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Elucidating the role of PRMTs in prostate cancer using open access databases and a patient cohort dataset

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Summary. Protein arginine methylation is an understudied epigenetic mechanism catalyzed by enzymes known as Protein Methyltransferases of Arginine (PRMTs), while the opposite reaction is performed by Jumonji domain- containing protein 6 (JMJD6). There is increasing evidence that PRMTs are deregulated in prostate cancer (PCa). In this study, the expression of two PRMT members, PRMT2 and PRMT7 as well as JMJD6, a demethylase, was analyzed in PCa. Initially, we retrieved data from The Cancer Genome Atlas (TCGA) project and the Gene Expression Omnibus (GEO) database to explore the differential expression of various PRMT family members in patients with PCa and then applied immunohistochemistry in a patient cohort across the spectrum of PCa, including non-neoplastic prostate tissue and lymph node metastatic foci. The results from the TCGA analysis revealed that PRMT7, PRMT6 and PRMT3 expression increased while PRMT2, PRMT9 and JMJD6 levels decreased in the tumor compared to non-neoplastic prostate. Results from the GEO datasets were similar, albeit not identical with the TCGA results, with PRMT7 and PRMT3 being upregulated and PRMT2 and JMJD6 being downregulated in the tumor compared to non-neoplastic tissue in some of them. In addition, PRMT7 levels decreased with stage and grade progression in the TCGA analysis. In the patient cohort, both PRMTs and JMJD6 were overexpressed in PCa compared to non-neoplastic tissue, and nuclear PRMT2 and JMJD6 were upregulated in lymph node metastasis, too. PRMT7 and JMJD6 expression were upregulated with the progression of stage and JMJD6 was also increased with the elevation of grade. After androgen ablation therapy, nuclear expression of PRMT7 and JMJD6 were elevated compared to untreated tumors. PRMT2, PRMT7 and JMD6 were also correlated with markers of EMT and cell cycle regulators. Finally, our findings indicate that PRMTs and JMJD6 are involved in prostate cancer progression and revealed a potential interplay of PRMTs with EMT mediators, underscoring the need for therapeutic targeting of arginine methylation in prostate cancer.

Key words: PRMTs, Prostate cancer, TCGA and GEO analysis validated with immunohistochemistry

Introduction

Prostate cancer is a major health care challenge and represents the second most common cancer in men, with 1,414,259 new cases worldwide in 2020 (Sung et al., 2021). Previous studies have shown that AR silencing and developmental reprogramming occurs during the evolution of the disease as a result of epigenetic modifications (Tzelepi et al., 2012; Kleb et al., 2016; Pomerantz et al., 2020). Epigenetics is a field of molecular biology that studies the inherited changes of gene expression without any alterations in DNA sequencing (Blanc and Richard, 2017). Among the mechanisms involved in epigenetics, methylation has been the focus of recent studies and its involvement in both physiological and pathological processes is widely recognized. We and others have shown that aberrant DNA methylation is involved in prostate cancer (Pca) progression (Tzelepi et al., 2019; Sugiura et al., 2021).

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Abbreviations. Pca, Prostate Cancer; PRMTs, Protein Methyltransferases of Arginine; JMJD6, Jumonji domain- containing protein 6; EMT, Epithelial to Mesenchymal Transition; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; ARv7, AR splice variant 7; PGG, prognostic grade group; RPS, Radical prostatectomy specimens; IMG, Ioanna Maria Grypari; VT, Vasiliki Tzelepi; TMA, tissue microarray; SD, standard deviation; polyS, polyserine

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However, recently the focus has shifted towards histone methylation as an important dynamic regulatory mechanism of transcription (Blanc and Richard, 2017; Lorton and Shechter, 2019).

Among the histone residues frequently methylated, arginine methylation is an understudied histone post-translational modification that is increasingly associated with cancer progression (Jarrold and Davies, 2019). Arginine methylation is mediated by enzymes known as Protein Methyltransferases of Arginine (PRMTs), whereas the opposite reaction is performed by Jumonji domain- containing protein 6 (JMJD6), which has a double role acting as both an arginine demethylase and a hydroxylase (Poulard et al., 2016; Blanc and Richard, 2017; Zhang et al., 2021). PRMTs have been implicated in various cellular processes such as DNA damage repair, transcription and translation, signal transmission, chromatin remodeling and protein to protein interactions (Lorton and Shechter, 2019; Zhang et al., 2019). These functions are often dysregulated in cancer (Mathioudaki et al., 2011; Falahi et al., 2015; Blanc and Richard, 2017; Raposo and Piller, 2018; Zhang et al., 2018), as neoplastic cells change their protein expression profile in order to escape apoptosis, enhance proliferation, and evade the immune system via upregulation of oncogene expression and silencing of tumor suppressor genes (Hsu et al., 2017). Thus, there is an emerging need for further studies regarding the involvement of PRMTs in PCa, as they can be potent prognostic biomarkers and can be used as therapeutic targets in the era of personalized therapy (Sugiura et al., 2021). Selective inhibitors for some of these enzymes have already been developed and are being tested in preclinical and clinical trials with promising results (Cheng et al., 2019).

Recently, our group showed that PRMT1 and PRMT4/CARM1 are upregulated early in PCa progression, with CARM1 being further upregulated after therapy, suggesting a role in therapy resistance (Grypari et al., 2021). These correlative observations support the hypothesis that an epigenetic network drives lethal PCa progression (Hong et al., 2004; Raposo and Piller, 2018; Grypari et al., 2021). Furthermore, an interplay between PRMTs and AR signaling, the cell cycle, and Epithelial to Mesenchymal Transition (EMT) was identified, linking PRMTs to specific milestones of PCa progression (Grypari et al., 2021).

In order to understand the role of PRMTs in PCa, gene expressions of all PRMT family members were analyzed based on data retrieved from The Cancer Genome Atlas (TCGA) project and the Gene Expression Omnibus (GEO) database. Then, to expand the findings of our previous studies, two additional family members of PRMTs (PRMT2 and PRMT7) as well as an enzyme with the opposite function (a demethylase, JMJD6) were analyzed by immunohistochemistry in a cohort of patients across the spectrum of prostate cancer progression: primary low and high grade PCa, treated PCa, lymph node metastatic foci and peripheral non-neoplastic prostate tissue. Specifically, we selected PRMT2, which has a weak methylation activity, participating in mRNA processing (Vhuiyan et al., 2017) and is highly expressed in androgen dependent tissues, such as skeletal muscle, prostate and heart, acting as a co-regulator of AR transportation in the nucleus (Meyer et al., 2007). We also examined PRMT7, the only member of type III PRMTs, and the only one having monomethylarginine/MMA as the final product. PRMT7 has been correlated with EMT, through suppression of E-cadherin expression (Haghandish et al., 2019) and has been associated with adverse prognosis in other hormone- dependent cancers, such as breast carcinoma (Baldwin et al., 2015; Szewczyk et al., 2020; Halabelian and Barsyte-Lovejoy, 2021). Finally, we studied JMJD6, which acts as a demethylase, as it has been shown to interact with AR splice variant 7 (ARv7) (Fan et al., 2018; Paschalis et al., 2021; Tong, 2021), and has been identified as a pivotal regulator of gene expression in PCa with potential prognostic utility (Cangiano et al., 2021).

Expression of theses markers was then correlated with markers of EMT (E-cadherin, ZEB1 and TWIST1) and cell cycle mediators (p53 and cyclin D1). According to the literature, there are more than 90 recognized mediators of EMT associated with acquisition of a mesenchymal phenotype in various types of cancer (Ishikawa et al., 2021). ZEB1 and TWIST1 are considered to be two of the three core transcriptional factors of EMT, while E-cadherin is a marker of the epithelial phenotype and its expression is directly suppressed by these two transcription factors (Park et al., 2008). Thus, ZEB1, TWIST1 and E-cadherin were selected as the most representative markers of EMT. Regarding the cell cycle, we selected Cyclin D1, as it regulates the transition from G1 to S phase through phosphorylation of the RB protein, and is known to be implicated and have prognostic value in various malignancies (Casimiro et al., 2016). p53 is another important regulator of the cell cycle, and is inactivated in 30% of PCa, linked with invasive behavior and metastatic potential in cancer cells (Liu et al., 2020).

