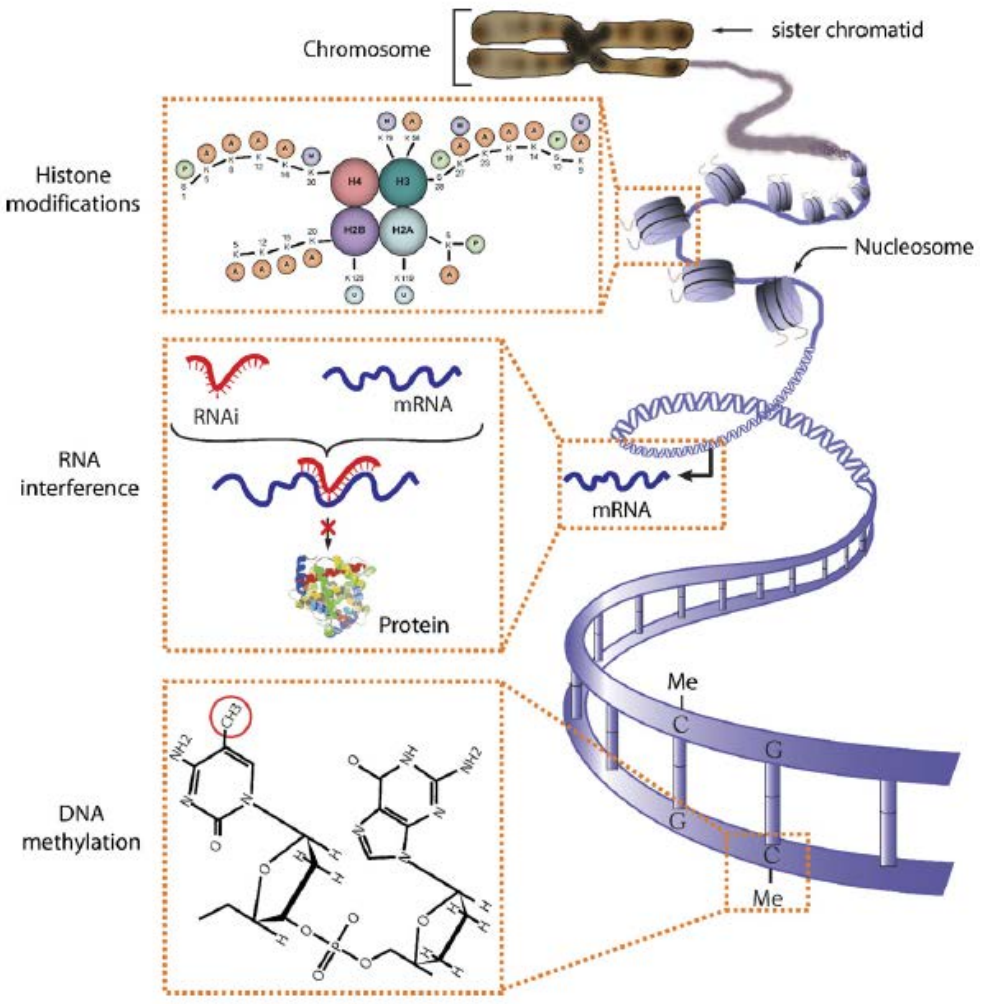

Figure 5 – Mechanisms of epigenetic regulation: DNA methylation, histone modifications, and non-coding RNAs. *Adapted from [50].*

DNA METHYLATION

DNA methylation consists in the addition of a methyl group at the 5' position of a cytosine ring within CpG dinucleotides. This alteration is catalyzed by DNA methyltransferases [51]. DNA methylation occurs almost exclusively in the context of CpG dinucleotides that tend to cluster in regions called CpG islands. The great majority of human gene promoters presents CpG islands which are frequently unmethylated in normal cells, even though during development and tissue differentiation, some of them can specifically be methylated [52].

Concerning gene expression regulation, DNA methylation has been associated with gene expression inhibition and with chromatin repressive states [52]. This transcription silencing might occur either by avoiding binding of RNA polymerase and transcription factors [51, 53] or indirectly through recruiting methyl-CpG-binding domain proteins that consequently promote recruitment of histone-modifying and chromatin-remodeling complexes [52].

Alterations on DNA methylation pattern have been widely described in cancer [54]. Tumor cells are characterized by a global loss of DNA methylation that promotes chromosomal instability, reactivation of transposable elements and loss of imprinting. Beyond global hypomethylation, there is also frequently observed hypermethylation at specific CpG islands, mainly in promoter regions of tumor-suppressor genes [49, 52].

COVALENT HISTONE POST-TRANSLATIONAL MODIFICATIONS

Histones are highly conserved proteins that are tightly enclosed with DNA and, therefore, involved in regulation of gene expression. Together, histones and DNA form the so-called nucleosomes, that comprise 146 bp of DNA coiled in sequence around a core of eight histonic proteins, two of each H2A, H2B, H3 and H4, linked by H1 [55, 56].

Histones maintain contact with DNA through their flexible globular domain and amino acid residue regions (lysine, serine and arginine) that protrude from the nucleosome: histone "tails". The majority of post-translational modifications (PTMs) in histones occur at this region [57]. The pattern of histone modifications determines chromatin status (euchromatin or heterochromatin), and accessibility of DNA to nuclear factors and subsequently gene transcription [58]. Heterochromatin has a highly package conformation comprising mostly inactive genes, whilst euchromatin is relatively uncondensed and represents *loci* being actively transcribed [56]. PTMs encompass methylation, acetylation, phosphorylation, ubiquitylation and sumoylation being the first two the most abundant and the better characterized, so far [59, 60]. Acetylated histones

are generally associated with a less compact chromatin that facilitates access to transcriptional machinery and thereby leads to gene activation. In contrast, methylated histones may be associated either to gene repression or activation, depending on the amino acid residue and the number of methyl groups added. The histone code comprises a combination of modifications on each histone and/or nucleosome and is strongly related to gene transcription (Figure 6). The primary protein families involved in this process are the enzymes responsible for adding acetyl or methyl groups to histone tails, termed writers, including histone acetyltransferases and histone methyltransferases (HMTs), and the erasers, responsible for the removal of these marks, and include histone deacetylases and demethylases (HDMs) [61, 62].

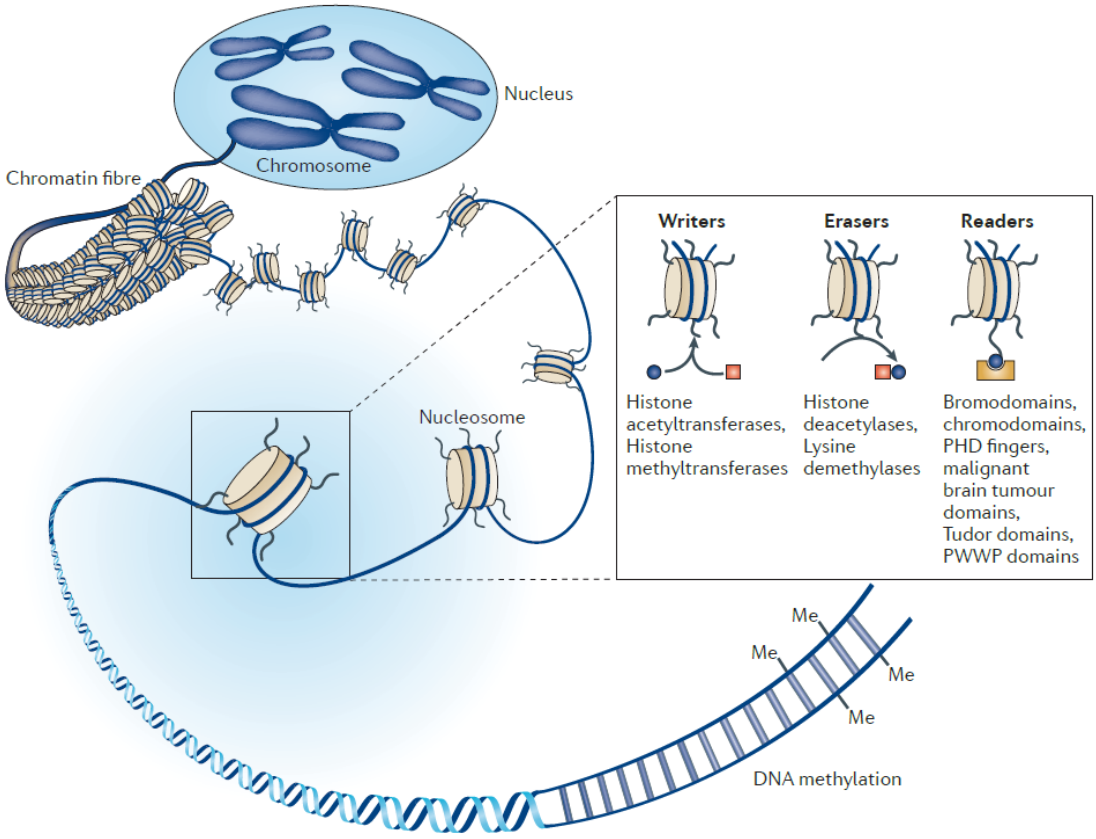

Figure 6 – Histone methylation and acetylation and the proteins responsible for the producing, removal or recognition of these marks. *Adapted from [61].*

Histone modifications are thought to alter the electrostatic charge of the histones resulting in a structural change or their binding affinity to DNA. Additionally, these modifications may serve as binding sites for protein recognition modules, such as the bromodomains or chromodomains, which are termed readers since they recognize acetylated lysines or methylated lysines, respectively. Abnormal patterns of histone modifications due to altered expression and/or activity of key chromatin-modifying enzymes were implicated in tumorigenesis [62]. Furthermore, a balance between HMTs

and HDMs is crucial for a normal cellular phenotype and its deregulation has been reported for several tumor models [63].

NON-CODING RNAS

Non-Coding RNAs (ncRNAs) are a class of RNAs that, although biological relevant and essential to a correct development, do not encode for proteins [64]. They are involved in several pathways, including chromosome dynamics, splicing, RNA editing, inhibition of translation and mRNA destruction [65]. Concerning ncRNAs, the most widely studied class is microRNAs (miRNAs). These molecules are responsible for repression of mRNA translation by mRNA degradation or inhibition of translation. It is presently acknowledged that each miRNA can target several mRNAs and a single mRNA can be targeted by multiple miRNAs. Hence, altered miRNAs can dramatically affect a variety of cellular mechanisms, namely proliferation, cell death, differentiation, development and metabolism [66].

Several mechanisms are involved in miRNAs deregulation, including genetic and epigenetic events [67]. Indeed, the miRNAs abnormal expression is a frequently event in cancer [64, 68], where they may act either as oncogenes (oncomiRs) or tumor-suppressors depending on target genes and tumor context [69]. Globally miRNAs are downregulated in cancer, but specific upregulation has also been described [70]. In tumor suppressor miRNAs a decreased expression might occur mainly due to defects at miRNAs biogenesis causing an abnormal expression of its targets which might lead to tumor formation. On the other hand, oncomiRs amplification and/or overexpression might also induce tumors by decreasing expression of a miRNA-target tumor-suppressor gene [69].

HISTONE METHYLATION AND PROSTATE CANCER

HISTONE METHYLATION

The role of histone methylation in PCa has been increasingly elucidated over the past decades. In contrast to histone acetylation, histone methylation does not alter histone tail ionic charge; instead it determines its basicity, hydrophobicity, and transcription factors affinity towards DNA [71]. Histone methylation can occur either in lysine residues, by lysine histone methyltransferases (KMT), or in arginine residues, by protein arginine methyltransferases (PRMT) and might positively or negatively regulate gene transcription. The reversibility of histone methylation has been established through the discovery of histone lysine and arginine demethylases, uncovering a new level of histone plasticity [72, 73]. Whereas lysine residues might be modified into mono- (me1), di- (me2), or trimethyl (me3) states, arginine can only be modified to mono- or dimethyl states (symmetric or asymmetrically) [74]. Thus, different degrees of methylation may be associated with distinct chromatin regions or transcriptional states [75] (Figure 7).

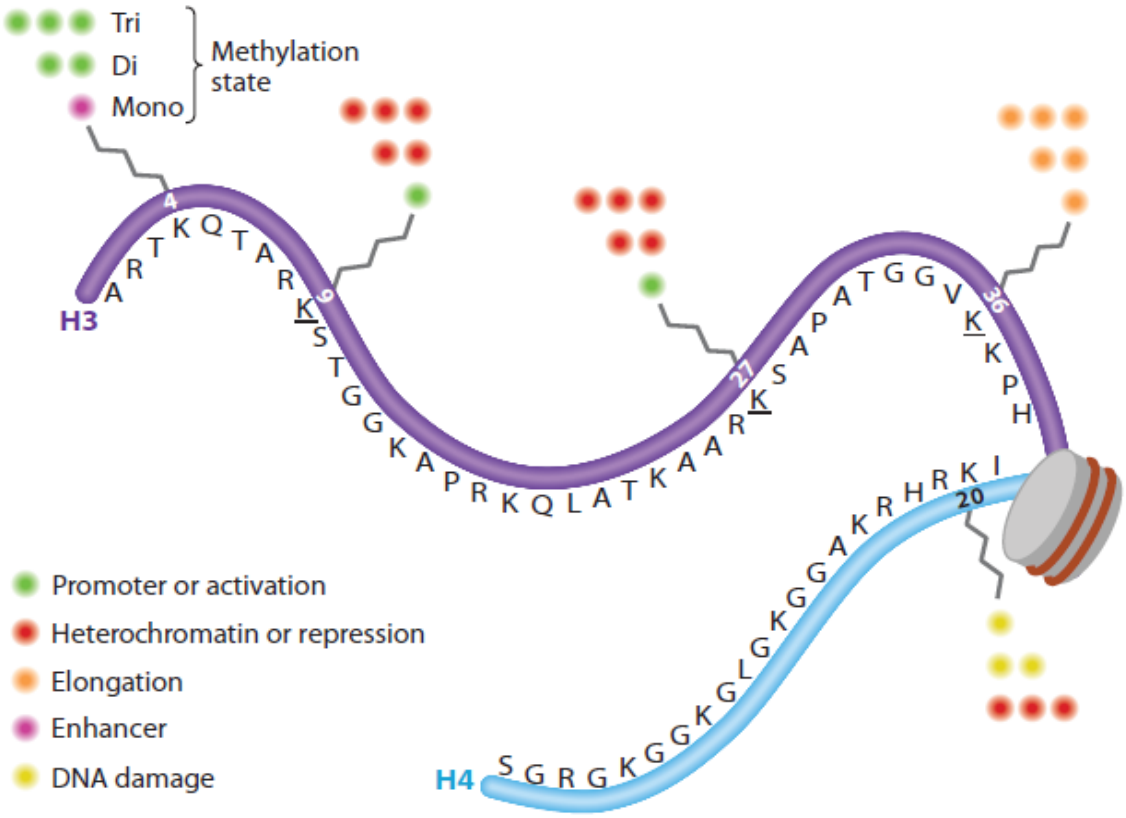

Figure 7 – Major lysine methylation marks on histones H3 and H4. The embedded numbers refer to the methylated amino acid residue on each histone. The general function of each mono-, di-, and trimethylation state is represented in dots of distinct colors according to its function. *Adapted from [76].*

H3K4me3 and H3K4me2 are highly enriched at transcriptionally competent or active gene promoters, but the mono-methylated form of H3K4 is associated with gene enhancers. H3K27me3 is present in transcriptionally repressed promoters whereas silent pericentric heterochromatin is marked by H3K9me3 [77]. Recent data have enlightened the role of histone arginine methylation in transcription regulation which was found to be able to promote or antagonize the interaction of nuclear factors with other nearby histone marks [78, 79].

Thus far, more than 50 HMTs and 30 HDMs were identified [61, 80-82] (Figure 8). Concerning methylation, all HMTs use S-adenosylmethionine as a co-substrate to transfer methyl groups and can be divided in three classes of histone-methylating enzymes: SET domain lysine methyltransferases, non-SET domain lysine methyltransferases, and arginine methyltransferases [83]. The specificity of these enzymes is questionable since they target non-histone proteins alongside with several histone residues [80]. The arginine methylases might be classified by its type of methylation: type I enzymes leads to symmetric arginine methylation while type II is responsible for the asymmetric process [78]. Regarding HDMs, they are categorized in two different groups: Lys-specific demethylases (LSD) and Jumonji C (JMJC) histone demethylases. The JMJC family demethylates mono-, di-, and trimethylates lysines enzymes, whereas LSD family proved to be unable to catalyze the demethylation of the trimethylated state [82, 84].

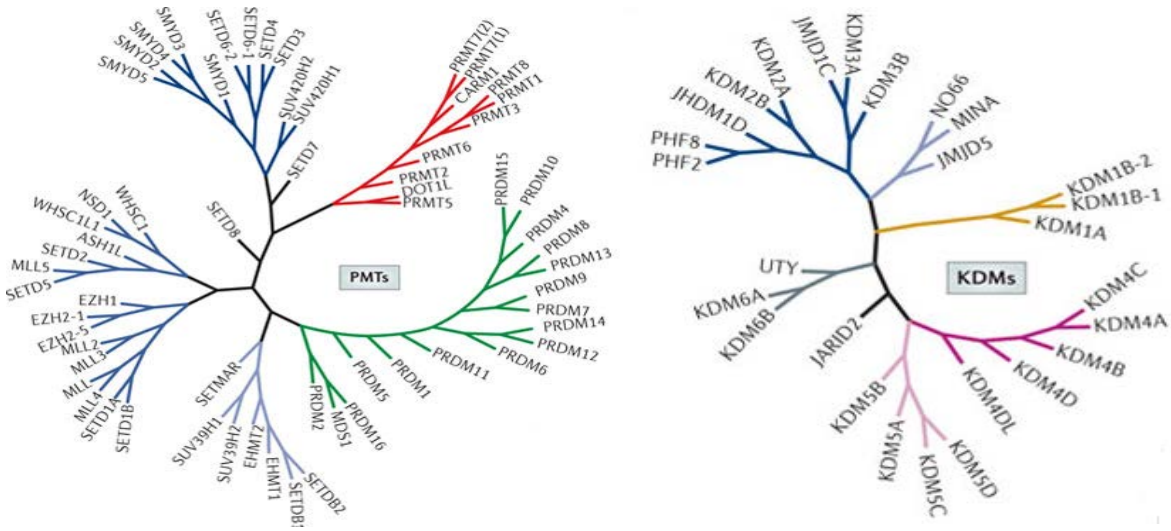

Figure 8 – Phylogenetic trees of epigenetic protein families: protein methyltransferases (PMTs) and lysine demethylases (KDMs). *Adapted from [61].*

Recent studies suggested that HMTs and HDMs deregulation might be crucial to cancer onset and progression. Indeed, in cancer cells, some of these enzymes can be altered resulting in abnormal expression and mechanisms underlying this deregulation may include genetic alterations such as chromosomal translocations, gene mutations or

fusion proteins. Genetic alterations in KMTs and KDMs may serve as patient stratification biomarkers for future potential treatment with specific inhibitors of these histone modifiers [85, 86].

HISTONE METHYLATION IN PCA

Histone post-translational modifications have been linked to prostate carcinogenesis but there is still much to explore concerning this issue [46]. Global patterns of histone modifications have been linked to risk of PCa recurrence. Regarding histone methylation, levels of H4R3me2 and H3K4me2 enables distinction between two groups of low-grade PCa (GS 6 or less) with different prognosis outcome [87]. Some histone modifying patterns, namely H3K4me2 and H3K4me1, were also associated with increased risk of PCa recurrence [88, 89]. Similarly, methylation of H3K4 and H3K27 were already correlated with tumor grade or recurrence [88, 90]. Nevertheless, it is important to note that the presence of multiple epigenetic marks should be interpreted carefully; indeed, it is currently recognized the importance of the balance of PTMs, especially bivalent marks such as H3K4/K27me3 [91].

Deregulation of some lysine histone methylases - Mixed-Lineage Leukemia 2 (MLL2), Mixed-Lineage Leukemia 3 (MLL3), nuclear receptor binding SET domain protein 1 (NSD1), enhancer of zeste homolog 2 (EZH2) or SET and MYND domain containing 3 (SMYD3) - in PCa tissues has also been demonstrated [88, 92]. However due to inappropriate tissue sampling and/or to the reduced number of samples tested, the reliability of most studies is rather limited. Nevertheless, EZH2, a member of the Polycomb complex components, was already proved to be a driver in prostate carcinogenesis [87, 93]. EZH2 is a HMT that catalyzes repressive marks such as H3K27me3 and occasionally H3K9me2, thereby associated with heterochromatinization, repression of genes and ability to influence DNA methylation [94-98]. Furthermore, EZH2 was found to be overexpressed in metastatic PCa, being associated with high proliferation rates and tumor aggressiveness [99, 100].

Since methylation of H3K9 is linked to repression of AR regulated genes in prostatic cells, silencing of its specific demethylases, namely LSD1, lysine-specific demethylase 3A (KDM3A) or lysine-specific demethylase 4C (KDM4C), leads to an increase the levels of these repressive marks on AR targeted genes regulatory regions and thus decreases its expression [101, 102]. In fact, LSD1 demethylates H3K4 and H3K9, and its upregulation is associated with aggressive and hormone-refractory PCa and with high risk of disease relapse [103-107].

THE METHYLTRANSFERASE SMYD3

SET and MYND domain-containing protein 3 (SMYD3) is a KMT that plays an important role in transcriptional regulation. Its KMT activity mainly relies on two conserved amino acid sequences, NHSC and EEL, located within the SET domain. It was firstly described as a histone modifier with dimethyl- and trimethyltransferase activity at lysine 4 of H3 (H3K4) activating transcription of a set of downstream genes, which elicits its oncogenic effect [108]. Currently, it is acknowledged that this enzyme also methylates H4K5 and H4K20 as well as other non-histonic proteins [109, 110]. Moreover, since SMYD3 can be found not only in the nucleus but also in the cytoplasm, it has been suggested that it could methylate cytoplasmic or membrane proteins. Indeed, it has been already reported that SMYD3 methylates lysine 831 of vascular endothelial growth factor receptor 1 (VEGFR1) enhancing its kinase activity [111]. SMYD3 also interacts with RNA helicase HELZ and recruits RNA polymerase II, thus affecting transcription [108]. Moreover, SMYD3 binds to specific DNA sequences (SBE), 5'-CCCTCC-3' or 5'-GGAGGG-3', within promoter regions of target genes, allowing their elongation [108]. In fact, SMYD3 showed to be capable of binding to those motifs and consequently leads to transcriptional activation of several downstream genes including NK2 homeobox 8 (*Nkx2.8*), wingless-type MMTV integration site family member 10B (*WNT10B*), PR Domain Containing 2 With ZNF Domain (*PRDM2 or RIZ1*), met proto-oncogene (*c-Met*) and 15-Lipoxygenase-1 (*15-LOX-1*) [108, 112-115]. Additionally, SMYD3 also regulates indirectly gene expression by altering the conformation of genes' promoters, impacting on recruitment of DNA methyltransferases and thus contributing to site specific DNA methylation [113].

Interestingly, SMYD3 deregulation has been previously reported in a wide range of human cancers with enhanced expression observed in colorectal, hepatocellular and breast carcinomas [108, 112]. Functional *in vitro* studies showed that SMYD3 knockdown is associated with growth inhibition, apoptosis and reduced migration/invasion potential in cancer cell lines of those tumors, thus supporting its oncogenic role [108, 112, 113, 116, 117]. Furthermore, it has also been reported that SMYD3 may directly interact with the ligand-binding domain of the estrogen receptor (ER) and be recruited to the proximal promoter regions of ER target genes inducing their expression by addition of di- and trimethylation of H3K4, through its KMT activity [118]. Therefore it is plausible that SMYD3 might interact with other nuclear receptors, including the AR, and consequently, it can be implicated in prostate carcinogenesis. However, the role of SMYD3 and respective histone marks on the abnormal expression of genes associated with PCa initiation and progression, as well as the cellular pathways affected, remains unknown.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61(2):69-90
2. Ferlay J SI, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr, accessed on 17/12/2013
3. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H, Forman D, Bray F. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer. 2013;49(6):1374-403
4. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin. 2013;63(1):11-30
5. Prasad SM, Drazer MW, Huo D, Hu JC, Eggener SE. 2008 US Preventive Services Task Force recommendations and prostate cancer screening rates. JAMA. 2012;307(16):1692-4
6. Patel AR, Klein EA. Risk factors for prostate cancer. Nat Clin Pract Urol. 2009;6(2):87-95
7. Boyd LK, Mao X, Lu YJ. The complexity of prostate cancer: genomic alterations and heterogeneity. Nat Rev Urol. 2012;9(11):652-64
8. Gronberg H. Prostate cancer epidemiology. Lancet. 2003;361(9360):859-64
9. Crawford ED. Epidemiology of prostate cancer. Urology. 2003;62(6 Suppl 1):3-12
10. Chornokur G, Dalton K, Borysova ME, Kumar NB. Disparities at presentation, diagnosis, treatment, and survival in African American men, affected by prostate cancer. Prostate. 2011;71(9):985-97
11. Brawley OW. Prostate cancer epidemiology in the United States. World J Urol. 2012;30(2):195-200
12. Bostwick DG, Burke HB, Djakiew D, Euling S, Ho SM, Landolph J, Morrison H, Sonawane B, Shifflett T, Waters DJ, Timms B. Human prostate cancer risk factors. Cancer. 2004;101(10 Suppl):2371-490
13. DeVita VT, Lawrence TS, Rosenberg SA. Cancer: Principles & Practice of Oncology: Wolters Kluwer/Lippincott Williams & Wilkins; 2008.
14. Shen MM, Abate-Shen C. Molecular genetics of prostate cancer: new prospects for old challenges. Genes Dev. 2010;24(18):1967-2000
15. Selman SH. The McNeal prostate: a review. Urology. 2011;78(6):1224-8
16. Hricak H, Scardino PT. Prostate cancer. Cambridge, UK: Cambridge University Press; 2009. xi, 230 p. p.

