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Original Article

TET1-mediated different transcriptional regulation in prostate cancer

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Abstract: The recent studies demonstrated that the global 5-hydroxymethylcytosine (5 hmC) level decreased in prostate cancer (PCa) involved the 5-methylcytosine (5 mC) hydroxymethylase, Ten-eleven translocation (TET)1 reduction. 5 hmC and TET1 were both revealed a dual function in bivalent domain associated with developmental regulators in embryonic stem cell model. However, the mechanism underlying the DNA methylation and hydroxymethylation change mediated by TET1 downregulation in PCa remains unclear. Herein, using BSP to assess the 5 mC level in promoters of ten specific marker gene in PCa, our results present that Cdh1, Gstp1, Pten, Apc, Runx3 and Mgmt are observed to be hypermethylated in promoters and lower expression while Cyr61, Sema3c and Ptgs2 are reversed patterns compared to the normal prostate tissues. Furthermore, using ChIP methods to investigate the H3K4me3 and H3K27me3 patterns in promoters, these four markers are all demonstrated to be associated with Polycomb-repressed characterization and upregulated in response to TET1/PRC2 reduction in PCa. Thus, our findings reveal a distinct activating and repressive function of TET1-mediated transcriptional regulation in prostate cancer.

Keywords: Prostate cancer, TET1, methylation, PRC2, 5 mC, 5 hmC

Introduction

The prostate cancer (PCa) is one of the most common cancer in the males in the world [1, 2]. Epigenetic regulation disorders have been demonstrated to exert in the process of neoplasia, invasion and metastasis of PCa [3-6]. DNA methylation, one of the most studied modifications of the cytosine in CpG dinucleotide conversion to a methylated cytosine (5 mC) is observed in promoters involving in gene silencing, genome stability and chromatin remodeling [7]. In PCa, the DNA methylation patterns aberrant change of some oncogenes and antioncogenes are thought as the biomarkers for cancer classifiers in diagnosis [8]. However, the mechanisms underlying the different DNA methylation patterns established in promoters during the PCa formation still remains unclear.

The recent discovery of the further oxidizing 5mC to 5-hydroxymethylcytosine (5 hmC) catalyzed by ten-eleven translocation (TET) enzymes reveals a new epigenetic state in genomic CpG islands [9, 10]. DNA hydroxymethylation is demonstrated to be involved in an intermediate of active demethylation process in zygote programming and embryonic stem cells (ESCs) self renewal [9, 11]. Subsequently, 5 hmC is testified high distribution in pluripotent stem cells, progenitor cells and terminal differentiated tissues but not in the differentiation process and cancers [12, 13]. However, TET1 and 5 hmC are demonstrated to be associated with a dual function in transcriptional reactivation and Polycomb-repression in ESCs [14, 15]. Despite the pluripotent gene transcriptional activities and hypomethylation patterns are maintained by TET1, the presence of other TET1-targeted genes having bivalent domains of both active histone marks H3K4me3 and repressive marks H3K27me3 upregulation mediated by Polycomb repressive complexes (PRC) loss in response to TET1 depletion raise the questions that whether TET1 plays a similar role of dual functions in PCa that regulates the DNA methylated modification in the promoter of different genes.

Table 1. The sample characterization of BPH and PCa patients

No.	Disease	Age (years)	Body mass index	Prostate-specific antigen (ng/ml)	Volume (ml) prostate	Previous treatment
1	BPH	57	N/A	13.77	74	None
2	BPH	73	2.63	24.38	89	None
3	BPH	61	17.25	21.63	103	None
4	BPH	66	3.08	1.96	56	None
5	BPH	72	20.14	11.30	65	None
6	BPH	64	5.47	5.07	163	None
7	BPH	60	N/A	3.95	105	None
8	BPH	81	32.78	7.61	79	None
9	BPH	71	5.52	14.32	66	None
10	BPH	69	2.98	8.64	49	None
11	BPH	79	14.97	5.23	78	None
12	PCa	66	26.48	15.63	16	None
13	PCa	63	21.34	18.61	45	None
14	PCa	59	19.57	20.14	74	None
15	PCa	67	23.33	17.40	61	None
16	PCa	74	18.69	12.36	32	None
17	PCa	63	28.53	20.64	39	None
18	PCa	78	24.30	14.33	51	None
19	PCa	68	25.92	13.62	42	None
20	PCa	65	23.67	17.71	40	None
21	PCa	71	18.21	12.09	38	None
22	PCa	70	19.46	14.57	68	None
23	PCa	64	21.69	13.68	52	None
24	PCa	75	36.02	20.51	41	None
25	PCa	69	21.78	19.00	36	None
26	PCa	68	29.33	14.95	29	None
27	PCa	59	27.56	13.63	33	None
28	PCa	62	28.41	16.92	54	None
29	PCa	63	26.94	13.34	43	None
30	PCa	68	25.35	15.21	91	None
31	PCa	74	28.76	17.02	70	None