Materials and methods

Databases analysis

Data from the TCGA-PRAD project were retrieved from The Cancer Genome Atlas database, using the 'TCGA biolinks' R package. (Colaprico et al., 2016) Prostate adenocarcinomas (N=375) and non-neoplastic prostate tissue (N=43) were analyzed.

Of the TCGA data, 140 samples were classified as T2 stage, 114 as T3a and 106 as T3b. Regarding prognostic grade group (PGG), 27 cases were assigned a PGG1, 106 cases had a PGG2, 77 cases had a PGG 3, 39 cases were assigned a PGG4, and 111 cases had a PGG5. Patients' characteristics from the TCGA are demonstrated in Table 1.

Gene expression data were also retrieved from the

Gene Expression Omnibus (GEO) database. Four separate datasets with prostate cancer cases were chosen based on the number of patients and availability of clinical information, such as Gleason score and stage. When feasible, only patients with Caucasian origin were included in the analysis, in order to match the patients in our cohort.

Specifically, data acquired from the dataset with ID number: GSE21034 included 150 neoplastic prostate samples, 29 samples of normal prostate tissue and 16 samples from metastatic prostate cancer foci. Within the metastatic foci group were locally aggressive cases infiltrating colon or bladder, as well as cases with lung, bone, node, neck, spine, testes and brain metastases. Regarding the clinicopathologic characteristics of the patients, only data on clinical stage was available. In addition, staging was performed using a previous version of the AJCC classification system, thus, correlation with stage was not possible in our analysis. Data on Gleason Grade/ Prognostic Grade Group was not available in this dataset.

Data reclaimed from the dataset with ID number: GSE46602 was also examined and included 36 samples from neoplastic and 14 samples from non-neoplastic prostate. Another dataset from GEO database (ID number: GSE32571) was analyzed, including 59 cases of prostate cancer and 39 samples from normal prostate tissue. Stage and Grade were not available for these datasets.

Finally, only one dataset (ID number: GSE134051) of 164 cases included information regarding the Gleason Score, albeit only the sum was known (that is the primary and secondary patterns were not specified in the dataset). Thus, it was not possible to subclassify the cases with Gleason score 7, in prognostic grade groups 2 or 3 according to the latest international classification guidelines, and the guidelines that we used in our cohort of patients. Instead, albeit not ideal, we decided to run a comparison between low and high grade cases. The "low grade" group consisted of 103 cases with Gleason score lower than or equal to 7 and the remaining 61 cases had a Gleason score higher than 7 and were identified as the "high grade" group.

Patient cohort

Radical prostatectomy specimens (RPS) from 101 patients with prostate cancer were retrieved from the archives of the Department of Pathology of the University Hospital of Patras, Greece (approval by the Ethics and Research Committee of the University Hospital of Patras, Protocol Number 195/6.4.2021). The study was conducted in accordance with the Declaration of Helsinki.

All the cases included were re-evaluated by two pathologists (IMG and VT). Both stage and Grade Group/Gleason Score were assigned according to the latest (8th) TNM and WHO classification, respectively (Buyyounouski et al., 2017; Moch et al., 2016). The pathological characteristics of the patients are shown in Table 2. In 48 of the N1 cases, tissue from the lymph node metastatic foci was also available. Finally, in 62 cases adjacent normal prostate tissue from the peripheral zone was also studied.

Table 1. Pathologic characteristics of the patients from the TCGA database.

Group	Stage			
	T2	T3a	T3b	Sum
1	20	6	1	27
2	72	29	5	106
3	27	35	15	77
4	7	16	16	39
5	14	28	69	111
Sum	140	114	106	

Tissue microarray construction

A tissue microarray (TMA) was constructed from the RPS as previously reported (Tzelepi et al., 2019). Areas that represented the different patterns of high-grade prostate carcinoma (i.e., fused glands, poorly formed glands, cribriform formations, and intraductal carcinoma) were sampled, as was benign prostate tissue from the peripheral zone.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Tzelepi et al., 2019). Antigen retrieval was performed at 600W in a microwave for 20 minutes. Endogenous peroxidase blocking was performed by incubating the slides in a 3% H_2O_2 solution for 15 minutes. Envision (Dako, Carpentaria, CA, USA) was used as the detection system. Sections were counterstained with Harris' acidified hematoxylin. All cases including the lymph node metastatic foci were stained with antibodies against PRMT2, PRMT7 and JMJD6. In an effort to elucidate the specific roles of PRMT2, PRMT7 and JMJD6, primary tumors were also stained with antibodies against markers of cell cycle

Table 2. Pathological characteristics of the patients in our cohort.

Group	Stage			
	T2	T3a	T3b	Sum
1	24	1	-	25
2	5	3	3	11
3	-	4	17	21
4	-	-	12	12
5	-	2	32	34
Sum	29	10	64	103
pN				
N0	15	5		20
pN1	10	5	64	79
pNx	4			4
Treated	1		12	13

signaling (p53, Cyclin D1) and EMT (ZEB1, TWIST1 and E-cadherin). Source and dilution for each of the antibodies used are shown in Table 3.

Evaluation of immunohistochemical stains

The whole stained slides for all markers were scanned with Pannoramic DESK Scanner (3DHISTECH Ltd., Hungary) and viewed with Panoramic Viewer 1.15.4 (3DHISTECH Ltd). The immunohistochemically stained slides were scored by two pathologists (IMG and VT). Each core was scored separately by dividing the number of positive epithelial cells by the total number of epithelial cells to define the percentage of positive cells in increments of 10 (i.e., 0, 10, 20 etc). At least 100 cells were evaluated in each core. The intensity of staining was scored as 1+, 2+, and 3+. The percentage of positive cells was then multiplied by the intensity of staining and a final score ranging from 0 to 300 was calculated. The mean expression of all cores per case was then evaluated. Nuclear, membrane and cytoplasmic staining were separately evaluated when present. For the markers p53, cyclin D1 and E-cadherin, intensity of staining was similar among all cases, thus, it was not included in the final score.

Statistical analysis

Patient characteristics and biomarker expression data were summarized with descriptive statistics and exploratory data analysis. Categorical data were described using contingency tables. Continuously scaled measures were summarized with descriptive statistical measures [e.g., median with standard deviation (SD)]. Kolmogorov Smirnoff /Lilliefors test was used to determine the pattern of data distribution. A Kruskal Wallis test was performed for comparisons of biomarker expression between variables. Wilcoxon signed rank test/Friedman test was used for paired comparisons. Spearman's correlation was used to compare the relationship between the biomarkers. All reported P values are two-sided at a significance level of 0.05. To adjust for multiple comparisons, a Bonferroni correction was used. Analyses were performed using IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp. Armonk, NY).

Data from the TCGA-PRAD project and GEO databases were analyzed using 'DESeq2' R package. The differential gene expression tests of DESeq2 are based on a negative binomial generalized linear model. We performed three separate analyses in order to match with the categories examined above. After the model was fit on the data for each gene, the log2 Fold Change for each sample group was calculated, along with the p-value and the Benjamini-Hochberg adjusted p-value. In each separate analysis, all the possible comparisons between the groups were performed, as well as Speraman's correlation to highlight potential correlations between the markers. In addition, as the genome TCGA base contained far more neoplastic than non-neoplastic tissue samples, the R statistical program restricted the excess number of neoplastic cases via a random selection of 150 adequate samples in order to make a reliable comparison of neoplastic versus non-neoplastic cases.

Results

PRMT expression is deregulated in PCa

We used the data from the Cancer Genome Atlas data base to explore the differential expression of various members of the PRMT family in patients with PCa. At first, we examined how gene expression differed in neoplastic tissues compared to normal prostate tissue. The results showed that PRMT7, PRMT6 and PRMT3 expression was higher and PRMT2, PRMT9 and JMJD6 levels were lower in the tumor compared to non-neoplastic tissue. P and p-adjusted values of the above comparisons are presented in Table 4.

Data retrieved from dataset GSE21034 were also analyzed. The results showed that PRMT1, PRMT2 and JMJD6 expression levels were lower in neoplastic tissue compared to non-neoplastic prostate. In addition, PRMT7, CARM1, PRMT5, PRMT1 and PRMT9 were overexpressed and JMJD6 was downregulated in metastasis compared to the primary tumor.

Dataset GSE46602 was also utilized to compare

Table 3. Dilution and source of the antibodies used. AR: androgen receptor. PSA: prostate-specific antigen.