17. Collins AT, Maitland NJ. Prostate cancer stem cells. Eur J Cancer. 2006;42(9):1213-8
18. Moyer VA. Screening for Prostate Cancer: U.S. Preventive Services Task Force Recommendation Statement. Ann Intern Med. 2012;157(2):120-34
19. Heidenreich A, Bellmunt J, Bolla M, Joniau S, Mason M, Matveev V, Mottet N, Schmid HP, van der Kwast T, Wiegel T, Zattoni F. EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and treatment of clinically localised disease. Eur Urol. 2011;59(1):61-71
20. Obort AS, Ajadi MB, Akinloye O. Prostate-Specific Antigen: Any Successor in Sight? Rev Urol. 2013;15(3):97
21. Eble JL, Sauter G, Epstein JI, Sesterhenn IA. Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs: WHO Classification of Tumours: World Health Organization; 2004.
22. Wolf AM, Wender RC, Etzioni RB, Thompson IM, D'Amico AV, Volk RJ, Brooks DD, Dash C, Guessous I, Andrews K, DeSantis C, Smith RA. American Cancer Society guideline for the early detection of prostate cancer: update 2010. CA Cancer J Clin. 2010;60(2):70-98
23. Tefekli A, Tunc M. Future Prospects in the Diagnosis and Management of Localized Prostate Cancer. ScientificWorldJournal. 2013;2013:347263
24. Catalona WJ, D'Amico AV, Fitzgibbons WF, Kosoko-Lasaki O, Leslie SW, Lynch HT, Moul JW, Rendell MS, Walsh PC. What the U.S. Preventive Services Task Force Missed in Its Prostate Cancer Screening Recommendation. Ann Intern Med. 2012;157(2):137-8
25. Epstein JI. An update of the Gleason grading system. J Urol. 2010;183(2):433-40
26. Stark JR, Perner S, Stampfer MJ, Sinnott JA, Finn S, Eisenstein AS, Ma J, Fiorentino M, Kurth T, Loda M, Giovannucci EL, Rubin MA, Mucci LA. Gleason score and lethal prostate cancer: does 3 + 4 = 4 + 3? J Clin Oncol. 2009;27(21):3459-64
27. Lotan TL, Epstein JI. Clinical implications of changing definitions within the Gleason grading system. Nat Rev Urol. 2010;7(3):136-42
28. Iczkowski KA, Lucia MS. Current perspectives on Gleason grading of prostate cancer. Curr Urol Rep. 2011;12(3):216-22
29. Cheng L, Montironi R, Bostwick DG, Lopez-Beltran A, Berney DM. Staging of prostate cancer. Histopathology. 2012;60(1):87-117
30. Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol. 2010;17(6):1471-4

31. Ramon J, Denis L. Prostate Cancer: Springer, Berlin; 2007.
32. Edge S, Byrd D, Compton C. AJCC Cancer Staging Manual 7th Edition: Springer, New York; 2010.
33. Montie JE. Staging of prostate cancer. Current TNM classification and future prospects for prognostic factors. Cancer. 1995;75(S7):1814-8
34. Bubendorf L, Schöpfer A, Wagner U, Sauter G, Moch H, Willi N, Gasser TC, Mihatsch MJ. Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. Hum Pathol. 2000;31(5):578-83
35. Falzarano SM, Magi-Galluzzi C. Prostate cancer staging and grading at radical prostatectomy over time. Adv Anat Pathol. 2011;18(2):159-64
36. Partin AW, Mangold LA, Lamm DM, Walsh PC, Epstein JI, Pearson JD. Contemporary update of prostate cancer staging nomograms (Partin Tables) for the new millennium. Urology. 2001;58(6):843-8
37. Chen FZ, Zhao XK. Prostate Cancer: Current Treatment and Prevention Strategies. Iran Red Crescent Med J. 2013;15(4):279-84
38. Jani AB, Hellman S. Early prostate cancer: clinical decision-making. Lancet. 2003;361(9362):1045-53
39. Ramsay AK, Leung HY. Signalling pathways in prostate carcinogenesis: potentials for molecular-targeted therapy. Clin Sci (Lond). 2009;117(6):209-28
40. Perlmutter MA, Lepor H. Androgen deprivation therapy in the treatment of advanced prostate cancer. Rev Urol. 2007;9 Suppl 1:S3-8
41. Damber JE, Aus G. Prostate cancer. Lancet. 2008;371(9625):1710-21
42. Carles J, Castellano D, Climent MA, Maroto P, Medina R, Alcaraz A. Castration-resistant metastatic prostate cancer: current status and treatment possibilities. Clin Transl Oncol. 2012;14(3):169-76
43. Sridhar SS, Freedland SJ, Gleave ME, Higano C, Mulders P, Parker C, Sartor O, Saad F. Castration-Resistant Prostate Cancer: From New Pathophysiology to New Treatment. Eur Urol. 2013
44. Chi KN, Bjartell A, Dearnaley D, Saad F, Schroder FH, Sternberg C, Tombal B, Visakorpi T. Castration-resistant prostate cancer: from new pathophysiology to new treatment targets. Eur Urol. 2009;56(4):594-605
45. Delcuve GP, Rastegar M, Davie JR. Epigenetic control. J Cell Physiol. 2009;219(2):243-50
46. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. Carcinogenesis. 2010;31(1):27-36
47. Rodriguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. Nat Med. 2011;17(3):330-9

48. Sandoval J, Esteller M. Cancer epigenomics: beyond genomics. Curr Opin Genet Dev. 2012;22(1):50-5
49. Esteller M. Epigenetics in cancer. N Engl J Med. 2008;358(11):1148-59
50. Sawan C, Vaissiere T, Murr R, Herceg Z. Epigenetic drivers and genetic passengers on the road to cancer. Mutat Res. 2008;642(1-2):1-13
51. Kanwal R, Gupta S. Epigenetic modifications in cancer. Clin Genet. 2012;81(4):303-11
52. Portela A, Esteller M. Epigenetic modifications and human disease. Nat Biotechnol. 2010;28(10):1057-68
53. Bird A. DNA methylation patterns and epigenetic memory. Genes Dev. 2002;16(1):6-21
54. Robertson KD. DNA methylation and human disease. Nature Reviews Genetics. 2005;6(8):597-610
55. Jenuwein T, Allis CD. Translating the histone code. Science. 2001;293(5532):1074-80
56. Dawson MA, Kouzarides T, Huntly BJ. Targeting epigenetic readers in cancer. N Engl J Med. 2012;367(7):647-57
57. Cosgrove MS, Boeke JD, Wolberger C. Regulated nucleosome mobility and the histone code. Nat Struct Mol Biol. 2004;11(11):1037-43
58. Lund AH, van Lohuizen M. Epigenetics and cancer. Genes Dev. 2004;18(19):2315-35
59. Kouzarides T. Chromatin modifications and their function. Cell. 2007;128(4):693-705
60. Zentner GE, Henikoff S. Regulation of nucleosome dynamics by histone modifications. Nat Struct Mol Biol. 2013;20(3):259-66
61. Arrowsmith CH, Bountra C, Fish PV, Lee K, Schapira M. Epigenetic protein families: a new frontier for drug discovery. Nat Rev Drug Discov. 2012;11(5):384-400
62. Miremadi A, Oestergaard MZ, Pharoah PD, Caldas C. Cancer genetics of epigenetic genes. Hum Mol Genet. 2007;16 Spec No 1:R28-49
63. Waldmann T, Schneider R. Targeting histone modifications-epigenetics in cancer. Curr Opin Cell Biol. 2013;25(2):184-9
64. Esteller M. Non-coding RNAs in human disease. Nat Rev Genet. 2011;12(12):861-74
65. Mattick JS, Makunin IV. Non-coding RNA. Human molecular genetics. 2006;15(suppl 1):R17-R29
66. Garzon R, Calin GA, Croce CM. MicroRNAs in cancer. Annual review of medicine. 2009;60:167-79

67. Melo SA, Esteller M. Dysregulation of microRNAs in cancer: playing with fire. FEBS Lett. 2011;585(13):2087-99
68. Batista PJ, Chang HY. Long noncoding RNAs: cellular address codes in development and disease. Cell. 2013;152(6):1298-307
69. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. Nature Reviews Cancer. 2006;6(4):259-69
70. Davalos V, Esteller M. MicroRNAs and cancer epigenetics: a macrorevolution. Curr Opin Oncol. 2010;22(1):35-45
71. Zhang Y, Reinberg D. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev. 2001;15(18):2343-60
72. Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell. 2004;119(7):941-53
73. Chang B, Chen Y, Zhao Y, Bruick RK. JMJD6 is a histone arginine demethylase. Science. 2007;318(5849):444-7
74. Brame CJ, Moran MF, McBroom-Cerajewski LD. A mass spectrometry based method for distinguishing between symmetrically and asymmetrically dimethylated arginine residues. Rapid Commun Mass Spectrom. 2004;18(8):877-81
75. Lee DY, Northrop JP, Kuo MH, Stallcup MR. Histone H3 lysine 9 methyltransferase G9a is a transcriptional coactivator for nuclear receptors. J Biol Chem. 2006;281(13):8476-85
76. Mosammaparast N, Shi Y. Reversal of histone methylation: biochemical and molecular mechanisms of histone demethylases. Annu Rev Biochem. 2010;79:155-79
77. Varier RA, Timmers HT. Histone lysine methylation and demethylation pathways in cancer. Biochim Biophys Acta. 2011;1815(1):75-89
78. Di Lorenzo A, Bedford MT. Histone arginine methylation. FEBS Lett. 2011;585(13):2024-31
79. Litt M, Qiu Y, Huang S. Histone arginine methylations: their roles in chromatin dynamics and transcriptional regulation. Biosci Rep. 2009;29(2):131-41
80. Spannhoff A, Hauser AT, Heinke R, Sippl W, Jung M. The emerging therapeutic potential of histone methyltransferase and demethylase inhibitors. ChemMedChem. 2009;4(10):1568-82
81. Dambacher S, Hahn M, Schotta G. Epigenetic regulation of development by histone lysine methylation. Heredity (Edinb). 2010;105(1):24-37

82. Cloos PA, Christensen J, Agger K, Helin K. Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. Genes Dev. 2008;22(9):1115-40
83. Smith BC, Denu JM. Chemical mechanisms of histone lysine and arginine modifications. Biochim Biophys Acta. 2009;1789(1):45-57
84. Kooistra SM, Helin K. Molecular mechanisms and potential functions of histone demethylases. Nat Rev Mol Cell Biol. 2012;13(5):297-311
85. Tian X, Zhang S, Liu HM, Zhang YB, Blair CA, Mercola D, Sassone-Corsi P, Zi X. Histone lysine-specific methyltransferases and demethylases in carcinogenesis: new targets for cancer therapy and prevention. Curr Cancer Drug Targets. 2013;13(5):558-79
86. Chi P, Allis CD, Wang GG. Covalent histone modifications--miswritten, misinterpreted and mis-erased in human cancers. Nat Rev Cancer. 2010;10(7):457-69
87. Seligson DB, Horvath S, Shi T, Yu H, Tze S, Grunstein M, Kurdistani SK. Global histone modification patterns predict risk of prostate cancer recurrence. Nature. 2005;435(7046):1262-6
88. Bianco-Miotto T, Chiam K, Buchanan G, Jindal S, Day TK, Thomas M, Pickering MA, O'Loughlin MA, Ryan NK, Raymond WA, Horvath LG, Kench JG, Stricker PD, Marshall VR, Sutherland RL, Henshall SM, Gerald WL, Scher HI, Risbridger GP, Clements JA, Butler LM, Tilley WD, Horsfall DJ, Ricciardelli C. Global levels of specific histone modifications and an epigenetic gene signature predict prostate cancer progression and development. Cancer Epidemiol Biomarkers Prev. 2010;19(10):2611-22
89. Ellinger J, Kahl P, von der Gathen J, Rogenhofer S, Heukamp LC, Gutgemann I, Walter B, Hofstadter F, Buttner R, Muller SC, Bastian PJ, von Ruecker A. Global levels of histone modifications predict prostate cancer recurrence. Prostate. 2010;70(1):61-9
90. Seligson DB, Horvath S, McBrian MA, Mah V, Yu H, Tze S, Wang Q, Chia D, Goodglick L, Kurdistani SK. Global levels of histone modifications predict prognosis in different cancers. Am J Pathol. 2009;174(5):1619-28
91. Ke XS, Qu Y, Cheng Y, Li WC, Rotter V, Oyan AM, Kalland KH. Global profiling of histone and DNA methylation reveals epigenetic-based regulation of gene expression during epithelial to mesenchymal transition in prostate cells. BMC Genomics. 2010;11:669
92. Ke XS, Qu Y, Rostad K, Li WC, Lin B, Halvorsen OJ, Haukaas SA, Jonassen I, Petersen K, Goldfinger N, Rotter V, Akslen LA, Oyan AM, Kalland KH. Genome-

wide profiling of histone h3 lysine 4 and lysine 27 trimethylation reveals an epigenetic signature in prostate carcinogenesis. PLoS One. 2009;4(3):e4687

93. Karanikolas BD, Figueiredo ML, Wu L. Comprehensive evaluation of the role of EZH2 in the growth, invasion, and aggression of a panel of prostate cancer cell lines. Prostate. 2010;70(6):675-88
94. Hoffmann MJ, Engers R, Florl AR, Otte AP, Muller M, Schulz WA. Expression changes in EZH2, but not in BMI-1, SIRT1, DNMT1 or DNMT3B are associated with DNA methylation changes in prostate cancer. Cancer Biol Ther. 2007;6(9):1403-12
95. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RG, Otte AP, Rubin MA, Chinnaiyan AM. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature. 2002;419(6907):624-9
96. Gibbons RJ. Histone modifying and chromatin remodelling enzymes in cancer and dysplastic syndromes. Hum Mol Genet. 2005;14 Spec No 1:R85-92
97. Jeronimo C, Bastian PJ, Bjartell A, Carbone GM, Catto JW, Clark SJ, Henrique R, Nelson WG, Shariat SF. Epigenetics in prostate cancer: biologic and clinical relevance. Eur Urol. 2011;60(4):753-66
98. Yang YA, Yu J. EZH2, an epigenetic driver of prostate cancer. Protein Cell. 2013;4(5):331-41
99. Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA, Salvesen HB, Otte AP, Akslen LA. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol. 2006;24(2):268-73
100. Yu J, Rhodes DR, Tomlins SA, Cao X, Chen G, Mehra R, Wang X, Ghosh D, Shah RB, Varambally S, Pienta KJ, Chinnaiyan AM. A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. Cancer Res. 2007;67(22):10657-63
101. Chin SP, Dickinson JL, Holloway AF. Epigenetic regulation of prostate cancer. Clin Epigenetics. 2011;2(2):151-69
102. Chen Z, Wang L, Wang Q, Li W. Histone modifications and chromatin organization in prostate cancer. Epigenomics. 2010;2(4):551-60
103. Opel M, Lando D, Bonilla C, Trewick SC, Boukaba A, Walfridsson J, Cauwood J, Werler PJ, Carr AM, Kouzarides T, Murzina NV, Allshire RC, Ekwall K, Laue ED. Genome-wide studies of histone demethylation catalysed by the fission yeast homologues of mammalian LSD1. PLoS One. 2007;2(4):e386

104. Metzger E, Wissmann M, Yin N, Muller JM, Schneider R, Peters AH, Gunther T, Buettner R, Schule R. LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature. 2005;437(7057):436-9
105. Kahl P, Gullotti L, Heukamp LC, Wolf S, Friedrichs N, Vorreuther R, Solleder G, Bastian PJ, Ellinger J, Metzger E. Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence. Cancer Res. 2006;66(23):11341-7
106. Scoumanne A, Chen X. The lysine-specific demethylase 1 is required for cell proliferation in both p53-dependent and -independent manners. J Biol Chem. 2007;282(21):15471-5
107. Hayami S, Kelly JD, Cho HS, Yoshimatsu M, Unoki M, Tsunoda T, Field HI, Neal DE, Yamaue H, Ponder BA, Nakamura Y, Hamamoto R. Overexpression of LSD1 contributes to human carcinogenesis through chromatin regulation in various cancers. Int J Cancer. 2011;128(3):574-86
108. Hamamoto R, Furukawa Y, Morita M, Iimura Y, Silva FP, Li M, Yagyu R, Nakamura Y. SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells. Nat Cell Biol. 2004;6(8):731-40
109. Foreman KW, Brown M, Park F, Emtage S, Harriss J, Das C, Zhu L, Crew A, Arnold L, Shaaban S, Tucker P. Structural and functional profiling of the human histone methyltransferase SMYD3. PLoS One. 2011;6(7):e22290
110. Van Aller GS, Reynoird N, Barbash O, Huddleston M, Liu S, Zmoos AF, McDevitt P, Sinnamon R, Le B, Mas G, Annan R, Sage J, Garcia BA, Tummino PJ, Gozani O, Kruger RG. Smyd3 regulates cancer cell phenotypes and catalyzes histone H4 lysine 5 methylation. Epigenetics. 2012;7(4):340-3
111. Kunizaki M, Hamamoto R, Silva FP, Yamaguchi K, Nagayasu T, Shibuya M, Nakamura Y, Furukawa Y. The lysine 831 of vascular endothelial growth factor receptor 1 is a novel target of methylation by SMYD3. Cancer Res. 2007;67(22):10759-65
112. Hamamoto R, Silva FP, Tsuge M, Nishidate T, Katagiri T, Nakamura Y, Furukawa Y. Enhanced SMYD3 expression is essential for the growth of breast cancer cells. Cancer Sci. 2006;97(2):113-8
113. Chen LB, Xu JY, Yang Z, Wang GB. Silencing SMYD3 in hepatoma demethylates RIZI promoter induces apoptosis and inhibits cell proliferation and migration. World J Gastroenterol. 2007;13(43):5718-24
114. Zou JN, Wang SZ, Yang JS, Luo XG, Xie JH, Xi T. Knockdown of SMYD3 by RNA interference down-regulates c-Met expression and inhibits cells migration and invasion induced by HGF. Cancer Lett. 2009;280(1):78-85

115. Liu C, Xu D, Han H, Fan Y, Schain F, Xu Z, Claesson HE, Bjorkholm M, Sjoberg J. Transcriptional regulation of 15-lipoxygenase expression by histone h3 lysine 4 methylation/demethylation. PLoS One. 2012;7(12):e52703
116. Luo XG, Zou JN, Wang SZ, Zhang TC, Xi T. Novobiocin decreases SMYD3 expression and inhibits the migration of MDA-MB-231 human breast cancer cells. IUBMB Life. 2010;62(3):194-9
117. Wang SZ, Luo XG, Shen J, Zou JN, Lu YH, Xi T. Knockdown of SMYD3 by RNA interference inhibits cervical carcinoma cell growth and invasion in vitro. BMB Rep. 2008;41(4):294-9
118. Kim H, Heo K, Kim JH, Kim K, Choi J, An W. Requirement of histone methyltransferase SMYD3 for estrogen receptor-mediated transcription. J Biol Chem. 2009;284(30):19867-77

Chapter II

Rational and Aims

RATIONAL AND AIMS

PCa is one of the most incident cancers worldwide, and currently these tumors are frequently overdiagnosed and overtreated. Nevertheless, mortality rates are not decreasing and it is expected, within the next years, a rising trend in prevalence mainly due to population aging. Prevention might have a slight impact on disease-related mortality, whereas diagnostic and prognostic evaluation is crucial both to an earlier identification of clinical relevant tumors and to better adjust therapeutic options in accordance with disease aggressiveness. Thus, a major challenge is to identify men with aggressive disease, for which suitable treatment might be crucial to attain a cure, and spare patients with clinically insignificant disease from unnecessary treatment morbidity.

Abnormalities in epigenetic mechanisms, namely histone methylation, might contribute to cancer initiation and progression. The acknowledged involvement of HMTs and HDMs deregulation in cancer allows to consider these enzymes as biomarkers intended for accurate diagnosis or for stratification of cancer patients into subgroups with different prognosis and tumor biology, aiming at appropriate selection for targeted therapies. Additionally, understanding the role of these enzymes in carcinogenesis through functional studies and addressing the mechanisms underlying cancer cells' phenotypic plasticity may allow for the discovery of novel therapeutic targets.

Hence, the main goal of this PhD project was to determine which HMTs and HDMs are relevant for prostate carcinogenesis and how their assessment might provide novel tools for patient management. To accomplish this objective, several tasks were established:

- Identify HMTs and HDMs displaying altered expression levels in a relatively large series of PCa patients submitted to RP comparing with normal prostate tissues;
- Evaluate expression levels of HMTs and HDMs and ascertain their correlation with clinicopathological parameters;
- Assess the clinical usefulness of HMTs and HDMs for prediction of disease progression.

Because SMYD3 deregulation was reported to play an important role in other solid tumors, and our data suggested that it may be relevant for PCa progression, we investigated its function using *in vitro* and *in vivo* cell assays, which allowed us to:

- Evaluate the effect of SMYD3 silencing in malignant phenotype of PCa cell lines;
- Assess the impact of SMYD3 knockdown in PTMs' patterns;
- Identify molecular pathways and putative target genes regulated by SMYD3;
- Determine the importance of SMYD3 methyltransferase catalytic activity in the modulation of PCa cells phenotype.

Chapter III

Deregulated expression of selected histone methylases and demethylases in prostate carcinoma

Filipa Quintela Vieira[1,2], Pedro Costa-Pinheiro[1], João Ramalho-Carvalho[1], Andreia Pereira[1], Francisco Duarte Menezes[3], Luís Antunes[4], Isa Carneiro[1,3], Jorge Oliveira[5], Rui Henrique[1,3,6*], Carmen Jerónimo[1,6*]

[1]Cancer Epigenetics Group – Research Center, Portuguese Oncology Institute – Porto, Portugal; [2]School of Allied Health Sciences (ESTSP), Polytechnic of Porto, Portugal; Departments of [3]Pathology, [4]Epidemiology and [5]Urology – Portuguese Oncology Institute – Porto, Portugal; [6]Department of Pathology and Molecular Immunology, Institute of Biomedical Sciences Abel Salazar (ICBAS) – University of Porto, Portugal

[*]Joint senior authors

ABSTRACT

Prostate cancer (PCa), a leading cause of cancer-related morbidity and mortality, arises through the acquisition of genetic and epigenetic alterations. Deregulation of histone methylases (HMTs) or demethylases (HDMs) has been associated with PCa development and progression. However, the precise influence of altered HMTs or HDMs expression and respective histone marks in PCa onset and progression remains largely unknown. To clarify the role of HMTs and HDMs in prostate carcinogenesis, expression levels of 37 HMTs and 20 HDMs were assessed in normal prostate and PCa tissue samples by RT-qPCR. *SMYD3*, *SUV39H2*, *PRMT6*, *KDM5A* and *KDM6A* were up-regulated, whereas *KMT2A-E* (*MLL1-5*) and *KDM4B* were downregulated in PCa, compared to normal prostate tissues. Remarkably, *PRMT6* was the histone modifier that best discriminated normal from tumorous tissue samples. Interestingly, *EZH2* and *SMYD3* expression levels significantly correlated with less differentiated and more aggressive tumors. Remarkably, *SMYD3* expression levels were of independent prognostic value for prediction of disease-specific survival of PCa patients with clinically localized disease submitted to radical prostatectomy. We concluded that expression profiling of HMTs and HDMs, especially *SMYD3*, might be of clinical usefulness for assessment of PCa patients and assist in pre-therapeutic decision-making.

INTRODUCTION

Prostate cancer (PCa) is the most frequently diagnosed cancer in men from developed countries and a leading cause of cancer-related morbidity and mortality worldwide [1, 2]. At its earliest stages, PCa is frequently asymptomatic, fostering the use of biomarkers, such as serum prostate-specific antigen (PSA), for screening and identification of asymptomatic low-stage tumors, followed by prostate biopsy for diagnosis confirmation [3]. However, prostate biopsy meets with several limitations, including sampling error as well as intra- and interobserver variability in Gleason grading [4, 5], which even in conjunction with other prognostic factors used for therapeutic decision (*e.g.*, clinical stage and pre-therapeutic serum PSA levels) are rather imperfect in predicting disease progression [6, 7]. Consequently, there is a significant degree of uncertainty concerning the threat that a PCa poses to an individual patient, entailing overtreatment [8].

The role of epigenetic modifications in cancer initiation and progression has been emphasized [9]. In addition to aberrant DNA methylation, alterations in chromatin modification patterns, due to histones post-translational modifications (PTMs), were implicated in carcinogenesis and have emerged as potential key regulators of cancer-related pathways [10]. Importantly, PTMs may be changed in cancer cells due to altered expression or activity of key chromatin-modifying enzymes [10].

Histone methylation, carried out by histone methylases (HMTs), requires different families of enzymes depending on the residue (lysine histone methylases (KMT) methylate lysine residues, whereas protein arginine methylase (PRMT) methylate arginines) and might positively or negatively regulate gene transcription. Whereas lysine residues might be modified into mono-, di-, or trimethyl states, arginine can only be modified to mono- or dimethyl states (symmetric or asymmetrically) [11]. Different degrees of methylation may be, thus, associated with distinct chromatin regions or transcriptional states (e.g., trimethylation of lysine 9 of histone H3 is associated with pericentromeric heterochromatin and transcriptional repression, whereas its dimethylation is linked to repressed genes in euchromatin [12]). Recently, the reversibility of histone methylation has been established through the discovery of histone lysine and arginine demethylases (HDMs), uncovering a new level of histone plasticity [13, 14].

Altered HMTs expression levels have been found in PCa, most notably EZH2 (enhancer of zeste homolog 2), a lysine methylase, which is increased in metastatic PCa, marking aggressive disease [15, 16]). Specific relationships between histone marks and tumor grade or recurrence (particularly methylation of H3K4 and H3K27) have been reported [17, 18] and deregulation of some lysine histone methylases - *MLL2*, *MLL3*,

NSD1, *EZH2* or *SMYD3* - in PCa tissues has been also demonstrated [18, 19]. However, the validity of most studies is limited due to inappropriate tissue sampling and/or to the reduced number of samples tested.

Because deregulation of HMTs and HDMs affects post-translational control of cellular proteins involved in cancer-relevant signaling networks, a better understanding of their function might lead to the identification of more accurate markers that might be useful to discriminate patients benefiting from a more aggressive treatment from those that might be spared unnecessary and potentially harmful interventions. Therefore, we sought to identify HMTs and HDMs displaying altered expression levels, in a relatively large series of PCa patients submitted to radical prostatectomy, and further test their clinical usefulness for prediction of disease progression.

MATERIAL AND METHODS

PATIENTS AND TISSUE COLLECTION

Primary tumors from 160 patients with clinically localized prostate adenocarcinoma, consecutively diagnosed and primarily treated with radical prostatectomy at the Portuguese Oncology Institute, Porto, Portugal, were prospectively collected. For control purposes, non-neoplastic prostate tissue samples were obtained from the peripheral zone of 15 prostates not harboring PCa collected from cystoprostatectomy specimens of bladder cancer patients [normal prostate tissue (NPT)]. All tissue specimens were promptly frozen immediately after surgery, following informed consent. Five-micron thick sections were cut and stained for the identification of the areas of PCa (*i.e.*, the index or dominant tumor) and normal tissue. Then, the tissue block was trimmed to maximize the yield of target cells (>70% of target cells). Subsequently, an average of fifty 12-µm thick sections was cut and every fifth section was stained to ensure a uniform percentage of target cells and to exclude contamination from neoplastic cells in normal tissue samples. Histological slides from formalin-fixed, paraffin embedded tissue fragments were routinely obtained from the same surgical specimens and assessed for Gleason score (GS) [20] and TNM stage [21]. Relevant clinical data were collected from the clinical records. These studies were approved by the institutional review board [Comissão de Ética para a Saúde (IRB-CES-IPOFG-EPE 019/08)] of Portuguese Oncology Institute - Porto, Portugal.

RNA ISOLATION

All tissue samples were suspended in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and, after addition of chloroform to the lysed cells, total RNA was purified from the aqueous phase of TRIzol extract using the PureLink RNA Mini Kit (Invitrogen) following manufacturer recommendations. The concentration, purity and integrity of RNA samples were determined on a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) and agarose gel electrophoresis.

