

Deregulated expression of selected histone methylases and demethylases in prostate carcinoma

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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

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

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- μ m 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)

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 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 (van Haaften *et al.* 2009), and analysis was extended 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, <i>n</i>	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, <i>n</i> (%)		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, <i>n</i> (%)		NA		NA
<7	3 (30.0)		57 (38.7)	
≥7	7 (70.0)		93 (62.0)	

PCa, prostate cancer; NA, not applicable.

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); arginine 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

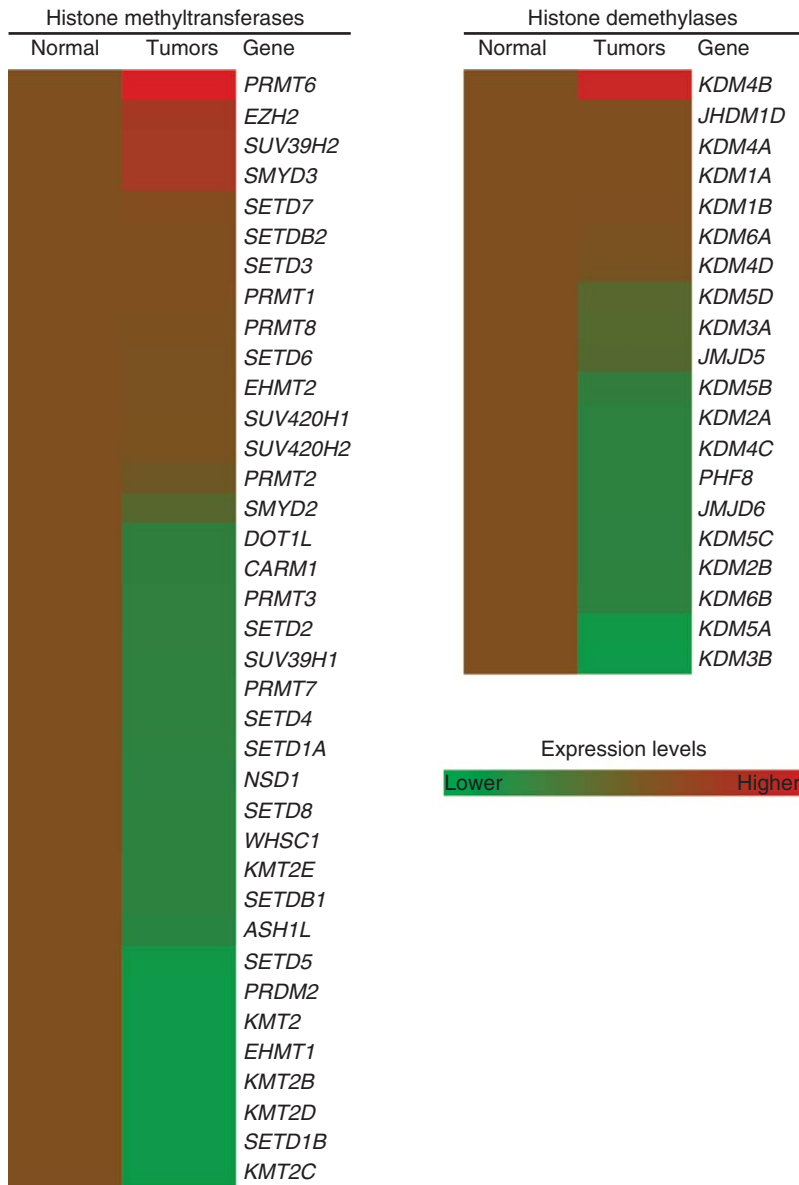


Figure 1

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

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

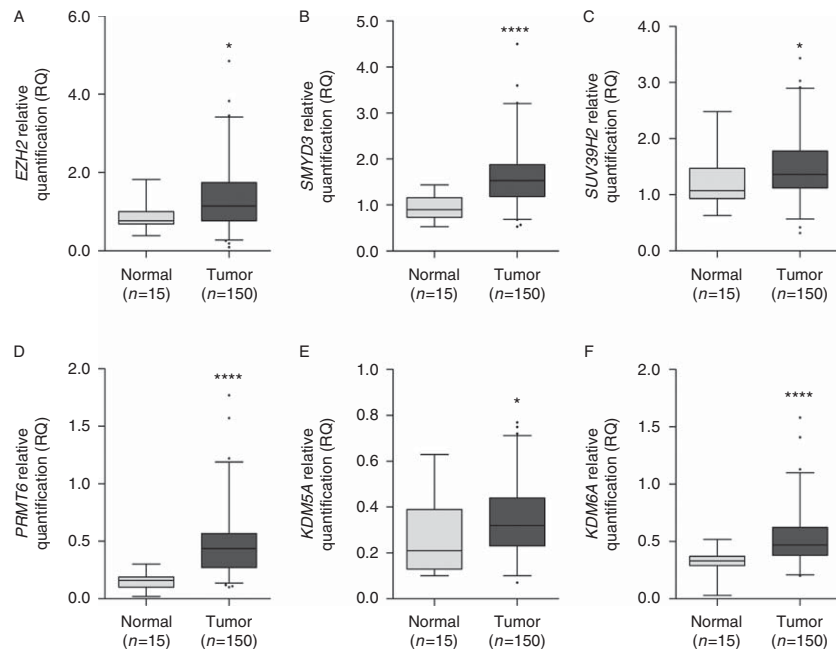
Gene	Normal	Tumor	P value, M-W	AUC
<i>EZH2</i>	0.77 (0.39–1.82)	1.15 (0.09–4.85)	0.014	0.692
<i>KMT2A</i>	0.69 (0.21–2.52)	0.29 (0.08–1.30)	< 0.001	0.212
<i>KMT2B</i>	3.23 (1.61–6.76)	2.05 (0.49–8.07)	0.004	0.272
