Integrative multi-Omics analysis depicts the methylome and hydroxymethylome of recurrent bladder cancers and identifies biomarkers for predicting PD-L1 expression

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Abstract

Background:

Urinary bladder cancer is one of the most common malignancies of the urinary tract; however, the mechanism of its high recurrence and responses to immunotherapy remains unclear, resulting in difficulties in clinical outcome prediction. Epigenetic alterations, especially DNA methylation, play important roles in bladder cancer development and are increasingly investigated as biomarkers for diagnostic or prognostic predictions. However, little is known regarding hydroxymethylation since previous studies based on bisulfite-sequencing approaches could not differentiate between 5mC and 5hmC signals, resulting in entangled methylation results.

Methods:

Here, we used a multi-omics approach to provide the genome, transcriptome, methylome, and hydroxymethylome landscape of both primary and recurrent bladder cancers.

Results:

By whole exome sequencing, we identified driver mutations that are involved in the UBC development, such as FGFR3, KDMTA and KDMT2C. However, few of these driver mutations are associated with the recurrence or the PD-L1 down-regulation in UBC. By integrating RRBS and oxRRBS-seq data, we identified fatty acid oxidation-related genes significantly enriched in 5hmC-associated transcription alterations in recurrent bladder cancers. We also observed a serial of 5mC hypomethylation DMRs in the gene body of NFATC1, a gene that is highly involved in the T-cell immune response, in bladder cancer samples with high expression of PD-L1. And since 5mC and 5hmC alternations are globally anti-correlated, RRBS-seq based markers which combine the 5mC and 5hmC signals, attenuate the cancer-related signals and therefore are not optimal to be used as clinical biomarkers.

Conclusions:

By multi-omics profiling of UBC samples, we showed that epigenetic alternations are more involved than genetic mutations in the recurrence and PD-L1 regulation of UBC. In addition, as a proof of principle, we demonstrated that the combined measurement of 5mC and 5hmC level by bisulfite-based method will compromise the prediction accuracy of epigenetic biomarkers.

Introduction

Urinary bladder cancer (UBC) is one of the most common malignancies of the urinary tract and one of the major causes of cancer-related deaths worldwide (1). Approximately 75% of UBCs were non-muscle-
invasive (2, 3). Although early-stage UBCs are treatable with transurethral resection of the bladder tumors, they are characterized by frequent recurrence rates as high as 60–80% (4). Guidelines recommend lifelong surveillance for recurrent UBC through cystoscopy (5). However, cystoscopy is invasive, painful, and costly. Furthermore, its unsatisfactory sensitivity could lead to missing 10–40% of bladder malignancies (6). Urine cytology is a complementary tool that is hampered by suboptimal sensitivity, especially in low-grade UBCs (7, 8). Therefore, there is an urgent need for an accurate, efficient, and preferably noninvasive screening and diagnostic method for UBC.

DNA methylation and hydroxymethylation are epigenetic mechanisms that covalently affect DNA and can be exploited as DNA biomarkers. DNA methylation is conventionally acknowledged as a silencing epigenetic marker, which is promoted by DNA methyltransferases (DNMTs), generating 5-methylcytosine (5-mC or 5mC) (9). In contrast, hydroxymethylation involves an oxidative process that converts 5-mC to 5-hydroxymethylcytosine (5-hmC or 5hmC). This reaction is promoted by a family of dioxygenases, namely ten-eleven translocation proteins (TETs). Through hydroxymethylation, TETs can help maintain an unmethylated state, thus playing the role of an activator in intracellular transcription (10). Previous studies have shown that 5hmC tends to exist in promoter regions with dual histone markers, including H3K4me3 for expression activation and H3K27me3 for expression repression. Thus, 5hmC may be involved in regulating gene expression by recruiting activators or repressors. Genomic hypermethylation, which may result from a disturbed balance between methylation and hydroxymethylation, can lead to aberrant silencing of tumor suppressors and DNA repair enzymes, resulting in accelerated carcinogenesis (11). 5hmC modification levels are decreased in cancers, including bladder cancer. However, few studies have investigated 5hmC in bladder cancers at base resolution.