Herein, we verified the presence of TET1 and 5hmC loss in human carcinoma of prostate, then we applied the bisulfite sequencing and quantitative polymerase chain reaction (qPCR) methods to investigate the DNA methylation and mRNA levels of ten studied PCa specific biomarkers, Cdh1, Gstp1, Pten, Apc, Runx3, Mgmt, Cyr61, Sema3c and Ptgs2. The observation of Cdh1, Gstp1, Pten, Apc, Runx3 and Mgmt display an obvious hypermethylation pattern and lower expression while Cyr61, Sema3c and Ptgs2 showed hypomethylated promoters and higher expression in PCa tissues as compared to the normal counterparts. Since these ten genes were all validated to be bound with TET1 in promoters in previous high-throughput data, however, the promoters of Cyr61, Sema3c and Ptgs2 were meanwhile demonstrated to be the target of H3K27me3 using chromatin immunoprecipitation (ChIP) analysis. Furthermore, these bivalent genes were observed Ezh2 (subunit of PRC2) reduction in PCa. Thus, our findings reveal a distinct activating and repressive function of TET1-mediated transcriptional regulation in prostate cancer and provide the evidence of the mechanism of partial genes hypomethylation and aberrant transcriptional level in tumor.

Materials and methods

Clinical sample materials

The studies were conducted using radical surgical specimens harvested from 31 males: 20 patients with adenocarcinoma of prostate (Gleason grade 6-7) and 11 patients with benign prostatic hyperplasia (BPH) treated from 2009 to 2011 (**Table 1**) and immediately frozen in liquid nitrogen and stored at -70°C. All patients gave the written informed consents.

Immunohistochemical (IHC) staining

The IHC experiments were performed by the methods of Yang H, et al. [16]. The antibodies information: TET1 (Active motif; 61443) and 5hmC (Active motif; 39769).

DNA extraction and BSP detection

Genomic DNA was isolated from samples using the QIAamp DNA Mini Kit (Qiagen) according to the provided protocol. The 1 ug DNA whose OD

Table 2. The BSP primers sequences used in this study

Gene ID	BSP primers sequences at the promoter	PCR length (bp)	TM (°C)
Apc	F: GGGTTAGGGTTAGGTAGGTTGT	229	58
	R: ACACCTCCATTCTATCTCCAATAAC		
Cdh1	F: GAATTGTAAAGTATTTGTGAGTTTG	167	55
	R: AATACCTACAACAACAACAACAAC		
Cyr61	F: TATTTTTGAGATGTTTGAGAATTTTG	183	58
	R: TTTCACTCAAAATCCCAAC		
Gstp1	F: GTGATTTAGTATTGGGG	149	58
	R: CTAAAAACTCTAAACCCCATCCC		
Mgmt	F: AATAAAGTTTTTGGGTAAGGGGA	129	55
	R: CCCTACCATCAATAAAAAACATACC		
Pten	F: GGGGAATTTTTAGGTAAAGGTTGT	204	58
	R: CAAATAAAAAAAACCAAATAACCAC		
Ptgs2	F: AGTTAAGTGTTTTTTTGTTTTTTTT	181	58
	R: ATAATCCCCACTCTCCTATCTAATC		
Runx3	F: GGTTTTGGGTTGTGGTATTG	238	58
	R: AACAAATCCTCCAAAATCAAATAAC		
Sema3c	F: GTTATTTAGGAGGTTGAGGTAGGAGA	226	58
	R: ACAACCTTTTAAAACAAAAAAATAC		

