Transcriptomic Analysis of Histone Methyltransferase Setd7 Knockdown and Phenethyl Isothiocyanate in Human Prostate Cancer Cells

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Abstract. *Background/Aim:* Transcriptomic analysis was performed to evaluate the differential gene expression profiles of Setd7 knockdown (KD) and the effects of phenethyl isothiocyanate (PEITC) in human prostate cancer (PCa) LNCaP cells. *Materials and Methods:* RNA isolated from wild-type and Setd7-KD LNCaP cells in the presence or absence of PEITC was subjected to microarray analysis followed by Ingenuity® Pathway Analysis (IPA). *Results:* Setd7 KD impacted a larger set of genes and caused a higher fold change compared to PEITC treatment. Several signaling pathways were altered particularly inflammation-related TNFR signaling and PTEN/PI3K/AKT signaling by Setd7 KD and PEITC. Interestingly, PEITC and Setd7 KD at a small subset of genes that could be potential molecular targets. *Conclusion:* This study offers new insights into the mechanisms of action of the epigenetic modifier Setd7 and the effects of PEITC treatment in PCa cells and enhances our understanding of the potential cancer preventive/treatment effects of isothiocyanate compounds such as PEITC in PCa.

In recent years, many epidemiological studies have suggested that the consumption of cruciferous vegetables can

reduce the incidence of PCa (1). Isothiocyanates (ITCs), such as phenethyl isothiocyanate (PEITC), have been reported to have chemoprotective, antitumor, anti-inflammatory, and HDAC- and/or DNA methyltransferase (DNMT)-inhibitory properties (2-11). In addition, PEITC reduced the risk of PCa in mouse models and suppressed the growth of PCa cell xenografts *in vivo* (12-14). PCa is one of the most common cancers among men in the United States. Approximately 1 out of 39 men will die of prostate cancer, and the risk increases as men age. Thus, new strategies are required to prevent, or slow the initiation and progression of PCa. Recent studies revealed that PEITC and Setd7 affect many targets involved in various signaling pathways. However, the mechanism of interaction of PEITC and Setd7 in PCa has not yet been previously explored.

Accumulating evidence suggests that epigenetic regulation contributes to cancer initiation and progression and may precede genetic mutation. Setd7 (also known as Set7/9) is a SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) domain-containing lysine methyltransferase that targets lysine 4 on histone H3 (H3K4). Setd7 exhibits monomethyltransferase activity and promotes an open chromatin architecture, transcriptional activation and target genes, expression (15). Gene transcription is regulated genetically through mutations and epigenetically through covalent histone modification and DNA methylation that either promote or restrict the gene promoters' accessibility to components of the basic transcriptional machinery (16, 17). Recently, many studies have shown that Setd7 plays multiple roles in regulating, not only histone assembly but also non-histone protein stability and/or activity, including that of Dnmt1, p65, ER α , AR, and Pcaf (18-22). Several groups have shown that Setd7 contributes to inflammation through interactions with components of the NF- κ B signaling pathway, thus leading to the down-regulation or up-regulation of NF- κ B activity depending on the cell type and/or physiological conditions (20, 23-25).

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In prostate cancer (PCa), Setd7 methylates the androgen receptor (AR), shows pro-proliferative and anti-apoptotic activity, and exhibits up-regulated nuclear expression (26). Recent whole-transcriptome sequencing results indicated that *Setd7* has frameshift mutations in castration-resistant prostate cancer (CRPC), which could lead to altered chromatin accessibility in co-transcriptional RNA processing (27). Only a few publications exist on Setd7 and PCa, but increasing evidence indicates the importance and potential of Setd7 in PCa therapeutics and diagnostics. A spate of recent studies on Setd7 and PEITC revealed that many targets involved in various signaling pathways can be influenced by these factors. However, whether PEITC affects epigenetic regulation by Setd7 in PCa and the global transcriptomic profiling has not yet been previously explored. In this study, Affymetrix™ oligonucleotide microarrays were used to analyze differential gene expression and signaling pathways induced by PEITC and the impact of Setd7 KD in human LNCaP cells.

Materials and Methods

Materials. RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/ml), puromycin, versene, and trypsin-EDTA were supplied by Gibco (Grand Island, NY, USA). PEITC and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture and treatment. The PCa LNCaP cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in RPMI 1640 with 10% FBS at 37°C in a humidified 5% CO₂ atmosphere. Cells were grown to approximately 80% confluence and treated with medium containing PEITC (5 µM) at the indicated concentrations for 24 h or other indicated times as previously described (28). DMSO (0.1%) was used as the vehicle control.

Setd7 knockdown in LNCaP cells. Lentivirus-mediated short hairpin RNAs (shRNAs) were used to establish stable mock (control, sh-Mock) and Setd7 knockdown (sh-SETD7) LNCaP cells. The shRNA clone sets were obtained from GeneCopoeia (Rockville, MD, USA), and lentiviral-mediated transduction was performed according to the manufacturer's protocol as previously described (28).

RNA isolation and oligonucleotide microarray analyses for transcriptome profiling. Total RNA was extracted from cells using a RNeasy Mini kit (Qiagen, Valencia, CA, USA). The quality and quantity of the extracted RNA samples were determined using an Agilent 2100 Bioanalyzer and a NanoDrop instrument, respectively. A total of 4 RNA samples [1 sample per group x 4 groups (DMSO- or PEITC-treated LNCaP and LNCaP Setd7-KD cells)] were sent to RUCDR for subsequent technical procedures including quality control, RNA concentration measurement, cDNA synthesis, cRNA biotin labeling, hybridization, and scanning of the arrays. In brief, the samples were applied to the GeneChip Human Gene 2.0 ST Array (Affymetrix, Santa Clara, CA, USA), which contains probes for the detection of >40,000 transcripts, >25,000 genes, and >11,000 long intergenic non-coding RNA (lincRNA) transcripts. The expression value for each gene was determined by calculating the average of differences in intensity (perfect match intensity minus mismatch

intensity) between its probe pairs and was stored in CEL data files. **Computational analyses of microarray data.** CEL data files were imported into R (version 3.4.2) using the package *oligo* (29). Pseudo-image plots were used to assess the spatial distribution of the data on the chips. Due to differences in the magnitude of the readings, rank-transformed data were plotted to avoid the masking of spatial features that may have been present on the array (Figure 1A). The data were normalized by applying the *robust multiple average* (RMA) algorithm (R function *rma* from package *oligo*) for background subtraction, quantile normalization, and summarization (via median-polish) (30, 31) and annotated with the *pd.hugene.2.0.st* R package (Figure 1B). The normalized values for the 150 top differentially expressed genes across 4 groups (WT, KD, WT PEITC, and KD PEITC) are presented in Figure 2.