As previously reported, aberrant DNA hydroxymethylation is a hallmark of various malignancies (12). Forloni et al. (13) demonstrated that TET was transcriptionally downregulated by oncogenic epidermal growth factor receptors in lung cancers, thus silencing diverse tumor suppressors. The same was valid for colorectal cancer, as indicated by Neri et al. (14). TET normally inhibits tumor growth by repressing the WNT signaling pathway via the demethylation of the promoters of WNT inhibitors. In colon cancer tissues, downregulation of TET led to insufficient DNA hydroxymethylation of WNT inhibitors and accelerated colon carcinogenesis. Other malignancies potentially affected by aberrant DNA hydroxymethylation include prostate cancer, breast cancer, ovarian cancer, skin cancer, and several hematopoietic malignancies (e.g., acute myeloid leukemia, chronic myelomonocytic leukemia, and T-cell lymphomas) (14–16). Notably, aberrant DNA hydroxymethylation is also considered one of the earliest events in urothelial carcinomas (16, 17). A global loss of 5-hmC has been observed in both UBC and UBC cell lines compared to controls or adjacent tissues (15). Therefore, similar to the conventional DNA methylation level measured based on bisulfite treatment, the hydroxymethylation level is also conceived to be a promising biomarker for UBC detection, especially in the repetitive surveillance of recurrent UBC.

In the present study, we used a multi-omics approach to investigate the possible use of 5mC and 5hmC levels (base-resolution) as urinary biomarkers for associating crucial clinical outcomes and investigating their diagnostic values in UBC.
Materials And Methods

Clinical samples

The study was performed at the Urology Surgery, Xuzhou Central Hospital, Xuzhou 221009, PR China. The subjects were recruited from 2020.09 to 2021.02. Tissue samples of bladder cancer patients who underwent laparoscopic radical cystectomy (LRC), partial cystectomy (PC) or transurethral resection of bladder tumor (TURBT) were selected and bladder tumor tissues and paracancerous tissues were collected. The tissues were cleaned with sterile normal saline immediately after the surgery and stored at minus 80 degrees (-80°C) for detection. A total of 45 bladder tissue specimens were collected from 45 patients, including 45 bladder cancer tissues and 18 paracancerous tissues.

RNA-seq library construction

The RNA-seq libraries were constructed by E-GENE Co. Ltd as follows. Briefly, the total RNA of each sample was extracted using Invitrogen TRIzol® Reagent and then treated with RNase-free DNase I for 30 min according to the manufacturer's protocols. The poly(A) containing mRNA were purified using Oligo(dT) Beads from about 1 ug total RNA. The captured mRNA was firstly fragmented into 100-200nt using divalent cations at elevated temperature. The fragmented mRNA was reverse transcribed with SuperScript II and then converted to double-stranded cDNA using RNaseH and DNA Pol I by random priming. After purification, the double-stranded cDNA was subjected to blunt-ending, dA addition to 3'-end and adapter ligation. Finally, PCR was carried out to enrich the adapter ligated cDNA and the libraries were analyzed by Agilent Bioanalyzer 2100 and quantified by qPCR before sequenced by the Illumina sequencing platform.

oxRRBS & RRBS-seq library construction

2µg genomic DNA was digested using MspI enzyme for 16 hours at 37°C. After digestion, libraries were constructed as the Illumina Pair-End protocol with some modifications. Briefly, purified digested DNA was subsequently treated with a mix of T4 DNA polymerase, Klenow Fragment and T4 polynucleotide kinase to repair, blunt and phosphorylate ends. After that, sequencing control DNA supplied by the TrueMethyl Seq Kit (CEGX) were mixed with the blunt DNA. The mixture DNA fragments were subsequently 3’ adenylated using Klenow Fragment (3’-5’ exo-) and following with ligation to adaptors synthesized with 5’-methylcytosine instead of cytosine using T4 DNA Ligase. the DNA was purified using QIAquick PCR purification kit (Qiagen) after reaction of each step.