SCREENING OF HISTONE METHYLASES AND DEMETHYLASES

Five NPTs and ten independent PCa samples were chosen to encompass the full spectrum of prostate carcinomas in this series considering the Gleason score and pathological stage (Table 1). After treatment with DNAse Turbo DNA-free (Ambion, Austin, TX, USA), a total of 1 µg of total RNA was reverse transcribed using the High

Capacity cDNA RT kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. TaqMan® Array 96-Well Plates were designed in order to evaluate expression levels of 37 HMTs and 20 HDMs. RT-qPCR protocol was performed on an ABI-7500 Real-Time PCR system (Applied Biosystems) according to manufacturer's instructions and each sample was run in triplicate.

The amount of mRNAs of the genes studied was normalized to that of the *GUSB* reference gene and the median value of NPTs and PCa samples was chosen to calculate fold-difference in gene expression between groups, using the comparative Ct method. Genes with a logarithmized fold change above 0.5 or below -0.5 were further considered. The expression of *KDM6A* was also included because it has been previously reported as deregulated in several tumor models [22], and analysis was extend to all members of the MLL family.

VALIDATION OF SELECTED ENZYMES

After gene selection, mRNA levels were confirmed in a large and independent group of 150 PCa tissues and 15 NPTs. A total of 300ng was reverse transcribed and amplified using TransPlex®Whole Transcriptome Amplification Kit (Sigma-Aldrich®, Germany) with subsequent purification using QIAquick PCR Purification Kit (QIAGEN, Germany), according to manufacturer's instructions. HMTs or HDMs mRNA levels were evaluated using TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA, supplementary table 1) and the most suitable endogenous control assays for analysis of prostate tissues [23], *GUSB* and *TFRC*, were also analyzed. To determine the relative expression levels in each sample, the values of the target gene were normalized using the median of the two internal reference genes to obtain a ratio (HMT or HDM/Mean of *TFRC* and *GUSB*). Each plate included multiple non-template controls and serial dilutions of a cDNA from human prostate RNA (Ambion, Invitrogen, Carlsbad, CA, USA) to construct a standard curve for each plate. All experiments were run in triplicate.

STATISTICAL ANALYSIS

For statistical analysis purposes, PCa samples were divided into two- or three-grade categories for GS (GS ≤ 6 and GS ≥ 7) and pathological stage (pT2, pT3a and pT3b), respectively. The Shapiro-Wilk's W test allowed for the examination of the appropriateness of a normal distribution assumption for each of the parameters (data not shown). Then, the median and range of the mRNA expression levels for each group of samples were determined and analyzed using Mann-Whitney U test. A Receiver Operator

Characteristics (ROC) curve was constructed by plotting the true positive rate (sensitivity) against the false positive rate (1-specificity) and the area under the curve (AUC) was calculated to assess diagnostic performance. Possible correlations between the expression levels and GS or pathological stage were assessed by the Kruskal-Wallis one-way analysis of variance (ANOVA), followed by Mann-Whitney U test when appropriate. For multiple comparisons the Bonferroni method was used to adjust P values. Spearman nonparametric correlation tests were additionally carried out to ascertain correlations between age, PSA levels and HMTs or HDMs expression levels. The prognostic significance of available clinical variables (pathological stage, GS, age, and serum PSA levels) was assessed by constructing disease-specific and disease-free survival curves using the Kaplan-Meier method with log-rank test (univariate test). A Cox-regression model comprising the four variables (multivariate test) was also constructed. Disease-free survival was calculated from the date of the radical prostatectomy to the date of biochemical relapse, or date of last follow-up or death if relapse-free. For the purposes of survival analyses, all cases were coded based on expression levels of each enzyme using the percentile 75 value as empirical threshold. Cases were also subdivided according to serum PSA levels (below and above median value) and age (above 60, between 60 and 70, and above 70). Statistical analysis was performed using SPSS for Windows, version 20.0 (SPSS, Chicago, IL, USA) and the level of significance was set to $p<0.05$. Graphs were built using GraphPad Prism 5.0 software for Windows (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

EVALUATION OF HISTONE METHYLASES AND DEMETHYLASES EXPRESSION LEVELS

Expression levels of 37 HMTs and 20 HDMs were assessed in ten PCa and five normal prostate samples (relevant clinical and pathological data is depicted in Table 1).

Table 1 – Clinical and pathological features of patients included in the testing set and in the validation series

	PCa	Normal	PCa	Normal
Number of patients, n	10	5	150	15
Age (years), median (range)	59 (53-71)	61 (49-66)	64 (49-75)	64 (45-80)
PSA levels (ng/mL), median (range)	12.3 (3.5-19.9)	n.a.	8.2 (2.9-23.0)	n.a.
Pathological stage, n (%)		n.a.		n.a.
pT2	4 (40.0)		89 (59.3)	
pT3a	2 (20.0)		50 (33.3)	
pT3b	4 (40.0)		11 (7.3)	
Gleason Score, n (%)		n.a.		n.a.
< 7	3 (30.0)		57 (38.7)	
≥7	7 (70.0)		93 (62.0)	

PCa, prostate cancer; n.a., not applicable.

Most enzymes were downregulated in PCa compared to NPT (Figure 1). Based on fold-variation, lysine histone methylases *SUV39H2*, *SMYD3*, *MLL1-5* and *EZH2* (the latter used as positive control according to the literature), argine histone methylase *PRMT6* and histone demethylases *KDM4B*, *KDM6A*, *KDM5A* and *KDM3B* were selected for validation (Supplementary Table 2). This was performed using RT-qPCR in a larger and independent series comprising 150 PCa samples and 15 normal prostate tissues (relevant clinical and histopathological data is displayed in Table 1). Statistically significant differences between NPT and PCa tissue samples were found for all candidate genes, except *KDM3B* (Table 2).

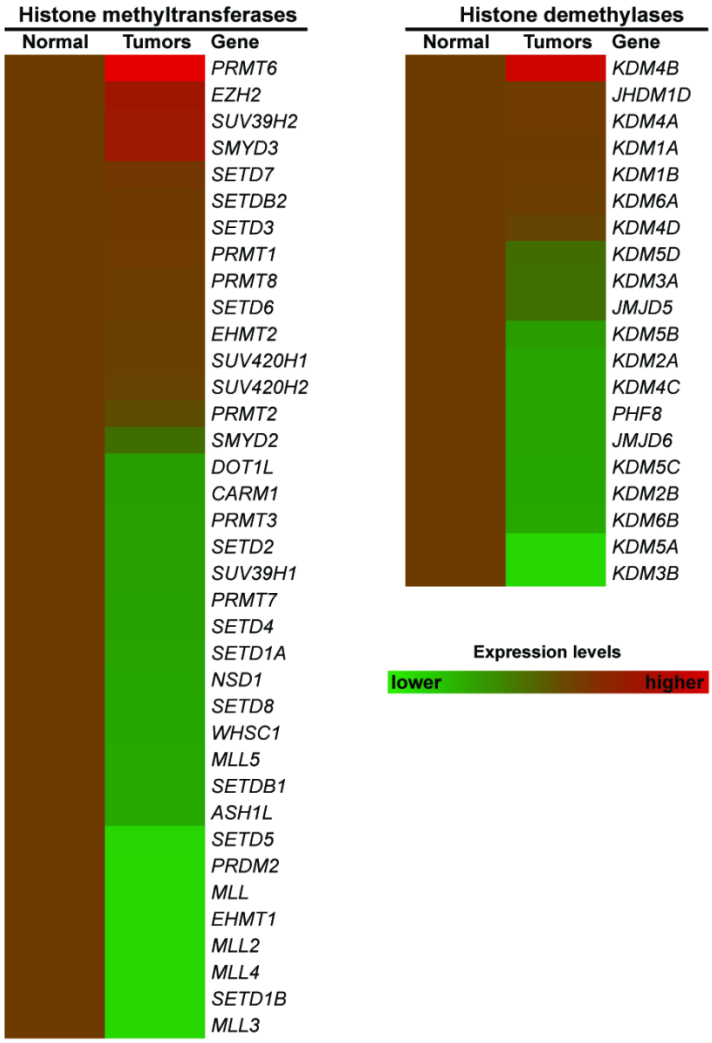

Figure 1 – Expression levels of 37 HMTs and 20 HDMs in normal and PCa tissues. Gene expression of five normal prostate tissues and ten PCa calculated using comparative Ct method. The results presented correspond to median value of each group.

Table 2 – Distribution of selected HMTs and HDMs expression levels in normal and PCa tissue samples

Gene	Normal	Tumor	p-value, M-W	AUC
EZH2	0.77 (0.39-1.82)	1.15 (0.09-4.85)	**0.014**	**0.692**
MLL1	0.69 (0.21-2.52)	0.29 (0.08-1.30)	**<0.001**	**0.212**
MLL2	3.23 (1.61-6.76)	2.05 (0.49-8.07)	**0.004**	**0.272**
MLL3	2.37 (1.24-4.40)	1.45 (0.39-4.06)	**<0.001**	**0.232**
MLL4	3.91 (1.36-10.21)	1.97 (0.36-8.66)	**<0.001**	**0.272**
MLL5	0.73 (0.58-3.46)	0.53 (0.21-1.55)	**<0.001**	**0.162**
PRMT6	0.16 (0.02-0.30)	0.43 (0.10-1.77)	**<0.001**	**0.923**
SMYD3	0.90 (0.53-1.44)	1.53 (0.53-4.50)	**<0.001**	**0.855**
SUV39H2	1.07 (0.63-2.48)	1.36 (0.32-3.43)	**0.044**	**0.657**
KDM3B	0.26 (0.03-0.45)	0.24 (0.11-0.78)	n.s.	**0.495**
KDM4B	2.24 (0.47-7.81)	0.47 (0.08-2.23)	**<0.001**	**0.098**
KDM5A	0.21 (0.10-0.63)	0.32 (0.07-0.77)	**0.026**	**0.675**
KDM6A	0.33 (0.03-0.52)	0.47 (0.20-1.58)	**<0.001**	**0.813**

HMT, histone methylase; HDM, histone demethylase; PCa, prostate cancer, M-W, Mann-Whitney test; AUC, area under the curve; n.s., not significant.

As expected, higher *EZH2* expression levels were observed in PCa compared to NPT and the same trend was verified for *SMYD3*, *SUV39H2*, *PRMT6*, *KDM5A* and *KDM6A* (Figure 2 and Table 2).

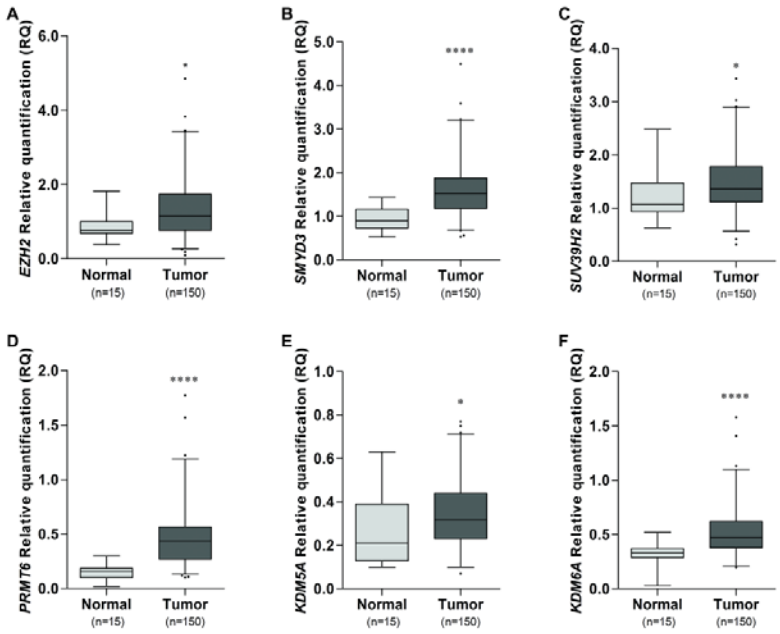

Figure 2 – Identification of HMTs and HDMs overexpressed in PCa. Relative quantification of *EZH2* (A), *SMYD3* (B), *SUV39H2* (C), *PRMT6* (D), *KDM5A* (E) and *KDM6A* (F), displaying higher expression levels in PCa compared to normal prostate tissues (**** $p < 0.0001$; * $p < 0.05$).

Contrarily, all selected members of MLL family and *KDM4B* were downregulated in PCa samples compared to NPT (Figure 3 and Table 2).

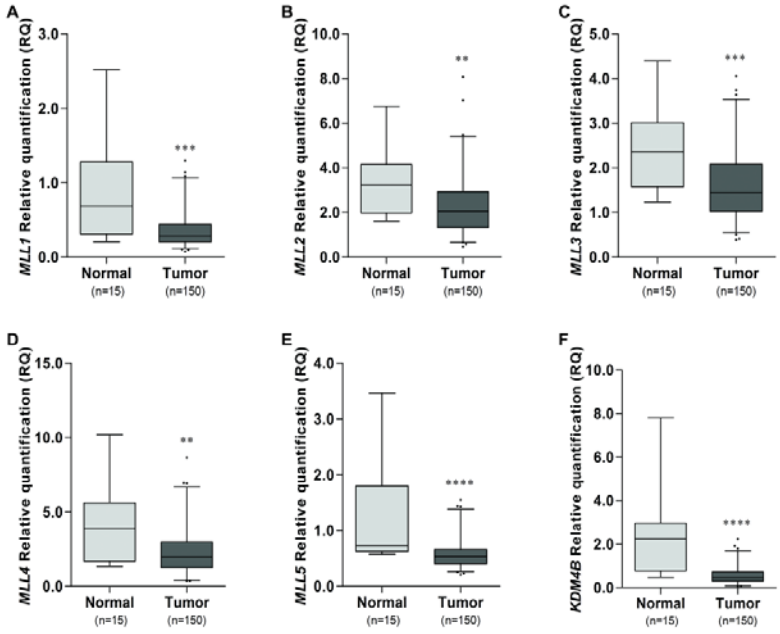

Figure 3 – Identification of HMTs and HDMs downregulated in PCa. Relative quantification of *MLL1* (A), *MLL2* (B), *MLL3* (C), *MLL4* (D), *MLL5* (E), and *KDM4B* (F) depicted lower levels in PCa compared to normal prostate tissues (**** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$).

Interestingly, significant positive correlations between several members of MLL family were found in PCa samples (Table 3).

Table 3 – Coefficient of correlation (r) between the expression levels of all members of MLL family in PCa tissue samples

	MLL1	***MLL2***	***MLL3***	***MLL4***	***MLL5***
MLL1	-	0.773	0.814	0.600	0.560
MLL2	-	-	0.844	0.729	0.468
MLL3	-	-	-	0.650	0.464
MLL4	-	-	-	-	0.458
MLL5	-	-	-	-	-

PCa, prostate cancer; Spearman Correlation; $p < 0.001$ for all comparisons.

To reinforce the oncogenic role of altered enzyme expression, transcript levels (categorized according to percentile 75) were tested as PCa biomarkers in tissue samples. Remarkably, *PRMT6* performed best in sensitivity (90.0%) and specificity (73.3%) for discriminating PCa from NPT, and ROC curve analysis showed an AUC of 0.923 (95% confidence interval: 0.870-0.977, $p<0.001$) (Figure 4).

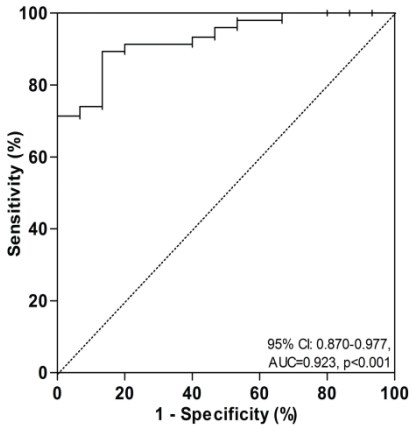

Figure 4 – Performance of *PRMT6* expression as biomarker for PCa. Receiver operator characteristic (ROC) curve evaluating the ability of PRMT6 expression levels in discriminating PCa from normal prostate tissues (AUC, area under the curve; CI, confidence interval).

No significant differences in age between PCa patients and normal tissue donors were apparent. Statistically significant associations between expression levels of *SMYD3* (p=0.044) or *MLL1* (p=0.041) and pathological stage were disclosed (higher levels in pT3b cases for both genes, Figure 5A and 5B). When the patient cohort was stratified according to GS, increased levels of *EZH2* (p=0.048) and *MLL3* (p=0.018) were associated with less differentiated tumors (Figure 5C and 5D). No statistically significant associations were found between gene expression levels and patients' age or PSA levels.

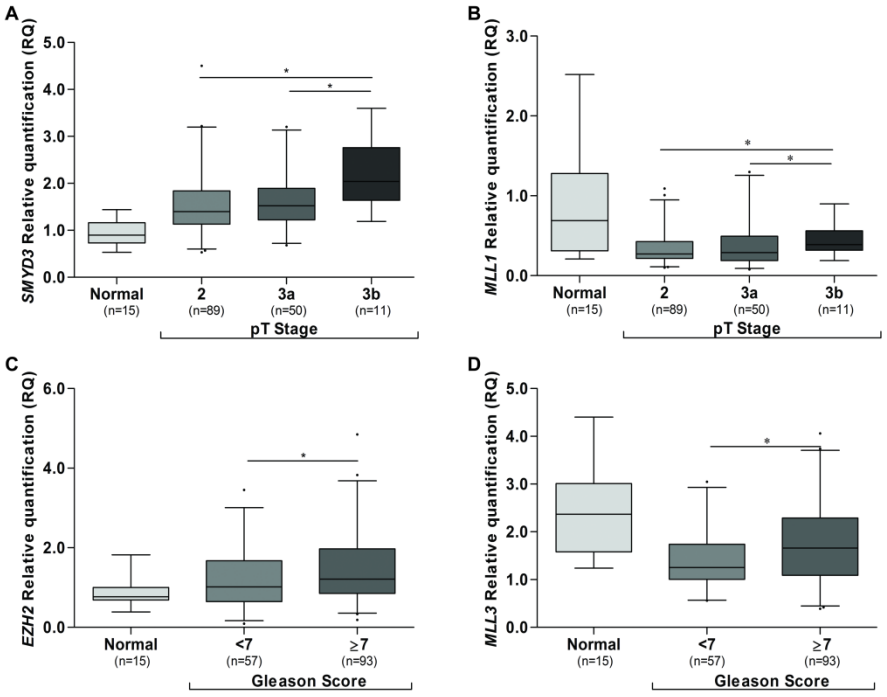

Figure 5 – Association of HMTs with clinicopathological parameters. Distribution of *SMYD3* (A) and *MLL1* (B) expression levels according to pathological stage, showing higher levels in locally advanced disease stage pT3b (* $p < 0.05$). Distribution of *EZH2* (C) and *MLL3* (D) expression levels according to Gleason score, displaying higher levels in tumors with Gleason score ≥ 7 (* $p < 0.05$).

SURVIVAL ANALYSIS

The median follow-up period of this series of PCa patients was 105 months (range: 3-145 months). At the time of the last follow-up, five patients (3.3%) had died from PCa and 45 of 136 (33%) presented biochemical recurrence. In 14 patients, serum PSA levels > 0.2ng/mL persisted following surgery and these were not further considered for disease-free survival analysis. Disease-specific survival curves using established clinical variables or expression levels of selected genes did not display prognostic value within the available follow-up time. However, disease-free survival (DFS) analysis showed that tumors with higher transcript levels of *EZH2* (p=0.001) or *SMYD3* (p=0.010) were significantly associated with a shorter time to relapse, in univariate analysis (Figure 6). Higher GS ($p < 0.001$), advanced pathological stage (pT3a p=0.016 and pT3b p=0.002) and higher PSA levels (p=0.029) were also associated with shorter DFS, whereas age was not of prognostic value within the available follow-up time. In multivariate analysis, higher GS, stage pT3b and high *SMYD3* expression levels retained statistical significance (p=0.001, p=0.027 and p=0.025 respectively) and were capable of independently predict prognosis, whereas *EZH2* expression, PSA and pathological stage pT3a did not show independent prognostic value, in this dataset (Table 4).

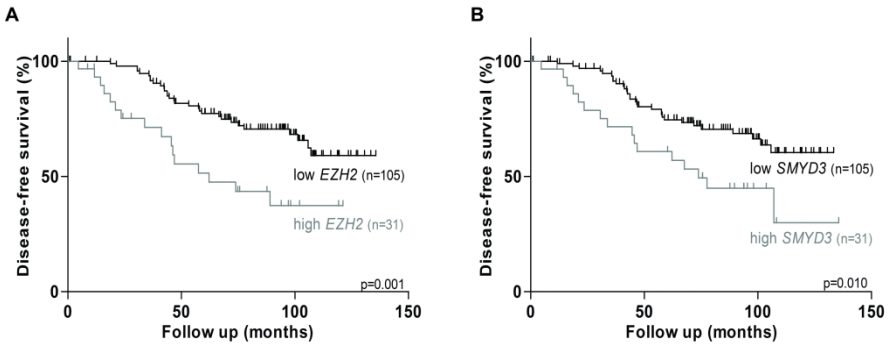

Figure 6 – Kaplan-Meier estimated disease-free survival curves for PCa patients. Disease-free survival curves of 136 PCa patients according to expression levels of *EZH2* (A) and *SMYD3* (B). The results of RT-qPCR presented were categorized using third quartile (75th percentile) value as the cutoff.

Table 4 – Cox regression models assessing the potential of clinical and epigenetic variables in the prediction of disease-free survival for 136 PCa patients

Gene	Variable	HR	95% CI for HR	P value (CR)
EZH2	PSA levels > med	1.652	0.914-2.986	0.096
	Gleason Score	4.206	1.820-9.718	**0.001**
	pT Stage > 2			0.148
	vs 3a	1.457	0.753-2.819	0.264
	vs 3b	2.397	0.980-5.864	0.055
	EZH2 expression > Q75	1.890	0.983-3.637	0.056
SMYD3	PSA levels > med	1.697	0.940-3.064	0.079
	Gleason Score	4.259	1.817-9.982	**0.001**
	pT Stage 2			0.086
	vs 3a	1.476	0.755-2.886	0.255
	vs 3b	2.662	1.115-6.356	**0.027**
	SMYD3 expression > Q75	2.049	1.096-3.832	**0.025**

PCa, prostate cancer; CR, Cox regression; HR, hazard ratio; CI, confidence interval; Med, median value; Q75, quartile 75 value; bold highlights statistical significance ($p < 0.05$).

DISCUSSION

Deregulation of histone PTMs patterns has been associated with PCa development and progression [15, 18, 19]. Because these modifications might be due to altered expression or activity of key chromatin-modifying enzymes [10], we attempted to globally characterize alterations of expression affecting HMTs and HDMs in PCa tissues and determine whether those might be of clinical and pathological relevance.

Overall, 37 HMTs and 20 HDMs expression levels were assessed, comprising most of the relevant members of each class. Owing to the relatively large number of genes tested, this panel was initially tested in a small series of tissue samples. This might underestimate the frequency and magnitude of changes in gene expression, but it allows for the selection of the most significantly altered. Thus, to confirm the initial findings in the arrays, a validation study was performed for the selected genes using RT-qPCR and only three out of twelve genes were not confirmed. Importantly, genes that were previously reported to be overexpressed in PCa, such as *EZH2* [16], surfaced in the array and were confirmed in the large series of PCa, thus validating our initial approach. Furthermore, *EZH2* expression was significantly increased in high GS cases, not associating with pathological stage, confirming previous observations [24].

Interestingly, some of the most significantly altered genes encode for enzymes that display antagonistic functions. Although this might result in a balance between repressive and active PTMs, it must be recalled that the effect in gene expression will depend on the specific genomic locations and how tumor suppressor genes or oncogenes are differentially affected [25, 26]. Contrarily, concerning the enzymes that have overlapping functions, the same trend was not apparent. This is most likely due to function redundancy, so that oncogenesis is already facilitated through the alteration of a single enzyme responsible for a specific PTM [27].

We found that H3K4 methylase *SMYD3* was upregulated in PCa, paralleling previous observations in colorectal, hepatocellular and breast carcinomas [28, 29], whereas MLL family members (which accomplish the same PTM) were downregulated. Remarkably, higher *SMYD3* transcript levels were associated with locally advanced disease, suggesting an association with more aggressive PCa. Interestingly, SMYD3 overexpression has been linked with enhanced proliferation and loss of differentiation [29-33] and this may support the association found in PCa. Moreover, SMYD3 also methylates H4K5 and H4K20 and other non-histone proteins, which may also contribute to its oncogenic role [34, 35]. We found that *KDM5A*, encoding an H3K4 demethylase, was also overexpressed in our PCa series. Remarkably, KDM5A has an antagonistic interaction with pRB, and it is also associated with *MYC* [36], a proto-oncogene which is

upregulated in PCa. This putative oncogenic activity, already demonstrated in gastric cancer [37], is also supported by our findings.

On the other hand, nearly all members of the MLL family were globally downregulated in PCa. This family also targets H3K4, but its downregulation might not impact in H3K4me3 levels owing to *SMYD3* overexpression. *MLL1* and *MLL3* displayed higher expression levels in PCa cases with higher GS and more advanced stage, although levels remained lower than those of normal prostate tissues. MLLs operate in complexes [38], a feature that may explain the observed correlation between some members of this family. Because not all MLL genes are present in the same complexes, a downstream mechanism responsible for their global downregulation in prostate carcinogenesis likely exists. Furthermore, a negative crosstalk between methylation of H3R2 by PRMT6 and H3K4 by MLL complex was described [39] and increased expression of *PRMT6* was identified in our set of PCa. The overexpression of this histone modifier, already reported in bladder and lung cancer, might lead to a decrease of p53 levels, fostering tumorigenesis [40, 41]. Interestingly, PRMT6 proved to be the HMT that best discriminated PCa from normal prostate tissues, further supporting a role for its deregulation in prostate carcinogenesis.

SUV39H2 and KDM4B methylate and demethylate H3K9, respectively. *SUV39H2*'s role in cancer depends on the model: in B-cell lymphomas it acts as a tumor suppressor [42], whereas in breast cancer it is regarded as an oncogene [43], as our results suggest for PCa. A positive correlation between *KDM4B* expression and increased PCa grade has been reported [44], but we were not able to confirm it, probably due to methodological differences. Coffey and co-workers used samples of benign prostate hyperplasia (BPH) as controls and this lesion is reported to be potentially linked with PCa arising in the transition zone [45]. Moreover, their analysis was based on a qualitative evaluation of cytoplasmic immunostaining in a small portion of tissue [44]. On the contrary, we used morphologically normal prostate tissue from the peripheral zone, were over 80% of PCa originate, and expression was quantitatively assessed at transcript level.