Ingenuity Pathway Analysis (IPA). Isoforms with log2 fold changes greater than 0.5 were subjected to Ingenuity Pathway Analysis (IPA 4.0, Ingenuity Systems, www.ingenuity.com). The input isoforms were mapped to IPA's knowledgebase, and the relevant biological functions, networks, and pathways related to Setd7 knockdown and PEITC treatments were identified.

Quantitative polymerase chain reaction (qPCR) for microarray data validation. qPCR was used to validate selected differentially expressed genes detected by microarrays. Following the manufacturer's instructions, first-strand complementary DNA was synthesized from 1 µg of RNA using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA, USA). qPCR analysis was performed on a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems) and complementary DNA as the template. The sequences of the primers used in PCR amplifications are listed in Table I. The PCR conditions were as follows: initial denaturation for 10 min at 95°C followed by 40 cycles at 95°C for 15 sec and 1 min at 60°C. Melting curve analysis was performed to ensure the specificity of the products. The relative gene expression levels were normalized to the GAPDH level using the $2^{-\Delta\Delta C_T}$ formula ($\Delta\Delta C_T = \Delta C_T \text{ sample} - \Delta C_T \text{ control}$).

Statistical analysis. Statistical significance was assessed with one-way ANOVA followed by Dunnett's *post-hoc* test among multiple experimental groups and Student's *t*-test between two experimental groups using GraphPad PRISM software. The values are presented as the means±standard deviation (SD), and *p*-values less than 0.05 were considered statistically significant.

Results

Top differentially expressed genes and canonical pathways affected in LNCaP Setd7-KD cells and LNCaP cells. Setd7, an epigenetic modifier plays a prominent role in histone and non-histone protein lysine methylation. To understand the effects of Setd7 KD in LNCaP PCa cells, the gene expression profile of wild-type LNCaP cells was compared to that of Setd7-KD cells. A total of 651 differentially expressed genes showed a log2 fold change greater than 0.5 or less than -0.5. The top 10 genes most down-regulated by Setd7 KD and the top 10 genes most up-regulated by Setd7 KD are presented in Table II. These genes had dramatic fold changes (as low as 0.150-fold to as high as 28.06-fold) when

Figure 1. Pseudo-image plots were used to assess the spatial distribution of the data on the chips. (A) Rank transformed data. (B) Normalized annotated data.

Figure 2. The 150 top differentially expressed genes with normalized values across 4 groups (WT, KD, WT PEITC, and KD PEITC).

Setd7 expression was KD. Interestingly, the tumor suppressor gene *Rarres3* and the antioxidant gene *Sod2* were up-regulated 28.06-fold, and 5.66-fold, respectively, when Setd7 was knocked down. Canonical pathway analysis in IPA was used to understand the possible biological functions associated with these differentially expressed genes. Based on the ratio between the number of differentially expressed genes within our datasets belonging to a given pathway and the total number of reference genes in the corresponding pathway in the IPA knowledgebase, Fisher's exact test was used to identify significantly affected canonical pathways. Using a *p*-value cutoff of less than 0.05, a total of 377 canonical pathways were identified as significantly

Table I. The primers used in qPCR.

Primer	Sequence (5'→3')
SI For	AAATCAGACACCCAATCGTTTCC
SI Rev	GGGCAACCTTCACATCATACAA
PCDHB13 For	CACCCTACTAACGGAGAGACC
PCDHB13 Rev	GTCATTGACATCGGCGATCAG
CD24 For	CTCCTACCCACGCAGATTTATTC
CD24 Rev	AGAGTGAGACCACGAAGAGAC
SDC4 For	GGACCTCCTAGAAGGCCGATA
SDC4 Rev	AGGGCCGATCATGGAGTCTT
RAB31 For	GGGGTTGGGAAATCAAGCATC
RAB31 Rev	GCCAATGAATGAAACCGTTTCCT
GADD45B For	TACGAGTCGGCCAAAGTTGATG
GADD45B Rev	GGATGAGCGTGAAGTGGATT
SACS For	GCGCGATGTGAAGGAACGTA
SACS Rev	TCTCCAATCTTGATCCAGTCAGA
GH2 For	GACACAAAATCGCACAAACGATG
GH2 Rev	GGGCAGCTAGAAGCCACAG
ID1 For	CTGCTCTACGACATGAACGG
ID1 Rev	GAAGGTCCCTGATGTAGTCGAT
LXN For	GAAATCCCGCCGACCAACTA
LXN Rev	CTGCACCTCAAACACCTGT
LRRTM1 For	TCGGGCAACGAGATCGAGTA
LRRTM1 Rev	GCTTGTCAGGGACTTCCAAGA
FCRL6 For	CTGGGCTTTACTGGTGTGAGG
FCRL6 Rev	TGAGCACAGGACGGGATACA
THSD7A For	TCAGTGTGAGCCCGTGATTTC
THSD7A Rev	GATGCACGCTATCTCCCTCA
APOM For	GCTACCATCCGCATGAAAGAT
APOM Rev	CTGGCCTGTCTCATTACAGCA
ARMC12 For	CAAAAGCGTAGTCAGCCTGG
ARMC12 Rev	GATGCAGATGGGTGAGTTGC
TKTL1 For	ACAAGCAGTCAGATCCAGAGA
TKTL1 Rev	TAGCTGGCCCTGTGGAAGTA
ZNF235 For	CCTCTTACCCAGCGTAGTATTCA
ZNF235 Rev	CCTGAGCTATAACTGGTGTCTC
TPM3 For	TGAAAACCGGGCCTAAAAGAT
TPM3 Rev	GATCACCAACTTACGAGCCAC
DCAF4L1 For	CAGCGACCAGCTCTTCGTAG
DCAF4L1 Rev	TGGGGACGTAGAGGTTTCTGA
CBLN2 For	GGCGTTTGGAGCAACTTCG
CBLN2 Rev	TTCAAGGCCAGGGTCGTTT
TAS2R19 For	GAAGATGCGGCTCCATAGCAA
TAS2R19 Rev	TGGCAAAGCAGGAGTACAAGT
OR9A2 For	ATGTATCGCTCAACTTTTCCTGT
OR9A2 Rev	CCCATGACACTATTACCACCCAA

