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PTEN deletion drives aberrations of DNA methylome and transcriptome in different stages of prostate cancer

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Abstract

PTEN is a tumor suppressor gene and one of the most frequently mutated/deleted genes in human prostate cancer (PCa). However, how PTEN deletion would impact the epigenome and transcriptome alterations remain unknown. This hypothesis was tested in a prostate-specific PTEN $-/-$ (KO) mouse prostatic adenocarcinoma model through DNA methyl-Seq and RNA-Seq analyses. Examination of cancer genomic datasets revealed that PTEN is expressed at lower levels in PTEN-deleted tumor samples than in normal solid tissue samples. Methylome and transcriptome profiling identified several inflammatory responses and immune response signaling pathways, including NF- κ B signaling, IL-6 signaling, LPS/IL-1-mediated inhibition of RXR Function, PI3K in B lymphocytes, iCOS-iCOSL in T helper cells, and the role of NFAT in regulating the immune response, were affected by PTEN deletion. Importantly, a small subset of genes that showed DNA hypermethylation or hypomethylation was correlated with decreased or increased gene expression including CXCL1. qPCR analyses of representative genes validated the RNA-Seq results. Histopathological examinations showed that the severity of prostatic intraepithelial neoplasia and inflammation development gradually increased as PTEN null mice aged. Collectively, these findings suggest that loss of PTEN drives global changes in DNA CpG methylation and transcriptomic gene expression and highly associated with several inflammatory

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Author contributions

CW and CYZ designed the research and performed experiments; CW, CYZ, YPF, DC, RYW, YQY, DS, and ANK conceived the idea, discussed the data and analyzed the results; YPF provided computational assistance and contributed to the data analysis; CW wrote the paper; ANK revised the paper. All authors reviewed the results and approved the final version of the manuscript.

#These authors contributed equally to this work.

Conflicts of interest statement

The authors declare no potential conflicts of interest.

and immune molecular pathways during PCa development. These biomarkers could be valuable molecular targets for cancer drug discovery and development against PCa.

Keywords

PTEN; prostate cancer; transcriptome; DNA methylation; CXCL1

Introduction

Prostate cancer (PCa) is one of the most common cancers among men in the United States. The PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor gene is one of the most frequently mutated/deleted genes in a variety of human cancers including PCa (1). In human PCa, PTEN mutation is one of the most common genetic alterations. A mouse model that uses the Pb-Cre promoter and Pten-flox has been commonly used to examine the role of PTEN deletion in prostate tumorigenesis (2). PTEN deletion has been highly linked to inflammation, in prostate-specific PTEN null mice, the expression of CXCL8/IL-8, a pro-inflammatory chemokine promoting tumorigenesis is increased (3). In a mouse PCa model, constitutive active expression of IKK2 alone was insufficient to promote prostate tumorigenesis, but when combined with heterozygous loss of PTEN, IKK2 activation led to an increase in tumor size that was accompanied by increases in inflammatory cytokine secretion from the stromal microenvironment of the prostate because PTEN loss drove epithelial prostate tumor cells toward metastasis (4). Furthermore, PTEN deficiency in the epithelium showed a dose-dependent transformation in mouse prostate tissue, and low-grade prostatic intraepithelial neoplasia (PIN) was observed in mice with monoallelic deletions, while lesions with the ability to form invasive and eventually metastasizing carcinomas were found in those with full deletions. In addition, in prostate-specific PTEN null mice, at week 10, the low-grade PIN was observed; at week 12, the high-grade PIN was observed, and adenocarcinoma developed starting from week 12 to week 20 (at week 20, 100 % of the mice had adenocarcinoma) (5–7).

It appears that majority of epigenetic CpG methylation changes detected in cancer cells are acquired during neoplastic development and therefore specific to cancer (8) and that epigenetic changes arise earlier than genetic defects. There is strong evidence indicating that epigenetic modifications, a stably heritable phenotype that does not require alterations in the DNA sequence, play an important role in PCa (9, 10). DNA methylation is the most well-studied epigenetic mechanism associated with aberrant gene expression in cancer and consists of the addition of a methyl group by DNA methyltransferases to the 5-cytosine residue within the CpG dinucleotide (11). Global DNA hypomethylation and regional hypermethylation are common features of cancer development and progression (12). Specifically, DNA hypermethylation in promoter regions plays a critical role in suppressing tumor suppressor gene expression partly by blocking the binding of transcription factors (13). It has been shown that tumor suppressor genes are frequently epigenetically silenced in mouse models with malignant tumors (14, 15). For instance, PTEN methylation has been associated with microsatellite instability-high sporadic colorectal cancers, endometrial carcinoma, sporadic breast cancer, melanoma, and multiple myeloma (16–21).

Evidence suggests that in PCa, tumor suppressor genes silenced by promoter hypermethylation are related to many important cellular events, including cell proliferation, apoptosis, DNA repair, hormonal responses, and cancer initiation and progression (22–24). However, it is not clear how silencing of tumor suppressor gene such as PTEN deletion would impact the CpG methylome, and transcriptome during PCa development.

Here, we report the results of genome-wide CpG methylome and transcriptome profiling in 12- to 20-week-old PTEN null mice and investigate the correlations between gene expression and methylation as well as canonical signaling pathways including several inflammatory response and immune response signaling pathways. These results could facilitate a better understanding of the molecular events and the epigenetic regulatory mechanisms that control the transition from inflammation to malignancy.