<i>KMT2C</i>	2.37 (1.24–4.40)	1.45 (0.39–4.06)	< 0.001	0.232
<i>KMT2D</i>	3.91 (1.36–10.21)	1.97 (0.36–8.66)	< 0.001	0.272
<i>KMT2E</i>	0.73 (0.58–3.46)	0.53 (0.21–1.55)	< 0.001	0.162
<i>PRMT6</i>	0.16 (0.02–0.30)	0.43 (0.10–1.77)	< 0.001	0.923
<i>SMYD3</i>	0.90 (0.53–1.44)	1.53 (0.53–4.50)	< 0.001	0.855
<i>SUV39H2</i>	1.07 (0.63–2.48)	1.36 (0.32–3.43)	0.044	0.657
<i>KDM3B</i>	0.26 (0.03–0.45)	0.24 (0.11–0.78)	NS	0.495
<i>KDM4B</i>	2.24 (0.47–7.81)	0.47 (0.08–2.23)	< 0.001	0.098
<i>KDM5A</i>	0.21 (0.10–0.63)	0.32 (0.07–0.77)	0.026	0.675
<i>KDM6A</i>	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* (Karaniakos *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 (**** $P < 0.0001$; * $P < 0.05$).

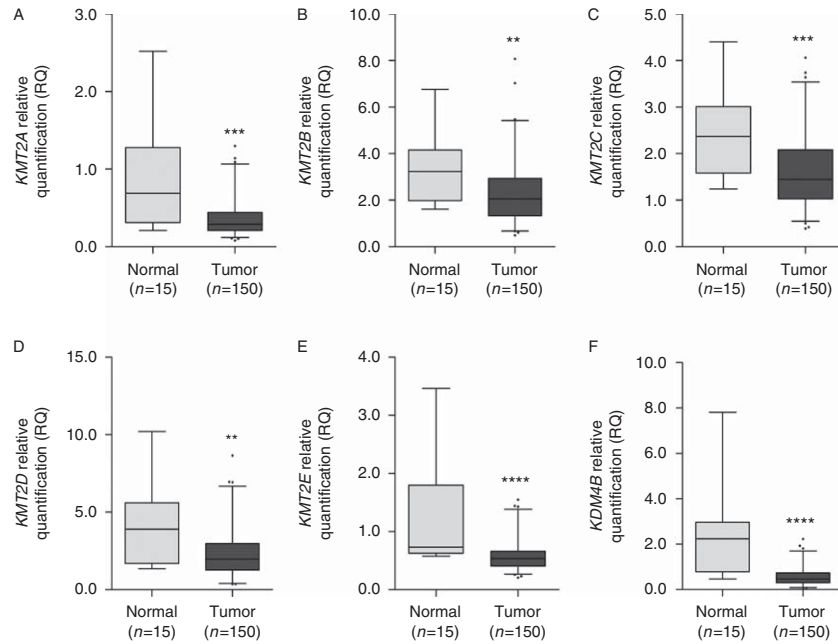


Figure 3

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

	<i>KMT2A</i>	<i>KMT2B</i>	<i>KMT2C</i>	<i>KMT2D</i>	<i>KMT2E</i>
<i>KMT2A</i>	–	0.773	0.814	0.600	0.560
<i>KMT2B</i>	–	–	0.844	0.729	0.468
<i>KMT2C</i>	–	–	–	0.650	0.464
<i>KMT2D</i>	–	–	–	–	0.458
<i>KMT2E</i>	–	–	–	–	–

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

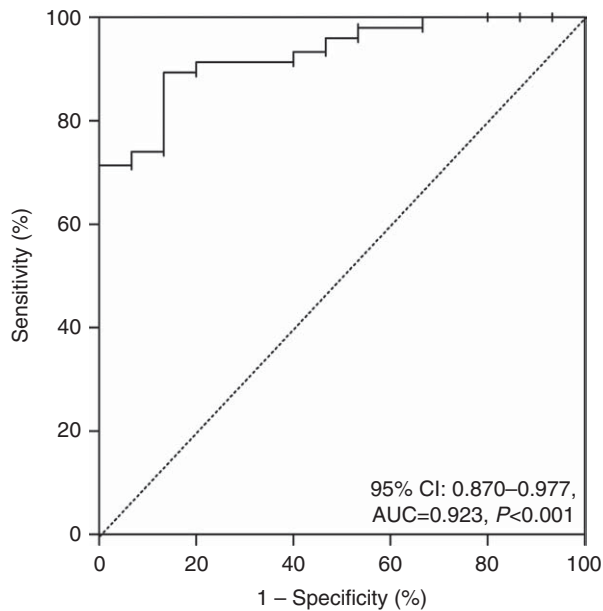


Figure 4
Performance of *PRMT6* expression as biomarker for PCa. Receiver operator characteristic (ROC) curve evaluating the ability of *PRMT6* expression levels in discriminating PCa from normal prostate tissues. AUC, area under the curve.