Before oxidation reaction, all products had been purified using Magnetic Beads Binding Solution 1 supplied by the TrueMethyl Seq Kit (CEGX) according to the manufacturer’s instructions. After purification, the oxidation reaction was conducted in a reaction volume of 25 µl with 1 µl of the Oxidant Solution (CEGX) for each oxRRBS library, and RRBS library sample added in 1ul of ultrapure water instead of Oxidant as control. Both two libraries were subjected to 40°C for 30 min treatment in a thermo cycler with the lid heated at 57°C. After that, centrifuged the reaction mixture at 14,000 X g for 10 min and then transferred the supernatant into a new 0.2 ml PCR tube for the further bisulfite treatment, respectively.
Bisulfite conversion treatment was performed using a TrueMethyl Seq Kit (CEGX) according to the manufacturer’s instructions. The final oxRRBS and RRBS libraries were generated by PCR amplification using adapter compatible barcode primers, quantified by an Agilent 2100 Bioanalyzer (Agilent Technologies) and real-time PCR assay and then sequenced by Illumina Hiseq.

5mC/5hmC specific qPCR

Clinical samples of 25 wax blocks were cut into 10μm thick wax rolls and sample nucleic acids were extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, USA). BisulPlus™ Loci 5mc & 5hmC Detection PCR Kit (Epigentek, USA) was used to modify the genomic DNA with bisulphite according to the manufacturer’s instructions. Bisulfite conversion of genomic DNA results in unmethylated cytosine being converted to uracil while methylated cytosines remain unchanged. The bisulfite-modified DNA was further treated with specific APOBEC deaminase, which converts 5mC to T, distinguishing it from 5hmC. Using online CPG Island software to analyse the EZHIP, ALKBH5 and TUBG1 genes (http://emboss.bioinformatics.nl/cgi-bin/emboss/cpgplot). The sequences of the more dense CGP dinucleotide region were selected to design the 5mC and 5hmC primers on Premier 5.0 software (Primer, Canada). Prepare the reaction system according to the manufacturer’s instructions using the amplification reagents in the BisulPlus™ Loci 5mc & 5hmC Detection PCR Kit (Epigentek, USA). The PCR Cycling conditions were as follows: 60 s at 95°C, followed by 45 cycles of 15 s at 95°C and 30 s at 60°C, and a final cycle of 15 s at 95°C, 60 s at 60°C, 30 s at 95°C and 15 s at 60°C. The sequences of PCR primers were as follows: EZHIP (5hmC): PCR forward CCGTTTTTCCGCTGCAGGC and PCR reverse CGTCAGCACAGCTATGATTCGAGGC; ALKBH5 (5hmC): PCR forward GGCGGCGCGCGTGAAGACAG and PCR reverse GCAGACAGGAACCGCTTGCCGTCCG; TUBG1 (5hmC): PCR forward GGGATTTCGTTGTGGGATTTTGGATTTTA and PCR reverse CCCTCAAAATTTAAACACTAAACTA.

Measuring serum HSV IgG level

The venous blood of patients was collected following uniform standards and sent to the laboratory department of Xuzhou Central Hospital for determination. HSV1/2 IgG expression levels were measured on an AutoLumo A6000 instrument (AutoBio, China) using a herpes simplex virus type 1/2 antibody kit (AutoBio, China) according to the manufacturer’s instructions.