Materials and methods

Chemicals

Antibodies were obtained from Abcam (Cambridge, MA, USA; Nrf2 and Nqo1 antibodies), Santa Cruz Biotechnology (Santa Cruz, CA, USA; actin (I-19) antibodies and all secondary antibodies).

Animal studies

Pb-Cre4 mice (strain: B6.Cg-Tg(Pbsn-cre)4Prb/Nci) were obtained from the National Cancer Institute, USA; and Pten(flox/flox) mice (C;129S4-Pten^{tm1Hwu}/J) were obtained from the Jackson Laboratories (25, 26). F2 prostate-specific Pten knockout male offspring (Pb-cre/Pten(flox/flox)) were generated by crossing male Pb-Cre4 mice with female Pten(flox/flox) mice. For simplicity, Pb-cre/Pten(flox/flox) mice are referred to as Pten-null, and Pten(flox/flox) mice are considered wild-type (WT). The mice were genotyped using PCR, and only the male mice that were cre carriers and homozygous Pten^{flox/flox} were used for the treatment groups. For Pb-Cre4 genotyping, a 393-bp product was produced (primers: 5'-CTGAAGAATGGGACAGGCATT-3' and 5'-CATCACTCGTTGCATCGACC-3'). Pten genotyping was determined based on the size of the product obtained from PCR amplification using the primers 5'-CAAGCACTCTGCGAACTGAG-3' and 5'-AAGTTTTTGAAGGCAAGATGC-3', with a 328-bp band indicating a mutant allele and a 156-bp band combined with a 328-bp band indicating a heterozygote.

Experimental procedure

All mice were housed in our animal facility in accordance with the protocol approved (01–016) by the Rutgers University Institutional Animal Care and Use Committee (IACUC). All mice were maintained under standard 12-h light/12-h dark cycles with water and diet provided *ad libitum* unless otherwise specified. As shown in Fig. 1A, mice were randomly assigned to two experimental groups (n=10): WT and Pten-null. Mice were sacrificed by CO₂ asphyxiation at two time-points: 12 weeks and 20 weeks. There were ten mice per group per time point. Mouse prostates were removed immediately and either dissected into ventral & lateral prostate (VLP) and dorsal & lateral prostate (DLP), snap-frozen and stored at –80 °C for epigenomics and biomarker analysis or fixed in 10 % phosphate-buffered

formalin at room temperature for 24 hours for histopathological evaluations and immunohistochemistry (IHC) staining.

Histopathological examination

The prostate samples were transferred to increasing concentrations of ethanol (70 %, 80 %, 95 %, and 100 %), cleared in xylene, and then embedded in Paraplast Plus (Thermo Fisher Scientific, Waltham, MA, USA). Tissue blocks were serially sectioned (4 μ m) and mounted on glass slides. The sections were stained with hematoxylin and eosin (H&E) or used for immunohistological staining. These sections were used for the measurement and morphological identification of prostatic intraepithelial neoplasia (PIN) in addition to the number of inflammatory cells and other histological changes. All histological identifications were captured at 200-fold magnification. The H&E sections were examined under a light microscope (Nikon Eclipse E600, Japan).

Isolation of nucleic acid and next-generation sequencing

Total RNA and DNA were extracted from ventral & lateral prostate tissues isolated from each animal using an AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA). The quality and concentration of the extracted RNA and DNA were determined using an Agilent 2100 Bioanalyzer and a NanoDrop spectrophotometer, respectively. Twelve RNA samples (2 samples per group) and 12 DNA samples (2 samples per group) were subjected to RNA-Seq and SureSelect Methyl-Seq, respectively. Library preparation and sequencing were performed by RUCDR Infinite Biologics. The RNA-Seq procedures were the same as those described previously (27). The DNA samples were further processed using an Agilent Mouse SureSelect Methyl-Seq Target Enrichment System (Agilent Technologies Inc., Santa Clara, CA, USA) and sequenced on an Illumina NextSeq 500 instrument with 76-bp single-end reads, resulting in the generation of 30–45 million reads per sample. For RNA-Seq, 75-bp paired-end reads produced approximately 30 million reads per sample.

Bioinformatics analyses

RNA-Seq—Cutadapt (28) was used to remove the Illumina Universal Adapter sequence. The reads were aligned by TopHat (version 2.1.1) to the mouse genome (mm10) (29), and PCR duplicates were removed. Reads overlapping with genomic features were counted by HTSeq-count (version 0.9.1) (30) and analyzed for differential expression with DESeq2 (version 1.14.1) in R (version 3.4.0) (31).

DNA SureSelect Methyl-Seq

The reads were aligned to the *in silico* bisulfite-converted mouse genome (mm10) with the Bismark (version 0.15.0) alignment algorithm (32). After alignment, Methylkit (version 3.8) was used to extract methylation counts for each CpG site. CpG sites were further clustered into DMRs in a 300 window using BEDTools (version 2.24.0) (33). Each DMR contained at least three CpG sites, all of which were hypermethylated or hypomethylated. Of the 3 or more CpG sites, at least 1 CpG methylation difference greater than 10 % and all other CpG site methylation differences greater than 5 % that had a *P* value smaller than 0.05 were

considered significant. Genomic annotation was performed with SnpEff (version SnpEff 4.3t) (34).