Antigen	Dilution	Source
Cyclin D1	prediluted	Thermo Scientific, Rockford, USA
E cadherin	1:50	DAKO, Carpinteria, USA
JMJD6	1:200	Abcam, Cambridge, UK
ki67	1:50	DAKO, Carpinteria, USA
PRMT2	1:100	Abcam, Cambridge, UK
PRMT7	1:500	Novus Biologicalis, Littleton, USA
p53	1:1000	DAKO, Carpinteria, USA
Rb	1:30	Calbiochem-EMD Chemicals. Inc. Gibbstown, USA
TWIST1	1:400	Merck KGaA. Darmstadt. Germany
ZEB1	1:250	Sigma Aldrich. Saint Louis, USA

Table 4. P and p-adjusted values of the comparison of tumor versus normal prostatic tissue based on the TCGA data.

Marker	Fold change (Tumor/Normal)	P value*	Padj*
PRMT2	0.80	<0.001	<0.001
PRMT3	1.14	0.008	0.029
PRMT6	1.35	<0.001	<0.001
PRMT7	1.31	<0.001	<0.001
PRMT9	0.88	0.001	0.005
JMJD6	0.86	0.01	0.033

*Differential expression in genes is significant when p value is <0.05 and padj <0.1

prostate cancer with normal prostate tissue. This analysis showed that PRMT6, PRMT5 and PRMT3 levels were increased and PRMT2 decreased in the tumor.

Finally, according to the GSE32571 dataset PRMT1, PRMT7, PRMT3, PRMT5, PRMT6 levels were higher and JMJD6 and PRMT2 levels were lower in neoplastic tissue compared to the non-neoplastic tissue samples.

The results from the GEO datasets are shown in Tables 5-7.

Then, we examined whether a further deregulation occurs with stage and grade progression. Statistical analysis of the TCGA dataset showed that PRMT7 levels decreased from T2 to T3b and CARM1/PRMT4 was upregulated in T3b compared to T3a. In addition, PRMT9 levels decreased from PGG2 to PGG3 and PGG5. PRMT3 was higher in PGG5 compared to PGG2 and PGG3. PRMT4/CARM1 expression was higher in PGG5 compared to PGG3. P and p-adjusted values of the above comparisons are presented in Table 8.

Data from GEO dataset (ID number: GSE134051) were also used to compare the differential expression of PRMTs in low and high-grade cases of prostate cancer. The only statistically significant result noticed was increased PRMT4/CARM1 expression, in high grade compared to low grade tumors. (fold Change Low/High grade: 0.891551, p <0.001, padj: 0.032)

PRMTs and JMJD6 are overexpressed in PCa, while PRMT2 is upregulated in lymph node metastasis too

To validate the results from the TCGA and GEO analysis on the protein level immunohistochemical analysis was performed for PRMT2, PRMT7 and JMJD6 in a cohort of patients across the spectrum of PCa. All markers tested demonstrated nuclear and cytoplasmic staining, whereas JMJD6 showed membrane staining as well, in both neoplastic and non-neoplastic peripheral zone prostate tissue. A faint membrane staining was also observed in both PRMTs, although staining was inconsistent and barely perceptible, and thus it was not further scored. Statistical analysis showed that cytoplasmic expression of PRMT2, PRMT7 and JMJD6 were elevated in neoplastic cells, compared with normal prostate glands (p_{PRMT2}=0.005, and p<0.001 for both PRMT7 and JMJD6). The differential expression in the other cellular compartments was not statistically important. Additionally, nuclear and cytoplasmic expression in PRMT2 was higher in lymph node

Table 5. P and p-adjusted values of the comparison of "tumor versus normal" and "tumor versus metastatic prostate tissue" based on the GSE21034 dataset.

Marker	Fold change (Neoplasm vs. normal)	P value*	Padj*
PRMT2	0.79599	<0.001	<0.001
JMJD6	0.691544	<0.001	<0.001
PRMT1	0.918231	0.031	0.092
	Fold change (Primary vs. Metastasis)	P value*	Padj*
JMJD6	1.544031	<0.001	<0.001
PRMT7	0.8784	0.002	0.018
PRMT4	0.876469	0.005	0.033
PRMT5	0.857289	0.012	0.062
PRMT1	0.875297	0.015	0.072
PRMT9	0.874504	0.02	0.092

*Differential expression in genes is significant when p value is <0.05 and padj <0.1

Table 6. P and p-adjusted values of the comparison of "tumor versus normal" based on the GSE46602.

Marker	Fold change (Neoplasm vs. normal)	P value*	Padj*
PRMT6	2.21574	<0.001	0.009
PRMT5	1.76568	0.001	0.015
PRMT3	1.64592	0.002	0.022
PRMT2	0.50274	0.004	0.039

*Differential expression in genes is significant when p value is <0.05 and padj <0.1

Table 7. P and p-adjusted values of the comparison of "tumor versus normal" based on the GSE32571.

Marker	Fold change (Neoplasm vs. normal)	P value*	Padj*
PRMT6	1.257	<0.001	<0.001
PRMT2	0.8259	<0.001	<0.001
PRMT5	1.1113	<0.001	0.007
JMJD6	0.8911	0.005	0.033
PRMT3	1.1547	0.005	0.034
PRMT7	1.1726	0.009	0.055
PRMT1	1.2691	0.012	0.071

*Differential expression in genes is significant when p value is <0.05 and padj <0.1

Table 8. P and p-adjusted values of the differential expression of PRMTs according to T stage and Grade Group based on the TCGA data.

Marker	Fold change	P value*	Padj*
PRMT3	G5/ G2= 1.1	0.015	0.049
	G5/ G3= 1.13	0.005	0.027
PRMT4/CARM1	T3a/T3b= 0.926	0.017	0.113
	G5/ G3=1.11	0.002	0.015
PRMT7	T2/T3b=1.136	0.0044	0.022
	G5/G1= 0.81	0.003	0.029
	G5/G2= 0.89	0.012	0.04
	G5/G3= 0.83	<0.001	0.003
PRMT9	G3/G2= 0.92	0.012	0.083
	G5/ G2=0.93	<0.001	0.0347

*Differential expression in genes is significant when p value is <0.05 and padj <0.1

metastasis compared with primary foci (p=0.039 and p=0.022 respectively), while nuclear expression of JMJD6 was lower in lymph node metastasis compared with the primary neoplasm (p=0.013). Table 9 summarizes the mean expression of the markers in peripheral zone non-neoplastic, neoplastic, and lymph node metastatic tissue. Figure 1 shows representative images of the markers in non-neoplastic tissue, primary