correlated with Setd7 KD in LNCaP cells. Table III displays the 10 most significant pathways, log(*p*-value) scores, the ratio of affected genes to total genes in that particular pathway, and details regarding significantly differentially expressed genes. The top 3 canonical pathways significantly influenced by Setd7 KD were "Death Receptor Signaling", "TNFR2 Signaling", and "Acute Phase Response Signaling". Based on these results, Setd7 KD significantly affected the expression of many genes.

Table II. Top 10 down-regulated and up-regulated genes in LNCaP Setd7-KD cell compared to LNCaP cell. Fold change larger than 1 indicates higher expression in LNCaP Setd7-KD cell. Fold change smaller than 1 indicates lower expression in LNCaP cell.

Gene ID	Gene name	Fold Change	Location
Down-regulated			
<i>BSG</i>	Basigin (Ok blood group)	0.150	Plasma Membrane
<i>PLOD2</i>	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	0.197	Cytoplasm
<i>DNAJC15</i>	DnaJ heat shock protein family (Hsp40) member C15	0.213	Cytoplasm
<i>TSPAN6</i>	Tetraspanin 6	0.242	Plasma Membrane
<i>SCD</i>	Stearoyl-CoA desaturase	0.248	Cytoplasm
<i>TMEFF2</i>	Transmembrane protein with EGF like and two follistatin like domains 2	0.268	Cytoplasm
<i>ACKR3</i>	Atypical chemokine receptor 3	0.274	Plasma Membrane
<i>DSEL</i>	Dermatan sulfate epimerase-like	0.278	Extracellular Space
<i>TUBB4A</i>	Tubulin beta 4A class IVa	0.289	Cytoplasm
<i>TMPRSS2</i>	Transmembrane protease, serine 2	0.311	Plasma Membrane
Up-regulated			
<i>RARRES3</i>	Retinoic acid receptor responder 3	28.06	Cytoplasm
<i>GBP2</i>	Guanylate binding protein 2	12.76	Cytoplasm
<i>ID1</i>	Inhibitor of DNA binding 1, HLH protein	8.43	Nucleus
<i>JUNB</i>	JunB proto-oncogene, AP-1 transcription factor subunit	7.47	Nucleus
<i>NMI</i>	N-myc and STAT interactor	7.31	Cytoplasm
<i>BIRC3</i>	Baculoviral IAP repeat containing 3	7.22	Cytoplasm
<i>OSMR</i>	Oncostatin M receptor	6.24	Plasma Membrane
<i>CASP4</i>	Caspase 4	5.77	Cytoplasm
<i>SOD2</i>	Superoxide dismutase 2	5.66	Cytoplasm
<i>TNFAIP3</i>	TNF alpha induced protein 3	5.44	Nucleus

Table III. The 10 most significant canonical pathways regulated by Setd7 knockdown compared to normal LNCaP cell. Genes in bold are down-regulated in LNCaP Setd7-KD cells.