Ingenuity Pathway Analysis (IPA)

Isoforms with false discovery rates ($q < 0.05$) and a $\log_2\text{FoldChange} > 1.0$ or < -1.0 were subjected to Ingenuity Pathway Analysis (IPA 4.0, Ingenuity Systems, www.ingenuity.com). The input isoforms were mapped to IPA's knowledge base, and the relevant biological functions, networks, and pathways related to PTEN KO were identified.

TCGA gene expression analysis

Level 3 RNA read counts for prostate adenocarcinoma data were downloaded from the TCGA portal (<https://portal.gdc.cancer.gov>). Copy number variation (CNV) data for prostate patients were downloaded from cBioPortal (<http://www.cbioportal.org>). The PTEN deletion of cancer samples were selected when CNV was -1 or -2 . Cancer samples without PTEN copy number alterations were selected when CNV was 0 . DESeq2 (version 1.14.1) in R (version 3.4.0) (31) was used to calculate the DEGs between solid tissue normal samples and primary tumor samples with PTEN CNV normal or PTEN deletion.

RNA isolation and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the treated cells using a GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA, USA). According to the manufacturer's instructions, first-strand complementary DNA was synthesized from $1\ \mu\text{g}$ of RNA using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA, USA). qPCR analysis was performed in an ABI7900HT system (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems) using complementary DNA as the template. The sequences of the primers used for PCR amplifications are listed in Table 1. The PCR conditions were as follows: initial denaturation for 10 min at $95\ ^\circ\text{C}$ followed by 40 cycles of 15 s at $95\ ^\circ\text{C}$ and 1 min at $60\ ^\circ\text{C}$. A melting curve analysis was performed to ensure the specificity of the products. The relative gene expression levels were normalized to GAPDH levels using the 2^{-C_T} formula ($C_T = C_{T\text{ sample}} - C_{T\text{ control}}$).

Western blotting

The cells were harvested using radioimmunoprecipitation assay (RIPA) buffer (Millipore, Billerica, MA, USA) supplemented with a protein inhibitor cocktail (Sigma, St. Louis, MO). Then, the cell lysates were sonicated at $4\ ^\circ\text{C}$ and centrifuged at 13,000 rpm for 15 min at $4\ ^\circ\text{C}$. The supernatants were collected and quantified using a Pierce™ bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Then, $40\ \mu\text{g}$ of total protein was diluted with Laemmli's sodium dodecyl sulfate sample buffer (Boston Bioproducts, Ashland, MA, USA) and denatured at $95\ ^\circ\text{C}$ for 10 min. The proteins were loaded and separated on 4–15% Criterion™ Tris-HCl Precast Gels (Bio-Rad, Hercules, CA, USA) and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA), followed by blocking with 5% bovine serum albumin (BSA) in Tris-buffered saline-0.1% Tween 20 (TBST) buffer for 1 h at room temperature. The membrane was sequentially incubated with specific primary antibodies and HRP-conjugated secondary

antibodies. The protein bands were visualized using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA) with a Gel Documentation 2000 system (Bio-Rad, Hercules, CA, USA).

ChIP assay

ChIP assays were performed using a MAGnify™ Chromatin Immunoprecipitation System (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. After the samples were harvested and washed twice with PBS, chromatin was cross-linked with 1% formaldehyde for 10 min at room temperature. Next, 1.25 M glycine was added to quench the excess formaldehyde. The cells were pelleted by centrifugation, resuspended in lysis buffer and sonicated to generate 200 to 500 bp DNA fragments using a Bioruptor sonicator (Diagenode Inc., Sparta, NJ, USA). The diluted chromatin solution was immunoprecipitated with 2 µg of anti-H3K27me3 or anti-H3K27Ac antibody (Abcam, Cambridge, MA, USA) or rabbit immunoglobulin G. After a wash, cross-link reversal, DNA elution, and DNA purification, the relative amount of immunoprecipitated DNA was quantified via qPCR using the primers listed in Table 1. The enrichment of the precipitated DNA was calibrated using a standard curve constructed from the serial dilution of the inputs, which were treated in parallel with the precipitated samples except antibody was not added in the immunoprecipitation procedure, and the data are presented as the fold changes in the signal-to-input ratio normalized to the control.

Statistical analysis

Statistical significance was tested with a one-way ANOVA followed by Dunnett's post hoc test between multiple experimental groups and the Student's t-test between two experimental groups using GraphPad PRISM software. The values are presented as the mean $\pm$ SD (standard deviation). p-values less than 0.05 were considered statistically significant.

Results

Prostate invasive adenocarcinoma in a prostate-specific PTEN KO mouse model

PTEN mutation is among the most common genetic alterations reported in human PCa and inflammation. To identify the PTEN mutation/deletion responsible for the development of PCa, we extracted a sample population ($n=498$) that included primary tumor samples and 52 solid tissue normal samples with gene expression data from 'The Cancer Genome Atlas' (TCGA) data set and the CNV results of the corresponding samples in the cBioPortal database. Among the included tumor samples, 158 PTEN-deleted primary tumor samples and 328 PTEN copy number normal primary tumor samples were found, consistent with a previous report showing that PTEN deletions and/or mutations are present in 30 % of primary PCa prostate tissue samples. Volcano plots showed that PTEN is not differentially expressed between PTEN copy number normal tumor samples vs. solid tissue normal samples, while the expression of PTEN was significantly lower in PTEN-deleted tumor samples than in solid tissue normal samples (Fig. 1A). The log₂-fold change was approximately -1, indicating that PTEN expression is probably controlled only by copy number alterations and not by transcription factor binding. This observation suggests that PTEN deletion may play a driving role in PCa development.