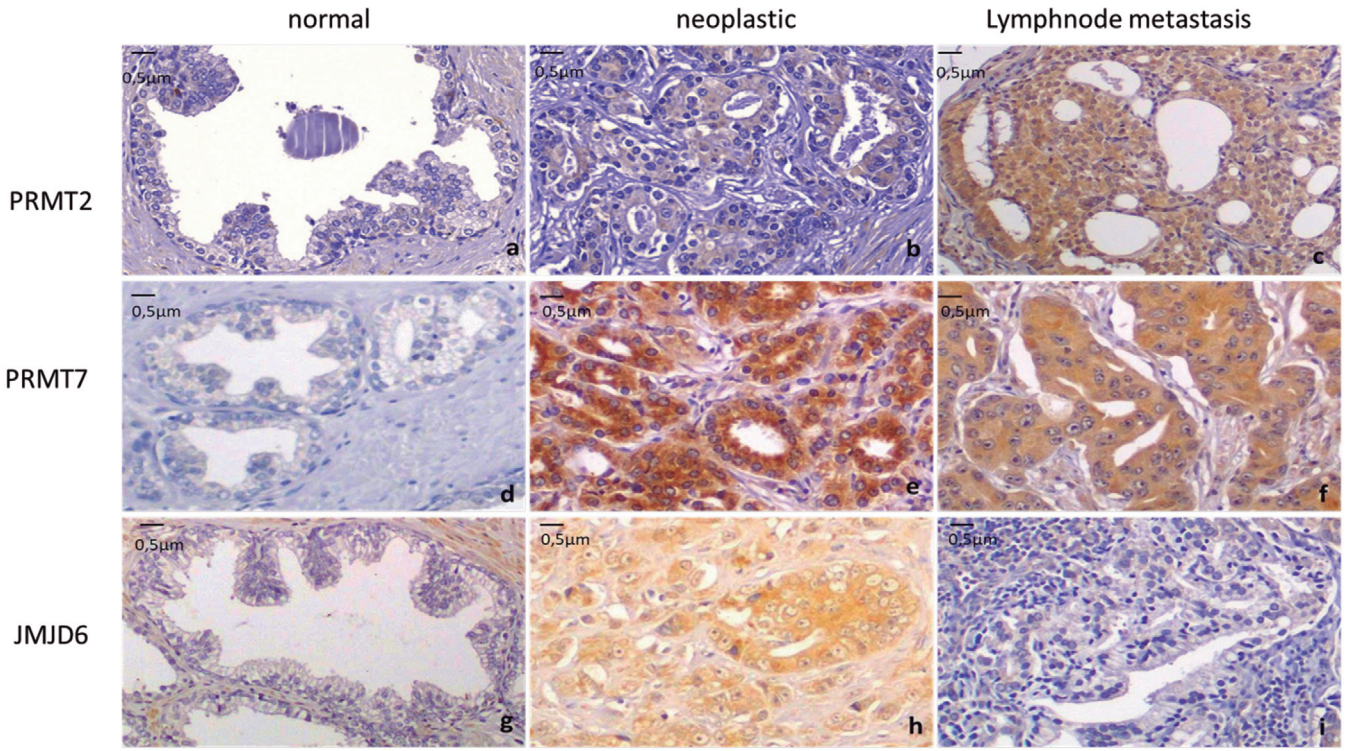

Fig. 1. Representative images of PRMT2, PRMT7 and JMJD6 expression in normal, neoplastic and metastatic PCa tissue. Magnification, x 20.

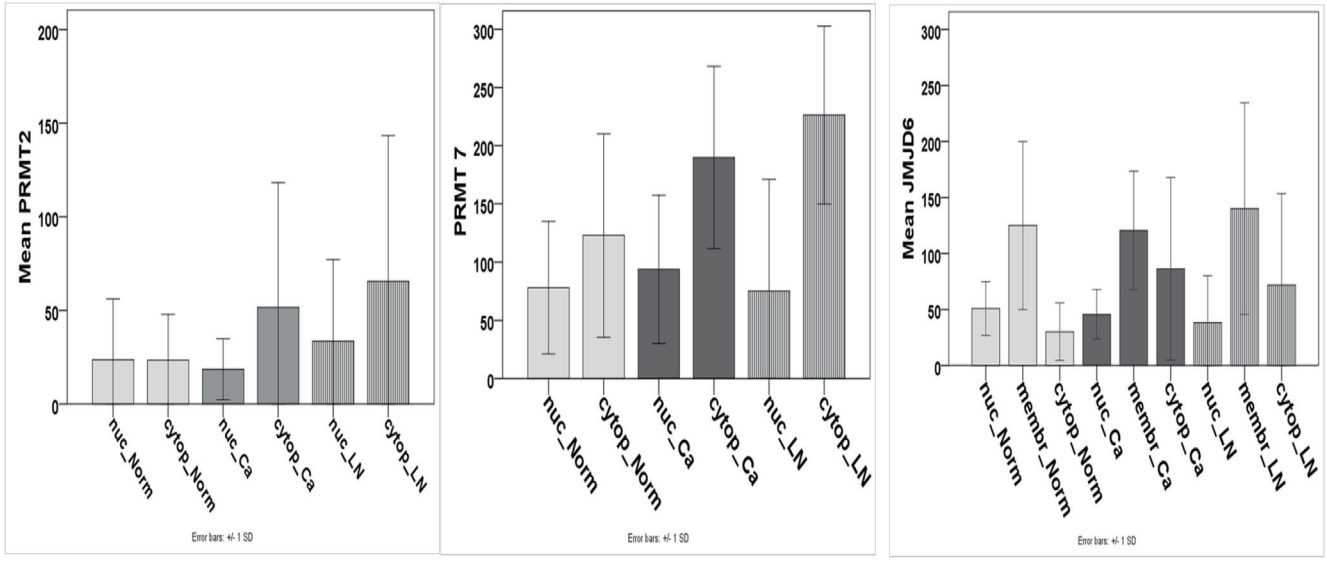

Fig. 2. Bar diagram of expression of the markers in normal, neoplastic and metastatic PCa tissue. Ca: neoplastic, cytop: cytoplasm; LN: Lymph Node; Norm: normal; nuc: nuclear; membr: membrane.

PCa and lymph node metastatic foci and Figure 2 shows the bar graph diagram of the marker expression.

PRMT7 and JMJD6 expression increase with progression of PGG and stage

Subgroup analysis according to stage revealed that JMJD6 expression increased with the progression of stage from pT2 to pT3a (p_{nucl}=0.001, p_{cytop}=<0,001 and p_{memb}=0,006) and from pT2 to pT3b (p_{nucl}=0,001 and p_{cytop}<0,001). PRMT7 expression increased from pT2 to pT3b (p=0,044). Additionally, JMJD6 expression was upregulated from PGG1 to PGG3 (p_{nucl}=0,021 and p_{cytop}=0,001) and PGG1 to PGG5 (p_{nucl}=0,011 and p_{cytop}<0,001). Table 10 and Table 11 show the mean expression of the markers in regards to PGG and stage, respectively. Figure 3 shows the bar graph diagram of the marker expression levels with regard to stage and grade.

Androgen ablation promotes nuclear expression of PRTM7 and JMJD6

Cases that had received antiandrogen treatment preoperatively showed higher PRMT7 and JMJD6 nuclear expression compared to untreated cases (p=0.002 and p=0.018 respectively). Table 12 shows the mean expression levels of the markers in treated and

Table 9. Mean and standard deviation of expression levels of PRMT2, PRMT7, JMJD6 in peripheral zone non neoplastic, neoplastic, and lymph node metastatic tissue.

	Non neoplastic		Neoplastic		Lymph Node metastasis		P#	P*
	Mean	SD	Mean	SD	Mean	SD		
PRMT2nu	26.56	26.10	23.19	24.20	31.84	37.42	>0.05	0.039
PRMT2cyt	27.90	39.58	49.04	62.72	83.44	80.93	0.005	0.022
PRMT7nu	65.35	43.59	72.18	46.61	177.38	85.78	>0.05	>0.05
PRMT7cyt	104.06	71.68	177.38	85.78	218.60	85.14	<0.001	>0.05
JMJD6nuc	35.67	25.28	37.62	22.98	38.46	49.96	>0.05	0.013
JMJD6cyt	26.33	35.05	72.60	71.91	83.14	80.81	<0.001	>0.05
JMJD6membr	126.47	70.97	120.28	66.08	137.4	91.02	>0.05	>0.05

nu=nuclear; cyt=cytoplasm; membr=membrane; SD=standard deviation. #Neoplastic vs. non-neoplastic. *Lymph node vs. neoplastic.

Table 10. Mean and standard deviation expression levels of PRMT2, PRMT7 and JMJD6 in carcinomas according to PGG

	PGG1		PGG2		PGG3		PGG4		PGG5	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PRMT2nu	25.92	20.46	27.33	25.81	19.67	17.09	17.65	6.52	23.82	32.91
PRMT2cyt	38.75	46.44	38.44	39.32	46.92	65.78	43.62	48.25	63.60	80.33
PRMT7nu	61.04	43.21	94.10	48.56	61.69	32.15	63.17	15.01	82.61	58.90
PRMT7cyt	138.13	79.11	196.67	76.87	172.87	83.48	193.87	87.94	195.82	88.66
JMJD6nuc	23.33	16.66	40.51	22.91	46.44	30.65	40.20	15.85	40.50	19.97
JMJD6cyt	34.17	54.45	57.26	48.77	83.33	54.92	65.06	60.41	100.28	88.78
JMJD6membrane	93.70	80.50	123.92	45.49	144.39	64.54	157.71	54.50	109.08	56.54

nu: nuclear; cyt: cytoplasm.

Table 11. Mean and standard deviation expression levels of PRMT2, PRMT7 and JMJD6 in carcinomas according to stage.