A major goal of our study was to determine the potential clinical usefulness of altered HMTs and HDMs expression in PCa. Only *EZH2* and *SMYD3* disclosed a significant association with disease-free survival, in univariate analysis. Similar results have been reported for *EZH2* expression, although assessed by immunohistochemistry [24, 46, 47], and it was found to independently predict PCa recurrence. Although we did not confirm this result for *EZH2* at transcript level, a statistical trend was apparent. It should be recalled that our series only incorporates patients with clinically localized PCa, submitted to radical prostatectomy, which represent a subset of the whole spectrum of PCa patients. Because these patients are selected for having clinically organ-confined

disease, the corresponding PCa are usually of low and intermediate grade (mostly Gleason score 6 and 7 in the biopsy) and low stage (cT1c and cT2). Thus, it does not comprise the full spectrum of PCa as clinically advanced and high grade cases at diagnosis will not be considered (in general) for curative-intent radical prostatectomy. Notwithstanding these limitations, high *SMYD3* expression retained prognostic significance in multivariate analysis, confirming its potential clinical usefulness. To more easily translate for routine practice, however, it would be important to determine if immunohistochemical assessment of SMYD3 expression would provide the same result. Though several commercially available antibodies were tested, none provided satisfactory results.

Concerning disease-specific survival, no statistically significant associations were apparent, probably due to relatively short follow-up data. A follow-up period of 15 or 20 years is usually required to detect differences in PCa survival in patients with localized disease submitted to radical prostatectomy [48]. Nonetheless, biochemical recurrence is also an important primary endpoint in many studies. As expected, GS and pathological stage were of prognostic significance in univariate analysis, although only the former and stage pT3b denoted independent prognostic value in multivariate analysis. The fact that stage pT3a did not surfaced as independent prognostic parameter for DFS in multivariate analysis is most likely due to the association between tumor stage and histological grade, as pT3a cancers were mostly of high GS.

In conclusion, we identified a set of HMTs and HDMs deregulated in prostate cancer that might contribute to the disease development and progression. To the best of our knowledge, this is the first study to demonstrate that HMT *SMYD3* expression levels are able to predict disease-specific survival of PCa patients with clinically localized disease, submitted to radical prostatectomy. Therefore, determination of SMYD3 expression levels in prostate biopsies might be able to convey relevant prognostic information in a pre-therapeutic setting. Functional studies are mandatory to ascertain the role of SMYD3 in prostate carcinogenesis.

FUNDING

This study was funded by research grants from Liga Portuguesa Contra o Cancro – Núcleo Regional do Norte, Research Center of Portuguese Oncology Institute – Porto (CI-IPOP 4-2008) and European Community's Seventh Framework Programme – Grant number FP7-HEALTH-F5-2009-241783. FQV and JRC are supported by FCT-Fundação para a Ciência e a Tecnologia grants (SFRH/BD/70564/2010 and SFRH/BD/71293/2010, respectively), and PCP is supported by a grant from Liga Portuguesa Contra o Cancro – Núcleo Regional do Norte.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61(2):69-90
2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012;62(1):10-29
3. Stamey TA. Making the most out of six systematic sextant biopsies. Urology. 1995;45(1):2-12
4. King CR, Long JP. Prostate biopsy grading errors: a sampling problem? Int J Cancer. 2000;90(6):326-30
5. Allsbrook WC, Jr., Mangold KA, Johnson MH, Lane RB, Lane CG, Epstein JI. Interobserver reproducibility of Gleason grading of prostatic carcinoma: general pathologist. Hum Pathol. 2001;32(1):81-8
6. Duffy MJ. Prostate-specific antigen: does the current evidence support its use in prostate cancer screening? Ann Clin Biochem. 2011;48(Pt 4):310-6
7. Lapointe J, Li C, Higgins JP, van de Rijn M, Bair E, Montgomery K, Ferrari M, Egevad L, Rayford W, Bergerheim U, Ekman P, DeMarzo AM, Tibshirani R, Botstein D, Brown PO, Brooks JD, Pollack JR. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. Proc Natl Acad Sci U S A. 2004;101(3):811-6
8. Moyer VA. Screening for prostate cancer: U.S. Preventive Services Task Force recommendation statement. Ann Intern Med. 2012;157(2):120-34
9. Hirst M, Marra MA. Epigenetics and human disease. Int J Biochem Cell Biol. 2009;41(1):136-46
10. Miremadi A, Oestergaard MZ, Pharoah PD, Caldas C. Cancer genetics of epigenetic genes. Hum Mol Genet. 2007;16 Spec No 1:R28-49
11. Brame CJ, Moran MF, McBroom-Cerajewski LD. A mass spectrometry based method for distinguishing between symmetrically and asymmetrically dimethylated arginine residues. Rapid Commun Mass Spectrom. 2004;18(8):877-81
12. Lee DY, Northrop JP, Kuo MH, Stallcup MR. Histone H3 lysine 9 methyltransferase G9a is a transcriptional coactivator for nuclear receptors. J Biol Chem. 2006;281(13):8476-85
13. Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell. 2004;119(7):941-53
14. Chang B, Chen Y, Zhao Y, Bruick RK. JMJD6 is a histone arginine demethylase. Science. 2007;318(5849):444-7

15. Seligson DB, Horvath S, Shi T, Yu H, Tze S, Grunstein M, Kurdistani SK. Global histone modification patterns predict risk of prostate cancer recurrence. Nature. 2005;435(7046):1262-6
16. Karanikolas BD, Figueiredo ML, Wu L. Comprehensive evaluation of the role of EZH2 in the growth, invasion, and aggression of a panel of prostate cancer cell lines. Prostate. 2010;70(6):675-88
17. Seligson DB, Horvath S, McBrian MA, Mah V, Yu H, Tze S, Wang Q, Chia D, Goodglick L, Kurdistani SK. Global levels of histone modifications predict prognosis in different cancers. Am J Pathol. 2009;174(5):1619-28
18. Bianco-Miotto T, Chiam K, Buchanan G, Jindal S, Day TK, Thomas M, Pickering MA, O'Loughlin MA, Ryan NK, Raymond WA, Horvath LG, Kench JG, Stricker PD, Marshall VR, Sutherland RL, Henshall SM, Gerald WL, Scher HI, Risbridger GP, Clements JA, Butler LM, Tilley WD, Horsfall DJ, Ricciardelli C. Global levels of specific histone modifications and an epigenetic gene signature predict prostate cancer progression and development. Cancer Epidemiol Biomarkers Prev. 2010;19(10):2611-22
19. Ke XS, Qu Y, Rostad K, Li WC, Lin B, Halvorsen OJ, Haukaas SA, Jonassen I, Petersen K, Goldfinger N, Rotter V, Akslen LA, Oyan AM, Kalland KH. Genome-wide profiling of histone h3 lysine 4 and lysine 27 trimethylation reveals an epigenetic signature in prostate carcinogenesis. PLoS One. 2009;4(3):e4687
20. Gleason DF, Mellinger GT. Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. J Urol. 1974;111(1):58-64
21. Wittekind CF, Greene FL, Hutter RVP, Klimpfinger M, Sobin LH. TNM Atlas: Illustrated guide to the TNM/pTNM classification of malignant tumors. Heidelberg, Germany: Springer-Verlag; 1997. 278-80 p.
22. van Haaften G, Dalgliesh GL, Davies H, Chen L, Bignell G, Greenman C, Edkins S, Hardy C, O'Meara S, Teague J, Butler A, Hinton J, Latimer C, Andrews J, Barthorpe S, Beare D, Buck G, Campbell PJ, Cole J, Forbes S, Jia M, Jones D, Kok CY, Leroy C, Lin ML, McBride DJ, Maddison M, Maquire S, McLay K, Menzies A, Mironenko T, Mulderrig L, Mudie L, Pleasance E, Shepherd R, Smith R, Stebbings L, Stephens P, Tang G, Tarpey PS, Turner R, Turrell K, Varian J, West S, Widaa S, Wray P, Collins VP, Ichimura K, Law S, Wong J, Yuen ST, Leung SY, Tonon G, DePinho RA, Tai YT, Anderson KC, Kahnoski RJ, Massie A, Khoo SK, Teh BT, Stratton MR, Futreal PA. Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nat Genet. 2009;41(5):521-3

23. de Kok JB, Roelofs RW, Giesendorf BA, Pennings JL, Waas ET, Feuth T, Swinkels DW, Span PN. Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. Lab Invest. 2005;85(1):154-9
24. Laitinen S, Martikainen PM, Tolonen T, Isola J, Tammela TL, Visakorpi T. EZH2, Ki-67 and MCM7 are prognostic markers in prostatectomy treated patients. Int J Cancer. 2008;122(3):595-602
25. Hake SB, Xiao A, Allis CD. Linking the epigenetic 'language' of covalent histone modifications to cancer. Br J Cancer. 2004;90(4):761-9
26. Chi P, Allis CD, Wang GG. Covalent histone modifications--miswritten, misinterpreted and mis-erased in human cancers. Nat Rev Cancer. 2010;10(7):457-69
27. Fullgrabe J, Kavanagh E, Joseph B. Histone onco-modifications. Oncogene. 2011;30(31):3391-403
28. Hamamoto R, Furukawa Y, Morita M, Iimura Y, Silva FP, Li M, Yagyu R, Nakamura Y. SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells. Nat Cell Biol. 2004;6(8):731-40
29. Hamamoto R, Silva FP, Tsuge M, Nishidate T, Katagiri T, Nakamura Y, Furukawa Y. Enhanced SMYD3 expression is essential for the growth of breast cancer cells. Cancer Sci. 2006;97(2):113-8
30. Ren TN, Wang JS, He YM, Xu CL, Wang SZ, Xi T. Effects of SMYD3 over-expression on cell cycle acceleration and cell proliferation in MDA-MB-231 human breast cancer cells. Med Oncol. 2011;28 Suppl 1:S91-8
31. Zou JN, Wang SZ, Yang JS, Luo XG, Xie JH, Xi T. Knockdown of SMYD3 by RNA interference down-regulates c-Met expression and inhibits cells migration and invasion induced by HGF. Cancer Lett. 2009;280(1):78-85
32. Wang SZ, Luo XG, Shen J, Zou JN, Lu YH, Xi T. Knockdown of SMYD3 by RNA interference inhibits cervical carcinoma cell growth and invasion in vitro. BMB Rep. 2008;41(4):294-9
33. Chen LB, Xu JY, Yang Z, Wang GB. Silencing SMYD3 in hepatoma demethylates RIZI promoter induces apoptosis and inhibits cell proliferation and migration. World J Gastroenterol. 2007;13(43):5718-24
34. Foreman KW, Brown M, Park F, Emtage S, Harriss J, Das C, Zhu L, Crew A, Arnold L, Shaaban S, Tucker P. Structural and functional profiling of the human histone methyltransferase SMYD3. PLoS One. 2011;6(7):e22290
35. Van Aller GS, Reynoird N, Barbash O, Huddleston M, Liu S, Zmoos AF, McDevitt P, Sinnamon R, Le B, Mas G, Annan R, Sage J, Garcia BA, Tummino PJ, Gozani O,

Kruger RG. Smyd3 regulates cancer cell phenotypes and catalyzes histone H4 lysine 5 methylation. Epigenetics. 2012;7(4):340-3

36. Rotili D, Mai A. Targeting Histone Demethylases A New Avenue for the Fight against Cancer. Genes & Cancer. 2011;2(6):663-79
37. Blair LP, Cao J, Zou MR, Sayegh J, Yan Q. Epigenetic regulation by lysine demethylase 5 (KDM5) enzymes in cancer. Cancers (Basel). 2011;3(1):1383-404
38. Ansari KI, Mandal SS. Mixed lineage leukemia: roles in gene expression, hormone signaling and mRNA processing. FEBS J. 2010;277(8):1790-804
39. Guccione E, Bassi C, Casadio F, Martinato F, Cesaroni M, Schuchlautz H, Luscher B, Amati B. Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive. Nature. 2007;449(7164):933-7
40. Neault M, Mallette FA, Vogel G, Michaud-Levesque J, Richard S. Ablation of PRMT6 reveals a role as a negative transcriptional regulator of the p53 tumor suppressor. Nucleic Acids Res. 2012;40(19):9513-21
41. Yoshimatsu M, Toyokawa G, Hayami S, Unoki M, Tsunoda T, Field HI, Kelly JD, Neal DE, Maehara Y, Ponder BA, Nakamura Y, Hamamoto R. Dysregulation of PRMT1 and PRMT6, Type I arginine methyltransferases, is involved in various types of human cancers. Int J Cancer. 2011;128(3):562-73
42. Cloos PA, Christensen J, Agger K, Helin K. Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. Genes Dev. 2008;22(9):1115-40
43. Franci C, Zhou J, Jiang Z, Modrusan Z, Good Z, Jackson E, Kouros-Mehr H. Biomarkers of residual disease, disseminated tumor cells, and metastases in the MMTV-PyMT breast cancer model. PLoS One. 2013;8(3):e58183
44. Coffey K, Rogerson L, Ryan-Munden C, Alkharaif D, Stockley J, Heer R, Sahadevan K, O'Neill D, Jones D, Darby S, Staller P, Mantilla A, Gaughan L, Robson CN. The lysine demethylase, KDM4B, is a key molecule in androgen receptor signalling and turnover. Nucleic Acids Res. 2013;41(8):4433-46
45. Guess HA. Benign prostatic hyperplasia and prostate cancer. Epidemiol Rev. 2001;23(1):152-8
46. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RG, Otte AP, Rubin MA, Chinnaiyan AM. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature. 2002;419(6907):624-9
47. Wolters T, Vissers KJ, Bangma CH, Schroder FH, van Leenders GJ. The value of EZH2, p27(kip1), BMI-1 and MIB-1 on biopsy specimens with low-risk prostate

cancer in selecting men with significant prostate cancer at prostatectomy. BJU Int. 2010;106(2):280-6

48. Popiolek M, Rider JR, Andren O, Andersson SO, Holmberg L, Adami HO, Johansson JE. Natural history of early, localized prostate cancer: a final report from three decades of follow-up. Eur Urol. 2013;63(3):428-35

SUPPLEMENTARY DATA

Supplementary Table 1 – TaqMan® Gene Expression Assays' references of the HMTs and HDMs analyzed

Gene	Assay reference
EZH2	Hs01016793_g1
GUSB	Hs99999908_m1
KDM3B	Hs00382671_m1
KDM4B	Hs00943636_m1
KDM5A	Hs00231908_m1
KDM6A	Hs00253500_m1
MLL1	Hs00610538_m1
MLL2	Hs00231606_m1
MLL3	Hs01005521_m1
MLL4	Hs00207065_m1
MLL5	Hs00218773_m1
PRMT6	Hs00250803_s1
SMYD3	Hs00224208_m1
SUV39H2	Hs00226596_m1
TFRC	Hs00951083_m1

Supplementary Table 2 – HMTs and HDMs (associated post translational modifications and function) with most significantly altered expression in prostate cancer

Enzyme	Main Activity	Function	Result
EZH2	H3K27&H3K9 HMT	Addition of repressive marks	Upregulated
SMYD3	H3K4, H4K5 & H4K20 HMT	Addition of active/repressive marks	Upregulated
SUV39H2	H3K9 HMT	Addition of repressive marks	Upregulated
PRMT6	H3R2 HMT	Addition of repressive marks	Upregulated
MLL1	H3K4 HMT	Addition of active marks	Downregulated
MLL2	H3K4 HMT	Addition of active marks	Downregulated
MLL3	H3K4 HMT	Addition of active marks	Downregulated
MLL4	H3K4 HMT	Addition of active marks	Downregulated
MLL5	H3K4 HMT	Addition of active marks	Downregulated
KDM5A	H3K4 HDM	Removal of active marks	Upregulated
KDM6A	H3K27 HDM	Removal of repressive marks	Upregulated
KDM4B	H3K9 HDM	Removal of repressive marks	Downregulated

HMT, histone methylase; HDM, histone demethylase.

Chapter IV

Effects of SMYD3 in prostate carcinogenesis

Filipa Quintela Vieira[1,2], Pedro Costa-Pinheiro[1], Susana Simões-Sousa[3,4], Inês Graça[1,2], Sara Monteiro-Reis[1], Diogo Almeida-Rios[1], Elsa Joana Sousa[1], Fátima Baltazar[3,4], Rui Henrique[1,5,6*], Carmen Jerónimo[1,6*]

[1]Cancer Epigenetics Group – Research Center, Portuguese Oncology Institute – Porto, Portugal; [2]School of Allied Health Sciences (ESTSP), Polytechnic of Porto, Portugal; [3]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal, [4]ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal [5]Departments of Pathology, Portuguese Oncology Institute – Porto, Portugal; [6]Department of Pathology and Molecular Immunology, Institute of Biomedical Sciences Abel Salazar (ICBAS) – University of Porto, Portugal

[*]Joint senior authors

ABSTRACT

Prostate cancer (PCa), one of the most incident cancers worldwide, remains a significant clinical challenge. Because current clinical and pathological parameters have a limited ability to discriminate between clinically significant and insignificant PCa, deeper understanding of its biology might improve clinical management and therapeutic decision. Altered expression of histone methyltransferases and histone methylation patterns are involved in prostate carcinogenesis. We previously demonstrated that *SMYD3* transcript levels have prognostic value and might discriminate among PCa with different clinical aggressiveness. Herein, we investigated the putative oncogenic role of SMYD3 in PCa cells. Knockdown of *SMYD3* on PCa cells attenuated the malignant phenotype of LNCaP and PC3 cell lines, both *in vitro* and *in vivo*. Furthermore, deletions affecting the SET domain showed a phenotypic impact similar to SMYD3 silencing, suggesting that the tumorigenic effect is mostly mediated through its histone methyltransferase activity. Moreover, cell cycle regulation surfaced as the main cellular pathway influenced by SMYD3, and *CCND2* was identified as a putative target gene for SMYD3 transcriptional regulation, through trimethylation of H4K20. Our results support a proto-oncogenic role for SMYD3 in prostate carcinogenesis, mainly due to its methyltransferase enzymatic activity. Thus, in addition to its usefulness as a biomarker for clinically aggressive disease, SMYD3 overexpression might also constitute an attractive therapeutic target in PCa. Nevertheless, a more comprehensive and detailed characterization of SMYD3 target genes is mandatory to fully elucidate its role in prostate tumorigenesis.

INTRODUCTION

Genetic alterations have been historically considered the main driving force of cancer initiation and progression, although more recently a prominent role for epigenetic modifications has been acknowledged [1]. In addition to aberrant gene promoter methylation, alterations in chromatin modification patterns due to post-translational modifications (PTMs) of histones have been demonstrated in cancer and emerged as potential key players in neoplastic transformation. Specifically, diverse PTMs might occur in amino tail domains, including lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, ADPribosylation, ubiquitination, and sumoylation [2]. Histone methylation is carried out by histone methyltransferases (HMTs) whereas histone demethylases (HDMs) antagonize their action [3, 4]. Depending on the target residue and the state of methylation (i.e., whether it is mono-, di- or trimethylated), PTMs may positively or negatively regulate gene transcription. In cancer cells, deregulation of HMTs or HDMs has been associated with altered post-translational control of cellular proteins affecting key signaling networks [5, 6].

Among HMTs, SET and MYND domain-containing protein 3 (SMYD3) belongs to a subfamily of SET domain-containing proteins with an important role in transcriptional regulation [7]. Its methyltransferase activity is highly dependent on two amino acid sequences, NHSC and EEL, located within the SET domain [7]. SMYD3 was firstly described as having dimethyl- and trimethyltransferase activity at lysine 4 of histone H3 (H3K4), but recently it has been reported that SMYD3 also methylates H4K5 and H4K20, as well as other non-histonic proteins, such as vascular endothelial growth factor receptor 1 (VEGFR1) [8-10]. An oncogenic role of SMYD3 has been suggested in several cancer models, including colorectal, hepatocellular, cervical and breast carcinomas [7, 11-14]. Indeed, SMYD3 was shown to induce transcriptional activation of several downstream genes, including *Nkx2.8*, *WNT10B*, *RIZ1*, *c-Met*, *15-LOX-1* and *MMP9* [7, 11, 12, 15-17]. Interestingly, since SMYD3 is able to promote either the active H3K4me3 or the repressive H4K20me3 marks, it has been suggested that it might act either by repressing tumor suppressor genes or inducing oncogenes' expression [8].

Prostate cancer (PCa), one of the most incident cancers worldwide, remains a significant clinical challenge as PSA screening led to substantial overdiagnosis and overtreatment of patients [18]. Thus, additional efforts are needed to better identify and characterize aggressive tumors, allowing for more appropriate therapeutic strategies that will avoid unnecessary and potentially harmful interventions [19]. We have previously reported that PCa tissues displayed higher *SMYD3* levels compared to normal prostate, especially at advanced disease stages. Moreover, we demonstrated that *SMYD3*

transcript levels convey prognostically important information and might discriminate among PCa with different clinical aggressiveness [20]. Nevertheless, how *SMYD3* deregulated expression and respective histone marks impact on PCa development and progression is still largely unknown. Herein, we sought to ascertain the impact of SMYD3 methyltransferase activity on PCa cells phenotype. Knockdown of *SMYD3* in LNCaP and PC3 cells attenuated the malignant phenotype, both *in vitro* and *in vivo*. This effect was mostly mediated through SMYD3 histone methyltransferase activity. Moreover, cell cycle regulation surfaced as the main cellular pathway influenced by SMYD3 methyltransferase activity, and *CCND2* was identified as a putative target gene for SMYD3 transcriptional regulation through trimethylation of H4K20. Thus, a proto-oncogenic role for SMYD3 in prostate carcinogenesis is suggested, mainly due to its methyltransferase enzymatic activity.

MATERIAL AND METHODS

CELL LINES AND CULTURE CONDITIONS

Human PCa cell lines LNCaP, PC3 and VCaP were kindly provided by Prof. Ragnhild A. Lothe from the Department of Cancer Prevention at the Institute for Cancer Research, Oslo, Norway, and DU145 was offered by Prof. Fátima Baltazar from the Life and Health Sciences Research Institute at the University of Minho, Braga, Portugal. All cell lines were maintained in recommended medium, supplemented with 10% Fetal Bovine Serum (FBS; GIBCO, Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin solution (GIBCO, Invitrogen), at 37ºC and 5% CO^2. PCa cell lines were karyotyped by G-banding (for validation purposes) and routinely tested for *Mycoplasma spp.* contamination (PCR Mycoplasma Detection Set, Clontech Laboratories Inc., Mountain View, CA, USA).

GENERATION OF SH-*SMYD3* SILENCED CELL LINES

SMYD3 knockdown was performed through viral transduction in LNCaP and PC3 cell lines using shRNA Lentiviral Particles (sc-61576-V; Santa Cruz Biotechnology Inc., Santa Cruz, CA) in the presence of polybrene (Santa Cruz Biotechnology Inc.) as described by the manufacturer. Additionally, control LNCaP and PC3 cells were generated using a non-target scramble shRNA (sc-108080; Santa Cruz Biotechnology Inc.). After transduction, stable clones with shRNA were selected with Puromycin dihydrochloride (cat. 631306, Clontech Laboratories Inc.) at a final concentration of 2µg/ml or 4µg/ml in LNCaP or PC3 cells, respectively.

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Cells were collected with a gum rubber-scraping device, lysed with RIPA buffer (sc-24948, Santa Cruz Biotechnology Inc.) and protein concentration was determined using BCA assay (Thermo Scientific, Waltham, MA, USA) according to manufacturer's information. Subsequently, 30µg of total protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated with antibodies against anti-SMYD3 (dilution: 1:500, Abcam, Cambridge, UK), anti-H3K4me1 (dilution: 1:1000, Abcam), anti-H3K4me2 (dilution: 1:1000, Abcam), anti-H3K4me3 (dilution: 1:400 Abcam), anti-H3K27me3 (dilution: 1:500 Millipore, Billerica, MA, USA) or the endogenous control β-actin (dilution: 1:8000, Sigma-Aldrich, Schnelldorf, Germany). The blots were developed

using Immun-Star™ WesternC™ Kit according to manufacturer's indications (BioRad, Hercules, CA, USA). All the experiments were performed in triplicate. Relative optical density determination was performed using QuantityOne® Software version 4.6.6. (Biorad) and proteins levels were normalized using beta-actin levels as reference.

CELL VIABILITY ASSAY

The effect of SMYD3 on cell viability was assessed in triplicates by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma-Aldrich) assay. The cells were seeded in 96-well plates at a density of $1x10^4$ cells/well and after 24, 48 and 72h, cells were incubated with MTT at 37ºC for 2h and the reaction was stopped by the addition of 100µl/well of Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) lysing for 10min. An automated plate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany) at 540nm with a reference filter of 630nm allowed for colorimetric quantification. The absorbance value was directly proportional to the number of viable cells.

APOPTOSIS ASSAY

Apoptosis was assessed using the APOPercentage™ kit (Biocolor Ltd., Newtownabbey, Northern Ireland, UK). LNCaP and PC3 cells were seeded in the same conditions as described for MTT assay. Following an incubation period of 72h, the APOPercentage assay was performed according to manufacturer's instructions. Quantification of apoptosis was achieved by measuring the optical density of the released dye at 550nm with a reference filter of 620nm using a FLUOstar Omega microplate reader. To normalize the OD measured in the apoptosis test to the cell number, the OD of apoptosis assay was divided by the OD of the viability assay, also performed in 96-well plates. The results of the apoptosis assay on the silenced cells were expressed as the ratio of the values obtained for scramble cells.

MIGRATION ASSAY

The ability of cells of each genotype to migrate into a defect in a monolayer culture was determined using the wound healing assay. Cells were grown to full confluence in 24-well plates and scratches were performed using a 100µL tip. The medium was removed, and cells were washed with PBS and medium replaced. Scratch closure was analyzed under the microscope and images were captured at different time points.

INVASION ASSAY

Invasion capacity was analyzed through Biocoat Matrigel Invasion Chambers (BD Biosciences, Franklin Lakes, NJ, USA) according to manufacturer's protocol. Briefly, cells were placed in Matrigel inserts and allowed to migrate for 24 or 48h at 37ºC. Non-migrating cells were removed from the top of the filter and cells that migrated were fixed in methanol and stained with DAPI. Migrated cells were manually counted and results were displayed as percentages of invasion relative to scramble.

CHICK CHORIOALLANTOIC MEMBRANE (CAM) ASSAY

To assess *in vivo* tumor formation and angiogenesis, the CAM assay was used, as previously described [21], with some modifications. Briefly, fertilized chicken eggs (Pinto Bar, n=5 for each experiment) were incubated at 37ºC and 70% humidity. On day 3 of development, after puncturing the air chamber, a hole in a specific region of the eggshell was performed and eggs were sealed with tape and returned to the incubator. On day 10, a small plastic ring was placed on the CAM and $5x10^6$ PCa cells (PC3 or LNCaP, control and sh-SMYD3), ressuspended in 20 µL of RPMI or RPMI/F12 medium, were injected in the ring over the CAM. On day 14, the tumor formed was photographed *in ovo* using a stereomicroscope (Olympus S2x16, Olympus, Tokyo, Japan) and, on day 16, chicks were sacrificed at -80ºC for 10 minutes. The CAM and tumors were fixed with 4% paraformaldehyde and photographed *ex ovo*. Samples were paraffin-embedded, sectioned, and stained with hematoxylin and eosin (HE) for histological analysis. The total area occupied by tumors was measured using the Cell B software (Olympus) and linear vessel density was assessed by calculating the ratio between the number of vessels and the total length of the membranes.