Pb-Cre/Pten (flox/flox) (PTEN KO) is a commonly used mouse model (2). To determine the driving role of PTEN-deletion with age, PTEN KO mice were generated for this study (Fig. 1B). The histology analysis of prostate tissues of 12-week-old PTEN null mice (Fig. 1C) showed that most glands developed high-grade PIN. Papillary or cribriform patterns grew into lumen ducts and were well-differentiated. The nuclear to cytoplasmic ratio was inverted. In the prostate tissues of 20-week-old PTEN null mice (Fig. 1D), more severe PIN had developed. All glands developed to high-grade PIN. Papillary or cribriform patterns grew into lumen ducts, and almost 90 % were filled, well-differentiated, shown glandular formations, cribriform patterns, frequent mitotic structures, a thick stromal layer, severe nuclear pleomorphism, and hyperchromasia. The above results indicated that the severity of PIN and inflammation development gradually increased as PTEN null mice aged. In the prostate tissues of PTEN WT mice (Fig. 1E), no atypical alteration was observed in epithelial cells throughout the entire gland. In addition, the sizes of prostates were significantly different between PTEN null mice and WT mice. Deletion of PTEN resulted in progressively enlarged prostate lobes (Fig. 1F).

We also found that the mRNA levels of Nrf2, Nqo1, and Nkx3.1 were significantly lower while the mRNA levels of COX2, iNOS, and IL6 were significantly higher in the prostate tissues of PTEN null mice than in those of WT mice (Fig. 2A & 2B). Global methylation status showed that there was significantly less DNA methylation in the VLPs of PTEN null mice than in those of PTEN WT mice (Fig. 2C). Therefore, the DNA and RNA used for next-generation sequencing (NGS) were all extracted from the VLPs of PTEN null and WT mice.

PTEN loss resulted in the activation of the AKT serine/threonine kinase, one of the primary targets of the PTEN-controlled signaling pathway (7). The protein expression of phosphorylated AKT was not detectable in WT prostates but was highly expressed in PTEN null cells (Fig. 2D). Nkx3.1 expression was directly correlated with PTEN loss and may be regulated by AKT activation. The protein expression level of Nkx3.1 was downregulated by PTEN loss (Fig. 2D), consistent with a previous report (7). To further investigate the effects of PTEN loss, proteins representing inflammation (iNOS), oxidative stress-related (Nrf2, Nqo1), epigenetic regulation (Ezh2), and cell proliferation (Cyclin A, Cyclin D1, and p27) were detected. We found that the protein levels of Nrf2, Nqo1, and P27 were significantly lower, while the protein levels of Ezh2, iNOS, Cyclin A, and Cyclin D1 were significantly higher in the prostate tissues of PTEN null mice than in those of WT mice (Fig. 2D). IHC analysis demonstrated that Nqo1 and Ezh2 remained negative in WT mice (Fig. 2E, right panel) and were positive in PTEN null mice (Fig. 2E, left panel). These results demonstrate that (1) the homozygous PTEN loss mouse model used in this study is useful for prostate cancer research and that (2) PTEN loss induces inflammation, histone methyltransferase activity, and cell proliferation.

PTEN deletion decreased H3K27me3 and H3K27Ac enrichment to the Nkx3.1 promoter region at 12 weeks and 20 weeks, respectively.

To provide insights into the molecular events associated with prostate tumorigenesis, we performed an in vivo chromatin immunoprecipitation (ChIP) assay on prostate tissues

obtained from 12-week-old and 20-week-old PTEN null and WT mice. The results showed that accumulation of H3K27me3, a transcription repressor, on the promoter region of Nkx3.1 was significantly lower in the PTEN null mice than in the WT controls at 12 weeks, suggesting that this prostatic tumor suppressor gene was transcriptionally activated. Moreover, there was also significantly less accumulation of H3K27Ac, a transcription activator, on the promoter region of Nkx3.1 in the PTEN null mice than in the WT controls at 20 weeks, suggesting that this prostatic tumor suppressor gene was transcriptionally repressed (Fig. 2F). These data indicate that PTEN deletion reduced H3K27me3 and H3K27Ac enrichment on the Nkx3.1 promoter region at different prostate cancer development stages, resulting in the subsequent activation or inhibition of Nkx3.1 expression. Thus, the mechanisms underlying Nkx3.1 loss in PTEN null mice may depend on differences in epigenetic and tumorigenic events between different stages.

PTEN KO and WT mice differ in prostate tissue gene expression

Our studies were performed using prostates harvested at 12- and 20-week-old PTEN null and WT mice. Two biological replicates from age-matched mice obtained from separate litters were harvested and collected in separate tubes for RNA and DNA extraction. Total RNA was extracted from both replicates (12 and 20 weeks old for a total of 8 samples) and prepared for RNA next-generation sequencing using an Illumina NextSeq 500 platform. Quality control metrics were performed and showed that there was good genome-wide coverage and that high-quality reads were produced. Between-strain correlation observations showed that gene expression measurements were highly correlated among replicates and ranged between 0.96 and 0.97 (Fig. 3A). A principal component analysis (PCA) showed clustering of samples based upon the first two principal components and indicated that there were clear differences between PTEN null mice and their wild-type counterparts (Fig. 3B). Similar to the PCA results, the clustering of samples in a heatmap constructed using DESeq2 demonstrated that there was a separation between PTEN null and WT control mice (Fig. 3C).