	T2		T3a		T3b	
	Mean	SD	Mean	SD	Mean	SD
PRMT2	27.04	19.78	26.65	25.14	20.82	25.94
PRMT2cyt	37.14	45.37	48.01	60.59	54.77	69.75
PRMT7nu	64.11	41.92	81.25	30.73	74.29	50.48
PRMT7cyt	143.39	80.76	163.07	67.12	194.49	86.69
JMJD6nuc	24.07	16.23	51.93	35.24	41.10	20.69
JMJD6cyt	28.70	50.05	88.60	45.14	88.62	75.87
JMJD6membrane	93.65	75.58	171.23	65.33	123.13	57.21

nu: nuclear; cyt: cytoplasm.

Table 12. Mean, Standard Error and Standard Deviation expression levels of PRMT2, PRMT7 and JMJD6 expression in treated and untreated tumors.

	Untreated Mean	Treated Mean	SE	SD	p
PRMT2 NU	50.04	45.63	92.183	24.20	0.614
PRMT2 CYT	51.34	36.29	92.227	62.72	0.086
PRMT7 NU	48.00	75.46	99.632	46.61	0.002
PRMT7 CYT	51.24	53.27	99.291	85.78	0.817
JMJD6 NU	48.35	68.96	98.501	22.98	0.018
JMJD6 CYT	50.58	53.85	98.523	71.91	0.707
JMJD6 MEMBRANE	51.97	40.65	97.108	66.08	0.187

nu: nuclear; cyt: cytoplasm; se: standard error, sd: standard deviation. *differential expression is significant when p value is <0.05.

untreated tumors and Figure 4 shows representative images from untreated and treated cases.

PRMT7 and JMJD6 correlate with markers of EMT and cell cycle regulators

A correlation between PRMTs and EMT markers was seen in our cohort. Specifically, cytoplasmic levels of PRMT2, PRMT7 and JMJD6 were associated with cytoplasmic levels of TWIST1, while PRMT7 and JMJD6 nuclear and cytoplasmic expression correlated with membranous E-cadherin expression. In addition, cytoplasmic levels of all three enzymes were positively correlated with cytoplasmic levels of ZEB1.

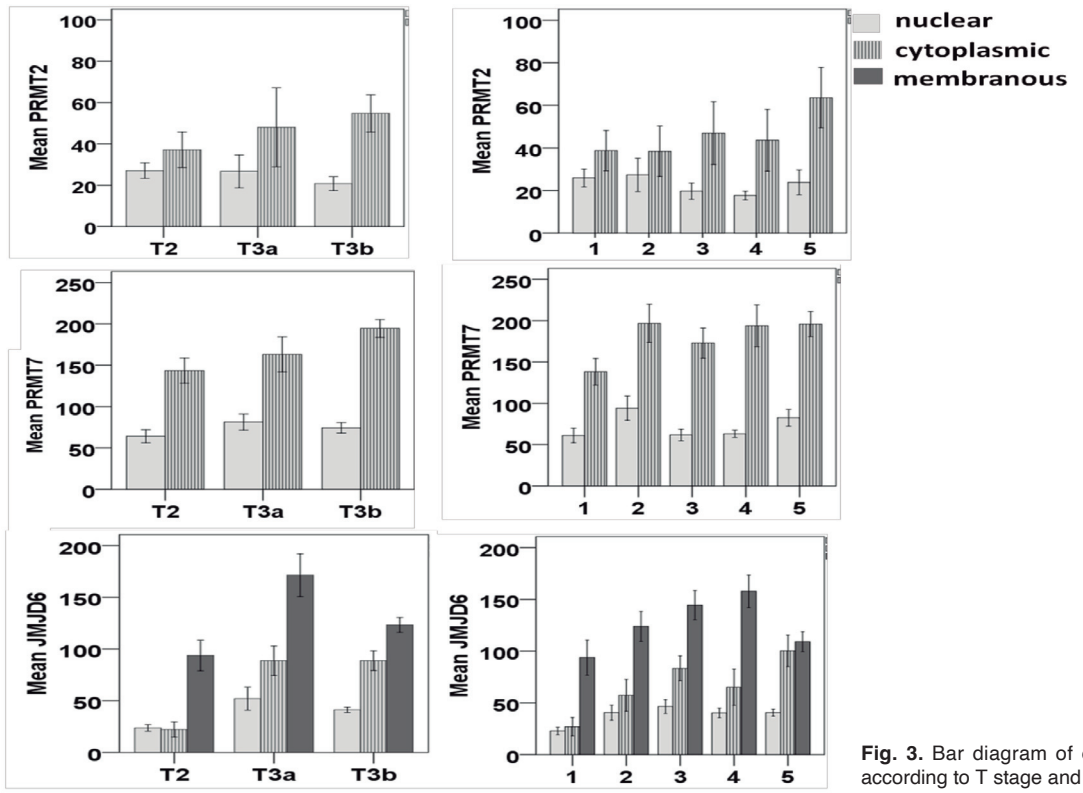

Fig. 3. Bar diagram of expression of the markers according to T stage and PGG

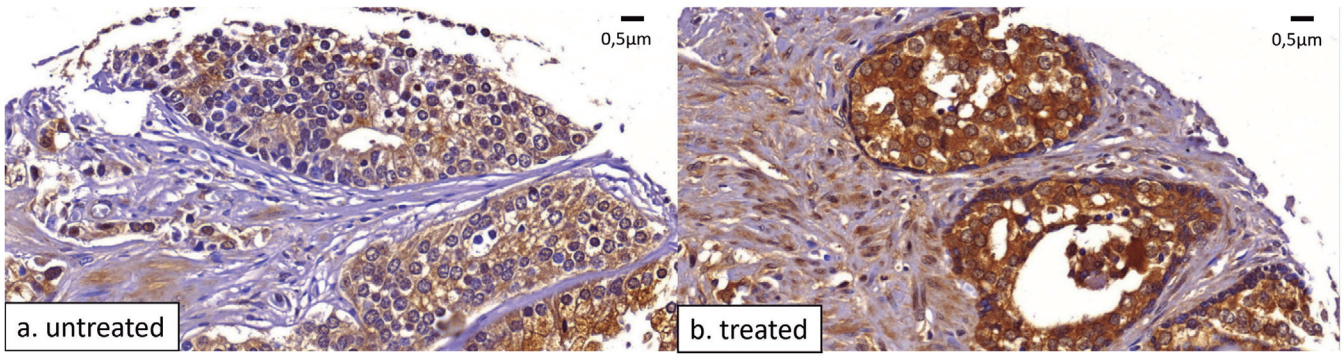

Fig. 4. Representative images of JMJD6 expression in untreated and treated PCa.

Correlation of PRMT expression with cell cycle regulators was also seen. Cytoplasmic levels of PRMT7 demonstrated a moderate association with cytoplasmic and nuclear expression of Cyclin D1 and nuclear PRMT7 demonstrated a weaker association with nuclear levels of Cyclin D1, while nuclear JMJD6 had a weak correlation with nuclear Cyclin D1. Finally, a weak negative association between JMJD6 and p53 was noticed.

PRMT levels were also compared with EMT and cell cycle markers in the TCGA data. A negative weak association was shown between Cyclin D1 and PRMT1, PRMT4 and PRMT7, as well as between TWIST1 and PRMT2. Finally, TWIST1 and PRMT1 levels showed a weak positive association. Analysis of the GSE32571 dataset revealed that PRMT2 was inversely and PRMT1 was positively associated with TWIST1.

Spearman's correlation coefficients (r) and statistically significant p values of the correlations between levels of expression of PRMTs and markers of EMT and cell cycle from our cohort are summarized in Table 13, while the results from the TCGA database are presented in Table 14, and the results from the GSE32571 dataset are shown in Table 15.

Correlations between PRMT family members

Analysis of expression between PRMTs showed a weak interplay between the abovementioned molecules. Specifically, cytoplasmic expression of PRMT2 had a weak association with cytoplasmic expression of both PRMT7 and JMJD6. Cytoplasmic levels of PRMT7 exhibited a moderate correlation with cytoplasmic expression of JMJD6. Nuclear levels of PRMT7 had also a weak correlation with membrane JMJD6 expression. Finally, to connect these results to our previous work, we

Table 13. Correlations between PRMTs, EMT mediators and cell cycle regulators based on the immunohistochemical results.