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,

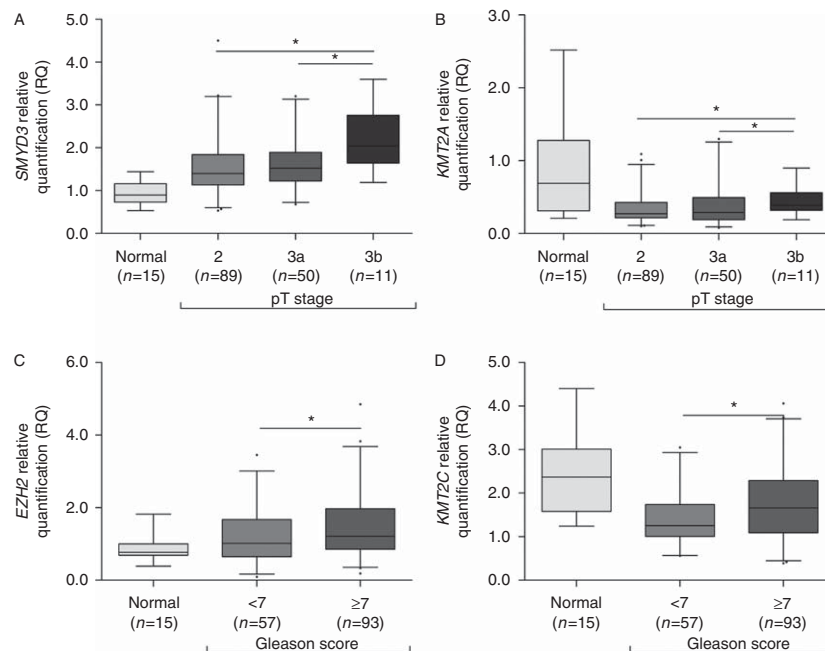


Figure 5
Association of HMTs with clinicopathological parameters. Distribution of *SMYD3* (A) and *KMT2A* (B) expression levels according to pathological stage, showing higher levels in locally advanced disease stage pT3b

(* $P < 0.05$). Distribution of *EZH2* (C) and *KMT2C* (D) expression levels according to Gleason score, displaying higher levels in tumors with Gleason score ≥ 7 (* $P < 0.05$).

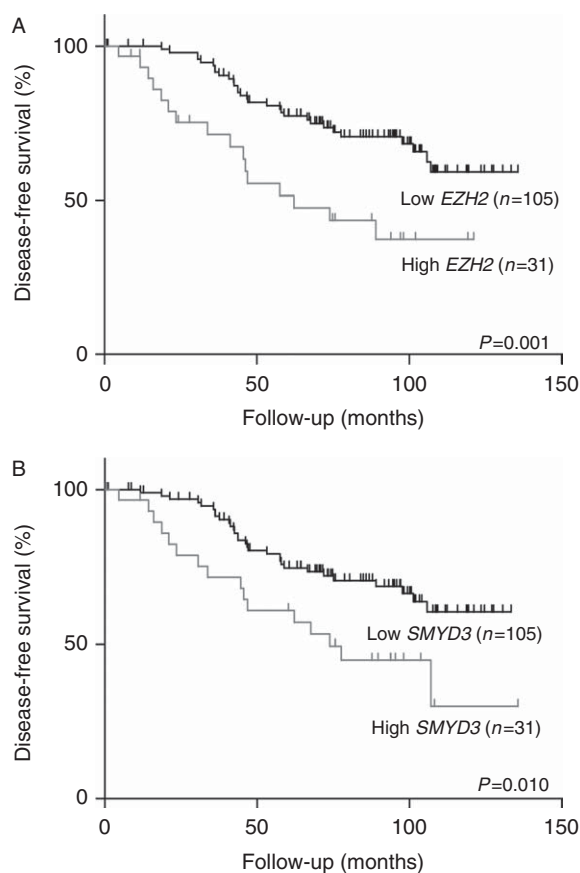


Figure 6
Kaplan–Meier estimated disease-free survival curves for PCa patients. Disease-free survival curves of 136 PCa patients according to expression levels of *EZH2* (A) and *SMYD3* (B). The results of RT-qPCR presented were categorized using third quartile (75th percentile) value as the cutoff.

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)
<i>EZH2</i>	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
	<i>EZH2</i> expression > Q75	1.890	0.983–3.637	0.056
<i>SMYD3</i>	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
	<i>SMYD3</i> 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 ($P < 0.05$).

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.

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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 analysis. 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.

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