To examine the effects of PTEN deletion in 12- and 20-week-old mice, we next focused on the gene expression profiles on PTEN null and WT mice at 12 and 20 weeks of age using a cutoff *P*-value of less than or equal to 0.05. A comparison between the 20-week-old PTEN null and WT control groups showed the greatest difference: 1278 genes were expressed at lower levels and 2645 genes were expressed at higher levels in the PTEN deletion-induced mice than in the WT control mice. At 12 weeks old, the loss of PTEN resulted in the downregulation of 1091 genes and the upregulation of 1812 genes (Fig. 4A). Of the 28 differentially expressed genes identified in both comparisons, 6 were downregulated in the 12-week group but upregulated in the 20-week group, and 22 were upregulated in the 12-week group but downregulated in the 20-week group. A total of 2657 genes were changed in the same direction (951 genes downregulated and 1706 genes upregulated). As shown in Fig. 4B, the clustering of the most differentially expressed genes showed clearly distinguishable patterns between the PTEN null and WT control groups. RNA-Seq data are also presented in Volcano plots for both the upregulated and the downregulated genes (Fig. 4C). Similarly, a comparison of 20-week-old mice showed that there were more significantly differentially

expressed genes at this age than at 12 weeks old. Hence, PTEN deletion led to an increase in the number of differentially expressed genes from 12 weeks to 20 weeks of age.

We further performed IPA with RNA-Seq data to identify the pathways regulated by PTEN deletion and/or its time-dependent effects. To achieve this goal, we used a broader list of differentially regulated genes that were generated by using a definition based on the false discovery rate ($q < 0.05$) and a $\log_2\text{FoldChange} > 1.0$ or < -1.0 . In these experiments, we analyzed 1810 genes identified in the comparison of 12-week-old PTEN null and WT control mice and 3587 genes identified in the comparison of 20-week-old PTEN null and WT control mice; an ingenuity pathway analysis was used. We then obtained two lists of regulated pathways from the two comparisons. Based on the $-\log(P\text{-value})$ of the pathways generated by ingenuity pathway analysis, the top 30 pathways that were common between the groups, all of which had a $-\log(P\text{-value}) > 4$ ($P < 0.0001$; Supplementary Tables 1), were selected, and their 'activation z scores' are shown in a heatmap (Fig. 4D). Among these pathways, 28 were activated by PTEN deletion in both the 12- and 20-week groups, while the other 2 pathways were inhibited. In addition, the effects exerted by PTEN deletion on the pathways were stronger at 20 weeks than at 12 weeks. Interestingly, most of these genes are involved in inflammatory responses and immune response pathways, such as NF- κ B signaling, IL-6 signaling, the LPS/IL-1-mediated inhibition of RXR function, PI3K signaling in B lymphocytes, iCOS-iCOSL signaling in T helper cells, and the role of NFAT in the regulation of the immune response. It was not surprising that these findings suggested that PTEN loss results in increases in the level of inflammation over time, especially via effects on genes that affect inflammatory responses and immune response pathways.

PTEN KO and WT mice exhibit different DNA methylation

Genomic DNA extracted from both replicates (12 and 20 weeks old for a total of 8 samples) was prepared for genome-wide DNA methylation next-generation sequencing using the Agilent SureSelect Mouse Methyl-Seq library preparation method, in which a capture-based approach is used to specifically enrich target sequences against the mouse methylome. Quality control metrics for the DNA methylation data showed that there was good genomic coverage and that a high percentage of reads were above the threshold. PCA showed that the samples were clustered based upon their first two principal components and that there were clear differences between PTEN null mice and their wild-type counterparts (Fig. 5C).

To improve our understanding of the involvement of DNA methylation in prostate tumorigenesis, we examined single-base-resolution DNA methylation of prostate samples obtained from the four groups as described in the above RNA-Seq section. We segmented our datasets according to gene substructure and found that methylation levels were unevenly distributed across the genome. The methylation ratios were much higher in exons, introns, 3'-untranslated regions (3'-UTRs) and intergenic regions than in 5'-untranslated regions (5'-UTRs) and promoter regions (Fig. 5A). However, the overall methylation levels in these four datasets were significantly different for the following two comparisons: 12-week-old PTEN null versus control and 20-week-old PTEN null versus control (Fig. 5B). We then focused on CpG-specific DNA methylation in two comparisons, PTEN null and WT mice at 12 and 20 weeks of age, using a cutoff for the methylation percentage difference of greater than or

equal to 25 % and a P -value of less than or equal to 0.01. Venn diagrams were generated to represent the intersections between the different comparisons. The comparison between the 20-week-old PTEN null and WT control groups showed the greatest difference: compared to the control animals, in the PTEN deletion-induced animals, 8557 loci were hypomethylated, and 14171 were hypermethylated. At 12 weeks old, compared to the control animals, in the PTEN null mice, 5292 loci were hypomethylated, and 5597 loci were hypermethylated (Fig. 5D). Of 1141 differentially methylated loci identified in both comparisons, 747 were hypomethylated in the 12-week-old group but hypermethylated in the 20-week-old group, and 394 were hypermethylated in the 12-week-old group but hypomethylated in the 20-week-old group. A total of 1708 loci had changed in the same direction (1052 hypermethylated and 656 hypomethylated). As shown in Fig. 5E, the clustering of top differentially methylated loci showed that there were clearly distinguishable patterns across PTEN null and WT control groups. These results clearly demonstrate that changes in DNA methylation are highly associated with PTEN loss in PCa development.