		PRMT1 nuc	PRMT2 cyt	PRMT2 nuc	CARM1nuc	CARM1cyto	PRMT7 cyt	PRMT7 nuc	JMJD6nucJ	MJD6 cyt
PRMT1 nuc	R	1	p>0.05	p>0.05	**0.369**	**0.260**	p>0.05	p>0.05	p>0.05	p>0.05
	P				**0.001**	**<0.001**				
PRMT1 cyt	R	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	**0.253**	p>0.05	p>0.05
	p							**0.033**		
CARM1 cyt	R	**0.260**	**0.361**	p>0.05	**0.613**	1	**0.402**	p>0.05	p>0.05	p>0.05
	p	**<0.001**	**0.003**		**<0.001**		**<0.001**			
CARM1 nuc	R	**0.369**	p>0.05	p>0.05	1	**0.613**	p>0.05	**0.354**	p>0.05	**-0.249**
	p	**0.001**				**<0.001**		**0.003**		**0.041**
PRMT2 nuc	R	p>0.05	**0.473**	1	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05
	p		<0.001							
PRMT2 cyt	R	p>0.05	1	**0.473**	p>0.05	**0.361**	**0.372**	p>0.05	p>0.05	**0.271**
	p			**<0.001**		**0.003**	**<0.001**			**0.007**
PRMT7 nuc	R	p>0.05	p>0.05	p>0.05	**0.354**	p>0.05	p>0.05	1	**0.338**	p>0.05
	p				**0.003**				**0.001**	
JMJD6 cyt	R	p>0.05	**0.271**	p>0.05	**-0.249**	p>0.05	**0.465**	p>0.05	**0.359**	1
	p		**0.007**		**0.041**		**<0.001**		**<0.001**	
JMJD6 mem	R	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	**0.203**	**0.343**	**0.197**
	p							**0.042**	**<0.001**	**0.049**
TWIST1 cyt	R	**0.365**	**0.361**	p>0.05	p>0.05	**0.310**	0.548	p>0.05	p>0.05	**0.545**
	p	**0.007**	**0.009**			**0.025**	<0.001			**<0.001**
TWIST1 nuc	R	**0.654**	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	**0.312**	**0.298**
	p	**0.000**							**0.022**	**0.028**
ZEB1 nuc	R	**0.595**	p>0.05	p>0.05	**0.655**	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05
	p	**0.000**			**0.000**					
ZEB1 cyt	R	**0.278**	**0.552**	**0.279**	p>0.05	p>0.05	**0.338**	p>0.05	p>0.05	**0.303**
	p	**0.042**	**<0.001**	**0.047**			**0.012**			**0.026**
E cadherin membrane	R	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	**0.364**	**0.291**	**0.338**	**0.413**
	p						**0.007**	**0.033**	**0.001**	**0.002**
Cyclin D1 nuc	R	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	**0.395**	**0.276**	**0.283**	p>0.05
	p						**0.003**	**0.043**	**0.038**	
Cyclin D1 cyt	R	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	**0.432**	p>0.05	p>0.05	p>0.05
	P						**0.001**			
P53 Nuc	R	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	**-0.331**	**-0.380**
	p								**0.014**	**0.005**

Differential expression is significant when p value is <0.05.

correlated these enzymes with PRMT1 and CARM1/PRMT4 expression and a weak but statistically important association was noted. Specifically, cytoplasmic expression of PRMT2, PRMT7 and JMJD6 correlated with cytoplasmic PRMT1 and cytoplasmic CARM1 expression.

We also examined the relationship between the PRMT expression levels, based on the TCGA data. Weak correlations were noted among most of the molecules and are shown in Table 14. Similarly to the results from our cohort, a weak positive association was shown between PRMT1-PRMT2, PRMT2-PRMT7 and JMJD6-PRMT7. Taken together these findings support the idea of a weak interplay among the PRMT family members. However, analysis of the GSE32571 dataset showed the opposite results, that is an inverse correlation between PRMT2-PRMT1, PRMT7-PRMT4, JMJD6-PRMT1 and PRMT7-JMJD6 (Table 15).

Discussion

Arginine methylation is an understudied epigenetic mechanism, crucial for cancer initiation and progression (Jarrold and Davies, 2019). In this study, using data from open access databases we showed that various PRMTs are deregulated in neoplastic cells compared to normal prostate tissue. In the TCGA base, PRMT7, PRMT6 and PRMT3 expression was enhanced and PRMT2, PRMT9 and JMJD6 levels were decreased in carcinoma compared to non-neoplastic tissue. Analysis of databases from GEO showed the same PRMTs up- and down-regulated in cancer, albeit in each database not all of them showed statistical significance, and in some of them additional PRMTs would show a deregulation in their expression levels. This depicts the complexity of methyltransferase expression and highlights the difficulty in drawing meaningful conclusions from available datasets, as the results of the gene expression are influenced by the design of each study and more importantly the number of samples included. The GEO datasets had significantly lower numbers of patients and non-neoplastic samples than the TCGA and our cohort, and may not have the power to identify all meaningful deregulations.

Previous studies have shown a similar deregulation of PRMTs in various carcinomas. For example, it has been demonstrated that PRMT2 expression is decreased (Oh et al., 2014), and PRMT7 is increased in breast carcinoma (Yao et al., 2014; Jain and Clarke, 2019), PRMT3 is upregulated in pancreatic cancer and PRMT6 is enhanced in prostate cancer (Almeida-Rios et al., 2016), the latter being in an accordance with the findings

Table 14. Spearman's correlations between expression levels of PRMTs and mediators of EMT and cell cycle based on the TCGA data.

MARKER		PRMT1	PRMT2	PRMT3	PRMT4	PRMT5	PRMT6	PRMT7	PRMT8	PRMT9	JMJD6
PRMT1	R	**1**	**0.267**	**-0.193**		**-0.138**	**-0.218**			**-0.352**	
	p		**<0.001**	**<0.001**	>0.05	**0.007**	**<0.001**	>0.05	>0.05	**<0.001**	>0.05
PRMT2	R	**0.267**	**1**	**-0.197**		**-0.299**	**-0.324**	**0.338**	**0.146**		
	p	**<0.001**		**<0.001**	>0.05	**<0.001**	**<0.001**	**<0.001**	**<0.001**	>0.05	>0.05
PRMT3	R	**-0.193**	**-0.197**	**1**		**0.378**	**0.219**	**-0.205**		**0.395**	**-0.221**
	p	**<0.001**	**<0.001**		>0.05	**<0.001**	**<0.001**	**<0.001**	>0.05	**<0.001**	**<0.001**
PRMT4	R				**1**	**0.15**				**0.11**	**-0.11**
	p	>0.05	>0.05	**0.015**		**0.003**	>0.05	>0.05	>0.05	**0.035**	**0.035**
PRMT5	R	**-0.138**	**-0.299**	**0.378**	**0.15**	**1**	**0.4**	**-0.158**		**0.305**	**-0.296**
	p	**0.007**	**<0.001**	**<0.001**	**0.003**		**<0.001**	**0.002**	>0.05	**<0.001**	**<0.001**
PRMT6	R	**-0.218**	**-0.324**	**0.219**		**0.4**	**1**	**-0.309**	**-0.182**	**0.202**	
	p	**<0.001**	**<0.001**	**<0.001**	>0.05	**<0.001**		**<0.001**	**<0.001**	**<0.001**	>0.05
PRMT7	R		**0.338**	**-0.21**		**-0.158**	**-0.309**	**1**		**-0.252**	**0.369**
	p	>0.05	<0.001	<0.001	>0.05	0.002	<0.001		>0.05	<0.001	<0.001
PRMT8	R		**0.146**				**-0.182**	>0.01	**1**		
	p	>0.05	**0.004**	**>0.05**	>0.05		**<0.001**	**>0.01**		>0.05	**>0.05**
PRMT9	R	**-0.352**		**0.395**	**0.11**	**0.305**	**0.202**	**-0.252**		**1**	**-0.175**
	p	**<0.001**	>0.05	**<0.001**	**0.035**	**<0.001**	**<0.001**	**<0.001**	>0.05		**<0.001**
JMJD6	R			**-0.221**	**-0.11**	**-0.296**		**0.369**		**-0.177**	**1**
	p	>0.05	>0.05	**<0.001**	**0.035**	**<0.001**	>0.05	**<0.001**	>0.05	**<0.001**	
TWIST1	R	**0.143**	**-0.168**							**-0.214**	
	p	**0.005**	**0.001**	>0.05	>0.05	>0.05	**0.013**	>0.05	>0.05	**<0.001**	>0.05
CYCLIN D1	R	**-0.255**		**-0.30**	**-0.155**	**-0.281**	**-0.187**	**-0.16**		**-0.175**	
	p	**<0.001**	>0.05	**<0.001**	**0.002**	**<0.001**	**<0.001**	**0.001**	>0.05	**<0.001**	>0.05
AR	R	**-0.536**	**-0.357**	**0.16**		**0.265**	**0.288**	**-0.403**	**-0.14**	**0.243**	
	p	**<0.001**	**<0.001**	**0.001**	>0.05	**<0.001**	**<0.001**	**<0.001**	**0.006**	**<0.001**	>0.05