Mutation data analysis

Fastq data were achieved and aligned with Burrows-Wheeler Aligner using hg38 genome. Samtools was used for sorting and GATK (4.2.5) best practice (Mutect2) was applied for analyzing the mutation data. Maftools was used for analyzing the tumor mutations and generating the oncoplots. TCGA datasets were used for comparing the driver mutation frequency.
RNA-seq data analysis

Trimmomatic (v0.39) was used to remove sequence with low-quality and adapter sequence from raw sequence files. Trimmed fastq files were mapped to hg38 genome reference by using STAR (v2.7.0a). Alignments were then cleaned by samtools (v1.9) view function with the parameter -F set as 268. The read counts mapped to each gene was calculated by Htseq-count (v2.0.2). Finally, DESeq2 (v1.36.0) was used to calculate the differentially expressed genes. The significant differentially expressed genes are defined by adjusted P value less than 0.05 and absolute value of log2FoldChange more than 1. Pathway enrichment and Gene ontology analysis were performed by using Enrichr.

oxRRBS-seq/RRBS-seq data analysis

TrimGalore (v0.6.5) was used to remove sequence with low-quality, adapter sequence and 5bp from 5’ end of read R2. The trimmed reads were then aligned to hg38 human genome reference by BSMAP (v2.90). Samtools view function was used to filter low quality alignments from the mapped bam file. The methratio.py provided by BSMAP was used for calculating the CpG methylation level from the cleaned bam file. The 5hmC methylation level of each CpG sites was calculated by subtracting the 5mC (oxRRBS-seq methylation level) level from RRBS-seq methylation level. The CpG 5mC or 5hmC ratio were finally processed by metilene (v0.2-8) to call de-novo 5mC or 5hmC DMRs, respectively. The significant 5mC DMRs are defined by absolute value of methylation difference more than 0.2 and adjust P value less than 0.05. The significant 5hmC DMRs are defined by absolute value of methylation difference more than 0.1 and adjust P value less than 0.05. Pathway enrichment and Gene ontology analysis were performed by using Enrichr.

Results

Genomic and transcriptomic profiling of the bladder cancer cohort

We examined the mutations (SNVs, indels) in our UBC samples with different statuses and grades (blue: recurrent, high-grade; red: primary, low-grade) (Figure S1A). The median number of variants per sample was 283.5, most of which were missense. The top ten genes with mutations were MUC16, TTN, HRNR, PRAG1, FAM8A1, LNP1, CBX3, KCNN2, DHDH, and TMPRSS13 (Figure S1B). All tumors were identified as microsatellite stable using genome-wide microsatellite analysis. The RTK-RAS, NOTCH, and WNT pathways were identified as the most significantly altered pathways in the enrichment analysis of the mutation datasets (Figure S1C). We next examined the mutation frequency of the known UBC driver mutations and determined that most allele frequencies ranged from 0.2 to 0.8 (Figure S1D, S1E). The mutation frequencies of these driver genes in our cohort were also comparable to those in the known TCGA datasets.
We further performed RNA-seq on 10 bladder paracancer tissues and 44 bladder cancer tissue samples to investigate transcriptomic alterations during bladder cancer oncogenesis. The RNA-seq data were processed using the STAR mapping tool. Using DESeq2, we identified 340 significantly upregulated and 458 downregulated genes (Figure S2A). The upregulated genes were most enriched in the herpes simplex virus 1 (HSV1) infection pathway, suggesting the presence of HSV1 infection in bladder cancer samples. Consistent with previous studies reporting that dysregulation of coagulation is associated with HSV infection, downregulated genes were enriched in the complement and coagulation cascade pathways. The downregulated genes were also enriched in common cancer signaling pathways, such as the PI3K-Akt and MAPK signaling pathways. In addition, we also observed the dysregulation of genes related to focal adhesion and ECM-receptor interaction, which are crucial to bladder cancer metastasis (Figure S2B). Overall, we here presented a high-quality mutation and RNA-seq dataset for the UBC cohort.

**Depicting the 5mC and 5hmC landscape alternation in bladder cancer through oxRRBS-seq and RRBS-seq**

The role of DNA methylation in bladder cancer oncogenesis has been extensively studied. However, most studies are based on the 450K methylation array, RRBS, or WGBS, which cannot distinguish 5mC from 5hmC. Oxidative RRBS (oxRRBS) was developed by Booth et al. (18) to further convert 5hmC into 5fC while keeping 5mC unconverted; therefore, it can profile the 5mC status on a genome-wide scale at base resolution. To illustrate the role of 5mC in bladder cancer development, we performed oxRRBS on 10 paracancerous and 44 cancerous bladder tissue samples. Using Metilene, we identified 1266 significantly hypermethylated 5mC differentially methylated regions (5mC DMRs) and 2666 hypomethylated 5mC DMRs (Fig. 1A). The DMRs had a minimal mean methylation change of 0.2 with an adjusted P-value of less than 0.05.