Correlation investigation of gene expression and DNA methylation

Given the impressive changes in gene expression and DNA methylation observed among the groups in this study, we next integrated the profiles to determine whether there are associations between methylation and gene expression. Most often, clusters of CpG loci form islands (also named DMR) that play an important role in the transcription of crucial and cancer-inhibiting genes. It is generally accepted that promoter methylation of DMRs is associated with the decreased transcription of downstream genes. Therefore, the DMRs were sorted into those containing at least 3 CpG sites, all of which were hypermethylated or hypomethylated. Of the 3 or more CpG sites, at least 1 CpG methylation difference greater than 10 % and all other CpG site methylation differences greater than 5 % that had a P value smaller than 0.05 were considered significant. Then, a cutoff q -value of less than 0.05 for the differentially expressed genes was applied for the combination with the significantly differentially methylated DMRs. Some but not all of the genes were involved in the correlation between gene expression and DNA promoter methylation. Surprisingly, seven genes stood out in the comparison of 12-week-old PTEN null versus WT control mice, and seventy-two genes were exacted for the 20-week-old PTEN null versus WT control group comparison (Supplementary Table 2). It was not surprising that many more genes were identified in the comparison of the 20-week-old groups than in that of the 12-week-old groups (Fig. 6). Moreover, 5 genes from the 12-week-old comparison and 6 genes from the 20-week-old comparison were selected for validation by qPCR (Fig. 7). Among these 11 genes, Cxcl1, Muc1, F3, Rnasel, and Cyr61 were upregulated, while I17d, Wnk4, Cietd2, Fry, and Eaf2 were downregulated by PTEN loss. It was noted that the angiogenesis-, inflammation-, and tumorigenesis-related gene CXCL1 (hypomethylation and upregulated gene expression) was confirmed in both the 12-week-old and the 20-week-old comparison by qPCR and western blotting. CXCL1 has been reported to play a critical role in prostate cancer progression. Collectively, our results suggest that an important subset of genes associated with PCa can be identified by investigating the correlations between gene expression and DNA methylation.

Discussion

In recent years, the role of PTEN in regulating normal development and physiology as well as tumorigenesis has been illustrated (35). PTEN has an important function in multiple tissues and organs. To address the role of PTEN in PCa, a PTEN prostate-specific deletion mouse model was used in this study in combination with RNA-Seq and DNA SureSelect methyl-Seq analysis. The correlation investigation of gene expression with methylation was performed in a genome-wide manner for the first time using unbiased methods in the mouse prostate. Some important PCa-related genes and DMRs were extracted from comparisons of 12-week-old and 20-week-old PTEN null and control wild type mice. Of note, CXCL1 was confirmed to be hypomethylated and upregulated in both 12-week-old and 20-week-old PTEN null mice. Additionally, some inflammatory response and immune response signaling pathways were identified in this integrated molecular study. Thus, studying how PTEN influences DNA methylation and gene expression during PCa development can increase our understanding of the molecular mechanisms that underlie PTEN-controlled prostate adenocarcinoma.

Our histopathological examination, IHC, qPCR, and western blotting results obtained from the PTEN KO mouse model are consistent with those presented in previous reports, demonstrating that the mouse model used by our group is suitable for performing such studies (7). Furthermore, our sequencing data obtained using this model have confirmed and extended previous observations by providing a plausible mechanistic explanation. Loss of PTEN was positively associated with a significantly higher risk of recurrence in PCa patients. The CpG sites that were aberrantly methylated in PTEN-deleted prostate tumors may mediate tumor progression (36). With the rapid development of techniques used to investigate the cancer methylome, several studies have identified candidate risk loci or DMRs for PCa (37, 38). However, it is not clear whether these epigenetic modifications are associated with gene function. Thus, DNA methyl-Seq and RNA-Seq were performed along with the correlation investigation.

An important subset of genes associated with PCa development was identified in our investigation of the correlation between gene expression and DNA methylation, and these results offer new insights that may facilitate the discovery of important genes that drive PCa and suggest potential targets and biomarkers for the prevention/treatment of PCa. To our surprise, when PTEN was deleted in the prostate, CXCL1 was upregulated, and the fold change notable increased from 12 weeks (9.50) to 20 weeks (260.72) according to the RNA-Seq results (Supplementary Tables 2). However, the role of CXCL1 in prostate-specific PTEN KO-induced PCa progression has been poorly investigated. CXCL1 plays important roles in inflammation, angiogenesis, tumorigenesis, and wound healing and has also been shown to be involved in the process of tumor formation (39). Increased CXCL1 protein levels were present in high-grade malignancies, in which it was associated with higher-grade prostate tumors (40). Increasing evidence shows that TAMs/CXCL1 promotes breast cancer metastasis via NF- κ B/SOX4 activation, and CXCL1-based therapy might become a novel strategy for breast cancer metastasis prevention (41). Also, targeting IL-6 and TIMP4 interactions with a CXCL1 monoclonal antibody offers a novel strategy to therapeutically manage prostate and bladder cancers (42). A recent study showed that obese patients with