Canonical pathways	-Log (p-Value)	Ratio	Target genes
Death receptor signaling	5.6	27/76 (0.355)	<i>MAP2K4</i> , <i>TNFSF10</i> , <i>PARP12</i> , <i>MAP3K5</i> , <i>NFKB1</i> , <i>PARP9</i> , <i>IKBKB</i> , <i>NFKBIA</i> , <i>TNFSF15</i> , <i>CASP8</i> , <i>BIRC3</i> , <i>PARP14</i> , <i>CASP10</i> , <i>TNFRSF21</i> , <i>TNFRSF1A</i> , <i>ACTB</i> , <i>PARP8</i> , <i>TBK1</i> , <i>PARP3</i> , <i>NFKB2</i> , <i>XIAP</i> , <i>DAXX</i> , <i>TRAF2</i> , <i>RIPK1</i> , <i>TNF</i> , <i>CASP7</i> , <i>HSPB1</i>
TNFR2 signaling	4.26	12/25 (0.480)	<i>MAP2K4</i> , <i>IKBKB</i> , <i>TRAF2</i> , <i>NFKBIA</i> , <i>JUN</i> , <i>TNFAIP3</i> , <i>TBK1</i> , <i>NFKB2</i> , <i>NFKB1</i> , <i>BIRC3</i> , <i>TNF</i> , <i>XIAP</i>
Acute phase response signaling	3.67	25/85 (0.294)	<i>MAP2K4</i> , <i>IL6ST</i> , <i>PIK3CA</i> , <i>FN1</i> , <i>TNFRSF1A</i> , <i>PDPK1</i> , <i>CEBPB</i> , <i>NFKB2</i> , <i>STAT3</i> , <i>MAP3K5</i> , <i>NFKB1</i> , <i>HNRNP</i> , <i>C5</i> , <i>HMOX1</i> , <i>IKBKB</i> , <i>TRAF2</i> , <i>SOD2</i> , <i>JUN</i> , <i>RBP7</i> , <i>NFKBIA</i> , <i>RIPK1</i> , <i>MAP2K2</i> , <i>SOC2</i> , <i>OSMR</i> , <i>TNF</i>
Neuroinflammation signaling pathway	3.4	38/155 (0.245)	<i>MAP2K4</i> , <i>B2M</i> , <i>TRAF3</i> , <i>PIK3CA</i> , <i>ICAM1</i> , <i>HLA-A</i> , <i>TGFB3</i> , <i>HLA-B</i> , <i>MFGE8</i> , <i>NFKB1</i> , <i>TGFB2</i> , <i>HMOX1</i> , <i>IKBKB</i> , <i>SOD2</i> , <i>JUN</i> , <i>FGFR4</i> , <i>TLR7</i> , <i>CASP8</i> , <i>STAT1</i> , <i>BIRC3</i> , <i>TNFRSF1A</i> , <i>CREB3</i> , <i>IFNGR2</i> , <i>GABBR1</i> , <i>IFNGR1</i> , <i>TBK1</i> , <i>NFKB2</i> , <i>PLA2G2A</i> , <i>ACVR1B</i> , <i>XIAP</i> , <i>APP</i> , <i>RIPK1</i> , <i>APH1A</i> , <i>GLUL</i> , <i>PTGS2</i> , <i>TNF</i> , <i>ACVR2A</i> , <i>IRAK2</i>
Agrin interactions at neuromuscular junction	3.15	15/44 (0.341)	<i>MAP2K4</i> , <i>ARHGEF7</i> , <i>ACTB</i> , <i>ITGA2</i> , <i>ITGA6</i> , <i>LAMC1</i> , <i>PTK2</i> , <i>ITGB2</i> , <i>JUN</i> , <i>ARHGEF6</i> , <i>UTRN</i> , <i>DAG1</i> , <i>ERBB2</i> , <i>CTTN</i> , <i>EGFR</i>
TNFR1 signaling	3.15	15/44 (0.341)	<i>MAP2K4</i> , <i>TNFRSF1A</i> , <i>TNFAIP3</i> , <i>NFKB2</i> , <i>NFKB1</i> , <i>XIAP</i> , <i>IKBKB</i> , <i>TRAF2</i> , <i>RIPK1</i> , <i>JUN</i> , <i>NFKBIA</i> , <i>CASP8</i> , <i>BIRC3</i> , <i>TNF</i> , <i>CASP7</i>
Role of RIG1-like receptors in antiviral innate immunity	3.02	10/24 (0.417)	<i>IKBKB</i> , <i>TRAF3</i> , <i>TRAF2</i> , <i>RIPK1</i> , <i>NFKBIA</i> , <i>TBK1</i> , <i>NFKB2</i> , <i>CASP8</i> , <i>NFKB1</i> , <i>CASP10</i>
OX40 signaling pathway	3.02	11/28 (0.393)	<i>B2M</i> , <i>MAP2K4</i> , <i>BCL2L1</i> , <i>TRAF3</i> , <i>TRAF2</i> , <i>NFKBIA</i> , <i>JUN</i> , <i>HLA-A</i> , <i>HLA-B</i> , <i>NFKB2</i> , <i>NFKB1</i>
T Helper cell differentiation	2.87	11/29 (0.379)	<i>IL6ST</i> , <i>TGFB2</i> , <i>TNFRSF1A</i> , <i>HLA-A</i> , <i>HLA-B</i> , <i>IFNGR2</i> , <i>IFNGR1</i> , <i>STAT3</i> , <i>STAT1</i> , <i>BCL6</i> , <i>TNF</i>
Induction of apoptosis 1 by HIV	2.78	16/52 (0.308)	<i>MAP2K4</i> , <i>TNFRSF1A</i> , <i>SLC25A3</i> , <i>MAP3K5</i> , <i>NFKB2</i> , <i>NFKB1</i> , <i>XIAP</i> , <i>BCL2L1</i> , <i>IKBKB</i> , <i>DAXX</i> , <i>TRAF2</i> , <i>RIPK1</i> , <i>NFKBIA</i> , <i>CASP8</i> , <i>BIRC3</i> , <i>TNF</i>

Table IV. Top 10 down-regulated and up-regulated genes in LNCaP Setd7-KD cell treated by PEITC compared to DMSO group. The expression of these genes in DMSO group was set to 1.

Gene ID	Gene name	Fold Change	Location
Down-regulated			
<i>TIMP1</i>	TIMP metalloproteinase inhibitor 1	0.369	Extracellular space
<i>TNKS</i>	Tankyrase	0.392	Nucleus
<i>HLA-DRB1</i>	Major histocompatibility complex, class II, DR beta 1	0.403	Plasma membrane
<i>GNAI2</i>	G protein subunit alpha i2	0.413	Plasma membrane
<i>H6PD</i>	Hexose-6-phosphate dehydrogenase/glucose 1-dehydrogenase	0.429	Cytoplasm
<i>PRKCA</i>	Protein kinase C alpha	0.439	Cytoplasm
<i>GRN</i>	Granulin precursor	0.439	Extracellular space
<i>NOP58</i>	NOP58 ribonucleoprotein	0.455	Nucleus
<i>mir-449</i>	microRNA 449a	0.457	Cytoplasm
<i>NMB</i>	Neuromedin B	0.459	Extracellular space
Up-regulated			
<i>RBBP4</i>	RB binding protein 4, chromatin remodeling factor	3.65	Nucleus
<i>CNOT7</i>	CCR4-NOT transcription complex subunit 7	3.21	Nucleus
<i>EXT2</i>	Exostosin glycosyltransferase 2	2.93	Cytoplasm
<i>PTK2</i>	Protein tyrosine kinase 2	2.58	Cytoplasm
<i>PHB</i>	Prohibitin	2.36	Nucleus
<i>RAB11B</i>	RAB11B, member RAS oncogene family	2.32	Cytoplasm
<i>ARFIP2</i>	ADP ribosylation factor interacting protein 2	2.23	Cytoplasm
<i>GNB2</i>	G protein subunit beta 2	2.11	Plasma membrane
<i>BCL2L2</i>	BCL2 like 2	2.08	Cytoplasm
<i>SERPINE1</i>	Serpin family E member 1	1.99	Extracellular space

Table V. The 10 most significant canonical pathways regulated by PEITC compared to DMSO in LNCaP Setd7-KD cell. Genes in bold are down-regulated in the PEITC treatment.