MUTAGENESIS AND TRANSIENT TRANSFECTION ASSAYS

For bacterial expression, *SMYD3* cDNA (Origene Technologies Rockville, MD, USA) was firstly deleted at NHSC or EEL motifs using specific primers (Supplementary table 1) of the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). After bacterial transformation, colonies were picked and the plasmids were purified using the Qiagen Plasmid Miniprep Kit (Qiagen, Hilden, Germany). Subsequently, deletions were confirmed by direct sequencing (Supplementary Table 1) in an ABI PRISM 310 automatic sequencer using Big Dye Terminator Chemistry (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations.

Expression plasmids encoding the mutant *SMYD3* (ΔNHSC or ΔEEL) or the original *SMYD3* cDNA were transiently transfected into sh-SMYD3 PC3 using LipofectAMINE2000 (Invitrogen), following manufacturer's instructions. Phenotypic assays for cell viability, apoptosis and invasion were performed as described above.

IDENTIFICATION OF PUTATIVE TARGET GENES

To assess whether SMYD3 was implicated in the regulation of selected genes involved in cell cycle, apoptosis, DNA repair, mTOR or MAPK/ERK pathways, a custom array panel (Roche Applied Science, Manheim, Germany) was designed for quantification of expression of those genes. Total RNA was extracted from all cell lines using TRIzol® (Invitrogen) according to manufacturer's instructions and cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to manufacturer's instructions. Expression levels were determined by real-time PCR in a LightCycler 480 (Roche Diagnostics) and the amounts of mRNA were normalized using *GUSB*, *TFRC* and *18S* as endogenous controls. The comparative Ct method [22] was used to calculate fold-difference in gene expression among groups and only genes with a logarithmized fold change above 1.25 or below -1.25 were further considered.

CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY

ChIP assay was performed using EZ-Magna ChIP G-Chromatin Immunoprecipitation Kit and the Magna Grip Rack (Millipore), according to the manufacturer's instructions. For each assay, anti-H3K4me3,anti-H3K27me3, anti-H4K20me3, anti-H3, anti-H4 (all from Abcam) and the negative control provided with the kit (normal mouse IgG), were used. DNA quantification was performed in a 7500 Real-Time PCR System (Applied Biosystems), using Power SYBR Green PCR Master Mix (Applied Biosystems). Three gene-specific pairs of primers for each gene promoter were used, in which primers A were located more distant upstream of Transcriptional Start-Site (TSS) and C those that were closer to TSS (Supplementary Table 1). The relative amount of promoter DNA was calculated for each histone mark over the core histone (H3 or H4) and normalized using Input Percent Method.

STATISTICAL ANALYSIS

The Shapiro-Wilk's W test allowed for the examination of the appropriateness of a normal distribution assumption for each of the parameters (data not shown). For *in vitro*

and *in vivo* assays, comparison between two groups was performed using the Mann–Whitney U-test. All statistical tests were 2-sided. Statistical analysis was carried out using Graph Pad Prism version 5. Significance level was set at $p<0.05$.

RESULTS

IMPACT OF *SMYD3* SILENCING ON THE MALIGNANT PHENOTYPE OF PCA CELLS

To select the most suitable *in vitro* model, *SMYD3* expression levels were assessed in PCa cell lines LNCaP, VCaP, DU145 and PC3. All cell lines expressed SMYD3, although at variable levels (Figure A). The cell lines displaying the highest expression levels among the androgen-sensitive and the androgen-refractory were then selected for further analysis (LNCaP and PC3, respectively). Lentiviral particles efficiently silenced *SMYD3* in those two cell lines, at transcript and protein level (Figure B).

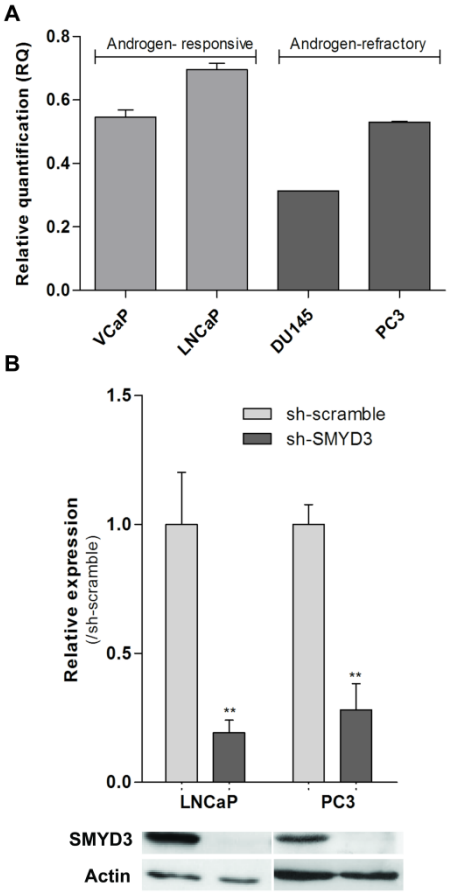

Figure 1 – *SMYD3* expression levels in PCa cell lines. The efficiency of SMYD3 knockdown was confirmed at mRNA level, using real-time RT-PCR (upper) and protein level, using Western-blot (lower), in LNCaP and PC3 cells. **p < 0.01 (Mann-Whitney U-test).

The MTT assay showed a 35% decrease in viability of PC3 cells, although the opposite was apparent in LNCaP cells, at 72h (Figure 2A and 2B). Moreover, knockdown of SMYD3 induced a significant increase in apoptosis in both cell lines (Figure 2C).

A significant decrease in migration rate as well as in invasiveness capacity was also demonstrated in SMYD3-depleted PC3 cells (Figure 2E and 2F), whereas for LNCaP a statistically significant reduction was only observed in cell migration (Figure 2D).

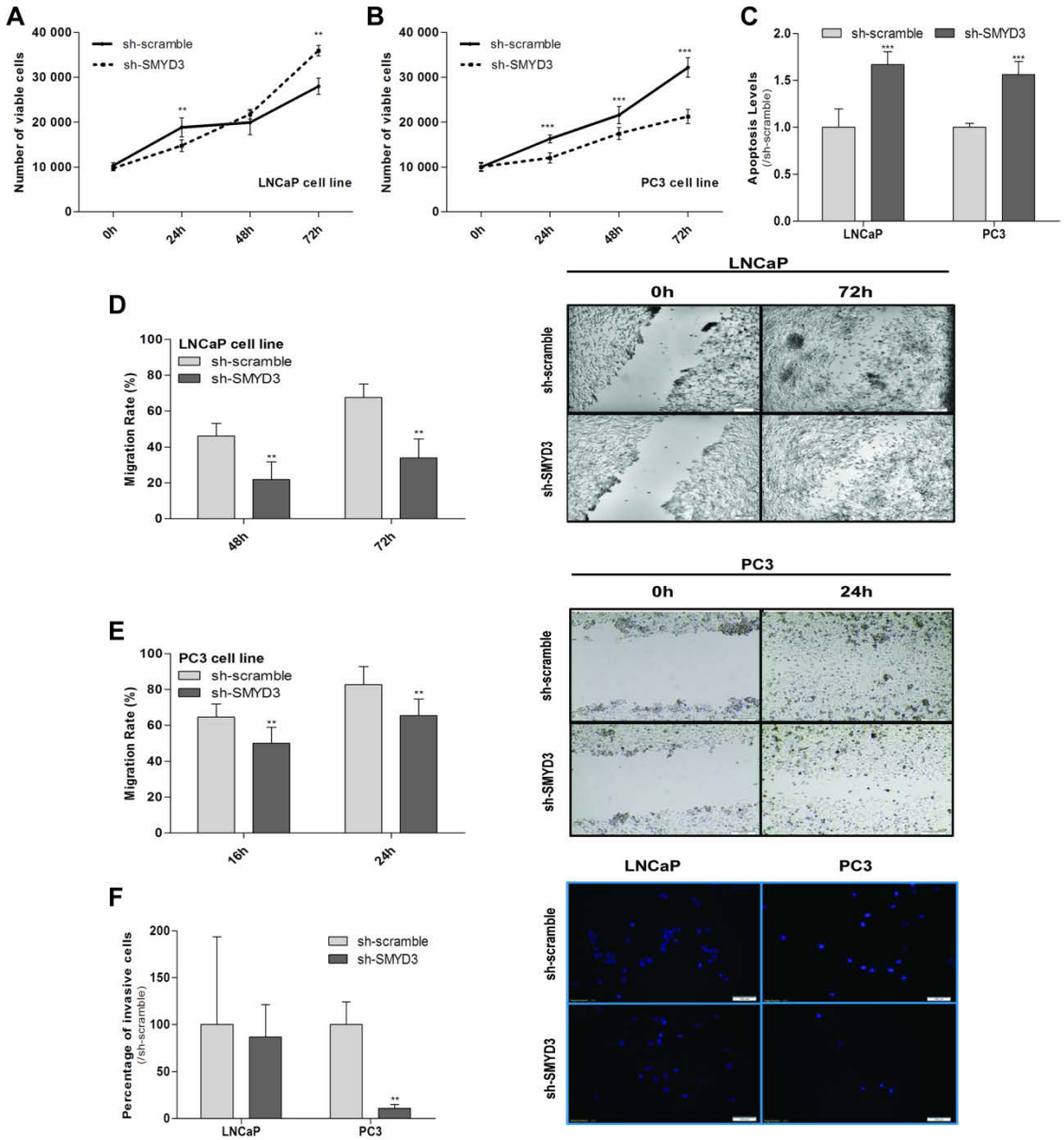

Figure 2 – Impact of SMYD3 silencing in the malignant phenotype of PCa cells. Cell viability in LNCaP (A) and PC3 (B): quantification of cell viability by MTT assay in sh-scramble and sh-SMYD3 at 0h, 24h, 48h and 72h in culture. Quantification of apoptosis (C) by APOPercentage kit of sh-Scramble, sh-SMYD3 LNCaP and sh-SMYD3 PC3 cells at 72h. Wound-healing scratch assay in sh-scramble and sh-SMYD3 LNCaP (D) and PC3 (E) cells: the left panels show the migration rate at 48h and 72h or 16h and 24h, in LNCaP and PC3, respectively, and the right panels display the illustrative images at the beginning and end point of the assay. Invasive ability was assessed by a Matrigel Invasion assay in sh-scramble and sh-SMYD3 LNCaP and PC3, cells at 48h and 24h, respectively. Results were normalized to the data obtained with sh-scramble cells. **p < 0.01, ***p < 0.001 (Mann-Whitney U-test).

EFFECTS OF SMYD3 KNOCKDOWN ON TUMOR FORMATION AND VESSEL DENSITY IN VIVO

The CAM assay was performed to evaluate the effect of SMYD3 on tumor growth and angiogenesis *in vivo* (Figure 3). The areas occupied by formed tumors were smaller in sh-SMYD3 LNCaP and PC3 compared to controls. Although statistical significance was not reached (Figure 3A), most likely due to small sample size, a trend was apparent in PC3 cells (p=0.056). No statistically significant difference was apparent in linear vessel density counted *ex ovo* between sh-scramble and sh-SMYD3 cells, for both cell lines.

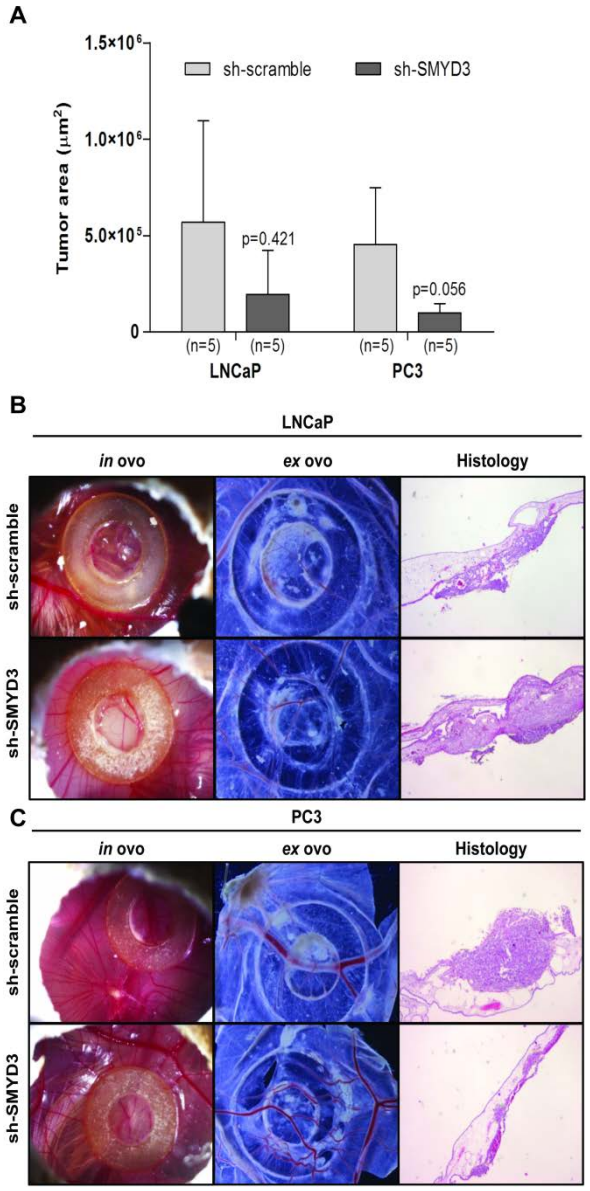

Figure 3 – *In vivo* effect of SMYD3 silencing in tumor formation and angiogenesis in LNCaP and PC3 cell lines. (A) Graphic depiction of tumor areas measured in histological sections. (B-C) Representative images of CAM assay 6 days after injection of LNCaP (B) or PC3 (C) sh-scramble or sh-SMYD3 cells. Images were taken *in ovo* and *ex ovo* (original magnification: x10), as well as from histological sections (original magnification: x40).

SMYD3 PUTATIVE ONCOGENIC FUNCTION IS ASSOCIATED WITH THE HISTONE METHYLTRANSFERASE ACTIVITY

The impact of SMYD3 knockdown on mono-, di- and trimethylation levels of H3K4, as well as on trimethylation of H3K27 was assessed using Western-blot (Figure 4). Both LNCaP and PC3 cells showed a paradoxical increment in H3Kme2 and H3K4me3 as well as a decrease in H3K4me1, but these differences did not reach statistical significance. Interestingly, SMYD3-silenced LNCaP cells displayed an increase (although not significant) in global levels of the repressive mark H3K27me3.

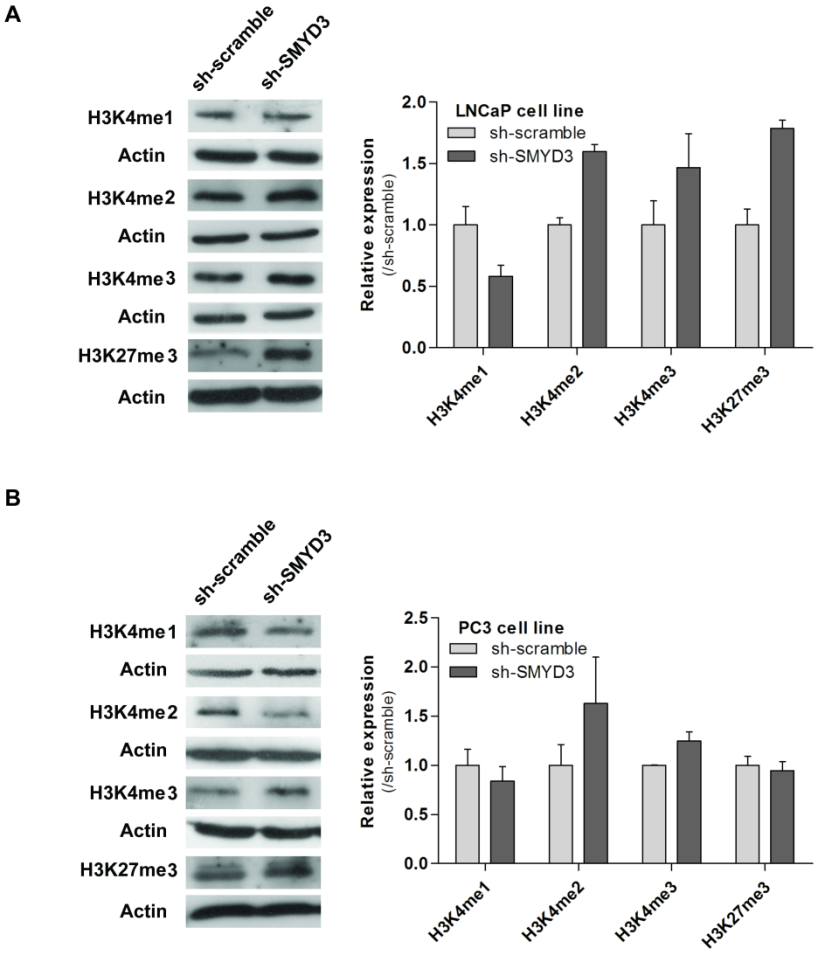

Figure 4 – Evaluation of global H3K4me1, H3K4me2, H3K4me3 and H3K27me3 in LNCaP (A) and PC3 (B) sh-scramble and sh-SMYD3 cells. The left panels show Western-blot images and the right panels depict relative density of bands by densitometric quantification, using β-actin as a reference.

To assess whether the previously documented phenotypic effects were due to SMYD3 methyltransferase activity, sh-SMYD3 PCa cells were transfected with mutant *SMYD3* deleted for main components of SMYD3 SET domain, the EEL and NHSC amino acid sequences. In the absence of one of these sequences, cell viability, apoptosis levels and invasion capacity were compromised (Figure 5). PC3 cells with mutant SMYD3

showed decreased cell viability and increased apoptotic levels (Figure 5A and 5B), being these effects more pronounced when SMYD3-EEL domain was deleted. Indeed, only SMYD3-ΔEEL-PC3 cells disclosed a statistically significant reduction in invasion capability when compared to the control cells (Figure 5C).

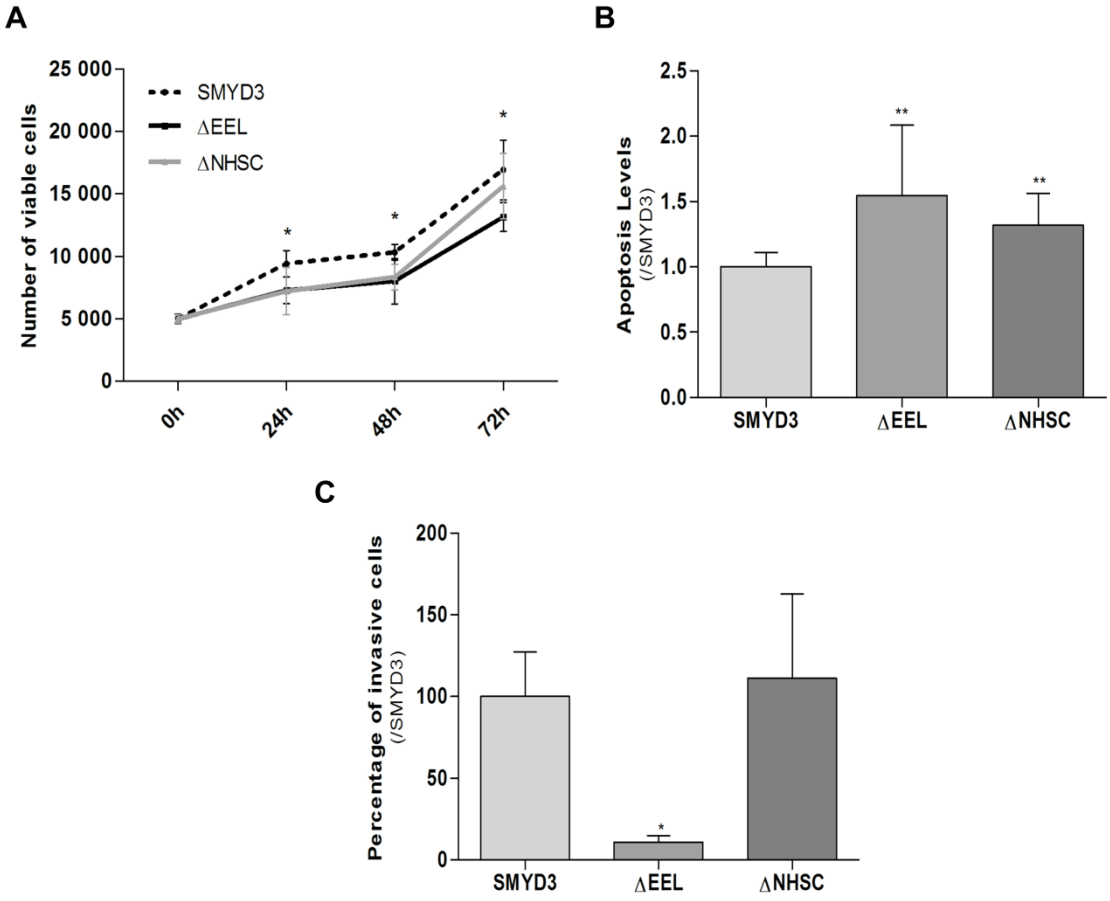

Figure 5 – Impact of normal or mutant SMYD3 in the malignant phenotype of PC3 cells. Cell viability, apoptosis levels and invasion capability were assessed in cells with normal expression of SMYD3 or with expression of a mutant protein with deletion of EEL (ΔEEL) or NHSC (ΔNHSC) region of SMYD3 SET domain. (A) Quantification of cell viability was performed by MTT assay after 72h of culture. (B) Quantification of apoptosis levels by APOPercentage kit was assessed after 72h in culture. (C) Invasive ability was evaluated by a Matrigel Invasion assay after 24h of culture. Results were normalized to the data obtained with the SMYD3 normal protein. *$p < 0.05$, **$p < 0.01$ (Mann-Whitney U-test).

SMYD3 KNOCKDOWN LEADS TO CCND2 RESTORED EXPRESSION THROUGH DOWNREGULATION OF H4K20ME3 MARK

To identify putative target genes of SMYD3 histone methyltransferase activity, expression profile of selected genes involved in cell cycle, apoptosis, DNA repair, mTOR and MAPK/ERK pathways was evaluated after SMYD3 knockdown (Figure 6A). Interestingly, most of the selected genes were found overexpressed in SMYD3-silenced PC3 cells, whereas the opposite was observed in LNCaP. Remarkably, the Cyclin D2

gene (*CCND2*) was overexpressed in both cell lines when SMYD3 expression was knockdown. ChIP analysis of *CCND2* promoter region showed a decrease in the H4K20me3 mark, concomitantly with the silencing of SMYD3 (Figure 6D), although no significant differences in H3K4me3 or H3K27me3 levels were apparent in none of the three regions tested (Figure 6A and 6B), in PC3 cells.

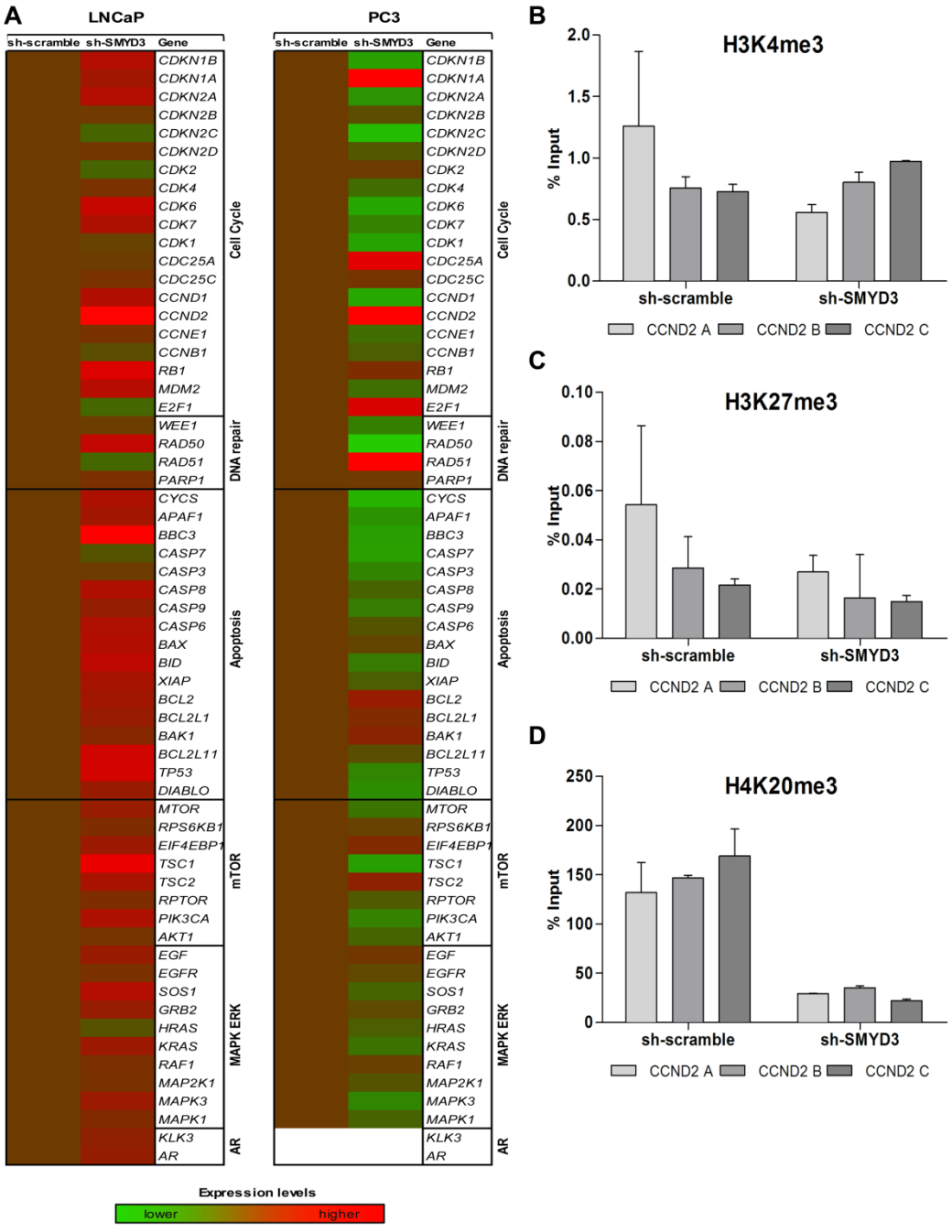

Figure 6 – Identification of putative SMYD3 target genes and its regulation by SMYD3 histone marks. (A) Expression quantification of genes involved in cell cycle, apoptosis, DNA repair, mTOR or MAPK/ERK pathways normalized to sh-scramble in LNCaP or PC3 cells. (B-C) Chromatin immunoprecipitation assay for H3K4me3 (B), H3K27me3 (C), and H4K20me3 (D) marks in *CCND2* promoter; primers A were located more distant upstream of Transcriptional Start-Site (-1734 bp), B (-1130 bp) and C (-840 bp) were the closest.