PCa have increased epithelial CXCL1 expression. Because it is driven by CXCL1 and CXCL8, adipose stromal cell recruitment to tumors could promote PCa progression (43). The inactivation of PTEN in smooth muscle promoted a pro-inflammatory phenotype and increased the production of CXCL1 under basal conditions (44). In addition, inhibiting PTEN expression in hepatocellular carcinoma HCC3 cells reduced their sensitivity to gefitinib in terms of its inhibitory effect on both Akt phosphorylation and the production of VEGF and CXCL1 (45). Our observation shows that the expression of CXCL1 was higher in PTEN null than in control mice agreed with findings presented in previous studies and indicates that CXCL1 could play a crucial role in PCa progression. Inhibition of CXCR2, the CXCL1 receptor, has been shown to block inflammation-driven tumorigenesis in PTEN-deficient mice (46). Additionally, our results provide the first evidence showing that the CXCL1 gene is remarkably hypomethylated in PTEN null mice, but the precise mechanisms underlying the role of CXCL1 in PCa remains unclear.

The top 30 significantly affected canonical pathways were predominantly inflammation-related and immune response-related pathways, including NF- κ B signaling, IL-6 signaling, the role of NFAT in the regulation of the immune response, and iCOS-iCOSL signaling in T helper cells. PTEN deletion activated these signaling pathways, and their levels were higher at 20 weeks than at 12 weeks (Fig. 4D). Inflammation is important to the pathogenesis of malignancies. Of note, two major proinflammatory cytokines, IL-1 β and TNF, were involved in most of the identified inflammation-related pathways. These factors could impact the biological behavior of prostatic tumors and could, therefore, be used to predict clinical outcomes in PCa, suggesting that IL-1 β and TNF signaling could be involved in PCa progression (47). Variation in IL-1 β expression was associated with PCa recurrence independent of pathologic prognostic factors (48). It has been reported that PTEN deletion in the epithelium results in the significant upregulation of genes within the inflammatory response and cytokine-cytokine receptor interaction pathways, including IL-1 β (49). Increasing evidence suggests that chronic inflammation is closely associated with epigenetic alterations mediated by DNA and histone modifications, thus driving changes in the expression of many inflammation-related genes, such as IL1R1, IL-1 β , toll-like receptor (TLR) 2, 15-lipoxygenase (15-LOX), cyclooxygenase-2 (COX2), CXCL14, CCL25, CXCL6, IL13, IL17C, and IL4R (50–57). In prostate carcinogenesis, growing evidence suggests a link between chronic or persistent inflammation and tumor development (5). Epidemiological data indicate that people diagnosed with chronic inflammatory prostatitis have an increased risk of developing PCa at a later age. Hence, our results further underscore the importance of inflammation in PCa progression and provide a plausible explanation based on whole-genome methylation profiling.

In summary, in this current study, we defined a quantitative global profile for the methylome and transcriptome of prostate-specific PTEN null mice using DNA SureSelect methyl-Seq and RNA-Seq analysis. Importantly, a subset of genes associated with PCa development was identified in our investigation of the correlation between gene expression and DNA methylation. It should be noted that some inflammatory response and immune response signaling pathways were implicated at both 12 weeks and 20 weeks of PTEN null mice. A limitation of the analysis is the small sample size included in the studies presented here, as this limited our ability to perform an integrative analysis of the changes in expression and

methylation that occurred between the PTEN null mice and their WT counterparts. However, our observations and conclusions offer new insights into how epigenetic modifications affect the progression of PCa, and these results could be translated into clinically useful applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviations

PCa	prostate cancer
PTEN	the phosphatase and tensin homolog located on chromosome 10
NQO1	NAD (P) H: quinone oxidoreductase 1
NRF2	NF-E2-related factor 2
PIN	prostatic intraepithelial neoplasia
qPCR	quantitative polymerase chain reaction
WT	wild-type; KO, knockout
TCGA	The Cancer Genome Atlas
NGS	next-generation sequencing

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Figure 1.

The severity of PIN and inflammation development gradually increased as PTEN null mice aged. (A) Volcano plots of the Cancer Genome Atlas (TCGA) RNA-Seq data indicating that PTEN expression levels are significantly lower in PTEN-deleted tumor samples than in solid tissue normal samples. The plots show that PTEN expression is unchanged between PTEN copy number normal tumor samples and solid tissue normal samples. The x-axis shows log2 expression fold-changes (FCs), and the y-axis shows log2 (padj values). The vertical dashed lines represent FC = 1.0; (B) Schematic diagram illustrating the generation of PTEN null mice from Pb-Cre4 and Pten(flox/flox) mice; (C) Histopathological examination of 12-week-old PTEN null mice with PIN progression lesions; (D) Histopathological examination of 20-week-old PTEN null mice with PIN progression lesions; (E) Histopathological examination of WT mice; (F) Representative photo of prostates obtained from PTEN null and WT mice.

Figure 2.