Table 3. The primers for qPCR used in this study

Gene ID	Primer sequences	PCR length (bp)	TM (°C)
Apc	F: GACTCGGAAATGGGGTCCAA	373	60
	R: GGAACAGGACTGCACTCTCC		
Cdh1	F: TCATGAGTGTCCCCCGGTAT	240	60
	R: TCTTGAAGCGATTGCCCCAT		
Cyr61	F: GCGTTTCCCTTCTACAGGCT	258	60
	R: GCGTTTCCCTTCTACAGGCT		
Gstp1	F: TATTTCCCAGTTCGAGGCCG	236	60
	R: TCCTGCTGGTCCTTCCCATA		
Mgmt	F: ACCGTTTGCGACTTGGTACT	268	60
	R: ACCGTTTGCGACTTGGTACT		
Pten	F: GTTCTCTCCTCTCGGAAGCTG	238	60
	R: GGAAGAGGCTGCACGGTTAG		
Ptgs2	F: GCCAAGCACTTTTGGTGGAG	356	60
	R: CCTTTCTCCGCAACAGGAGT		
Runx3	F: ACACTCTGCATACGCTTCTGT	354	60
	R: GATGCTGTTCGATGCCATGC		
Sema3c	F: GAAAGGAGCAGGGTTGCGGA	317	60
	R: TCGCTCAATCAAGCACCTCG		
β-Actin	F: ATGATGATATCGCCGCGCTC	211	60
	R: TCGATGGGGTACTTCAGGGT		

cycle), 94°C for 5 s, 60°C for 10 s, 72°C for 30 s (40 cycles). The results were collected at the

of absorbance at 260/280 determined between 1.6 and 1.8 was performed for chemical conversion using Epitect Bisulfite Kit (Qiagen) according to the provided protocol. The amplicons of ten PCa specific marks' promoter in the converted DNA template were amplified using BSP primers (**Table 2**) designed by MethPrimer software (www.urogene.org/methprimer/index1.html), purified and sequenced.

RNA extraction

Total RNA was isolated from samples using RNeasy Mini Kit (Qiagen) according to the provided protocol. 100 ng RNA whose OD of absorbance at 260/280 determined more than 1.8 was then for single strand cDNA synthesis using Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the provided protocol. The primers designed for the ten PCa specific marks expression detection were listed in **Table 3**.

ChIP

The tissue samples were diagnosed by 0.1% collagenase IV/DMEM for 10min and terminated by isochoric 10% serum on ice. ChIP assay of the cell suspension solution for Ezh2 and Tet1 was carried out using ChIP-IT Express Kit (Active motif) according to the provided protocol. The information of antibodies: Ezh2 (Cell Signaling Technology, CST-4905), H3K4me3 (Active motif, 61379), H3K27me3 (Active motif, 61017). ChIP-qPCR assay was performed using the primers listed in **Table 4**.

qPCR assay

qPCR was performed using FastStart Universal SYBR Green Master Mix (Roche). The protocol was as follows: 94°C for 30 s (first threshold in which the amplification was linear and analyzed by comparative Ct methods.

Table 4. The primers for ChIP-qPCR assay used in this study

Gene ID	Primer sequences
Cyr61	F: CATTCCTGAGATGTTTGAGAATTCTG
	R: TTTCGCTCGAGGTCCCGGC
Ptgs2	F: AGCCAAGTGTCCTTCTGCCCTCCCC
	R: GTAGTCCCCACTCTCCTGTCTGATC
Sema3c	F: GCTATTCAGGAGGCTGAGGCAGGAGA
	R: GCAGCCTTTTAAGGCAAAGAGATGC

Results

Total 5hmC and TET1 levels are reduced in PCa tissues compared to the counterparts

Tumors underwent a differentiation phenotype loss and immortality attribution achievement during the tumorigenesis of the normal terminal differentiated cells. The global 5 mC level was demonstrated somewhat lower in tumor than in normal tissues, while 5 hmC level was extremely low in less differentiated cells as well as cancer [12]. Consistent with the previous studies, the presence of 5 hmC was validated to be sparsely distributed in PCa compared to the normal prostate tissues and there is no significant correlation of change between 5 mC and 5 hmC (**Figure 1**). Then, the observation of TET1 profound reduction in PCa account for 5 hmC change due to the TET1 loss (**Figure 2**). These results above suggest that 5 hmC and TET are efficiently inhibited in the adenocarcinoma of prostate.