DISCUSSION

PCa is one of the most prevalent cancers and a leading cause of mortality and morbidity [18]. The growing concern about overdiagnosis and consequent overtreatment of PCa patients due to PSA screening should be addressed through the identification of those cancers that are most likely to cause clinically aggressive disease. Since current clinical and pathological parameters have a limited ability to discriminate between clinically significant and insignificant PCa, emphasis should be placed on a deeper understanding of the biology of this neoplasm, which might ultimately result in the development of more efficient biomarkers. These are mandatory to improve disease management and therapeutic decision. Because histone methylation seems to play a major role in gene expression regulation, epigenetic modifying enzymes or histone modification patterns may serve as biomarkers, suitable for diagnostic, prognostic or predictive purposes in PCa patients. We have previously reported that higher expression levels of histone methyltransferase *SMYD3* associate with more advanced stage PCa and these may predict unfavorable prognosis independently of Gleason score (GS) or pathological stage. Thus, in the present study, we sought to investigate the biological role of SMYD3 and the corresponding post-transduction modifications of histones, to determine how these might impact on the malignant phenotype of PCa cells.

Because higher expression levels of *SMYD3* were found in more aggressive PCa, our strategy was based on the knockdown of this enzyme to determine its phenotypic impact in PCa cells. After achieving a stable decrease in *SMYD3* expression, cell viability, apoptosis, migration and invasion ability assays were carried out in two PCa cell lines that are thought to largely represent *in vivo* heterogeneity of this neoplasm. Decreased expression of *SMYD3* attenuated the highly malignant phenotype of the androgen-insensitive PC3 cells, whereas, surprisingly, cell viability of the androgen-sensitive LNCaP cell line was not apparently compromised. Furthermore, *SMYD3* silencing was associated with increased cell death by apoptosis, although the underlying mechanism was not elucidated. Concerning LNCaP cells, results of cell viability and invasive capability assays contrast with those recently reported by Liu et al. [23], but parallel the results of cell migration and apoptosis levels. The discrepancies might be due to differences in methodology because the effectiveness of *SMYD3* silencing following transfection was confirmed in our study and that of Liu and co-workers [23]. Interestingly, the effect of *SMYD3* knockdown in LNCaP cells reported by Liu et al. mirror those we observed in PC3 cells, which are acknowledged as representing a more aggressive phenotype of PCa cells. It is noteworthy that LNCaP cells display a low invasion potential, a feature that may explain the lack of impact of *SMYD3* knockdown in the invasion assay of this cell line.

Globally, however, the results reported for SMYD3 silencing in PCa cells are in line with those reported for hepatocellular, colorectal, cervical and breast cancers [7, 11-14], which further supports an oncogenic role for SMYD3.

To further characterize the effect of SMYD3 silencing in PCa cells, an *in vivo* model (the CAM assay) was conducted. Although a trend for a decrease in tumor formation was depicted in sh-SMYD3 PC3 cells, no statistically significant differences were found in both cell lines tested. The same result was depicted for linear microvessel density. In a mouse model, *SMYD3* knockdown was able to reduce tumor formation by LNCaP cells [23]. Although mammalian mice models are more close to the biological conditions found in humans, the CAM assay has been used as an alternative owing to its lower cost and less strict regulations [24]. There are, however, some flaws in the assessment of phenotypic effects using the CAM model (including those related with sampling size), which preclude a direct comparison with well-established mice models. Thus, we believe that our results with the CAM assay are not definitive and require further refinements.

Since SMYD3 main activity is di- and trimethylation of H3K4, we checked global levels of the three states of methylation of this residue in LNCaP and PC3 cells. Furthermore, H3K27me3 levels were also assessed because it was previously hypothesized that SMYD3 could inappropriately bind to its target genes and competing with the silencing activity of repressive complexes [25]. Thus, repressive marks such as H3K27me3 might be converted to the activating mark H3K4me3 by SMYD3 and consequently promote inappropriate gene expression [25]. Interestingly, higher global levels of H3K27me3 were observed in SMYD3-silenced LNCaP, although no significant differences were apparent for H3K4 methylation status both in LNCaP and PC3. On one hand, our findings support the aforementioned hypothesis [25], although, on the other, no direct effect on the global levels of the marks directly catalyzed by SMYD3 was apparent. Because phenotypic effects were apparent in both tested cell lines, it is plausible that altered SMYD3 expression could only specifically affect the H3K4me status of its target genes, which might not be of sufficient magnitude to significantly alter the global H3K4 methylation levels.

In accordance with previous findings in other tumor models [7, 26], we hypothesized that the oncogenic properties of SMYD3 could depend on its histone methyltransferase activity. We tested this hypothesis by means of transfecting sh-SMYD3 cancer cells with a *SMYD3* gene mutated at the either the EEL or the NHSC amino acid sequences of the SET domain. Phenotypic assays demonstrated that the effects of a mutated SET domain largely overlap those of *SMYD3* silencing. Although it has been reported that the two regions of the SET domain have similar roles [26], we found that the

EEL domain was more closely associated with SMYD3 tumorigenic properties in PCa cells. Although this experiment strongly suggests that SMYD3 histone methyltransferase activity might be the most important for oncogenesis, the possible contribution of its enzymatic activity on cytoplasmic proteins can not be excluded.

Owing to the phenotypic effects observed due to *SMYD3* silencing, we then searched for possible target genes, focusing on some critical genes from relevant cellular pathways frequently deregulated in tumorigenesis. Surprisingly, opposite trends in gene expression were observed in LNCaP and PC3 cells, probably reflecting the different biology and malignant phenotype of these two PCa cell lines, which may explain, at the least partially, the dissimilar results of *SMYD3* silencing concerning the phenotype of LNCaP and PC3 cells. Interestingly, one gene involved in cell cycle regulation – *CCND2* – showed a similar expression pattern in both cell lines, *i.e.*, its expression was restored following *SMYD3* knockdown. These results strongly suggested that *CCND2* was a putative target gene of SMYD3. Remarkably, CCND2 is frequently downregulated in PCa [27], and both aberrant promoter methylation and histone acetylation have been implicated [27-29]. Furthermore, the *CCND2* promoter contains DNA sequences5'-CCCTCC-3' or 5'-GGAGGG-3', which are specifically recognized by SMYD3 for its transcriptional regulatory functions [7]. Thus, we interrogated the histone marks in the promoter region of *CCND2* containing those motifs, using the ChIP assay. Although no significant differences were found for H3K4 or H3K27 methylation levels, a significant decrease in the H4K20me3 mark was apparent in sh-SMYD3 PCa cells. Because the H4K20me3 repressive mark is also catalyzed by SMYD3 [8], our results indicate that SMYD3 overexpression might be also involved in *CCND2* silencing in PCa. Interestingly, epigenetic deregulation of androgen receptor expression due to SMYD3 overexpression has been reported in LNCaP cells [23] and SMYD3 may also directly interact with other nuclear receptors, such as estrogen receptor [26], further reinforcing its oncogenic role.

In conclusion, our data provide further evidence to sustain an oncogenic role for SMYD3 in prostate carcinogenesis. In addition to its usefulness as a biomarker for clinically aggressive disease, SMYD3 overexpression might also constitute an attractive therapeutic target in PCa because its tumor-promoting properties are mostly due to its histone methyltransferase activity. Indeed, *SMYD3* silencing might be able not only to refrain the expression of proto-oncogenes but also to restore the expression of genes inadequately silenced in PCa. A more comprehensive and detailed characterization of SMYD3 target genes is, however, mandatory to fully elucidate its role in prostate tumorigenesis.

FUNDING

This study was funded by research grants from Liga Portuguesa Contra o Cancro – Núcleo Regional do Norte, Research Center of Portuguese Oncology Institute – Porto (CI-IPOP 4-2008) and European Community's Seventh Framework Programme – Grant number FP7-HEALTH-F5-2009-241783. FQV, IG and SS are supported by FCT-Fundação para a Ciência e a Tecnologia grants (SFRH/BD/70564/2010, SFRH/BD/64082/2009 and PTDC/SAU-MET/113415/2009, respectively), and PCP is supported by a grant from Liga Portuguesa Contra o Cancro – Núcleo Regional do Norte.

REFERENCES

1. Hirst M, Marra MA. Epigenetics and human disease. Int J Biochem Cell Biol. 2009;41(1):136-46
2. Miremadi A, Oestergaard MZ, Pharoah PD, Caldas C. Cancer genetics of epigenetic genes. Hum Mol Genet. 2007;16 Spec No 1:R28-49
3. Suzuki T, Terashima M, Tange S, Ishimura A. Roles of histone methyl-modifying enzymes in development and progression of cancer. Cancer Sci. 2013
4. Albert M, Helin K. Histone methyltransferases in cancer. Semin Cell Dev Biol. 2010;21(2):209-20
5. Tian X, Zhang S, Liu HM, Zhang YB, Blair CA, Mercola D, Sassone-Corsi P, Zi X. Histone lysine-specific methyltransferases and demethylases in carcinogenesis: new targets for cancer therapy and prevention. Curr Cancer Drug Targets. 2013;13(5):558-79
6. Chi P, Allis CD, Wang GG. Covalent histone modifications--miswritten, misinterpreted and mis-erased in human cancers. Nat Rev Cancer. 2010;10(7):457-69
7. Hamamoto R, Furukawa Y, Morita M, Iimura Y, Silva FP, Li M, Yagyu R, Nakamura Y. SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells. Nat Cell Biol. 2004;6(8):731-40
8. Foreman KW, Brown M, Park F, Emtage S, Harriss J, Das C, Zhu L, Crew A, Arnold L, Shaaban S, Tucker P. Structural and functional profiling of the human histone methyltransferase SMYD3. PLoS One. 2011;6(7):e22290
9. Van Aller GS, Reynoird N, Barbash O, Huddleston M, Liu S, Zmoos AF, McDevitt P, Sinnamon R, Le B, Mas G, Annan R, Sage J, Garcia BA, Tummino PJ, Gozani O, Kruger RG. Smyd3 regulates cancer cell phenotypes and catalyzes histone H4 lysine 5 methylation. Epigenetics. 2012;7(4):340-3
10. Kunizaki M, Hamamoto R, Silva FP, Yamaguchi K, Nagayasu T, Shibuya M, Nakamura Y, Furukawa Y. The lysine 831 of vascular endothelial growth factor receptor 1 is a novel target of methylation by SMYD3. Cancer Res. 2007;67(22):10759-65
11. Hamamoto R, Silva FP, Tsuge M, Nishidate T, Katagiri T, Nakamura Y, Furukawa Y. Enhanced SMYD3 expression is essential for the growth of breast cancer cells. Cancer Sci. 2006;97(2):113-8
12. Chen LB, Xu JY, Yang Z, Wang GB. Silencing SMYD3 in hepatoma demethylates RIZI promoter induces apoptosis and inhibits cell proliferation and migration. World J Gastroenterol. 2007;13(43):5718-24

13. Luo XG, Zou JN, Wang SZ, Zhang TC, Xi T. Novobiocin decreases SMYD3 expression and inhibits the migration of MDA-MB-231 human breast cancer cells. IUBMB Life. 2010;62(3):194-9
14. Wang SZ, Luo XG, Shen J, Zou JN, Lu YH, Xi T. Knockdown of SMYD3 by RNA interference inhibits cervical carcinoma cell growth and invasion in vitro. BMB Rep. 2008;41(4):294-9
15. Zou JN, Wang SZ, Yang JS, Luo XG, Xie JH, Xi T. Knockdown of SMYD3 by RNA interference down-regulates c-Met expression and inhibits cells migration and invasion induced by HGF. Cancer Lett. 2009;280(1):78-85
16. Liu C, Xu D, Han H, Fan Y, Schain F, Xu Z, Claesson HE, Bjorkholm M, Sjoberg J. Transcriptional regulation of 15-lipoxygenase expression by histone h3 lysine 4 methylation/demethylation. PLoS One. 2012;7(12):e52703
17. Cock-Rada AM, Medjkane S, Janski N, Yousfi N, Perichon M, Chaussepied M, Chluba J, Langsley G, Weitzman JB. SMYD3 promotes cancer invasion by epigenetic upregulation of the metalloproteinase MMP-9. Cancer Res. 2012;72(3):810-20
18. Ferlay J SI, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr, accessed on 17/12/2013
19. DeVita VT, Lawrence TS, Rosenberg SA. Cancer: Principles & Practice of Oncology: Wolters Kluwer/Lippincott Williams & Wilkins; 2008.
20. Vieira FQ, Costa-Pinheiro P, Ramalho-Carvalho J, Pereira A, Menezes FD, Antunes L, Carneiro I, Oliveira J, Henrique R, Jerónimo C. Deregulated expression of selected histone methylases and demethylases in prostate carcinoma. Endocr Relat Cancer. 2014;21(1):51-61
21. Hagedorn M, Javerzat S, Gilges D, Meyre A, de Lafarge B, Eichmann A, Bikfalvi A. Accessing key steps of human tumor progression in vivo by using an avian embryo model. Proc Natl Acad Sci U S A. 2005;102(5):1643-8
22. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-8
23. Liu C, Wang C, Wang K, Liu L, Shen Q, Yan K, Sun X, Chen J, Liu J, Ren H, Liu H, Xu Z, Hu S, Xu D, Fan Y. SMYD3 as an Oncogenic Driver in Prostate Cancer by Stimulation of Androgen Receptor Transcription. J Natl Cancer Inst. 2013;105(22):1719-28

24. Tufan AC, Satiroglu-Tufan NL. The chick embryo chorioallantoic membrane as a model system for the study of tumor angiogenesis, invasion and development of anti-angiogenic agents. Curr Cancer Drug Targets. 2005;5(4):249-66
25. Sims RJ, 3rd, Reinberg D. From chromatin to cancer: a new histone lysine methyltransferase enters the mix. Nat Cell Biol. 2004;6(8):685-7
26. Kim H, Heo K, Kim JH, Kim K, Choi J, An W. Requirement of histone methyltransferase SMYD3 for estrogen receptor-mediated transcription. J Biol Chem. 2009;284(30):19867-77
27. Henrique R, Costa VL, Cerveira N, Carvalho AL, Hoque MO, Ribeiro FR, Oliveira J, Teixeira MR, Sidransky D, Jeronimo C. Hypermethylation of Cyclin D2 is associated with loss of mRNA expression and tumor development in prostate cancer. J Mol Med (Berl). 2006;84(11):911-8
28. Kobayashi T, Nakamura E, Shimizu Y, Terada N, Maeno A, Kobori G, Kamba T, Kamoto T, Ogawa O, Inoue T. Restoration of cyclin D2 has an inhibitory potential on the proliferation of LNCaP cells. Biochem Biophys Res Commun. 2009;387(1):196-201
29. Witt D, Burfeind P, von Hardenberg S, Opitz L, Salinas-Riester G, Bremmer F, Schweyer S, Thelen P, Neesen J, Kaulfuss S. Valproic acid inhibits the proliferation of cancer cells by re-expressing cyclin D2. Carcinogenesis. 2013;34(5):1115-24

SUPPLEMENTARY DATA

Supplementary Table 1 **-** List of primers used in (A) Site-Directed Mutagenesis for deletion of EEL and NHSC domains of *SMYD3*, (B) direct sequencing of *SMYD3* and (C) DNA quantification of several regions of *CCND2* promoter in Chromatin Immunoprecipitation

(A) Site-Directed Mutagenesis	
Primers EEL	F: CGAGACATCGAGGTGGGAACCATCTGCTACCT
	R: AGGTAGCAGATGGTTCCCACCTCGATGTCTCG
Primers NHSC	F: CCAGTATCTCTTTGCTCGACCCCAACTGTTCGAT
	R: ATCGAACAGTTGGGGTCGAGCAAAGAGATACTGG
(B) Direct Sequencing of *SMYD3*	
Primers	F: GCCTCAGGCAACTCGTAATG
	R: GAACAAGGCTTCCTCCAACA
(C) Chromatin Immunoprecipitation for *CCND2*	
Primers A	F: CCAAACTCTTCCTCACCCTTT
	R: CGTACACTAGGTTCCCTGCAA
Primers B	F: GGAAGGGGTGGTGGTGTTT
	R: CCCTGCATCTGCTGACAAG
Primers C	F: GGTTTCTGCTCGAGGATCAC
	R: GTTTCGAAAGCCCCGATTA

F: Forward; R: Reverse

Chapter V

General Discussion

GENERAL DISCUSSION

Prostate cancer (PCa) is a leading cause of cancer-related morbidity and mortality, worldwide. The widespread use of serum PSA for early detection led to a decrease in mortality but at the expense of overtreatment [1-3]. Current clinical and pathological parameters used to predict the threat that a given PCa poses to the patient's life are rather imperfect. Hence, the identification of accurate markers to discriminate PCa patients benefiting from a more aggressive treatment from those that might be spared unnecessary and potentially harmful intervention it is now demanded [4]. The role of epigenetic alterations in tumorigenesis, including histone onco-modifications, has been increasingly recognized [5, 6]. Indeed, deregulated expression and/or altered activity of key chromatin-modifying enzymes, including those that catalyze methylation or demethylation of specific histone residues [histone methyltransferases (HMTs) and histone demethylases (HDMs)], have been reported for several tumors [7]. Moreover, previous studies showed that both aberrant activity of epigenetic modifying enzymes and respective post-translational modifications (PTMs), may serve as markers of prognosis in different cancers, including PCa [7-10]. However, much is yet to be uncovered concerning the influence of altered expression of those two families of enzymes and respective histone marks in the abnormal expression of genes critical to prostate carcinogenesis and disease progression.

In this Thesis, we sought to characterize expression levels of HMTs and HDMs in normal and cancerous prostate tissues. Although, recently, additional enzymes belonging to these families have been identified, when this project was initiated, there were only 37 HMTs and 20 HDMs described in the literature, and these were the focus of our study. Firstly, we evaluated mRNA expression levels using a customized platform, including all the designated genes coding for HMTs and HDMs. We acknowledge two major limitations of this methodology: the reduced number of tested samples and the presence of unique probe/primers per gene that might influence the analysis, because in case a mutation is present in the recognized sequence it might preclude the amplification. Sampling of cases and heterogeneity of tumors might also introduce bias in interpretation of results. Notwithstanding those limitations, we were able to identify novel genes differentially expressed in PCa, in addition to the confirmation of *EZH2* overexpression which was previously reported [11, 12], providing indirect confirmation of the validity of our approach.

Interestingly, several enzymes responsible for establishing the same histone marks display a dissimilar pattern of expression. This finding was not entirely surprising, because although those enzymes catalyze modifications in the same residues, they probably affect different gene promoters and may, thus, have different consequences in

cancer initiation and/or progression. Therefore, alongside with full identification of the deregulated enzymes, it is also imperative to characterize their target genes.

Concerning the enzymes that we found to be deregulated in PCa, *KDM5A*, *PRMT6*, *MLL* family and *SMYD3* were highlighted. Except for PRMT6 that is an arginine methyltransferase, all the remaining enzymes target H3K4: SMYD3 and MLL add methyl residues and KDM5A catalyzes demethylation. Interestingly, SMYD3 was recently shown to methylate other histonic residues such as H4K5 and H4K20, as well as non-histonic proteins [13-15]. Higher expression levels of *KDM5A* were also found in PCa samples and were suggested to be critical for PCa tumorigenesis, owing to its association with relevant genes (e.g., pRB or MYC) implicated in that process [16]. Our results also indicate that *PRMT6* expression levels were able to accurately distinguish normal from neoplastic prostatic tissues, and may thus constitute a putative ancillary tool for diagnosis of PCa. Remarkably, *PRMT6* was also reported to be a negative regulator of H3K4 methylation, catalyzed by MLL family members [17]. Moreover, and concomitantly with an increase in *PRMT6* expression, downregulation of all members of the MLL family was observed, although the mechanism underlying this finding is still elusive.

One of the most interesting findings was SMYD3 overexpression in PCa, especially at advanced disease stages, suggesting a biological association with more aggressive PCa. We further disclosed a potential clinical usefulness for these findings as high *SMYD3* expression levels were able to predict biochemical relapse after radical prostatectomy. Importantly, this prognostic ability was independent of the GS or pathological stage and performed better than *EZH2* for prediction of disease-free survival, an HMT that has been acknowledged as an important driver in prostatic carcinogenesis [11, 12]. Thus, *SMYD3* expression emerged from this study as a promising biomarker for stratification of patients according to the aggressiveness of PCa. Validation studies, using different patient's cohorts, are now required to confirm these data. Furthermore, the translation of these observations to routine practice would be facilitated if SMYD3 protein levels, assessed by immunohistochemistry, provided results similar to those observed at transcript level.

These findings led us to re-direct the course of the Ph.D. project towards a better understanding of the biological role of SMYD3 and the impact of its altered expression in prostate carcinogenesis. To accomplish this, we evaluated SMYD3 impact on the malignant phenotype of PCa cells using *in vitro* and *in vivo* models. Two different strategies were adopted: permanent knockdown of *SMYD3* and deletion of regions critical for the catalytic activity of the SET domain.

Permanent knockdown of *SMYD3* was established in LNCaP and PC3 cells. Phenotypic assays showed that SMYD3 was involved in regulation of cell viability,

apoptosis, migration, invasion and tumor formation, and results were more expressive for the most aggressive tested cell line, *i.e.*, PC3. These results were largely corroborated and complemented by a recent publication [18], in which other assays confirmed the involvement of SMYD3 in the above mentioned pathways and also its role in tumor formation in a mouse model. Taken together, these results support the condition of *SMYD3* as a proto-oncogene in PCa, in similarity to other tumors such as hepatocellular, colorectal, cervical and breast carcinomas [19-23].

To further elucidate how SMYD3 histone methyltransferase activity impacted on PCa cells genome, we assessed global H3K4 and H3K27 methylation levels in the presence or absence of SMYD3 expression. However, no significant differences were disclosed between control cells from those depleted of *SMYD3*. These results might seem paradoxical but it should be reminded that the evaluation of histone marks was made at a global level, at this stage of the study. It is likely that the alterations due to *SMYD3* overexpression might only affect a relatively small subset of specific genes (activating oncogenes or repressing tumor suppressor genes), preventing the identification of significant global alterations. It would be also interesting to evaluate the H4K5 and H4K20 methylation marks, also catalyzed by SMYD3, but, unfortunately, the antibodies required are either unavailable or lacked specificity for Western-blot.

Nevertheless, we also determined if SMYD3 action was mainly due to its methylation function. Thus, transient transfection of normal or mutant SMYD3 (i.e., carrying deletions in the SET domain) into sh-SMYD3 PCa cells was performed. In contrast with other studies [24], a more significant role of EEL domain in the oncogenic properties of SMYD3, in contrast with the NHSC domain, was suggested. Whether redundancy exists concerning both EEL and NHSC sequences is still unknown and it will be investigated through the evaluation of the impact of a double deletion. Although an association of methyltransferase activity with SMYD3 oncogenic properties was disclosed, an involvement of methylation of non-histone proteins can not be excluded.

As mentioned above, the identification of the genes targeted by SMYD3 activity is critical to understand how its altered expression affects the biology of PCa. Intriguingly, the pattern of gene expression deregulation after *SMYD3* silencing in LNCaP and PC3 cells was substantially different, suggesting that the oncogenic properties of SMYD3 depend on the cellular context. Notwithstanding, following SMYD3 knockdown, both cell lines displayed *CCND2* overexpression, which suggests that this gene is a relevant target of SMYD3. Remarkably, although H3K4me3 levels remained unaltered, we found high levels of H4K20me3 (a repressive mark also catalyzed by SMYD3) within *CCND2* promoter region in PCa cells overexpressing *SMYD3*. In addition to the involvement of aberrant promoter methylation in CCND2 silencing [25], these data also suggest that

SMYD3 might also be involved in *CCND2* silencing in prostate carcinogenesis, unraveling a new layer of complexity on gene expression deregulation in PCa.

REFERENCES

1. Ferlay J SI, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr, accessed on 17/12/2013
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61(2):69-90
3. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012;62(1):10-29
4. DeVita VT, Lawrence TS, Rosenberg SA. Cancer: Principles & Practice of Oncology: Wolters Kluwer/Lippincott Williams & Wilkins; 2008.
5. Tian X, Zhang S, Liu HM, Zhang YB, Blair CA, Mercola D, Sassone-Corsi P, Zi X. Histone lysine-specific methyltransferases and demethylases in carcinogenesis: new targets for cancer therapy and prevention. Curr Cancer Drug Targets. 2013;13(5):558-79
6. Chi P, Allis CD, Wang GG. Covalent histone modifications--miswritten, misinterpreted and mis-erased in human cancers. Nat Rev Cancer. 2010;10(7):457-69
7. Miremadi A, Oestergaard MZ, Pharoah PD, Caldas C. Cancer genetics of epigenetic genes. Hum Mol Genet. 2007;16 Spec No 1:R28-49
8. Bianco-Miotto T, Chiam K, Buchanan G, Jindal S, Day TK, Thomas M, Pickering MA, O'Loughlin MA, Ryan NK, Raymond WA, Horvath LG, Kench JG, Stricker PD, Marshall VR, Sutherland RL, Henshall SM, Gerald WL, Scher HI, Risbridger GP, Clements JA, Butler LM, Tilley WD, Horsfall DJ, Ricciardelli C. Global levels of specific histone modifications and an epigenetic gene signature predict prostate cancer progression and development. Cancer Epidemiol Biomarkers Prev. 2010;19(10):2611-22
9. Ke XS, Qu Y, Rostad K, Li WC, Lin B, Halvorsen OJ, Haukaas SA, Jonassen I, Petersen K, Goldfinger N, Rotter V, Akslen LA, Oyan AM, Kalland KH. Genome-wide profiling of histone h3 lysine 4 and lysine 27 trimethylation reveals an epigenetic signature in prostate carcinogenesis. PLoS One. 2009;4(3):e4687
10. Seligson DB, Horvath S, Shi T, Yu H, Tze S, Grunstein M, Kurdistani SK. Global histone modification patterns predict risk of prostate cancer recurrence. Nature. 2005;435(7046):1262-6

11. Karanikolas BD, Figueiredo ML, Wu L. Comprehensive evaluation of the role of EZH2 in the growth, invasion, and aggression of a panel of prostate cancer cell lines. Prostate. 2010;70(6):675-88
12. Laitinen S, Martikainen PM, Tolonen T, Isola J, Tammela TL, Visakorpi T. EZH2, Ki-67 and MCM7 are prognostic markers in prostatectomy treated patients. Int J Cancer. 2008;122(3):595-602
13. Foreman KW, Brown M, Park F, Emtage S, Harriss J, Das C, Zhu L, Crew A, Arnold L, Shaaban S, Tucker P. Structural and functional profiling of the human histone methyltransferase SMYD3. PLoS One. 2011;6(7):e22290
14. Van Aller GS, Reynoird N, Barbash O, Huddleston M, Liu S, Zmoos AF, McDevitt P, Sinnamon R, Le B, Mas G, Annan R, Sage J, Garcia BA, Tummino PJ, Gozani O, Kruger RG. Smyd3 regulates cancer cell phenotypes and catalyzes histone H4 lysine 5 methylation. Epigenetics. 2012;7(4):340-3
15. Kunizaki M, Hamamoto R, Silva FP, Yamaguchi K, Nagayasu T, Shibuya M, Nakamura Y, Furukawa Y. The lysine 831 of vascular endothelial growth factor receptor 1 is a novel target of methylation by SMYD3. Cancer Res. 2007;67(22):10759-65
16. Rotili D, Mai A. Targeting Histone Demethylases A New Avenue for the Fight against Cancer. Genes & Cancer. 2011;2(6):663-79
17. Guccione E, Bassi C, Casadio F, Martinato F, Cesaroni M, Schuchlautz H, Luscher B, Amati B. Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive. Nature. 2007;449(7164):933-7
18. Liu C, Wang C, Wang K, Liu L, Shen Q, Yan K, Sun X, Chen J, Liu J, Ren H, Liu H, Xu Z, Hu S, Xu D, Fan Y. SMYD3 as an Oncogenic Driver in Prostate Cancer by Stimulation of Androgen Receptor Transcription. J Natl Cancer Inst. 2013;105(22):1719-28
19. Hamamoto R, Furukawa Y, Morita M, Iimura Y, Silva FP, Li M, Yagyu R, Nakamura Y. SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells. Nat Cell Biol. 2004;6(8):731-40
20. Hamamoto R, Silva FP, Tsuge M, Nishidate T, Katagiri T, Nakamura Y, Furukawa Y. Enhanced SMYD3 expression is essential for the growth of breast cancer cells. Cancer Sci. 2006;97(2):113-8
21. Chen LB, Xu JY, Yang Z, Wang GB. Silencing SMYD3 in hepatoma demethylates RIZI promoter induces apoptosis and inhibits cell proliferation and migration. World J Gastroenterol. 2007;13(43):5718-24

22. Luo XG, Zou JN, Wang SZ, Zhang TC, Xi T. Novobiocin decreases SMYD3 expression and inhibits the migration of MDA-MB-231 human breast cancer cells. IUBMB Life. 2010;62(3):194-9
23. Wang SZ, Luo XG, Shen J, Zou JN, Lu YH, Xi T. Knockdown of SMYD3 by RNA interference inhibits cervical carcinoma cell growth and invasion in vitro. BMB Rep. 2008;41(4):294-9
24. Kim H, Heo K, Kim JH, Kim K, Choi J, An W. Requirement of histone methyltransferase SMYD3 for estrogen receptor-mediated transcription. J Biol Chem. 2009;284(30):19867-77
25. Henrique R, Costa VL, Cerveira N, Carvalho AL, Hoque MO, Ribeiro FR, Oliveira J, Teixeira MR, Sidransky D, Jeronimo C. Hypermethylation of Cyclin D2 is associated with loss of mRNA expression and tumor development in prostate cancer. J Mol Med (Berl). 2006;84(11):911-8

Chapter VI

Conclusions and Future Perspectives

CONCLUSIONS AND FUTURE PERSPECTIVES

In this study we were able to identify several HMTs and HDMs implicated in prostate carcinogenesis, namely KDM5A, PRMT6, MLL family and SMYD3. PRMT6 might constitute a good biomarker to assist in PCa diagnosis, whereas SMYD3 expression levels associated with tumor aggressiveness and predicted disease-specific survival in clinically localized PCa patients submitted to radical prostatectomy.