Canonical pathways	-Log (p-Value)	Ratio	Target genes
tRNA charging	2.42	7/35 (0.200)	<i>CARS, RARS2, HARS, LARS2, TARS, AARS, EPRS</i>
Glutaryl-CoA degradation	1.63	3/11 (0.273)	<i>ACAT2, HSD17B4, HADH</i>
Phosphatidylethanolamine biosynthesis II	1.51	2/5 (0.400)	<i>CEPT1, PIGF</i>
Aspartate degradation II	1.51	2/5 (0.400)	<i>GOT1, MDH2</i>
Glioma signaling	1.44	10/89 (0.122)	<i>CDKN2A, RAF1, IGF2, IRS1, CDKN1A, IGF1R, EGF, MDM2, CDKN2B, PRKCA</i>
Tryptophan degradation III (Eukaryotic)	1.43	3/13 (0.231)	<i>ACAT2, HSD17B4, HADH</i>
Nur77 Signaling in T lymphocytes	1.42	5/32 (0.156)	<i>HLA-DRB1, CASP3, MEF2D, NR4A1, CYCS</i>
Hepatic fibrosis/Hepatic stellate cell activation	1.41	10/90 (0.111)	<i>SMAD2, IGF2, FN1, TIMP1, SMAD7, IGF1R, EGF, ECE1, SERPINE1, AGT</i>
Unfolded protein response	1.33	6/45 (0.133)	<i>SEL1L, SCAP, PDIA6, VCP, CANX, NFE2L2</i>
Superpathway of cholesterol biosynthesis	1.25	4/25 (0.160)	<i>SQLE, ACAT2, DHCR24, HMGCS1</i>

Top differentially expressed genes and canonical pathways modulated by PEITC in LNCaP Setd7 KD cells. To further determine differentially expressed genes in LNCaP Setd7-KD cells treated with PEITC or DMSO, we identified the top 10 down-regulated or up-regulated genes which are shown in

Table IV. A fold change less than 1 indicates that the expression of the gene was decreased by PEITC treatment compared to the expression in the control group, whereas a fold change greater than 1 indicates elevated expression in PEITC-treated LNCaP Setd7-KD cells. Among the top 10

Figure 3. Overview of the genes regulated by Setd7 knockdown and PEITC treatment. (A) Heat map of 68 genes with differential expression that appeared in WT versus KD group and KD PEITC versus KD group. (B, C) Venn diagrams comparing the up-regulated and down-regulated genes between WT versus KD group and KD PEITC versus KD group. Genes with log2 fold changes greater than 0.5 were counted. WT, LNCaP; KD, LNCaP Setd7-KD.

genes down-regulated by PEITC in the absence of Setd7, *Timp1*, *Tnks*, *Hla-drb1*, *Gnai2*, and *H6pd* were down-regulated by more than 2.3-fold, and of the top 10 up-regulated genes, *Rbbp4*, *Cnot7*, *Ext2*, *Ptk2*, *Phb*, and *Rab11b* were up-regulated by at least 2.3-fold relative to their expression in the DMSO-treated Setd7-KD group. In addition, canonical pathway analysis was performed using IPA software. A total of 274 canonical pathways were significantly associated with the genes differentially expressed between the PEITC treated group and the control DMSO group. The top 10 most significant canonical pathways are shown in Table V and include “tRNA Charging”, “Glutaryl-CoA Degradation”, and “Phosphatidylethanolamine Biosynthesis II”.

Overview of differentially expressed genes regulated by Setd7 knockdown and PEITC treatment. To examine the impact of

PEITC treatment and Setd7 knockdown in LNCaP cells, the gene expression profiles between wild-type LNCaP cells (WT) and LNCaP Setd7-KD (KD) cells as well as between PEITC-treated KD cells and DMSO-treated KD cells were compared. A cut-off log2 fold change greater than 0.5 was used to identify the differentially expressed genes. Overall, 2,405 differentially expressed genes were identified between the WT and KD group (1,302 genes were down-regulated by Setd7 knockdown, and 1,103 genes were up-regulated by Setd7 knockdown). A total of 2,117 differentially expressed genes were also observed between the PEITC-treated KD group and the DMSO-treated KD group (1,114 genes were up-regulated by PEITC treatment, and 1,003 genes were down-regulated by PEITC treatment) (Figure 3). Among the genes regulated by Setd7 knockdown and by PEITC treatment of LNCaP Setd7-KD cells, a total of 68 genes were

Figure 4. Validation of the mRNA changes of selected genes regulated by *Setd7* knockdown and PEITC treatment. mRNA isolated from WT, KD, and KD PEITC groups was subjected to qPCR analysis. Black bar: the qPCR results are presented as the fold changes of WT versus KD group and KD PEITC versus KD group using *Gapdh* as the endogenous control. White bar: fold change from the microarray analysis. The bars represent the mean values of triplicate samples (mean $\pm$ SD). WT, LNCaP; KD, LNCaP *Setd7*-KD.

changed in the same direction. Specifically, 25 genes were down-regulated by *Setd7* KD (25/1,302) compared to the WT group; these genes were also down-regulated in the PEITC-treated KD group (25/1,003) compared to the KD group. Forty-three up-regulated genes in the KD group (43/1,103) were also up-regulated by PEITC treatment (43/1,114). As shown in the heat map (Figure 3A), the 68 genes commonly differentially expressed by *Setd7* KD and by PEITC treatment in *Setd7*-KD cells include the following 3 types of non-coding RNAs: microRNA, long non-coding RNA, and small nucleolar RNA. This result suggests that some of the non-coding RNAs are modulated by *Setd7* knockdown and by PEITC treatment in *Setd7*-KD cells and that they may have relevant functions in chemoprevention. We selected 18 genes from the 68 shared genes and validated their expression in the WT, KD, and PEITC-treated KD groups by qPCR (Figure 4, black bars). The microarray findings were confirmed in fifty-six percent (10 out of 18 selected genes) of the tested genes (Figure 4, white bars).

Even though the fold changes deduced from the qPCR results in the expression of *Si* (KD PEITC vs. KD), *SDC4* (KD PEITC vs. KD), and *ID1* (KD vs. WT) were not consistent with those from the microarray results, the trend was to the same direction. According to the qPCR and microarray results, among these 10 genes, *Pcdh13*, *Cd24*, *Sdc4*, *Gadd45b*, *Sacs*, *Rab31*, and *Gh2* were up-regulated in the KD versus the WT group but decreased in the KD PEITC versus KD group. Furthermore, according to the qPCR and microarray results, the mRNA expression levels of *Id1* and *Lxn* were decreased in the KD versus the WT group but increased in the KD PEITC versus KD group (Figure 4).