Differential expression is significant when p value is <0.05.

in our study. The expression pattern of JMJD6 and PRMT9 in PCa was quite unexpected, as there is evidence of their overexpression in various other carcinomas such as hepatocellular carcinoma (Jiang et al., 2018; Zhang et al., 2021), oral squamous cell carcinoma (Lee et al., 2016a), lung carcinoma (Vangimalla et al., 2017) and even in prostate cancer (Tong, 2021). These discrepancies may reflect a different role of these molecules in the different tumors or may be attributed to different populations studied and different methodologies used.

Of interest, a recurrent finding from both GEO and TCGA databases, also confirmed with our immunohistochemical studies, was PRMT7 overexpression in neoplastic tissue compared to non-neoplastic prostate. This finding has also been shown in a small cohort of clinical PCa samples (Li et al., 2021). In addition, common in all databases was the decrease of JMJD6 and PRMT2 expression in the tumor compared to non-neoplastic samples, a finding that we were not able to confirm with immunohistochemistry as we showed PRMT2 to be overexpressed in neoplastic cells compared to normal prostate, and further enhanced in lymph node metastatic foci. Regarding JMJD6, it was increased in neoplastic cells, though it declined in lymph node metastatic foci. In addition, in our previous work we have found PRMT1 and CARM1 to be enhanced in carcinoma compared to non-neoplastic tissue (Grypari et al., 2021). These discrepancies between the databases and our immunohistochemical studies may reflect differences in analyzing protein vs. mRNA expression and highlight the need to examine not only mRNA expression but also the functional product for meaningful conclusions to be drawn. In addition, even though in our immunohistochemical studies only the tumor cells were analyzed, the databases are based on whole tissue (cancer and cancer-associated stroma) analysis. This may further explain the discrepancies seen in our study. Finally, the limited number of samples examined in some of the datasets, along with structural differences in the databases made it difficult to match the pathology features from external studies with our cohort. For instance, the metastatic foci in the datasets included tissue from locally advanced PCa, lymph node metastatic foci and remote metastasis, while our cohort studied only lymph node metastasis. However, our findings together with previous reports of PRMT deregulation in PCa (Majumder et al., 2006; Vieira et al., 2014; Almeida-Rios et al., 2016; Deng et al., 2017; Grypari et al., 2021), underscore a potential role of PRMTs in the malignant transformation of prostate cells.

In line with our previous work (Tzelepi et al., 2019; Grypari et al., 2021), we provide evidence that PRMTs are differentially expressed during the progression of

Table 15. Spearman's correlations between mRNA expression levels of PRMTs and mediators of EMT and cell cycle based on GSE32571 dataset.

MARKER		PRMT1	PRMT2	PRMT3	PRMT4	PRMT5	PRMT6	PRMT7	PRMT8	PRMT9	JMJD6
PRMT1	R	**1**	**-0.317**							**-0.371**	**-0.439**
	p		0.014	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	**0.004**	**<0.001**
PRMT2	R	**-0.317**	**1**	**-0.343**			**-0.29**				
	p	0.014		**0.007**	>0.05	>0.05	**0.026**	>0.05	>0.05	>0.05	>0.05
PRMT3	R		**-0.343**	**1**				**-0.438**			
	p	>0.05	**0.007**		>0.05	>0.05	>0.05	**<0.001**	>0.05	>0.05	>0.05
PRMT4	R				**1**	**-0.439**	**-0.624**	**-0.38**			
	p	>0.05	>0.05	>0.05		**<0.001**	**<0.001**	**0.003**	>0.05	>0.05	>0.05
PRMT5	R				**-0.439**	**1**	**0.446**		**-0.304**		
	p	>0.05	>0.05	>0.05	**<0.001**		**<0.001**	>0.05	**0.019**	>0.05	>0.05
PRMT6	R		**-0.29**		**-0.624**	**0.446**	**1**	**0.397**			
	p	>0.05	**0.026**	>0.05	**<0.001**	**<0.001**		**0.002**	>0.05	>0.05	>0.05
PRMT7	R			**-0.438**	**-0.38**		**0.397**	**1**	**-0.263**	**-0.263**	**-0.276**
	p	>0.05	>0.05	**<0.001**	**0.003**	>0.05	**0.002**		**0.044**	**0.044**	**0.034**
PRMT8	R					**-0.304**		**-0.263**	**1**		
	p	>0.05	>0.05	>0.05	>0.05	**0.019**	>0.05	**0.044**		>0.05	>0.05
PRMT9	R	**-0.371**						**-0.263**		**1**	**0.361**
	p	**0.004**	>0.05	>0.05	>0.05	>0.05	>0.05	**0.044**	>0.05		**0.005**
JMJD6	R	**-0.439**						**-0.276**		**0.361**	**1**
	p	**<0.001**	>0.05	>0.05	>0.05	>0.05	>0.05	**0.034**	>0.05	**0.005**	
TWIST1	R	**0.264**	**-0.476**								
	p	**0.043**	**<0.001**	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
CYCLIN D1	R				**0.282**	**-0.543**	**-0.36**	**-0.387**			
	p	>0.05	>0.05	>0.05	**0.03**	**<0.001**	**0.005**	**0.002**	>0.05	>0.05	>0.05
AR	R			**-0.443**	**-0.33**	**0.29**	**0.377**	**0.287**			
	p	>0.05	>0.05	**<0.001**	**0.01**	**0.026**	**0.003**	**0.028**	>0.05	>0.05	>0.05

PCa, further supporting the notion that an epigenetic network is activated in PCa. Again a discrepancy between the TCGA data and the immunohistochemical results was noted. TCGA analysis highlighted PRMT7 as inversely correlated with aggressive pathologic characteristics (stage and grade), while an enhancement of PRMT7 cytoplasmic expression with advanced stage was observed with immunohistochemistry. These findings underscore a potential disagreement between mRNA and protein levels, as has been noted in other proteins (Tzelepi et al., 2012) and highlight the complexity of gene expression regulation. In breast cancer, Baldwin et al. demonstrated that PRMT7 is upregulated in both primary and lymph node metastatic foci and they associated its expression with tumor grade in a proportionate way (Baldwin et al., 2015). In addition, various in vitro reports have linked PRMT7 with metastasis in breast and lung carcinoma (Geng et al., 2017; Cheng et al., 2018). Regarding PCa, expression of PRMT7 in relation to stage and grade has not been studied before. An in vitro study showed that PRMTs 4, 5 and 7 co-regulate alternative splicing events and downregulation of any of them diminished cell proliferation (Li et al., 2021). Taken together, these findings are in agreement with our immunohistochemical results and indicate that PRMT7 acts as a tumor-promoting gene.

In our cohort, JMJD6 nuclear levels increased with adverse pathologic parameters, that is stage and grade. JMJD6 levels have been correlated with the androgen receptor splicing variant 7 (ARv7), a variant of AR capable of activating AR mediated transcription even in androgen depleted environments, facilitating the transition to castrate resistant PCa (Paschalis et al., 2021). ARv7 is linked with adverse prognosis and therapy resistance. It has been demonstrated that JMJD6 is upregulated as PCa evolves to castrate resistant state along with the levels of ARV7. Recent studies proposed that JMJD6 hydroxylates U2AF65, which is a splicing factor targeting AR mRNA and subsequently generates ARV7 through alternative splicing (Paschalis et al., 2021; Tong, 2021). Nuclear levels of JMJD6 were enhanced in treated tumors in our cohort, further supporting its role in an androgen depleted environment. In addition, JMJD6 has been incorporated in a multiomics panel associated with prostate cancer recurrence (Wang et al., 2021). Similarly, overexpression of JMJD6 has been associated with poor outcome in various malignancies, such as colon (Kwok et al., 2017; Yang et al., 2020), lung (Vangimalla et al., 2017), hepatocellular (Wan et al., 2019), renal cell (Zhang et al., 2021) and oral carcinoma (Lee et al., 2016b). Especially in colon cancer, JMJD6 downregulates p53 activity through posttranslational alterations and activates the WNT and MAP kinase pathways (Yang et al., 2020). In addition to these, it inhibits apoptosis through suppression of c-myc and induces activation of the EMT (Yang et al., 2020). A correlation of JMJD6 with markers of EMT was also found in our study, however, the significance of these findings remains to be elucidated. Thus, JMJD6 seems to be involved in tumor progression and therapy resistance and may represent an attractive therapeutic target in PCa.