Different DNA methylation levels change in the promoters of TET1-target genes

Since the 5 hmC catalyzed by TET required 5 mC as a substrate for oxidation, however, a modest downregulation of global 5 mC was demonstrated to be no significant correlation with 5 hmC loss in PCa. We speculated that the presence of opposite change of DNA methylation levels in many oncogene and anti-oncogene promoters offset with each other in the overall 5 mC weighting. The relationship between 5 mC and 5 hmC in detailed genes in solid tumors still remains unclear and raises the possibility that TET1 reduction may impact on both 5 hmC and 5 mC in PCa. Previous studies reported that TET1 binding in two categories of targeted genes, one group was associated with bivalent domains including both H3K4me3 and H3K27me3 [17], the other one was only associated with active histone marks. Therefore, TET1 knockdown resulted in both genes upregulaion and downregulation in ESCs [14]. In accordance with these reasons, we aimed to investigate the DNA methylation of ten TET1-mediated genes listed in Wu, et al' work [14] which were widely utilized as the biomarkers in clinical PCa diagnosis: Apc, Cdh1, Cyr61, Gstp1, Mgmt, Pten, Ptgs2, Runx3 and Sema3c (**Table 5**). Interestingly, the observation of mRNA expression (**Figure 3**) and DNA methylation levels (**Figure 4**, Figure S1) of these genes in prostate cancer exhibit the consistent trend in previous studies of embryonic stem cells. The only TET-1 targeted genes, Cdh1, Gstp1, Pten, Apc, Runx3 and Mgmt, were demonstrated to be diminished transcriptional level and hypermethylated in promoters. In contrast, the bivalent genes, Cyr61, Sema3c and Ptgs2 display high expressed and hypomethylation in PCa.

H3K4me3 and H3K27me3 modified bivalent genes in PCa

The presence of only TET1-targeted gene mRNA expression and DNA methylation patterns in PCa attributed to the TET1-mediated hydroxymethylation loss and methylation accumulation. Nevertheless, with regard to the bivalent genes, Cyr61, Sema3c and Ptgs2, TET1 reduction resulted to the reversed higher transcriptional expression. It did raise the questions of what the histone modifications at proximal promoters of bivalent genes were in PCa. We investigated the binding profiles of H3K4me3 and H3K27me3, a group of antipodal function of chromatin activity in Cyr61, Sema3c and Ptgs2 promoters in PCa. ChIP-qPCR confirmed the effect of TET1 loss in PCa gave rise to H3K27me3 downregulation, but no obvious change of H3K4me3 binding (**Figure 5**) than the normal prostate tissues.

Discussion

The hydroxymethylase TET1 has been demonstrated to catalyzed 5 mC to 5 hmC for demethylation process in early embryo preimplantation development [9], somatic cell reprogramming [18], ESCs self-renewal [19] and tumorigenesis [20]. This type of active demethylation mediated by TET enzymes plays an important role in facilitating a transcriptional