Differences between PTEN null and WT mice at the molecular level. (A) Relative mRNA expression levels of Nrf2, Nqo1, and Nkx3.1; (B) Relative mRNA expression levels of COX2, iNOS and IL6; (C) The percentage of global DNA methylation; (D) Protein levels of markers of inflammation, oxidative stress, cell proliferation, and epigenetic mechanisms; (E) IHC staining for Nqo1 and Ezh2 in 20-week-old PTEN KO prostate tissues; (F) ChIP assays were performed to analyze the enrichment of H3K27me3 and H3K27Ac on the promoters and transcription start site, respectively, of Nkx3.1 in PTEN KO prostates. Data are expressed as the mean $\pm$ SD (n=3; normalized with β -actin and compared with the control value). * $p < 0.05$; # $p < 0.01$.

Figure 3.

RNA-Seq comparison of PTEN null and WT mice. (A) Between-strain correlation observations showed that there was a high level of reproducibility between samples; (B) PCA of RNA expression levels in PTEN null and WT mice showed that there were clear differences; (C) Hierarchical clustering dendrogram also showing similar clustering according to gene expression.

Figure 4.

Selective gene expression and signaling pathways in PTEN null and WT mice. (A) Venn diagram comparing the upregulated and downregulated genes in the 12-week-old PTEN null versus WT group and the 20-week-old PTEN null versus WT group; (B) Heatmap illustrating the clustering of the top differentially expressed genes showing that there were clearly distinguishable patterns across the PTEN null and WT control groups; (C) Volcano plots showing DEGs in response to PTEN loss at 12 weeks and 20 weeks with cutoffs of $q < 0.05$ and $\log_2(\text{fold change}) > 1$ or < -1 ; (D) Heatmap showing the top 30 regulated pathways that were shared in both comparisons (12 week/20 week PTEN null vs. WT group). Inflammatory responses and immune response pathways were affected by PTEN loss.

Figure 5.

Methyl-Seq comparison of PTEN null and WT mice. (A) Changes in DNA methylation observed between the PTEN null and WT mice by DNA region, including exons, introns, 5'-UTRs, 3'-UTRs, promoters, TTSs, and intergenic regions; (B) The percentage of global DNA methylation in the 12-week-old PTEN null versus WT group and the 20-week-old PTEN null versus WT group; (C) PCA of DNA methylation in PTEN null and WT mice showing that there were clear differences between the groups; (D) Venn diagram comparing the hypo- or hypermethylated CpG loci in the 12-week-old PTEN null versus WT groups and the 20-week-old PTEN null versus WT groups; (E) Heatmap illustrating the clustering of the top differentially methylated loci; the results revealed clearly distinguishable patterns across the PTEN null and WT control groups.

Figure 6.

Investigation of correlations between gene expression and DNA methylation. Starburst plots showing the 7 genes identified in the 12-week-old PTEN null versus WT comparison and the 72 genes identified in the 20-week-old PTEN null versus WT comparison based on a cutoff of 0.1 for DNA methylation in the promoter region and a 2-fold change threshold for RNA expression. Hypermethylated and downregulated genes are indicated in red, and hypomethylated and upregulated genes are indicated in blue.

Figure 7.

Validation of the mRNA changes observed for selected genes and their regulation by PTEN loss. mRNA and protein isolated from 12-week-old/20-week-old PTEN null and WT mice was subjected to qPCR analysis and western blotting analysis for CXCL1, respectively. Black bar: the qPCR results are presented as fold changes of 12-week-old/20-week-old PTEN null vs. WT groups obtained using GAPDH as the endogenous control. White bar: fold changes obtained in the RNA-Seq analysis. Bars represent the mean values of triplicate samples (mean $\pm$ SD).

Table 1:

The primers used in qPCR.

Primer Sequence (5' 3')	
CXCL1 For	CTGGGATTCACCTCAAGAACATC
CXCL1 Rev	CAGGGTCAAGGCAAGCCTC
MUC1 For	GGCATTCTGGGCTCCTTTCTT
MUC1 Rev	TGGAGTGGTAGTCGATGCTAAG
IL17D For	AGCACACCCGTCTTCTCTC
IL17D Rev	GCTGGAGTTCGCACTGTCC
WNK4 For	TCGGGTTTTGCCTTGTCTATTC
WNK4 Rev	ACAACCTCTGTCGCTCTGGTCT
CITED2 For	CGCCAGGTTTAACAACCTCCCA
CITED2 Rev	TGCTGGTTTGTCCCGTTCAT
EAF2 For	GCAGAAGCTAGTCTTATGGACCA
EAF2 Rev	GGGTGCTCCGAGCTGTATTTT
FRY For	AGCCTGGACAAGTGTGATATGC
FRY Rev	TGACTCATCGGAATCCTCGTG
RNASEL For	TAGGCGAACACATCAATGAGGA
RNASEL Rev	CTGCCTCTGGAACGCTGAG
F3 For	AACCCACCAACTATACCTACACT
F3 Rev	GTCTGTGAGGTCGCACTCG
CYR61 For	CTGCGCTAAACAACCTCAACGA
CYR61 Rev	GCAGATCCCTTTCAGAGCGG
GAPDH For	ACATCGCTCAGACACCATG
GAPDH Rev	TGTAGTTGAGGTCAATGAAGGG
NKX3.1-CHIP1 For	TGCGGATAAAGGAACCACCA
NKX3.1-CHIP1 Rev	AGGCATGACAAGTAGGTGCAGC
NKX3.1-CHIP2 For	TGAAAAGCATGCCCTGGTG
NKX3.1-CHIP2 Rev	CGCGGTGAGAAAATCAGTGTC