Top differentially expressed genes and canonical pathways modulated by PEITC in LNCaP cells. To determine the impact of PEITC on the transcriptome of LNCaP cells, the gene expression profiles of the PEITC-treated group were compared with those of the control DMSO-treated group. A total of 217 differentially expressed genes showed a log₂

Table VI. Top 10 down-regulated and up-regulated genes in LNCaP cells treated with PEITC compared to DMSO group. The expression of the genes in the DMSO group was set to 1.

Gene ID	Gene name	Fold Change	Location
Down-regulated			
<i>SLC25A3</i>	Solute carrier family 25 member 3	0.366	Cytoplasm
<i>LAMC1</i>	Laminin subunit gamma 1	0.405	Extracellular space
<i>CDC14B</i>	Cell division cycle 14B	0.426	Nucleus
<i>ATP5C1</i>	ATP synthase	0.505	Cytoplasm
<i>HSD17B7</i>	Hydroxysteroid 17-beta dehydrogenase 7	0.51	Cytoplasm
<i>IGHA1</i>	Immunoglobulin heavy constant alpha 1	0.513	Extracellular space
<i>mir-130</i>	microRNA 130a	0.515	Cytoplasm
<i>ST13</i>	ST13, Hsp70 interacting protein	0.515	Cytoplasm
<i>MINPP1</i>	Multiple inositol-polyphosphate phosphatase 1	0.521	Cytoplasm
<i>DARS</i>	Aspartyl-tRNA synthetase	0.524	Cytoplasm
Up-regulated			
<i>mir-515</i>	MicroRNA 520c	2.81	Cytoplasm
<i>NUDT5</i>	Nudix hydrolase 5	2.17	Cytoplasm
<i>CLDN12</i>	Claudin 12	2.13	Plasma membrane
<i>PDPK1</i>	3-phosphoinositide dependent protein kinase 1	2.07	Cytoplasm
<i>BCL2L2</i>	BCL2 like 2	2.07	Cytoplasm
<i>AMFR</i>	Autocrine motility factor receptor	2.06	Plasma Membrane
<i>RRM2B</i>	Ribonucleotide reductase regulatory TP53 inducible subunit M2B	2.04	Nucleus
<i>ABCB9</i>	ATP binding cassette subfamily B member 9	2.04	Cytoplasm
<i>CD59</i>	CD59 molecule (CD59 blood group)	2.03	Plasma Membrane
<i>SEN2</i>	SUMO1/sentrin/SMT3 specific peptidase 2	2.01	Nucleus

fold change greater than 0.5. The top 10 down-regulated and top 10 up-regulated genes in this comparison are presented in Table VI. Of the top 10 down-regulated genes, *Slc25a3*, *Lamc1*, *Cdc14b* were down-regulated by more than 2.3-fold upon PEITC treatment. Among the top 10 up-regulated genes, *mir-515*, *Nudt5*, *Cldn12* were up-regulated by 2.1-fold or higher upon PEITC treatment. IPA was used to analyze the canonical pathways regulated by PEITC. We identified a total of 341 canonical pathways significantly associated with the differentially expressed genes in the PEITC-treated group compared with the control DMSO group. The top 10 most significant canonical pathways are displayed in Table VII. Interestingly, “Cancer Drug Resistance by Drug Efflux”, “PI3K/AKT Signaling”, and “PTEN Signaling” are listed in the top 10 canonical pathways. The up-regulated gene *Pdpk1* and the down-regulated gene *Nfkb1* are strictly related to these pathways.

Overview of differentially expressed genes regulated by PEITC treatment compared with the control DMSO group in LNCaP cells and in LNCaP Setd7-KD cells. Differential gene expression profiling after treatment of LNCaP cells and in LNCaP Setd7 KD cells with PEITC was next examined by comparing: the PEITC-treated WT cells *versus* DMSO-treated WT cells and the PEITC-treated KD cells *versus* DMSO-treated KD cells. A log₂ fold change cut-off of 0.5

was used to identify the differentially expressed genes. A total of 1,998 differentially expressed genes were identified in the comparison of the PEITC-treated WT *versus* DMSO-treated WT group (1,079 genes were up-regulated by PEITC treatment, and 919 genes were down-regulated by PEITC treatment). Furthermore, 2,117 differentially expressed genes were identified in the comparison of the PEITC-treated KD *versus* untreated KD group (1,114 genes were up-regulated by PEITC treatment, and 1,003 genes were down-regulated by PEITC treatment) (Figure 5). Of the genes regulated by PEITC treatment, expression of a total of 76 genes was found to be affected in opposite directions. Specifically, 48 genes that were significantly up-regulated by PEITC treatment in WT cells (48/1,079) were down-regulated by PEITC treatment in KD cells (48/1,003). Twenty-eight significantly down-regulated genes in the PEITC-treated WT group (28/919) were increased by PEITC treatment in the KD group (28/1,114). These 76 genes might represent a set of molecular targets that explain the differences between PEITC-treated wild-type LNCaP cells and Setd7-KD cells, indicating that Setd7 may play an important role in the chemopreventive effects of PEITC in PCa cells. Given the potential function of the genes presented in Figure 5, 16 genes were selected for further validation by qPCR (black bars). Seventy-five percent (12 out of 16 selected genes) of genes tested by qPCR confirmed microarray findings (white

Table VII. The 10 most significant canonical pathways regulated by PEITC compared to DMSO in LNCaP cell. Genes in bold are down-regulated in the PEITC treatment.