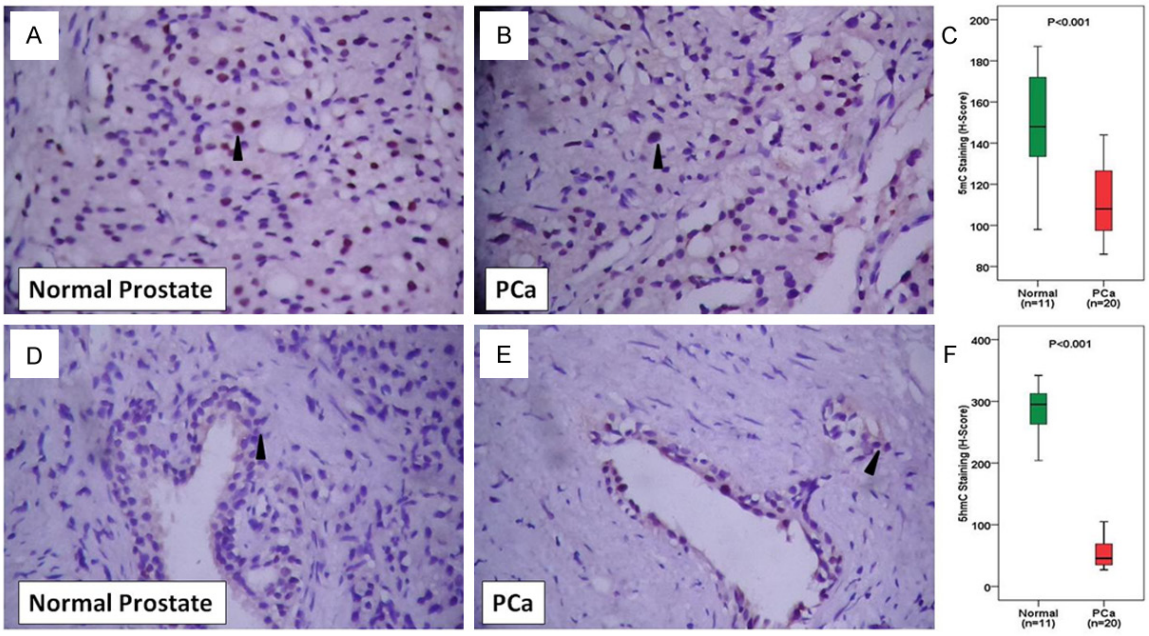

Figure 1. The 5 mC and 5 hmC distribution in normal prostate and PCa. Micrographs of 5mC staining patterns in normal human prostate (A) and PCa (B) as well as 5 hmC in prostate (D) and PCa (E). (C and F) represent the box plot of distribution of semi-quantitative intensities scores. Arrowheads show the positive 5 mC or 5 hmC staining.

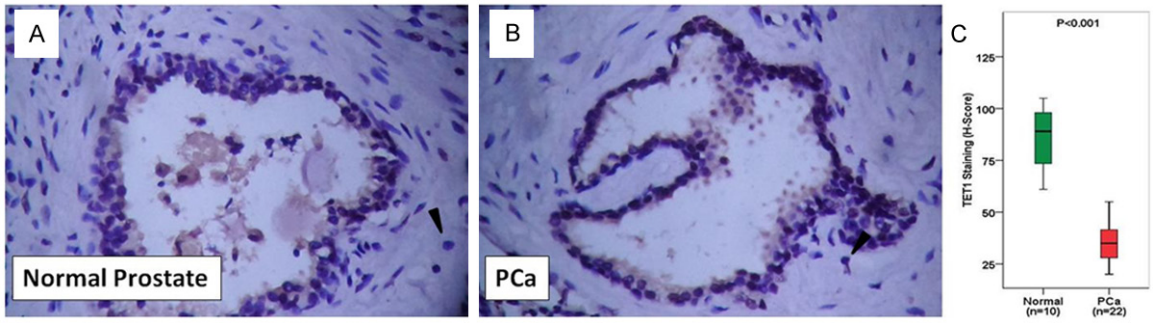

Figure 2. The IHC staining of TET1 in normal prostate and PCa. TET1 distribution in normal prostate tissues (A) and PCa (B). (C) Means the statistical box plot of distribution of semi-quantitative intensities scores. Arrowheads show the positive TET1 staining.

Table 5. The chromatin state information of Tet1-targeted genes in this study

	TET1 targeted	H3K-4me3	H3K-27me3	Bivalent	Status in cancer
Apc	√	√	×	×	↓ [22]
Cdh1	√	√	×	×	↓ [23]
Cyr61	√	√	√	√	↑ [24, 25]
Gstp1	√	√	×	×	↓ [26, 27]
Mgmt	×	√	×	×	↓ [28]
Pten	√	√	×	×	↓ [29]
Ptgs2	√	√	√	√	↓ [30] ↑ [31, 32]
Runx3	√	×	×	×	↓ [22]
Sema3c	√	√	√	√	↑ [33, 34]

active state of TET1-targeted genes with hypomethylated promoters for pluripotency maintenance in ESCs. Furthermore, the recent studies answer the other side of this question that the developmental regulators and somatic lineage differentiation genes expression are restrained due to the silencing mechanism of Polycomb proteins recruited by TET1 [14, 15]. These Polycomb targeted genes are thought as the bivalent genes whose properties are simultane-