Although the bladder cancer samples had increased hypomethylated DMRs, a similar number of hyper- and hypo-DMRs were annotated to the promoter, exon, and TTS regions. In contrast, more hypo-DMRs were annotated in the intron and intergenic regions (Fig. 1B). This finding is consistent with previous studies suggesting that hypermethylation occurs in promoter regions, whereas hypomethylation occurs in the gene body and intergenic regions. Hypermethylated genes are highly enriched in urogenital system development and branching involved in ureteric bud morphogenesis, suggesting that bladder cancer progression is accompanied by the disruption of the morphology of the urogenital system. These genes are also enriched in kidney development, such as kidney epithelium and metanephros development, consistent with chronic kidney disease is common in older patients with bladder cancer (Fig. 1C).

The hypo-DMRs are mainly enriched for positive regulation of anoikis, engulfment of apoptotic cells, and apoptotic cell clearance. Additionally, hypo-DMRs are enriched in PI3K pathway activity and cell cycle regulation, which contributes to the increased proliferative ability of bladder cancer cells. Notably, hypo-DMRs are the most enriched in male gonad development. Given that bladder cancer prevalence is three to four times higher in men, it will be interesting to further investigate whether DNA methylation is involved in the difference in bladder cancer incidence between sexes (Fig. 1D).
Next, we performed RRBS and oxRRBS on the same 10 bladder paracancer tissue and 44 bladder cancer tissue samples. The 5hmC level is calculated by subtracting the oxRRBS methylation level from RRBS, which profiles both 5hmC and 5mC at the same time. By comparing bladder cancer tissues to paracancerous tissues, we identified 287 hyper-5hmC DMRs and 534 hypo-5hmC DMRs. The 5hmC DMRs had a minimal mean methylation change of 0.1 and an adjusted P-value of less than 0.05. In contrast to 5mC DMRs, we observed a comparable number of 5hmC hyper-DMRs and hypo-DMRs in the exon, intergenic, intron, and TTS regions, but approximately five times more 5hmC hypo-DMRs in promoter regions than 5hmC hyper-DMRs (Fig. 1A and 1B).

Both 5hmC hyper- and hypo-DMRs are enriched in pathways related to the urinary system, such as ureteric bud development and kidney development, indicating that 5hmC alterations are likely involved in disrupting the urinary system during bladder cancer development. Additionally, 5hmC hypo-DMRs were enriched in nervous system development, regulation of the FGFR signaling pathway, positive regulation of IL-13 and IL-4 production, mesenchymal cell development, and negative regulation of epithelial cell proliferation suggesting that 5hmC also plays a role in regulating the proliferation, immune escape, and EMT of bladder cancer cells (Fig. 1E and 1F). We also observed 5hmC hypo-DMRs in many common transcriptional regulators, such as HDAC2, CHD8, E2F3, and KMT2A (Supplementary Table S3). These results further support the hypothesis that 5hmC hypomethylation is a crucial regulator of bladder cancer gene expression.

**Profiling transcriptional and methylation alternations in recurrent bladder cancer**

By comparing the RNA-seq data of 31 primary and nine recurrent bladder cancers using DESeq2, we identified 695 significantly upregulated genes and 197 downregulated genes (Figure S3A). The upregulated genes were mainly related to extracellular structure organization, ECM organization, angiogenesis, growth factor activity, and inflammatory responses. The downregulated genes were enriched in oxidative stress, purine catabolism, and the PPAR signaling pathway (Figure S3B). These results suggest that restructuring the ECM, inducing the generation of blood vessels, and repressing the stress response pathway are crucial for bladder cancer progression.