The results from *in vitro* and *in vivo* assays sustain an oncogenic role for SMYD3 in prostate carcinogenesis. Additionally, we demonstrated that its oncogenic properties are dependent on the histone methyltransferase activity and that *CCND2* is targeted by SMYD3, causing its repression through H4K20 methylation. The characterization of SMYD3 role in prostate carcinogenesis, mediated by its catalytic methyltransferase activity, indicates that this histone modifier might be an attractive target for personalized therapy of PCa patients.

Several questions remain unanswered, however, and further studies are planned to complement the results presented herein. We plan to assess *SMYD3* expression levels in prostate biopsies to very whether its expression levels convey prognostic information in a pre-therapeutic setting. Additionally, continued follow-up of the series of PCa patients submitted to radical prostatectomy is mandatory to determine if *SMYD3* expression is predictive of cancer-specific mortality.

Furthermore, the identification of SMYD3 target genes is a requirement to fully understand its role in prostate tumorigenesis. Chromatin immunoprecipitation sequencing (ChIP-Seq) will allow for the identification and characterization of putative target genes whose expression is regulated through the histone marks established by SMYD3.

Recently, several other enzymes involved in histone methylation or demethylation were identified and, thus, it would be interesting to complete our study through the evaluation of its expression in normal and cancerous prostate tissues. Considering the interesting results observed for KDM5A and PRMT6 and the available data from other neoplasms, we intend to explore their role in PCa, using a similar approach to that used for SMYD3. Additionally, it would be interesting to characterize the upstream mechanism(s) responsible for global downregulation of the MLL family and clarify their possible association with PRMT6 deregulation.

Appendix

Deregulated expression of selected histone methylases and demethylases in prostate carcinoma

Endocrine-Related Cancer. 2014;21(1):51-61

Research | F Q Vieira et al. | Deregulated expression of HMTs and HDMs in PCa | 21:1 | 51–61

Deregulated expression of selected histone methylases and demethylases in prostate carcinoma

Filipa Quintela Vieira[1,2], Pedro Costa-Pinheiro[1], João Ramalho-Carvalho[1], Andreia Pereira[1], Francisco Duarte Menezes[3], Luís Antunes[4], Isa Carneiro[1,3], Jorge Oliveira[5], Rui Henrique[1,3,6,*] and Carmen Jerónimo[1,6,*]

[1]Cancer Epigenetics Group, Portuguese Oncology Institute, Research Center, Rua Dr António Bernardino Almeida, 4200-072 Porto, Portugal
[2]School of Allied Health Sciences (ESTSP), Polytechnic of Porto, Portugal
Departments of [3]Pathology [4]Epidemiology [5]Urology, Portuguese Oncology Institute, Porto, Portugal
[6]Department of Pathology and Molecular Immunology, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto, Portugal
*(R Henrique and C Jerónimo are joint senior authors)

Correspondence should be addressed to C Jerónimo
Email
carmenjeronimo@ipoporto.min-saude.pt or cljeronimo@icbas.up.pt

Abstract

Prostate cancer (PCa), a leading cause of cancer-related morbidity and mortality, arises through the acquisition of genetic and epigenetic alterations. Deregulation of histone methyltransferases (HMTs) or demethylases (HDMs) has been associated with PCa development and progression. However, the precise influence of altered HMTs or HDMs expression and respective histone marks in PCa onset and progression remains largely unknown. To clarify the role of HMTs and HDMs in prostate carcinogenesis, expression levels of 37 HMTs and 20 HDMs were assessed in normal prostate and PCa tissue samples by RT-qPCR. *SMYD3*, *SUV39H2*, *PRMT6*, *KDM5A*, and *KDM6A* were upregulated, whereas *KMT2A-E (MLL1-5)* and *KDM4B* were downregulated in PCa, compared with normal prostate tissues. Remarkably, *PRMT6* was the histone modifier that best discriminated normal from tumorous tissue samples. Interestingly, *EZH2* and *SMYD3* expression levels significantly correlated with less differentiated and more aggressive tumors. Remarkably, *SMYD3* expression levels were of independent prognostic value for the prediction of disease-specific survival of PCa patients with clinically localized disease submitted to radical prostatectomy. We concluded that expression profiling of HMTs and HDMs, especially *SMYD3*, might be of clinical usefulness for the assessment of PCa patients and assist in pre-therapeutic decision-making.

Key Words
- prostate
- neoplasia
- molecular biology
- biomarker
- microarray

Endocrine-Related Cancer (2014) **21**, 51–61

Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer in men from developed countries and a leading cause of cancer-related morbidity and mortality worldwide (Jemal *et al.* 2011, Siegel *et al.* 2012). At its earliest stages, PCa is frequently asymptomatic, fostering the use of biomarkers, such as serum prostate-specific antigen (PSA), for screening and identification of asymptomatic low-stage tumors, followed by prostate biopsy for diagnosis confirmation (Stamey 1995). However, prostate biopsy meets with several limitations, including sampling error as well as intra- and interobserver variability in Gleason grading (King & Long 2000, Allsbrook *et al.* 2001), which even in conjunction with

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other prognostic factors used for therapeutic decision (e.g., clinical stage and pre-therapeutic serum PSA levels) are rather imperfect in predicting disease progression (Lapointe *et al.* 2004, Duffy 2011). Consequently, there is a significant degree of uncertainty concerning the threat that a PCa poses to an individual patient, entailing overtreatment (Moyer 2012).

The role of epigenetic modifications in cancer initiation and progression has been emphasized (Hirst & Marra 2009). In addition to aberrant DNA methylation, alterations in chromatin modification patterns, due to histones post-translational modifications (PTMs), were implicated in carcinogenesis and have emerged as potential key regulators of cancer-related pathways (Miremadi *et al.* 2007). Importantly, PTMs may be changed in cancer cells due to altered expression or activity of key chromatin-modifying enzymes (Miremadi *et al.* 2007).

Histone methylation, carried out by histone methyltransferases (HMTs), requires different families of enzymes depending on the residue (lysine HMTs (KMT) methylate lysine residues, whereas protein arginine methyltransferase (PRMT) methylate arginines) and might positively or negatively regulate gene transcription. Although lysine residues might be modified into mono-, di-, or trimethyl states, arginine can only be modified to mono- or dimethyl states (symmetric or asymmetrically) (Brame *et al.* 2004). Different degrees of methylation may be, thus, associated with distinct chromatin regions or transcriptional states (e.g., trimethylation of lysine 9 of histone H3 is associated with pericentromeric heterochromatin and transcriptional repression, whereas its dimethylation is linked to repressed genes in euchromatin (Lee *et al.* 2006)). Recently, the reversibility of histone methylation has been established through the discovery of histone lysine and arginine demethylases (HDMs), uncovering a new level of histone plasticity (Shi *et al.* 2004, Chang *et al.* 2007).

Altered HMTs expression levels have been found in PCa, most notably enhancer of zeste homolog 2 (EZH2), a lysine methyltransferase, which is increased in metastatic PCa, marking aggressive disease (Seligson *et al.* 2005, Karanikolas *et al.* 2010). Specific relationships between histone marks and tumor grade or recurrence (particularly methylation of H3K4 and H3K27) have been reported (Seligson *et al.* 2009, Bianco-Miotto *et al.* 2010) and deregulation of some lysine HMTs – KMT2B, KMT2C, NSD1, EZH2 or SMYD3 – in PCa tissues has been also demonstrated (Ke *et al.* 2009, Bianco-Miotto *et al.* 2010). However, the validity of most studies is limited due to inappropriate tissue sampling and/or to the reduced number of samples tested.

Because deregulation of HMTs and HDMs affects post-translational control of cellular proteins involved in cancer-relevant signaling networks, a better understanding of their function might lead to the identification of more accurate markers that might be useful to discriminate patients benefiting from a more aggressive treatment from those that might be spared unnecessary and potentially harmful interventions. Therefore, we sought to identify HMTs and HDMs displaying altered expression levels, in a relatively large series of PCa patients submitted to radical prostatectomy, and further test their clinical usefulness for the prediction of disease progression.

Materials and methods

Patients and tissue collection

Primary tumors from 160 patients with clinically localized prostate adenocarcinoma, consecutively diagnosed, and primarily treated with radical prostatectomy at the Portuguese Oncology Institute, Porto, Portugal, were prospectively collected. For control purposes, non-neoplastic prostate tissue samples were obtained from the peripheral zone of 15 prostates not harboring PCa collected from cystoprostatectomy specimens of patients with bladder cancer (normal prostate tissue (NPT)). All tissue specimens were promptly frozen immediately after surgery, following informed consent. Five-micron thick sections were cut and stained for the identification of the areas of PCa (i.e., the index or dominant tumor) and normal tissue. Then, the tissue block was trimmed to maximize the yield of target cells (>70% of target cells). Subsequently, an average of fifty 12-μm thick sections was cut and every fifth section was stained to ensure a uniform percentage of target cells and to exclude contamination from neoplastic cells in normal tissue samples. Histological slides from formalin-fixed, paraffin embedded tissue fragments were routinely obtained from the same surgical specimens and assessed for Gleason score (GS; Gleason & Mellinger 1974) and TNM stage (Hermanek *et al.* 1997). Relevant clinical data were collected from the clinical records. These studies were approved by the institutional review board (Comissão de Ética para a Saúde-(IRB-CES-IPOFG-EPE 019/08)) of Portuguese Oncology Institute, Porto, Portugal.

RNA isolation

All tissue samples were suspended in TRIzol reagent (Invitrogen) and, after addition of chloroform to the lysed cells, total RNA was purified from the aqueous phase of TRIzol extract using the PureLink RNA Mini Kit (Invitrogen)

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following manufacturer recommendations. The concentration, purity, and integrity of RNA samples were determined on a Nanodrop ND-1000 (ThermoScientific, Wilmington, DE, USA) and agarose-gel electrophoresis.

Screening of HMTs and HDMs

Five NPTs and ten independent PCa samples were chosen to encompass the full spectrum of prostate carcinomas in this series considering the GS and pathological stage (Table 1). After treatment with DNase Turbo DNA-free (Ambion, Austin, TX, USA), a total of 1 μg total RNA was reverse transcribed using the High Capacity cDNA RT kit (Applied Biosystems) according to the manufacturer's instructions. TaqMan Array 96-Well Plates were designed in order to evaluate expression levels of 37 HMTs and 20 HDMs. RT-qPCR protocol was performed on an ABI- 7500 Real-Time PCR system (Applied Biosystems) according to manufacturer's instructions and each sample was run in triplicate.

The amount of mRNAs of the genes studied was normalized to that of the GUSB reference gene and the median value of NPTs and PCa samples was chosen to calculate fold-difference in gene expression between groups, using the comparative *C*t method. Genes with a logarithmized fold change above 0.5 or below −0.5 were further considered. The expression of *KDM6A* was also included because it has been previously reported as deregulated in several tumor models (van Haaften *et al.* 2009), and analysis was extend to all members of the KMT2 family.

Validation of selected enzymes

After gene selection, mRNA levels were confirmed in a large and independent group of 150 PCa tissues and 15 NPTs. A total of 300 ng was reverse transcribed and amplified using TransPlex Whole Transcriptome Amplification Kit (Sigma–Aldrich) with subsequent purification using QIAquick PCR Purification Kit (Qiagen), according to manufacturer's instructions. HMTs or HDMs mRNA levels were evaluated using TaqMan Gene Expression Assays (Applied Biosystems, Supplementary Table 1, see section on supplementary data given at the end of this article) and the most suitable endogenous control assays for the analysis of prostate tissues (de Kok *et al.* 2005), GUSB, and TFRC were also analyzed. To determine the relative expression levels in each sample, the values of the target gene were normalized using the median of the two internal reference genes to obtain a ratio (HMT or HDM/Mean of *TFRC* and *GUSB*). Each plate included multiple non-template controls and serial dilutions of a cDNA from human prostate RNA (Ambion, Invitrogen) to construct a standard curve for each plate. All experiments were run in triplicate.

Statistical analysis

For statistical analysis purposes, PCa samples were divided into two- or three-grade categories for GS (GS $\leq$6 and GS $\geq$7) and pathological stage (pT2, pT3a, and pT3b) respectively. The Shapiro–Wilk's *W*-test allowed for the examination of the appropriateness of a normal distribution assumption for each of the parameters (data not shown). Then, the median and range of the mRNA expression levels for each group of samples were determined and analyzed using Mann–Whitney *U* test. A receiver operator characteristics (ROC) curve was constructed by plotting the true positive rate (sensitivity) against the false positive rate (1-specificity) and the area

Table 1 Clinical and pathological features of patients included in the testing set and in the validation series

	PCa	Normal	PCa	Normal
Number of patients, *n*	10	5	150	15
Age (years), median (range)	59 (53–71)	61 (49–66)	64 (49–75)	64 (45–80)
PSA levels (ng/ml), median (range)	12.3 (3.5–19.9)	NA	8.2 (2.9–23.0)	NA
Pathological stage, *n* (%)		NA		NA
pT2	4 (40.0)		89 (59.3)	
pT3a	2 (20.0)		50 (33.3)	
pT3b	4 (40.0)		11 (7.3)	
Gleason score, *n* (%)		NA		NA
<7	3 (30.0)		57 (38.7)	
≥7	7 (70.0)		93 (62.0)	

PCa, prostate cancer; NA, not applicable.

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under the curve (AUC) was calculated to assess diagnostic performance. Possible correlations between the expression levels and GS or pathological stage were assessed by the Kruskal–Wallis one-way ANOVA, followed by Mann–Whitney *U* test when appropriate. For multiple comparisons, the Bonferroni method was used to adjust *P* values. Spearman nonparametric correlation tests were additionally carried out to ascertain correlations between age, PSA levels, and HMTs or HDMs expression levels. The prognostic significance of available clinical variables (pathological stage, GS, age, and serum PSA levels) was assessed by constructing disease-specific and disease-free survival (DFS) curves using the Kaplan–Meier method with log-rank test (univariate test). A Cox-regression model comprising the four variables (multivariate test) was also constructed. DFS was calculated from the date of the radical prostatectomy to the date of biochemical relapse, or date of last follow-up, or death if relapse-free. For the purposes of survival analyses, all cases were coded based on the expression levels of each enzyme using the percentile 75 value as empirical threshold. Cases were also subdivided according to serum PSA levels (below and above median values) and age (above 60, between 60 and 70, and above 70). Statistical analysis was performed using SPSS for Windows, version 20.0 (SPSS) and the level of significance was set to $P<0.05$. Graphs were built using GraphPad Prism 5.0 software for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Evaluation of HMTs and HDMs expression levels

Expression levels of 37 HMTs and 20 HDMs were assessed in ten PCa and five normal prostate samples (relevant clinical and pathological data are depicted in Table 1). Most enzymes were downregulated in PCa compared with NPT (Fig. 1). Based on fold-variation, lysine HMTs *SUV39H2*, *SMYD3*, *KMT2A-E*, and *EZH2* (the latter used as positive control according to the literature); argine HMT *PRMT6*; and HDMs *KDM4B*, *KDM6A*, *KDM5A*, and *KDM3B* were selected for validation (Supplementary Table 2, see section on supplementary data given at the end of this article). This was performed using RT-qPCR in a larger and independent series comprising 150 PCa samples and 15 NPTs (relevant clinical and histopathological data are displayed in Table 1). Statistically significant differences between NPT and PCa tissue samples were found for all candidate genes, except *KDM3B* (Table 2). As expected, higher *EZH2* expression levels were observed in PCa compared with NPT and the same trend was verified for *SMYD3*, *SUV39H2*, *PRMT6*, *KDM5A*, and *KDM6A* (Fig. 2 and Table 2). Contrarily, all selected members of KMT2 family and *KDM4B* were downregulated in PCa samples compared with NPT (Fig. 3 and Table 2). Interestingly, significant positive correlations between several members of KMT2 family were found in PCa samples (Table 3). To reinforce the oncogenic role of altered enzyme expression, transcript levels (categorized according to percentile 75) were tested as PCa biomarkers in tissue samples. Remarkably, *PRMT6* performed best in sensitivity (90.0%) and specificity (73.3%) for discriminating PCa from NPT, and ROC curve analysis showed an AUC of 0.923 (95% CI 0.870–0.977, $P<0.001$) (Fig. 4). No significant differences in age between PCa patients and normal tissue donors were apparent. Statistically significant associations between expression levels of *SMYD3* ($P=0.044$) or *KMT2A* ($P=0.041$) and pathological stage were disclosed (higher levels in pT3b cases for both genes, Fig. 5A and B). When the patient cohort was stratified according to GS, increased levels of *EZH2* ($P=0.048$) and *KMT2C* ($P=0.018$) were associated with less differentiated tumors (Fig. 5C and D). No statistically significant associations were found between gene expression levels and patients' age or PSA levels.

Survival analysis

The median follow-up period of this series of PCa patients was 105 months (range: 3–145 months). At the time of the last follow-up, five patients (3.3%) had died from PCa and 45 of 136 (33%) presented biochemical recurrence. In 14 patients, serum PSA levels >0.2 ng/ml persisted following surgery and these were not further considered for DFS analysis. Disease-specific survival curves using established clinical variables or expression levels of selected genes did not display prognostic value within the available follow-up time. However, DFS analysis showed that tumors with higher transcript levels of *EZH2* ($P=0.001$) or *SMYD3* ($P=0.010$) were significantly associated with a shorter time to relapse, in a univariate analysis (Fig. 6). Higher GS ($P<0.001$), advanced pathological stage (pT3a $P=0.016$ and pT3b $P=0.002$), and higher PSA levels ($P=0.029$) were also associated with shorter DFS, whereas age was not of prognostic value within the available follow-up time. In multivariate analysis, higher GS, stage pT3b, and high *SMYD3* expression levels retained statistical significance ($P=0.001$, $P=0.027$, and $P=0.025$ respectively) and were capable of predicting prognosis independently, whereas *EZH2* expression, PSA, and

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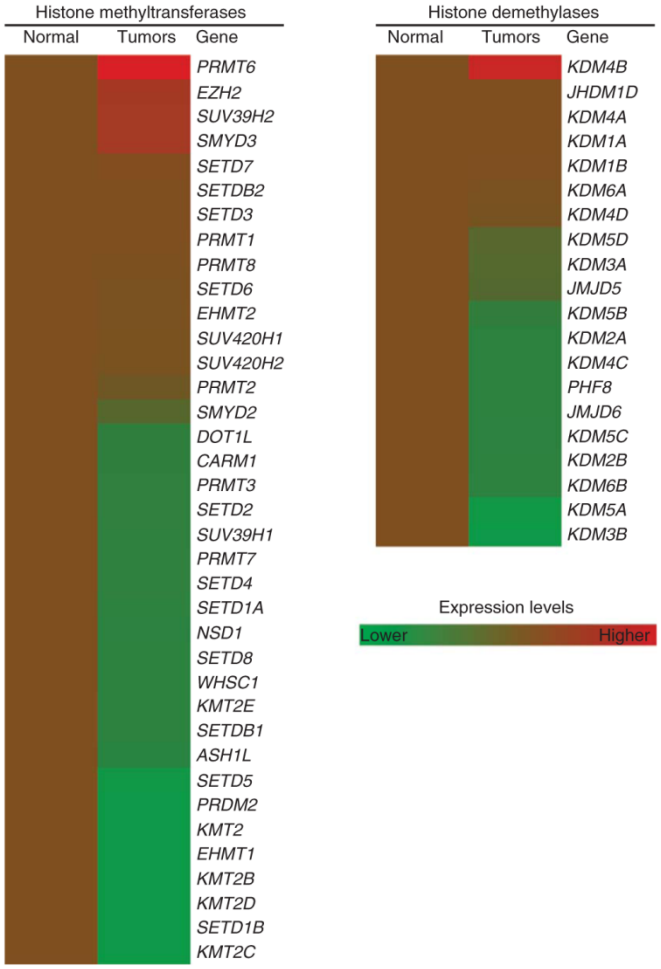

Figure 1
Expression levels of 37 HMTs and 20 HDMs in normal and PCa tissues. Gene expression of five normal prostate tissues and ten PCa calculated using comparative Ct method. The results presented correspond to median value of each group.

pathological stage pT3a did not show independent prognostic value, in this dataset (Table 4).

Discussion

Deregulation of histone PTM patterns has been associated with PCa development and progression (Seligson *et al.* 2005, Ke *et al.* 2009, Bianco-Miotto *et al.* 2010). Because these modifications might be due to altered expression or activity of key chromatin-modifying enzymes (Miremadi *et al.* 2007), we attempted to globally characterize alterations in expression affecting HMTs and HDMs in PCa tissues and determine whether those might be of clinical and pathological relevance.

Overall, 37 HMTs and 20 HDMs expression levels were assessed, comprising most of the relevant members of each class. Owing to the relatively large number of genes tested, this panel was initially tested in a small series of tissue samples. This might underestimate the frequency and magnitude of changes in gene expression, but it allows for

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Table 2 Distribution of selected HMTs and HDMs expression levels in normal and PCa tissue samples

Gene	Normal	Tumor	*P* value, M–W	AUC
EZH2	0.77 (0.39–1.82)	1.15 (0.09–4.85)	**0.014**	**0.692**
KMT2A	0.69 (0.21–2.52)	0.29 (0.08–1.30)	**<0.001**	**0.212**
KMT2B	3.23 (1.61–6.76)	2.05 (0.49–8.07)	**0.004**	**0.272**
KMT2C	2.37 (1.24–4.40)	1.45 (0.39–4.06)	**<0.001**	**0.232**
KMT2D	3.91 (1.36–10.21)	1.97 (0.36–8.66)	**<0.001**	**0.272**
KMT2E	0.73 (0.58–3.46)	0.53 (0.21–1.55)	**<0.001**	**0.162**
PRMT6	0.16 (0.02–0.30)	0.43 (0.10–1.77)	**<0.001**	**0.923**
SMYD3	0.90 (0.53–1.44)	1.53 (0.53–4.50)	**<0.001**	**0.855**
SUV39H2	1.07 (0.63–2.48)	1.36 (0.32–3.43)	**0.044**	**0.657**
KDM3B	0.26 (0.03–0.45)	0.24 (0.11–0.78)	NS	**0.495**
KDM4B	2.24 (0.47–7.81)	0.47 (0.08–2.23)	**<0.001**	**0.098**
KDM5A	0.21 (0.10–0.63)	0.32 (0.07–0.77)	**0.026**	**0.675**
KDM6A	0.33 (0.03–0.52)	0.47 (0.20–1.58)	**<0.001**	**0.813**

HMT, histone methyltransferase; HDM, histone demethylase; PCa, prostate cancer; M–W, Mann–Whitney *U* test; AUC, area under the curve; NS, not significant.

the selection of the most significantly altered. Thus, to confirm the initial findings in the arrays, a validation study was performed for the selected genes using RT-qPCR and only three out of 12 genes were not confirmed. Importantly, genes that were previously reported to be overexpressed in PCa, such as *EZH2* (Karanikolas *et al.* 2010), surfaced in the array and were confirmed in the large series of PCa, thus validating our initial approach. Furthermore, *EZH2* expression was significantly increased in high GS cases, not associating with pathological stage, confirming previous observations (Laitinen *et al.* 2008).

Interestingly, some of the most significantly altered genes encode for enzymes that display antagonistic functions. Although this might result in a balance between repressive and active PTMs, it must be recalled that the effect in gene expression will depend on the specific genomic locations and how tumor suppressor genes or oncogenes are differentially affected (Hake *et al.* 2004,

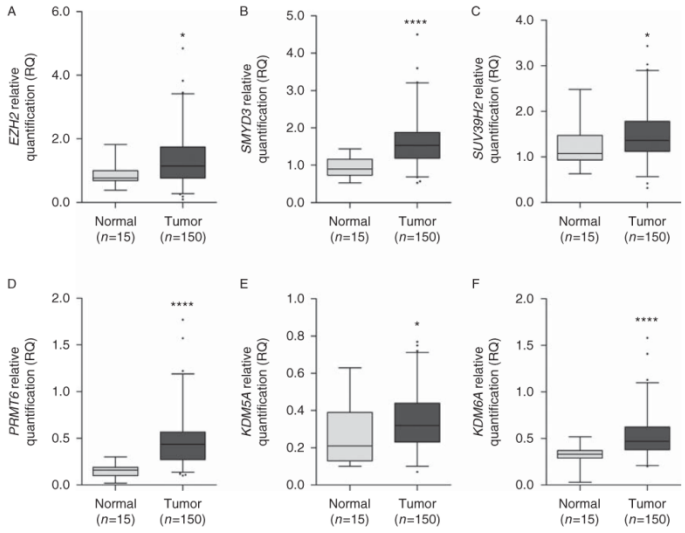

Figure 2
Identification of HMTs and HDMs overexpressed in PCa. Relative quantification of *EZH2* (A), *SMYD3* (B), *SUV39H2* (C), *PRMT6* (D), *KDM5A* (E), and *KDM6A* (F), displaying higher expression levels in PCa compared with normal prostate tissues (****$P<0.0001$; *$P<0.05$).