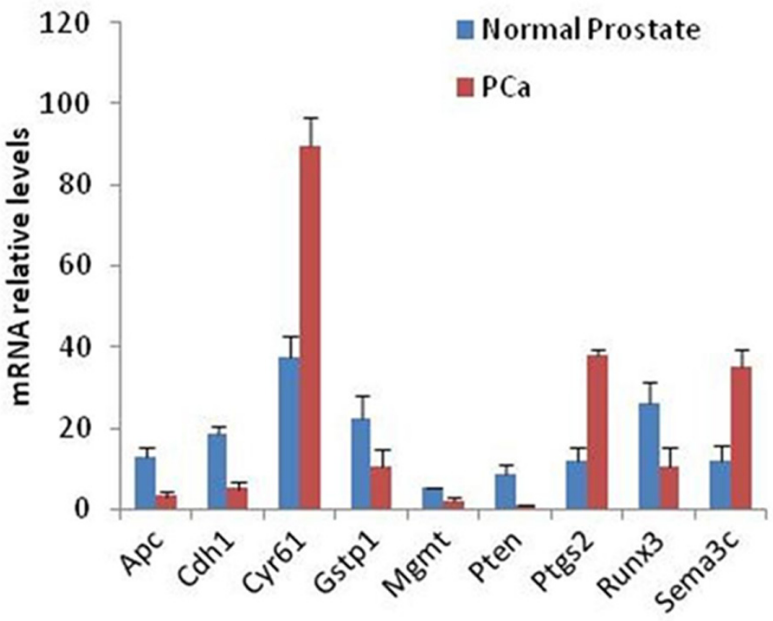

Figure 3. The mRNA relative level of nine biomarkers in PCa. The transcriptional level of tissues were investigated using realtime PCR analysis. The samples size is 11 normal prostate and 20 PCa. Error bars represent SD.

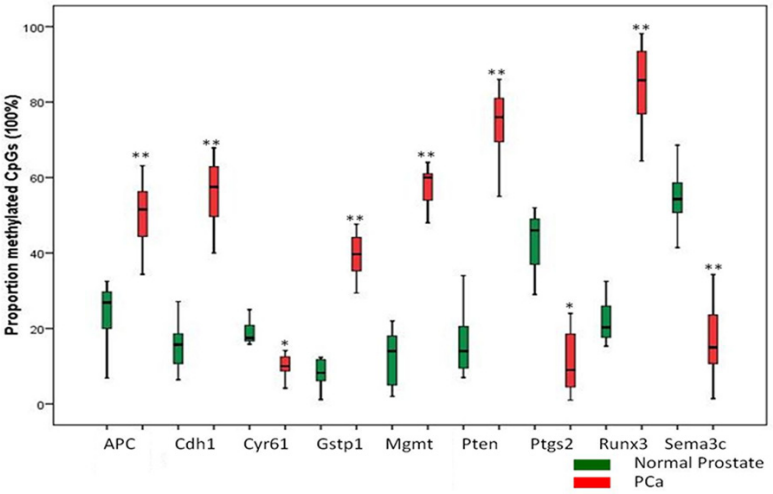

Figure 4. The CpGs methylation ratio at proximal promoters of nine biomarkers in PCa. The methylation patterns of each sample from 11 normal prostate and 20 PCa tissues were detected at least 10 clones for sequencing using BSP. "*" and "**" represent $P < 0.05$ and $P < 0.001$ respectively.

ously bound with H3K4me3 and H3K27me3. It might conclude that TET1 promote the pluripotent genes transcription, meanwhile inhibit the differentiated ones. It reminds of us a similar model of cancer that the oncogenes and anti-oncogenes exhibit reversed patterns of mRNA expression. Since some of the developmental regulators have been demonstrated as the bivalent genes and meanwhile the significant biomarkers in cancers, it interests us to validate the regulation of downstream targeted genes resulted from TET1 reduction in cancer.

We searched a list of TET1-targeted genes having a potential different trend of DNA methylation during the tumorigenesis of prostate (**Table 5**). The expression of only TET1-targeted genes and Mgmt not TET1-targeted but H3K4me3-targeted are all downregulated (**Figure 3**) demonstrated that TET1 reduction result in H3K4me3 loss to impact on transcriptional process of binding genes. Whereas the presence of bivalent genes all upregulated in PCa exhibit unapparent H3K4me3 loss (**Figure 5**) indicates that H3K27me3 is prior to H3K4me3 for transcriptional regulation in the model of TET1 mediated Polycomb repressive complex recruitment for bivalent gene silencing [21]. Taken together, we demonstrated the TET1's dual function for the different types of genes transcriptional regulation in tumor.