Similarly, by comparing primary and recurrent bladder cancer, we identified 1940 5mC hyper-DMRs, 516 5mC hypo-DMRs, 122 5hmC hyper-DMRs, and 742 5hmC hypo-DMRs (Fig. 2A). Consistent with the transcriptomic alterations, 5mC hyper- and hypo-DMRs were enriched in GO terms related to epithelial-to-mesenchymal transition, morphogenesis of epithelial tubes, cell-cell adhesions, and mesenchymal cell proliferation. In addition, 5mC hypo-DMRs are also enriched in the Wnt signaling pathway, which is usually exploited by cancer cells for survival and proliferation. Interestingly, the 5hmC hypo-DMRs were uniquely enriched in pathways related to lipolysis regulation (Figure S4).

Since targeting fatty acid metabolism has been reported to inhibit the malignant phenotype of bladder cancer (19), it is worth investigating whether 5hmC can directly regulate fatty acid metabolism, which may reveal new strategies or targets for drug development. We identified 5hmC DMRs associated with
fatty acid oxidation-related genes, including TWIST1, CGA, PTGER3, NPR1, GNAS, IRS2, and GNAI1. The 5hmC DMRs associated with PTGER3, NPR1, GNAS, IRS2, and GNAI1 were directly annotated to promoter regions. The 5hmC DMRs associated with TWIST1 and CGA were annotated to intergenic regions but overlapped with promoter-like cis-regulatory elements predicted by the ENCODE project (Figure S5). These observations suggest that 5hmC is directly involved in the regulation of the transcriptional activity of these genes. 5hmC DMRs are hypomethylated for TWIST1, NPR1, IRS2, and CGA, which was anti-correlated with their increased transcription levels. In contrast, the 5hmC DMR of PTGER3 is hypermethylated, whereas its expression is also highly upregulated in recurrent bladder cancers. GNAS and GNAI1 were not differentially expressed in recurrent bladder cancer samples, although they were associated with the 5hmC DMR in the promoter regions (Fig. 2B). However, we noticed that both genes had different transcript isoforms, suggesting that 5hmC DMRs may play a role in regulating alternative splicing (Fig. 2B and Figure S5). In summary, these results indicate that 5hmC can directly regulate the transcriptional activity of fatty acid metabolism genes in recurrent bladder cancer. Consistent with previous studies, the transcription factor to which 5hmC binds would decide the mechanism by which it regulates the expression.

Since the profiling of the 5hmC level was indirect, which was calculated by subtracting oxRRBS methylation from RRBS methylation, we performed 5hmC-specific PCR to validate one of the most hypermethylated 5hmC DMRs that is annotated to TUBG1, and a top hypomethylated 5hmC DMR annotated to EZHIP. Consistent with the sequencing results, we observed that the 5hmC level of TUBG1 was significantly increased in recurrent bladder samples, while the 5hmC level of EZHIP was decreased. To examine the relationship between 5mC and 5hmC, we performed 5mC-specific PCR on these two 5hmC DMRs. For both genes, 5mC levels were decreased in recurrent bladder cancer samples, suggesting that 5hmC alternations are not always anti-correlated with 5mC alternations (Fig. 2C).

Finally, five patients were diagnosed with recurrent bladder cancer within one year after the collection of UBC tissue samples. We then compared the 5mC and 5hmC profiling of the tissue samples of these five patients with the primary bladder cancers tissues of patients who haven't developed recurrent bladder cancers. Interestingly, the differential 5mC methylation of these five patients mimics the methylation alternations observed in the recurrent bladder cancer tissues (Fig. 2D). In contrast, the differential 5hmC methylation profile of these five patients is not correlated with recurrent bladder cancer tissues (Fig. 2E). These results demonstrate that 5mC alternations related to recurrent bladder cancer occur at the early stage during the bladder cancer progression and can be exploited as biomarkers for predicting bladder cancer prognosis. However, 5hmC alternations of recurrent bladder cancer cannot be observed in advance of the disease progression, therefore are less appropriate for being used as prognosis biomarkers.