Canonical pathways	-Log (p-Value)	Ratio	Target genes
Cancer drug resistance by drug efflux	4.11	9/36 (0.25)	<i>mir-154, mir-130, PIK3CA, RRAS2, mir-181, RRAS, PTGS2, NFKB1, mir-27</i>
Agrin interactions at neuromuscular junction	3.39	9/44 (0.205)	<i>LAMC1, PAK1, RRAS2, RRAS, ITGA2, RAC1, ITGA5, ACTG1, CTTN</i>
PI3K/AKT signaling	2.9	13/94 (0.138)	<i>PIK3CA, CDC37, RRAS2, RRAS, ITGA2, YWHAZ, ILK, ITGA5, PDPK1, PTGS2, GSK3B, NFKB1, BCL2</i>
Tetrahydrofolate salvage from 5, 10-methenyltetrahydrofolate	2.87	3/5 (0.6)	<i>MTHFD1, MTHFD2L, GART</i>
PTENsignaling	2.7	12/87 (0.138)	<i>PIK3CA, RRAS2, RRAS, TGFBR3, ITGA2, RAC1, ILK, ITGA5, PDPK1, GSK3B, NFKB1, BCL2</i>
PAK signaling	2.64	10/66 (0.152)	<i>PAK1, PIK3CA, RRAS2, RRAS, ITGA2, RAC1, ITGA5, LIMK2, NCK1, GIT1</i>
CCR3 signaling in eosinophils	2.55	11/79 (0.139)	<i>PAK1, PIK3CA, RRAS2, RRAS, RAC1, GNB5, PPP1CB, LIMK2, PLA2G2A, PLA2G12A, PRKCA</i>
Paxillin signaling	2.5	10/69 (0.145)	<i>PAK1, PIK3CA, RRAS2, ARFIP2, RRAS, ITGA2, RAC1, ITGA5, NCK1, ACTG1</i>
Role of MAPK signaling in the pathogenesis of influenza	2.41	7/39 (0.179)	<i>RRAS2, RRAS, PTGS2, PLA2G2A, PLA2G12A, BCL2, PRKCA</i>
Rac signaling	2.3	11/85 (0.129)	<i>ACTR2, PAK1, PIK3CA, RRAS2, ARFIP2, RRAS, ITGA2, RAC1, ITGA5, LIMK2, NFKB1</i>

bars). Among these 12 genes, the relative mRNA expression of *Lrrtm1*, *Fcrl6*, *Thsd7a*, *Apom*, *Armc12*, *Tktl1*, and *Zfp235* was increased in PEITC-treated WT cells, whereas *Setd7* knockdown prevented this induction. Furthermore, the mRNA expression levels of *Tpm3*, *Or9a2*, *Dcaf411*, *Cbln2*, and *Tas2r19* were all decreased in PEITC-treated WT cells, but *Setd7* knockdown prevented this suppression (Figure 6).

Discussion

In the current study, the impact of the epigenetic modifier histone methyltransferase *Setd7* KD and the biological effects of PEITC were investigated in LNCaP PCa cells. Accumulating evidence suggests that *Setd7* can influence chromatin conformation and transcriptional activity in a number of ways. PEITC has been shown to possess therapeutic effects *in vitro* and *in vivo*, including anticancer, antioxidant, and anti-inflammatory activities, and it may prevent PCa through epigenetic modifications. Therefore, it is of great interest to explore the role of *Setd7* in PEITC-treated PCa cells by obtaining transcriptome profiles with oligonucleotide microarray analysis. Notably, *Setd7* knockdown was found to dramatically affect the transcriptome profiles of LNCaP cells and to influence the expression of a higher number of genes and with greater fold changes than PEITC treatment.

The top 10 up-regulated and top 10 down-regulated genes presented in the tables, offer new insights into the discovery

of important genes driving PCa, and may also suggest potential candidate therapeutic targets and biomarkers for the prevention, treatment and/or diagnosis of PCa. To our surprise, when *Setd7* expression was inhibited in LNCaP cells, the tumor suppressor gene *Rarres3* (retinoic acid receptor responder 3) was up-regulated with a notably large fold change (28.06) (Table II). *Rarres3* was reported to be down-regulated by *Esrrb* (estrogen related receptor β) and by its synthetic ligand DY131 in PCa DU145 cells based on RNA-seq analysis (32). Additionally, using RNA sequencing, a recent work demonstrated that *Rarres3* can be epigenetically silenced by the histone methyltransferase *G9a*, which promotes liver cancer development (33).

Among the top regulated genes in PEITC-treated LNCaP *Setd7*-KD cells, *Rbbp4* (RB binding protein 4, chromatin remodeling factor) is the most significantly up-regulated gene (fold change=3.65, Table IV). *Rbbp4* is a component of co-repressor complexes involved in histone deacetylation and chromatin assembly that lead to transcriptional repression. *Rbbp4* can also regulate cell proliferation. When *Rbbp4* is overexpressed in LNCaP cells, it induces cell death (34). Nevertheless, no studies have been performed addressing the relationship between PEITC and *Rbbp4* in PCa. Our results indicated that the role of *Rbbp4* in cells treated with PEITC may be worth exploring in PCa cells. Of the genes that were most significantly up-regulated in PEITC-treated LNCaP cells, *miR-515* (fold change=2.81, Table VI) has been reported as a tumor suppressor in breast cancer and non-

Figure 5. Overview of the genes regulated by PEITC treatment in WT and KD groups. (A) Heat map of 76 genes with differential expression that appeared in WT PEITC versus WT group and KD PEITC versus KD group. (B, C) Venn diagrams comparing the up-regulated and down-regulated genes between WT PEITC versus WT group and KD PEITC versus KD group. Genes with log2 fold changes greater than 0.5 were counted. WT, LNCaP; KD, LNCaP Setd7-KD.

small cell lung cancer (NSCLC) based on its inhibition of proliferation and induction of apoptosis (35, 36). miR-515-5p is transcriptionally repressed by ER α and can directly target Mark4 to lead to inhibition of cancer cell migration and metastasis (35). Clinical data have shown that high miR-515-5p and low Mark4 expression are associated with increased survival of breast and lung cancer patients (36). Overall, these *in vivo* and *in vitro* results strongly suggest that PEITC may play a crucial role in PCa prevention by perturbing the molecular targets newly identified in our study, such as miR-515.