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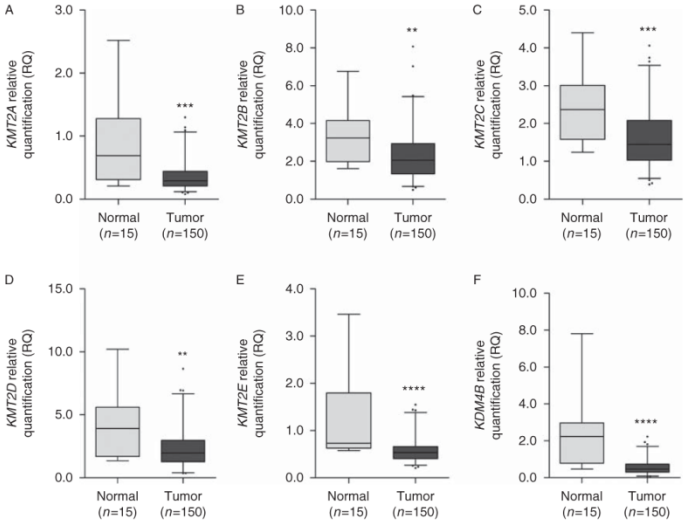

Figure 3
Identification of HMTs and HDMs downregulated in PCa. Relative quantification of *KMT2A* (A), *KMT2B* (B), *KMT2C* (C), *KMT2D* (D), *KMT2E* (E), and *KDM4B* (F) depicted lower levels in PCa compared with normal prostate tissues (****$P<0.0001$; ***$P<0.001$; **$P<0.01$).

Chi *et al.* 2010). Contrarily, concerning the enzymes that have overlapping functions, the same trend was not apparent. This is most likely due to function redundancy, so that oncogenesis is already facilitated through the alteration of a single enzyme responsible for a specific PTM (Fullgrabe *et al.* 2011).

We found that H3K4 methyltransferase *SMYD3* was upregulated in PCa, paralleling previous observations in colorectal, hepatocellular, and breast carcinomas (Hamamoto *et al.* 2004, 2006), whereas KMT2 family members (which accomplish the same PTM) were downregulated. Remarkably, higher SMYD3 transcript levels were associated with locally advanced disease, suggesting an association with more aggressive PCa. Interestingly, SMYD3 overexpression has been linked with enhanced proliferation and loss of differentiation (Hamamoto *et al.* 2006, Chen *et al.* 2007, Wang *et al.* 2008, Zou *et al.* 2009, Ren *et al.* 2011) and this may support the association found in PCa. Moreover, SMYD3 also methylates H4K5 and H4K20 and other non-histone proteins, which may also contribute to its oncogenic role (Foreman *et al.* 2011, Van Aller *et al.* 2012). We found that *KDM5A*, encoding an H3K4 demethylase, was also overexpressed in our PCa series. Remarkably, KDM5A has an antagonistic interaction with pRB, and it is also associated with *MYC* (Rotili & Mai 2011), a proto-oncogene which is upregulated in PCa. This putative oncogenic activity, already demonstrated in gastric cancer (Blair *et al.* 2011), is also supported by our findings.

On the other hand, nearly all members of the KMT2 family were globally downregulated in PCa. This family also targets H3K4, but its downregulation might not impact in H3K4me3 levels owing to SMYD3 overexpression. KMT2A and KMT2C displayed higher expression levels in PCa cases with higher GS and more advanced stage, although levels remained lower than those of NPTs. KMT2s operate in complexes (Ansari & Mandal 2010), a feature that may explain the observed correlation between some members of this family. Because not all KMT2 genes are present in the same complexes, a downstream mechanism responsible for their global downregulation in prostate carcinogenesis likely exists. Furthermore,

Table 3 Coefficient of correlation (*r*) between the expression levels of all members of KMT2 family in PCa tissue samples

	KMT2A	*KMT2B*	*KMT2C*	*KMT2D*	*KMT2E*
KMT2A	–	0.773	0.814	0.600	0.560
KMT2B	–	–	0.844	0.729	0.468
KMT2C	–	–	–	0.650	0.464
KMT2D	–	–	–	–	0.458
KMT2E	–	–	–	–	–

PCa, prostate cancer; Spearman correlation; $P<0.001$ for all comparisons.

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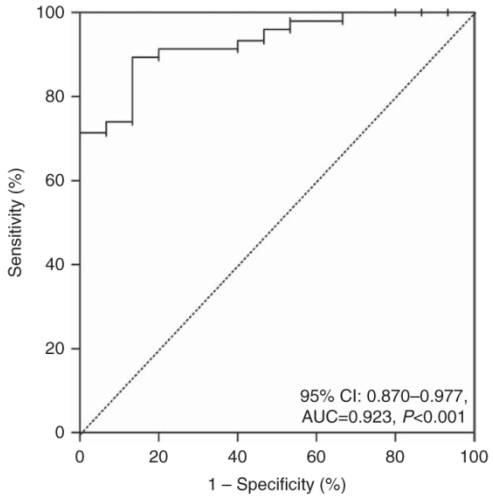

Figure 4
Performance of *PRMT6* expression as biomarker for PCa. Receiver operator characteristic (ROC) curve evaluating the ability of *PRMT6* expression levels in discriminating PCa from normal prostate tissues. AUC, area under the curve.

a negative crosstalk between methylation of H3R2 by PRMT6 and H3K4 by KMT2 complex was described (Guccione *et al.* 2007) and increased expression of PRMT6 was identified in our set of PCa. The overexpression of this histone modifier, already reported in bladder and lung cancer, might lead to a decrease in p53 levels, fostering tumorigenesis (Yoshimatsu *et al.* 2011, Neault *et al.* 2012). Interestingly, PRMT6 proved to be the HMT that best discriminated PCa from NPTs, further supporting a role for its deregulation in prostate carcinogenesis.

SUV39H2 and KDM4B methylate and demethylate H3K9, respectively. *SUV39H2*'s role in cancer depends on the model: in B-cell lymphomas it acts as a tumor suppressor (Cloos *et al.* 2008), whereas in breast cancer it is regarded as an oncogene (Franci *et al.* 2013), as our results suggest for PCa. A positive correlation between KDM4B expression and increased PCa grade has been reported (Coffey *et al.* 2013), but we were not able to confirm it, probably due to methodological differences. Coffey *et al.* used samples of benign prostate hyperplasia as controls and this lesion is reported to be potentially linked with PCa arising in the transition zone (Guess 2001). Moreover, their analysis was based on a qualitative evaluation of cytoplasmic immunostaining in a small portion of tissue (Coffey *et al.* 2013). On the contrary,

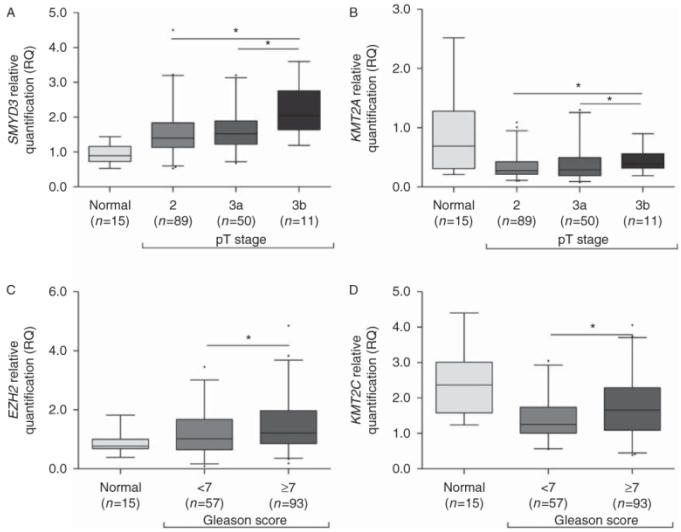

Figure 5
Association of HMTs with clinicopathological parameters. Distribution of *SMYD3* (A) and *KMT2A* (B) expression levels according to pathological stage, showing higher levels in locally advanced disease stage pT3b (*$P<0.05$). Distribution of *EZH2* (C) and *KMT2C* (D) expression levels according to Gleason score, displaying higher levels in tumors with Gleason score ≥7 (*$P<0.05$).

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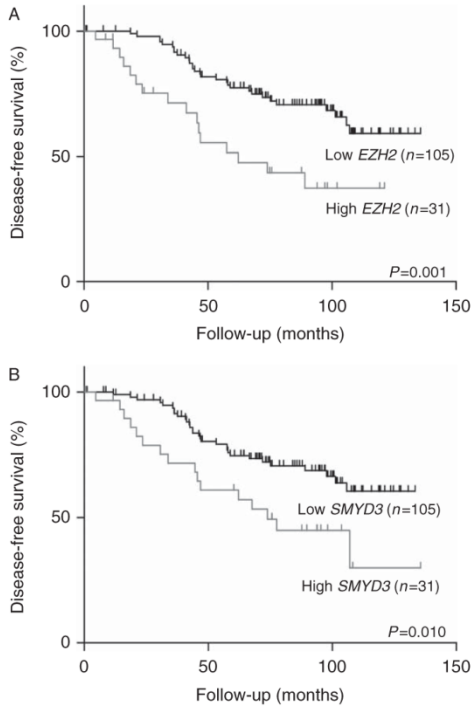

Figure 6
Kaplan–Meier estimated disease-free survival curves for PCa patients. Disease-free survival curves of 136 PCa patients according to expression levels of *EZH2* (A) and *SMYD3* (B). The results of RT-qPCR presented were categorized using third quartile (75th percentile) value as the cutoff.

we used morphologically NPT from the peripheral zone, were over 80% of PCa originate, and expression was quantitatively assessed at transcript level.

A major goal of our study was to determine the potential clinical usefulness of altered HMTs and HDMs expression in PCa. Only EZH2 and SMYD3 disclosed a significant association with DFS, in univariate analysis. Similar results have been reported for EZH2 expression, although assessed by immunohistochemistry (Varambally *et al.* 2002, Laitinen *et al.* 2008, Wolters *et al.* 2010), and it was found to independently predict PCa recurrence. Although we did not confirm this result for EZH2 at transcript level, a statistical trend was apparent. It should be recalled that our series only incorporates patients with clinically localized PCa, submitted to radical prostatectomy, which represent a subset of the whole spectrum of PCa patients. Because these patients are selected for having clinically organ-confined disease, the corresponding PCas are usually of low and intermediate grade (mostly GS 6 and 7 in the biopsy) and low stage (cT1c and cT2). Thus, it does not comprise the full spectrum of PCa, as clinically advanced and high-grade cases at diagnosis will not be considered (in general) for curative-intent radical prostatectomy. Notwithstanding these limitations, high *SMYD3* expression retained prognostic significance in multivariate analysis, confirming its potential clinical usefulness. To more easily translate for routine practice, however, it would be important to determine if immunohistochemical assessment of SMYD3 expression would provide the same result. Though several commercially available antibodies were tested, none provided satisfactory results.

Concerning disease-specific survival, no statistically significant associations were apparent, probably due to relatively short follow-up data. A follow-up period of 15 or 20 years is usually required to detect differences in PCa survival in patients with localized disease submitted to radical prostatectomy (Popiolek *et al.* 2013). Nonetheless, biochemical recurrence is also an important primary endpoint in many studies. As expected, GS and pathological stage were of prognostic significance in univariate analysis, although only the former and stage pT3b denoted independent prognostic value in multivariate analysis. The fact that stage pT3a did not surfaced as independent prognostic parameter for DFS in multivariate analysis is most likely due to the association between tumor stage and histological grade, as pT3a cancers were mostly of high GS.

Table 4 Cox regression models assessing the potential of clinical and epigenetic variables in the prediction of disease-free survival for 136 PCa patients

Gene	Variable	HR	95% CI for HR	*P* value (CR)
EZH2	PSA levels > med	1.652	0.914–2.986	0.096
	Gleason score	4.206	1.820–9.718	**0.001**
	pT stage >2			0.148
	vs 3a	1.457	0.753–2.819	0.264
	vs 3b	2.397	0.980–5.864	0.055
	EZH2 expression > Q75	1.890	0.983–3.637	0.056
SMYD3	PSA levels > med	1.697	0.940–3.064	0.079
	Gleason score	4.259	1.817–9.982	**0.001**
	pT stage 2			0.086
	vs 3a	1.476	0.755–2.886	0.255
	vs 3b	2.662	1.115–6.356	**0.027**
	SMYD3 expression > Q75	2.049	1.096–3.832	**0.025**

PCa, prostate cancer; CR, Cox regression; HR, hazard ratio; Med, median value; Q75, quartile 75 value; bold highlights statistical significance ($P<0.05$).

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In conclusion, we identified a set of HMTs and HDMs deregulated in PCa that might contribute to the disease development and progression. To the best of our knowledge, this is the first study to demonstrate that HMT *SMYD3* expression levels are able to predict disease-specific survival of PCa patients with clinically localized disease, submitted to radical prostatectomy. Therefore, determination of SMYD3 expression levels in prostate biopsies might be able to convey relevant prognostic information in a pre-therapeutic setting. Functional studies are mandatory to ascertain the role of *SMYD3* in prostate carcinogenesis.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-13-0375.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was funded by research grants from Liga Portuguesa Contra o Cancro – Núcleo Regional do Norte, Research Center of Portuguese Oncology Institute – Porto (CI-IPOP 4-2008) and European Community's Seventh Framework Programme – grant number FP7-HEALTH-F5-2009-241783. F Quintela Vieira and J Ramalho-Carvalho are supported by FCT-Fundação para a Ciência e a Tecnologia grants (SFRH/BD/70564/2010 and SFRH/BD/71293/2010 respectively), and P Costa-Pinheiro is supported by a grant from Liga Portuguesa Contra o Cancro – Núcleo Regional do Norte.

Author contribution statement

F Q Vieira, J Ramalho-Carvalho, R Henrique, and C Jerónimo contributed to experimental design. F Q Vieira, A Pereira, and I Carneiro performed RT-qPCR anaysis. F D Menezes and J Oliveira collect relevant clinical information. L Antunes assisted in statistical analysis. R Henrique performed tumor collection and histopathological classification. F Q Vieira, P Costa-Pinheiro, R Henrique, and C Jerónimo wrote the manuscript with input from co-authors. R Henrique and C Jerónimo are joint senior authors.

Acknowledgements

The authors are grateful to the patients who volunteered to provide samples and also to all the personnel of the Department of Urology, Portuguese Oncology Institute – Porto who kindly collaborated in this study.

References

Allsbrook WC Jr, Mangold KA, Johnson MH, Lane RB, Lane CG & Epstein JI 2001 Interobserver reproducibility of Gleason grading of prostatic carcinoma: general pathologist. *Human Pathology* **32** 81–88. (doi:10.1053/hupa.2001.21135)

Ansari KI & Mandal SS 2010 Mixed lineage leukemia: roles in gene expression, hormone signaling and mRNA processing. *FEBS Journal* **277** 1790–1804. (doi:10.1111/j.1742-4658.2010.07606.x)

Bianco-Miotto T, Chiam K, Buchanan G, Jindal S, Day TK, Thomas M, Pickering MA, O'Loughlin MA, Ryan NK, Raymond WA *et al.* 2010 Global levels of specific histone modifications and an epigenetic gene signature predict prostate cancer progression and development. *Cancer Epidemiology, Biomarkers & Prevention* **19** 2611–2622. (doi:10.1158/1055-9965.EPI-10-0555)

Blair LP, Cao J, Zou MR, Sayegh J & Yan Q 2011 Epigenetic regulation by lysine demethylase 5 (KDM5) enzymes in cancer. *Cancer* **3** 1383–1404. (doi:10.3390/cancers3011383)

Brame CJ, Moran MF & McBroom-Cerajewski LD 2004 A mass spectrometry based method for distinguishing between symmetrically and asymmetrically dimethylated arginine residues. *Rapid Communications in Mass Spectrometry* **18** 877–881. (doi:10.1002/rcm.1421)

Chang B, Chen Y, Zhao Y & Bruick RK 2007 JMJD6 is a histone arginine demethylase. *Science* **318** 444–447. (doi:10.1126/science.1145801)

Chen LB, Xu JY, Yang Z & Wang GB 2007 Silencing SMYD3 in hepatoma demethylates RIZI promoter induces apoptosis and inhibits cell proliferation and migration. *World Journal of Gastroenterology* **13** 5718–5724.

Chi P, Allis CD & Wang GG 2010 Covalent histone modifications–miswritten, misinterpreted and mis-erased in human cancers. *Nature Reviews. Cancer* **10** 457–469. (doi:10.1038/nrc2876)

Cloos PA, Christensen J, Agger K & Helin K 2008 Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. *Genes and Development* **22** 1115–1140. (doi:10.1101/gad.1652908)

Coffey K, Rogerson L, Ryan-Munden C, Alkharaif D, Stockley J, Heer R, Sahadevan K, O'Neill D, Jones D, Darby S *et al.* 2013 The lysine demethylase, KDM4B, is a key molecule in androgen receptor signalling and turnover. *Nucleic Acids Research* **41** 4433–4446. (doi:10.1093/nar/gkt106)

Duffy MJ 2011 Prostate-specific antigen: does the current evidence support its use in prostate cancer screening? *Annals of Clinical Biochemistry* **48** 310–316. (doi:10.1258/acb.2011.010273)

Foreman KW, Brown M, Park F, Emtage S, Harriss J, Das C, Zhu L, Crew A, Arnold L, Shaaban S *et al.* 2011 Structural and functional profiling of the human histone methyltransferase SMYD3. *PLoS ONE* **6** e22290. (doi:10.1371/journal.pone.0022290)

Franci C, Zhou J, Jiang Z, Modrusan Z, Good Z, Jackson E & Kouros-Mehr H 2013 Biomarkers of residual disease, disseminated tumor cells, and metastases in the MMTV-PyMT breast cancer model. *PLoS ONE* **8** e58183. (doi:10.1371/journal.pone.0058183)

Fullgrabe J, Kavanagh E & Joseph B 2011 Histone onco-modifications. *Oncogene* **30** 3391–3403. (doi:10.1038/onc.2011.121)

Gleason DF & Mellinger GT 1974 Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. *Journal of Urology* **111** 58–64.

Guccione E, Bassi C, Casadio F, Martinato F, Cesaroni M, Schuchlautz H, Luscher B & Amati B 2007 Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive. *Nature* **449** 933–937. (doi:10.1038/nature06166)

Guess HA 2001 Benign prostatic hyperplasia and prostate cancer. *Epidemiologic Reviews* **23** 152–158. (doi:10.1093/oxfordjournals.epirev.a000782)

van Haaften G, Dalgliesh GL, Davies H, Chen L, Bignell G, Greenman C, Edkins S, Hardy C, O'Meara S, Teague J *et al.* 2009 Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. *Nature Genetics* **41** 521–523. (doi:10.1038/ng.349)

Hake SB, Xiao A & Allis CD 2004 Linking the epigenetic 'language' of covalent histone modifications to cancer. *British Journal of Cancer* **90** 761–769. (doi:10.1038/sj.bjc.6601575)

Hamamoto R, Furukawa Y, Morita M, Iimura Y, Silva FP, Li M, Yagyu R & Nakamura Y 2004 SMYD3 encodes a histone methyltransferase

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involved in the proliferation of cancer cells. *Nature Cell Biology* **6** 731–740. (doi:10.1038/ncb1151)

Hamamoto R, Silva FP, Tsuge M, Nishidate T, Katagiri T, Nakamura Y & Furukawa Y 2006 Enhanced SMYD3 expression is essential for the growth of breast cancer cells. *Cancer Science* **97** 113–118. (doi:10.1111/j.1349-7006.2006.00146.x)

Hermanek P, Hutter RVP, Sobin LH, Wagner G & Wittekind CF 1997 Prostate. In *TNM Atlas: Illustrated guide to the TNM/pTNM classification of malignant tumors*, pp 278–280. Eds P Hermanek, RVP Hutter, LH Sobin, G Wagner & CF Wittekind. Heidelberg, Germany: Springer-Verlag.

Hirst M & Marra MA 2009 Epigenetics and human disease. *International Journal of Biochemistry & Cell Biology* **41** 136–146. (doi:10.1016/j.biocel.2008.09.011)

Jemal A, Bray F, Center MM, Ferlay J, Ward E & Forman D 2011 Global cancer statistics. *CA: A Cancer Journal for Clinicians* **61** 69–90. (doi:10.3322/caac.20107)

Karanikolas BD, Figueiredo ML & Wu L 2010 Comprehensive evaluation of the role of EZH2 in the growth, invasion, and aggression of a panel of prostate cancer cell lines. *Prostate* **70** 675–688. (doi:10.1002/pros.21112)

Ke XS, Qu Y, Rostad K, Li WC, Lin B, Halvorsen OJ, Haukaas SA, Jonassen I, Petersen K, Goldfinger N *et al.* 2009 Genome-wide profiling of histone h3 lysine 4 and lysine 27 trimethylation reveals an epigenetic signature in prostate carcinogenesis. *PLoS ONE* **4** e4687. (doi:10.1371/journal.pone.0004687)

King CR & Long JP 2000 Prostate biopsy grading errors: a sampling problem? *International Journal of Cancer* **90** 326–330. (doi:10.1002/1097-0215(20001220)90:6<326::AID-IJC3>3.0.CO;2-J)

de Kok JB, Roelofs RW, Giesendorf BA, Pennings JL, Waas ET, Feuth T, Swinkels DW & Span PN 2005 Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. *Laboratory Investigation* **85** 154–159. (doi:10.1038/labinvest.3700208)

Laitinen S, Martikainen PM, Tolonen T, Isola J, Tammela TL & Visakorpi T 2008 EZH2, Ki-67 and MCM7 are prognostic markers in prostatectomy treated patients. *International Journal of Cancer* **122** 595–602. (doi:10.1002/ijc.23145)

Lapointe J, Li C, Higgins JP, van de Rijn M, Bair E, Montgomery K, Ferrari M, Egevad L, Rayford W, Bergerheim U *et al.* 2004 Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *PNAS* **101** 811–816. (doi:10.1073/pnas.0304146101)

Lee DY, Northrop JP, Kuo MH & Stallcup MR 2006 Histone H3 lysine 9 methyltransferase G9a is a transcriptional coactivator for nuclear receptors. *Journal of Biological Chemistry* **281** 8476–8485. (doi:10.1074/jbc.M511093200)

Miremadi A, Oestergaard MZ, Pharoah PD & Caldas C 2007 Cancer genetics of epigenetic genes. *Human Molecular Genetics* **16** R28–R49. (doi:10.1093/hmg/ddm021)

Moyer VA 2012 Screening for prostate cancer: U.S. Preventive Services Task Force recommendation statement. *Annals of Internal Medicine* **157** 120–134. (doi:10.7326/0003-4819-157-2-201207170-00459)

Neault M, Mallette FA, Vogel G, Michaud-Levesque J & Richard S 2012 Ablation of PRMT6 reveals a role as a negative transcriptional regulator of the p53 tumor suppressor. *Nucleic Acids Research* **40** 9513–9521. (doi:10.1093/nar/gks764)

Popiolek M, Rider JR, Andren O, Andersson SO, Holmberg L, Adami HO & Johansson JE 2013 Natural history of early, localized prostate cancer: a final report from three decades of follow-up. *European Urology* **63** 428–435. (doi:10.1016/j.eururo.2012.10.002)

Ren TN, Wang JS, He YM, Xu CL, Wang SZ & Xi T 2011 Effects of SMYD3 over-expression on cell cycle acceleration and cell proliferation in MDA-MB-231 human breast cancer cells. *Medical Oncology* **28** (Suppl 1) S91–S98. (doi:10.1007/s12032-010-9718-6)

Rotili D & Mai A 2011 Targeting histone demethylases a new avenue for the fight against cancer. *Genes & Cancer* **2** 663–679. (doi:10.1177/1947601911417976)

Seligson DB, Horvath S, Shi T, Yu H, Tze S, Grunstein M & Kurdistani SK 2005 Global histone modification patterns predict risk of prostate cancer recurrence. *Nature* **435** 1262–1266. (doi:10.1038/nature03672)

Seligson DB, Horvath S, McBrian MA, Mah V, Yu H, Tze S, Wang Q, Chia D, Goodglick L & Kurdistani SK 2009 Global levels of histone modifications predict prognosis in different cancers. *American Journal of Pathology* **174** 1619–1628. (doi:10.2353/ajpath.2009.080874)

Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA & Casero RA 2004 Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119** 941–953. (doi:10.1016/j.cell.2004.12.012)

Siegel R, Naishadham D & Jemal A 2012 Cancer statistics, 2012. *CA: A Cancer Journal for Clinicians* **62** 10–29. (doi:10.3322/caac.20138)

Stamey TA 1995 Making the most out of six systematic sextant biopsies. *Urology* **45** 2–12. (doi:10.1016/S0090-4295(95)96168-2)

Van Aller GS, Reynoird N, Barbash O, Huddleston M, Liu S, Zmoos AF, McDevitt P, Sinnamon R, Le B, Mas G *et al.* 2012 Smyd3 regulates cancer cell phenotypes and catalyzes histone H4 lysine 5 methylation. *Epigenetics* **7** 340–343. (doi:10.4161/epi.19506)

Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RG, Otte AP *et al.* 2002 The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* **419** 624–629. (doi:10.1038/nature01075)

Wang SZ, Luo XG, Shen J, Zou JN, Lu YH & Xi T 2008 Knockdown of SMYD3 by RNA interference inhibits cervical carcinoma cell growth and invasion *in vitro*. *BMB Reports* **41** 294–299. (doi:10.5483/BMBRep.2008.41.4.294)

Wolters T, Vissers KJ, Bangma CH, Schroder FH & van Leenders GJ 2010 The value of EZH2, p27(kip1), BMI-1 and MIB-1 on biopsy specimens with low-risk prostate cancer in selecting men with significant prostate cancer at prostatectomy. *BJU International* **106** 280–286. (doi:10.1111/j.1464-410X.2009.08998.x)

Yoshimatsu M, Toyokawa G, Hayami S, Unoki M, Tsunoda T, Field HI, Kelly JD, Neal DE, Maehara Y, Ponder BA *et al.* 2011 Dysregulation of PRMT1 and PRMT6. Type I arginine methyltransferases, is involved in various types of human cancers. *International Journal of Cancer* **128** 562–573. (doi:10.1002/ijc.25366)

Zou JN, Wang SZ, Yang JS, Luo XG, Xie JH & Xi T 2009 Knockdown of SMYD3 by RNA interference down-regulates c-Met expression and inhibits cells migration and invasion induced by HGF. *Cancer Letters* **280** 78–85. (doi:10.1016/j.canlet.2009.02.015)

Received in final form 22 October 2013
Accepted 6 November 2013
Made available online as an Accepted Preprint
7 November 2013

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-13-0375

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