**Profiling transcriptional and methylation alternations in PD-L1 overexpression in bladder cancer**

To investigate the mechanism by which PD-L1 is regulated in bladder cancers, we split bladder cancer samples into PD-L1-high and -low groups based on PD-L1 expression levels. We identified 532 significantly upregulated genes, and 23 significantly downregulated genes in the PD-L1-high group
(Figure S6A). As expected, the upregulated genes were enriched in the negative regulation of inflammation and negative regulation of T cell activation, suggesting that PD-L1 overexpression in bladder cancer can inhibit the immune response. In addition, the upregulated genes were most enriched in an extracellular matrix organization and cell adhesion. Similarly, these genes were also most enriched in the focal adhesion-PI3K-Akt-mTOR-signaling pathway. Given that a previous study demonstrated that the focal adhesion kinase (FAK) could control PD-L1 expression and induce immune escape, the acquisition of mobility by downregulation of focal adhesion genes is likely associated with the overexpression of PD-L1 in bladder cancers (Figure S6B).

To investigate whether DNA methylation is involved in the immune escape process, we identified 1589 hyper 5mC DMRs, 2149 hypo 5mC DMRs, 370 hyper 5hmC DMRs and 1725 hypo 5hmC DMRs in samples with high PD-L1 expression levels (Fig. 3A). The hyper 5mC DMRs were highly enriched to maturity-onset diabetes of the young, basal cell carcinoma, and Hippo signaling pathways. In contrast, hypo 5mC DMRs were highly enriched in adherens junction assembly and negative regulation of epithelial-to-mesenchymal transition, and positive regulation of the apoptotic signaling pathway (Figure S7). In addition, among the 532 upregulated genes in PD-L1 highly expressed samples, 49 genes corresponded to 5mC hypo DMRs. These genes, including CCL11, CCL20, and S1PR1, were significantly enriched in the cellular response to cytokine stimuli and T-cell migration (Figure S8A).

The hyper 5hmC DMRs were enriched in the bladder cancer pathway, toll-like receptor signaling, and stress-activated MAPK cascade. In contrast, the hypo 5hmC DMRs were enriched in the insulin signaling pathway, regulation of lipolysis in adipocytes, focal adhesion, and the AMPK signaling pathway (Figure S7B). Among the transcriptionally upregulated genes in samples with highly expressed PD-L1 levels, 51 genes were associated with 5hmC hypo DMRs. These genes were enriched in the extracellular matrix organization and negatively regulated the inflammatory response to antigenic stimuli (Figure S8B).

**DNA methylation biomarkers for predicting immunotherapy response**

Here, we compared the RRBS-seq and oxRRBS-seq data between PD-L1 high and low bladder cancer samples and identified methylation markers that had optimal performance in predicting samples with a highly expressed PD-L1 gene. By univariate analysis, we identified 102 5mC DMR biomarkers with significant methylation differences and predicted an AUC score of more than 0.8 (Fig. 3B). In contrast, there were only 21 5hmC DMRs with AUC scores higher than 0.8, and their methylation difference was less significant than that of 5mC DMRs, indicating that 5mC biomarkers are more appropriate for predicting high PD-L1 expression in bladder samples.

In most cell-free DNA methylation biomarker studies, the methylation level is profiled using bisulfite-based or similar enzymatic technologies that convert both 5mC and 5hmC into uracil and therefore cannot distinguish between 5mC and 5hmC signals. Because 5hmC is a product of the active demethylation process of 5mC, 5hmC methylation alterations are globally anti-correlated with 5mC methylation alterations, which was indeed observed when comparing PD-L1-high and PD-L1low samples (Fig. 3C).
Given that 5hmC has a worse predicted AUC score and undergoes opposite methylation alterations than 5mC biomarkers, the oxRRBS-seq, which only profiles 5mC, can then be used to identify better markers than the commonly used RRBS-seq. As a proof-of-principle, we examined one of the top-performing DMR markers annotated to the TET gene. This DMR is 5mC-hypomethylated, whereas 5hmC is hypermethylated in PD-L1-high bladder cancer samples. When the methylation alternation was profiled using RRBS, this TET DMR was also hypomethylated, but the methylation difference was less significant than the 5mC alternation (Fig. 4A). Correspondingly, the 5mC level of this TET DMR had the highest predicted AUC score compared to those of the 5hmC and RRBS methylation levels (Fig. 4B).