Besides a reduced TET1 level in PCa, 5hmC is also revealed downregulated conclusively but not in accordance with 5 mC (**Figures 1**, **2**) which is consistent with the previous results [12]. From the BSP results, the CpG islands of only TET1-targeted gene promoters are observed hypermethylated in PCa than normal prostate tissues, while the bivalent ones display a hypomethylated landscape (**Figure 4**). Since BSP cannot efficiently distinguish 5 mC and 5 hmC, DNA methylation ratio represent the aggregation of 5 mC and 5 hmC. Thus, 5 mC level of the only TET-1 targeted gene promoters apparently increase in PCa. The most widely view is that TET1 reduction restrain from 5 mC to 5 hmC

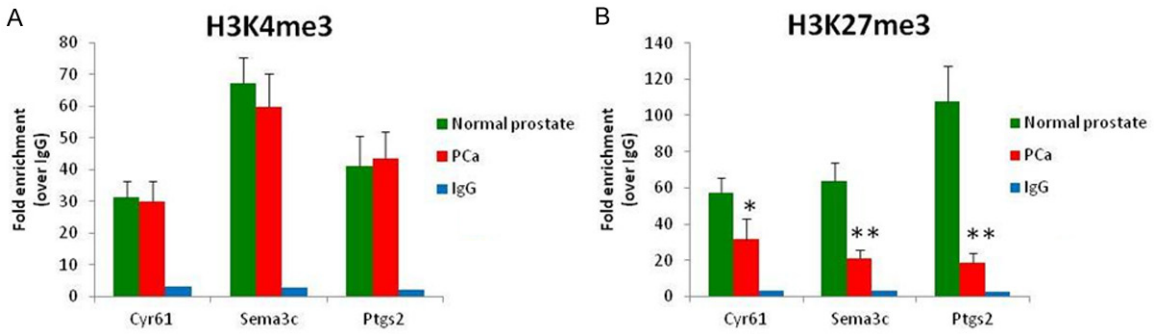

Figure 5. The triple-methylation state of H3K4 and H3K27 in proximal promoters of the three bivalent genes in PCa. ChIP-qPCR analysis of H3K4me3 and H3K27me3 occupancy at the promoters of Cyr61, Sema3c and Ptgs2, these three bivalent genes. "*" and "**" represent $P < 0.05$ and $P < 0.001$ respectively.

process [12]. Nevertheless, it cannot beconcluded that how 5 mC level in the bivalent genes change. With the presence of the total 5 mC level modest decreased in PCa, there may be somewhat augment of 5 mC level to compensate its loss in other genes such as only TET1 targeted ones. But it is also in conflict with the hypermethylated promoters resulted in gene silencing. Certainly, the genomic 5 mC distribution is not completely associated with TET1 and the 5 mC level change in the bivalent gene promoter in tumorigenesis still need to be further investigated. Collectively, our study identifies TET1 have a dual regulative function for genes transcription in PCa and provides evidence for the mechanism of cancer-associated methylation modification change.

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Disclosure of conflict of interest

None.

Abbreviations

PCa, prostate cancer; 5 mC, 5-methylcytosine; 5 hmC, 5-hydroxymethylcytosine; TET, ten-eleven translocation; ESCs, embryonic stem cells; PRC, polycomb repressive complexes; qPCR, quantitative polymerase chain reaction; BSP, bisulfite sequencing PCR; ChIP, chromatin immunoprecipitation.

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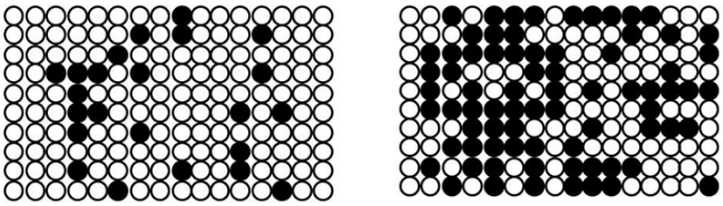

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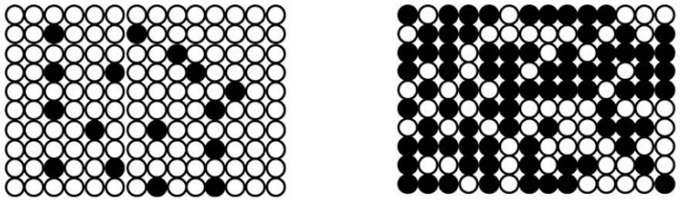