We also examined the subcellular localization of the enzymes and found that all three enzymes were located in both cytoplasm and nucleus, as has been shown by others (Meyer et al., 2007; Yao et al., 2014; Yang et al., 2020). JMJD6 was also expressed in the cell membrane. JMJD6, has multiple isoforms, created as a result of alternative splicing, and each of them is located in different cellular compartments and has unique interactions with other proteins (Böttger et al., 2015; Vangimalla et al., 2017). Interestingly, JMJD6 was initially recognized as a phospatidylserine receptor with a membrane localization and a role in phagocytosis (Böttger et al., 2015; Vangimalla et al., 2017; Oh et al., 2019; Yang et al., 2020; Tong, 2021). This might explain the membrane staining that we noticed in our experiments. Moreover, crystallography models of JMJD6 have revealed that in the c-terminal region, the protein contains a highly conserved polyserine (polyS) domain, comprised of 16 serine residues, which determines the exact localization of the enzyme within the cell (Wolf et al., 2013; Yang et al., 2020). Alternative splicing variants of JMJD6 are deficient of the polyS region, thus, the final JMJD6 product is located in the nucleolus, an area rich in splicing variant factors for pre mRNA (Böttger et al., 2015; Kwok et al., 2017; Vangimalla et al., 2017; Yang et al., 2020). When polyS region is present, JMJD6 is detected in the nucleus (Wolf et al., 2013; Kwok et al., 2017; Zheng et al., 2019; Yang et al., 2020). Further study is needed to elucidate the role and exact localization of the various JMJD6 isoforms in cancer cells.

PRMT2 has been shown to act as an AR co-activator and its localization depends on androgens (Meyer et al., 2007). In an androgen depleted environment, both PRMT2 and AR remain in the cytoplasm. In the presence of androgens, PRMT2 and AR are transferred to the nucleus, while in the presence of an AR antagonist, PRMT2 remains in the cytoplasm, and AR is translocated to the nucleus (Meyer et al., 2007). This is the first time, to the best of our knowledge, that the expression of PRMT2 has been tested in clinical samples from treated patients. In our cohort, there was no alteration in PRMT2 levels after androgen ablation, although this may be attributed to the small number of cases that were included in the treated group. Further study with a larger number of cases is needed to validate the results of pre-clinical studies in the clinical setting.

PRMT7 is located primarily in the cytoplasm and secondarily in the nucleus, whereas its enzymic action is encountered in the nucleus (Jain and Clarke, 2019). Thus, it is speculated that there may be a transportation enzyme that mediates its translocation between the two compartments. Alternatively, PRMT7 mediated-methylation may require only a small amount of the enzyme (Yao et al., 2014; Jain and Clarke, 2019). We

have shown that nuclear expression of PRMT7 increased in treated samples. Thus, the enzyme may be activated under androgen deprivation, further enhancing its translocation to the nucleus. In depth analysis of how the microenvironment of the nucleus or the cytoplasm affects the enzyme's catalytic action is needed.

We (Grypari et al., 2021) and others (Avasarala et al., 2015; Gao et al., 2016) have shown a role of PRMTs in the induction of EMT. Similarly, in this study, we showed an important positive association of cytoplasmic levels of PRMT7, PRMT2 and JMJD6 with cytoplasmic expression of TWIST and ZEB1. Even though both ZEB1 and TWIST are transcriptional factors, and thus are mainly detected in the nucleus, it has been previously reported that both of them can be located in the cytoplasm too, specifically in prostate cancer cells (Kwok et al., 2005; Haider et al., 2016; Wang et al., 2018; Børretzen et al., 2021). Both cytoplasmic and nuclear expression have also been correlated with higher Gleason score, advanced stage and presence of lymph node metastasis (Kwok et al., 2005; Wang et al., 2018). Thus, both cytoplasmic and nuclear expression of the markers was further analyzed. RB1 regulates AR in AR-positive breast carcinomas (Graham et al., 2009) and probably this role is extended to PCa, where AR is regulated by various epigenetic modulators, such as JMJD6 (Tong, 2021). As for the regulation of ZEB1 by post translational modifications, mainly phosphorylation, ubiquitination, sumoylation and acetylation have been studied so far, while methylation is understudied and needs to be investigated (Kang et al., 2021). Parallel studies of gene expression and classical widely available techniques, such as immunohistochemistry should be combined in the same patient cohort in order to understand better the results of gene expression and minimize the discrepancies we find when we compare our findings with databases from other research groups.

Until now, PRMT1 is the only member of the PRMT family that has been found to methylate TWIST1 (Kang et al., 2021). When TWIST1 is methylated, it suppresses E-cadherin, enhancing the migration ability of neoplastic cells in non-small cell lung carcinomas (Kang et al., 2021). Mutations on the arginine residue of TWIST1 lead to a cytoplasmic gathering of the mutant product, thus implying that PRMT1 participates in the regulation of the subcellular localization of TWIST1 (Kang et al., 2021). Our correlative associations suggest another potential regulatory role of PRMT2, PRMT7 and JMJD6 in regard to TWIST1, which has to be further characterized.

A correlation of PRMT7 and Cyclin D1 was shown in our study. In our previous study, markers of the cell cycle were correlated with PRMT expression, implying that PRMTs are involved in cell cycle regulation. Cyclin D1 is a driver of cell cycle regulation and cell division (Tchakarska and Sola, 2020). In prostate cancer cells, Cyclin D1 has been found to accumulate in either the cytoplasm or the nucleus and its cytoplasmic localization is shown to be inversely associated with low proliferation rate (Comstock et al., 2007; Tchakarska and Sola, 2020). However, there are controversies in the literature as some research groups have reported that cytoplasmic Cyclin D1 expression is linked with adverse prognosis in many neoplasms, including PCa, proposing a role in tumor cell invasiveness and extraprostatic extension of tumor cells (Fusté et al., 2016). Earlier studies demonstrated that its expression pattern alters in low and high grade tumors and it was suggested that Cyclin D1 status influences the expression of other proteins in an AR dependent manner (Comstock et al., 2007). It has also been shown that Cyclin D1 lessens the transactivation of AR (Montalto and De Amicis, 2020). Further studies need to verify the exact role of PRMT7 in the regulation of Cyclin D1 expression.

Additionally, we showed an inverse correlation of JMJD6 and p53 expression. This is in agreement with the evidence that JMJD6 downregulates p53 activity in colon cancer (Yang et al., 2020) by hydroxylating p53 and antagonizing the p300 mediated acetylation (Kwok et al., 2017). In depth analysis of the role of p53 in prostate cancer is required to strengthen this association.

Finally, although the association between the PRMT family members is not well established yet, there is evidence that some of them share substrates. For example, PRMT7, PTMT5 and PRMT4 methylate hnRNPA1, although each enzyme recognizes different sequences with the molecule. HnRNPA1 is an alternative splicing factor, whose overexpression is linked with multiple malignancies, such as prostate, breast and colon cancer (Li et al., 2021). A correlation among the various PRMTs was noted in our cohort, supporting a synergistic function, though we cannot clarify the mechanism of this interaction.

In conclusion, our immunohistochemical findings suggest that PRMT2, PRMT7 and JMJD6 are part of the epigenetic network activated in PCa. JMJD6 and PRMT2 seem to be important mediators of prostate cancer progression and a potential interplay between PRMTs and EMT was identified. Further studies elucidating the functional role of these molecules are needed in light of therapeutic targeting of arginine methylation.

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