The most significantly affected canonical pathway regulated by Setd7 knockdown in LNCaP cells is “Death Receptor Signaling” (Table III). Death receptors have an approximately 80-amino-acid-long death domain in the cytoplasmic portion, and form a subgroup within the TNFR (tumor necrosis factor receptor) superfamily (37). The TNFR

superfamily includes more than 20 proteins and functions in the immune system. Our results showed that 5 of the 27 molecules in “Death Receptor Signaling” are down-regulated, whereas other molecules, such as the NF- κ B-related and TNF-related genes, are up-regulated. Of the 5 down-regulated molecules, Ikbkb, a subunit of I κ B kinase, triggers immune responses associated with cytokine-activated intracellular pathways. Additionally, Ikbkb can activate NF- κ B. Notably, Ikbkb belongs also in other significant canonical pathways, such as “TNFR2 Signaling”, “Acute Phase Response Signaling”, “Neuroinflammation Signaling Pathway”, “TNFR1 Signaling”, “Role of RIGI-like Receptors in Antiviral Innate Immunity”, and “Induction of Apoptosis by HIV1”. TRAF2 directly interacts with TNF receptors and forms complexes with other Traf proteins. Traf2 is required for the TNF α -mediated activation of Mapk8/Jnk and NF- κ B. Correspondingly, our preliminary

Figure 6. Validation of the mRNA changes of selected genes regulated by PEITC treatment. mRNA isolated from WT, KD, WT PEITC and KD PEITC groups was subjected to qPCR analysis. Black bar: the qPCR results are presented as fold changes of WT PEITC versus WT group and KD PEITC versus KD group using *Gapdh* as the endogenous control. White bar: fold changes from the microarray analysis. The bars represent the mean values of triplicate samples (mean $\pm$ SD). WT, LNCaP; KD, LNCaP Setd7-KD.

data showed that Setd7 knockdown in LNCaP cells dramatically elevates the expression of inflammatory genes such as TNF. A previous study has shown that Setd7 deficiency prolongs the DNA binding of NF- κ B and induces TNF α -mediated expression of NF- κ B target genes (24). Together, our results further indicate the negative regulation of NF- κ B-mediated inflammatory responses by Setd7 and suggest additional potential targets of Setd7 in LNCaP cells. Another canonical pathway highlighted by Setd7 knockdown was “Agrin Interactions at Neuromuscular Junction” (Table III). Interestingly, this pathway was also identified as the most significant pathway affected by PEITC in LNCaP cells (Table VII). In addition, the canonical pathways most impacted by PEITC in LNCaP Setd7-KD cells are distinct from those regulated by PEITC treatment in wild-type LNCaP cells or those perturbed by Setd7 knockdown alone (Tables III, V, and VII). These results indicate that Setd7 has

an impact on PEITC-regulated genes in LNCaP cells as well.

Additionally, a total of 76 genes showed altered expression in opposite directions after PEITC treatment in wild-type LNCaP cells or in Setd7-KD LNCaP cells (Figure 5). Other studies have also shown changes in some of these genes (Figure 6) including *Fcrl6* (38), *Thsd7a* (39), *Apom* (40), *Mpeg1* (41), and *Tktl1* (42, 43). *Tktl1* has been reported as a potential biomarker for PCa. The expression of *Tktl1* is increased in monocytes of tumor patients and decreased in the serum of PCa patients (44). Another recent study has shown that surgical removal of prostate primary tumors resulted in the significant inhibition of *Tktl1* in macrophages (45). Our observations expand the suggestion that cross-talk might occur between Setd7 and *Tktl1* in PCa cells exposed to chemopreventive compounds such as PEITC. We also noticed non-coding RNAs in our list, including microRNAs, long non-coding RNAs, and small nucleolar RNAs. A

growing body of evidence suggests that the non-coding RNAs play a crucial role in modulating fundamental biological processes. For instance, a recent study demonstrated that the long non-coding RNA LOC283070 induces the transition of LNCaP cells into androgen-independent cells, possibly through Camk1d (46). From our observations, it is worth noting that the pseudogene Pdia3p1, a long non-coding RNA, is up-regulated by PEITC in LNCaP cells but down-regulated in LNCaP Setd7-KD cells. A recent *in vivo* and *in vitro* study demonstrated that Pdia3p1 promotes the progression of hepatocellular carcinoma and increases tumor proliferation while suppressing apoptosis through the regulation of the p53 signaling pathway in hepatocellular carcinoma cells. Pdia3p1 acts as an oncogene and a possible prognostic marker in hepatocellular carcinoma (47). Interestingly, our data showed that PEITC induced the expression of Pdia3p1 in LNCaP cells, which was prevented by Setd7 knockdown. Together, these results indicate that Setd7 may play an important role in LNCaP cells exposed to PEITC by regulating Pdia3p1 expression.

In addition, a subset of 68 genes that were differentially expressed in the same direction by Setd7 knockdown in wild-type LNCaP cells and by combined PEITC treatment and Setd7 knockdown were identified (Figure 3). The altered expression of some of these genes was confirmed by qPCR analysis (Figure 4). Alterations of several genes were noticed in our list, including *Rab31* (48), *Gadd45b* (49), *Adirf* (50), *Id1* (51), *Pca3* (52), and *Pcnt2* (53). Rab31 is a member of the monomeric GTP-binding proteins, that play essential roles in intracellular vesicle and granule transport (54). Rab31 influences cancer progression and is associated with various types of cancer (48). Our results supply further evidence that Rab31 can be inhibited by Setd7 knockdown in LNCaP cells with or without PEITC treatment. Also, two other targets, *Pca3* and *Pcat2*, which are affiliated with the non-coding RNA class, attracted our attention. *Pca3* is used as a urine-based PCa marker because it is only expressed in human prostate tissue and is highly overexpressed in PCa (55-57). The most commonly used biomarker for PCa is serum prostate-specific antigen (PSA), but *Pca3* has a higher specificity and a better positive and negative predictive value (58). In addition, *Pca3* knockdown can significantly suppress epithelial ovarian carcinoma cells and LNCaP cell proliferation, migration, and invasion and might be a novel diagnostic biomarker of epithelial ovarian carcinoma and a potential therapeutic target for inhibiting PCa (52, 59). However, these results are not in accordance with our observations because Setd7 knockdown and PEITC treatment both elevate the expression of *Pca3* in LNCaP cells. Thus far, many conflicting results have been obtained regarding *Pca3* in PCa, including its correlation with adverse tumor volume, grading (Gleason score), and extracapsular extension (60-62).

Our study provided the first quantitative gene expression profiling of Setd7-knockdown and PEITC treatment in PCa LNCaP cells. Altered biological pathways impacted by Setd7 KD and PEITC treatment were identified. A set of these genes are considered possible molecular targets involved in the prevention and treatment of PCa. These findings offer new insights on the molecular mechanisms underlying Setd7 and PEITC treatment in PCa which could be translated to the clinical setting.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

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