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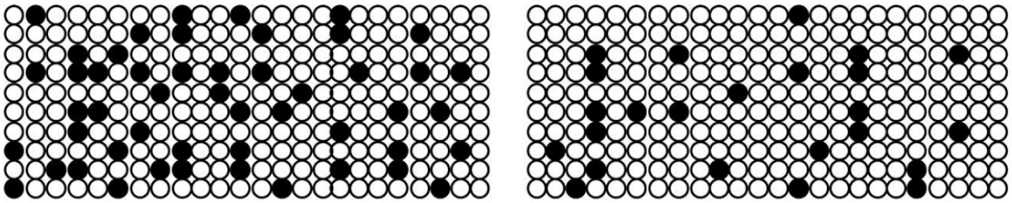

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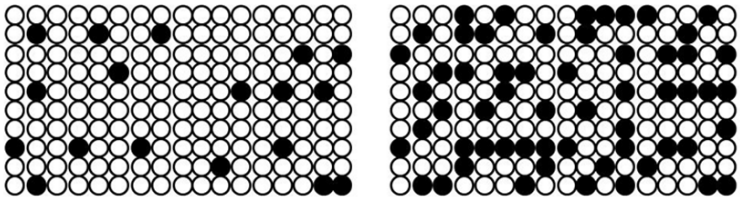

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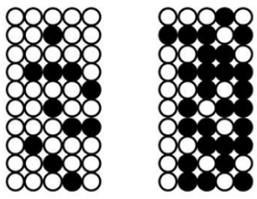

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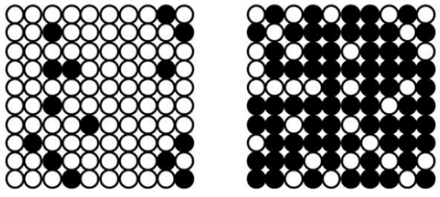

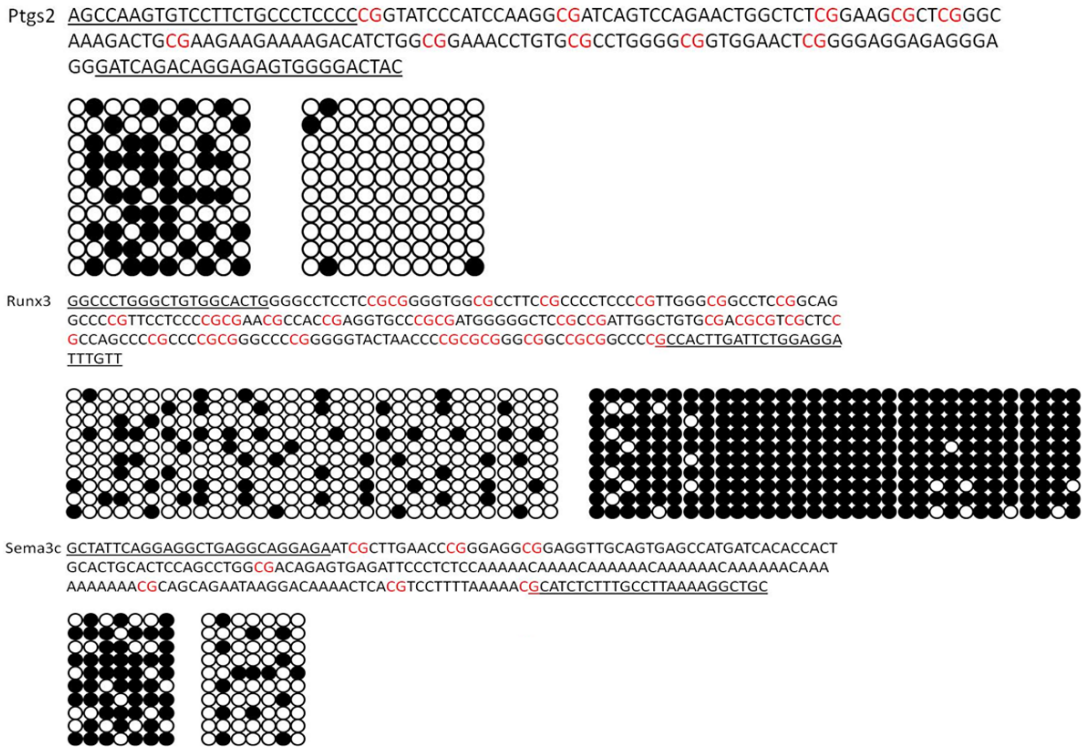

Figure S1. The DNA methylation patterns of the gene promoter region in 11 normal prostate and 20 PCa samples.