Notably, among the 102 identified 5mC DMR biomarkers, 5 DMRs are annotated to the NFATC1 gene, which plays a crucial role in T cell activation and is involved in the regulation of PD-1/PD-L1 signaling. By lowering the AUC score cutoff, 16 significant 5mC DMRs were identified in the NFATC1 gene body. Most of these DMRs are hypomethylated, co-localized with the NFATC1 exons, and encode cis-regulatory element regions, suggesting that these 5mC alterations are involved in the transcriptional regulation of NFATC1. The best-performing DMR (chr18:79461612–79461992), which is close to the seventh exon of NFATC1, had an AUC score of 0.884 when predicting PD-L1-high samples (Fig. 4C). Altogether, we demonstrated the potential of using DNA methylation markers for predicting immunotherapy response in patients with bladder cancer and the advantages of using oxRRBS-seq for methylation marker discovery.

**Discussion**

To comprehensively investigate the development of recurrent bladder cancer and the PD-L1 overexpression resulting in tumor immune escape, we performed multi-omics experiments to delineate the genetic, 5mC and 5hmC alternations during the above processes. By whole exome sequencing, we identified mutations in many known UBC driver genes, such as KDM6A, TP53 and FGFR3. However, few of these driver mutations are enriched to the recurrent bladder cancer or PD-L1 over-expression patients (Supplementary Figure S9). In contrast, significant 5mC and 5hmC DMRs can be identified in various pathways that are associated with cancer progression and immune response. These results indicates that epigenetic alternations are more involved in the progression of UBC.

The hydroxymethylation level, as mediated by ten-eleven translocation proteins (TET), could help regulate fatty acid metabolism. TET1 can be recruited by peroxisome proliferator-activated receptors α and γ (PPARα and PPARγ) to induce the demethylation of their response element region and interfere with fatty acid metabolism (20, 21). A decrease in TET1 could lead to the upregulation of genes involved in lipogenesis and fatty acid uptake, as well as the downregulation of genes related to lipolysis and fatty acid oxidation (21). Notably, elevated lipogenesis was predictive of poor prognosis in certain tumor types (22). Previous studies on the metabolic characteristics of cancer cells have demonstrated that, unlike normal cells, cancer cells require larger amounts of fatty acids because of the higher demand for the synthesis of signaling molecules, cellular structural elements, and adenosine triphosphate (23, 24). The fatty acid synthase (FASN) expression in bladder cancer is significantly upregulated and is regarded as an adverse prognostic factor for bladder cancer recurrence and progression (25–27). Tao et al. found that
a small interfering RNA of FASN could upregulate E-cadherin expression and downregulate Snail expression in bladder cancer cells, suggesting that recurrent bladder cancer may be associated with FASN-induced epithelial-mesenchymal transition (28). In this study, we specifically identified that fatty acid oxidation-related genes were significantly associated with 5hmC-induced transcription alteration in recurrent bladder cancers. These results enrich the current knowledge on how 5hmC contributes to metabolism regulation through epigenetic regulation.

Escaping immune surveillance is one of the hallmarks of cancer progression. One way of escaping the immune system is through PD-L1 overexpression by cancer cells, which binds to PD1 on the immune cell surface and inactivates the cancer immune response. Given its promising anti-tumor effect, the PD1/PD-L1 inhibitor (pembrolizumab) has been approved to treat bladder cancers. However, less than half of bladder cancer patients respond to immunotherapy. PD-L1 expression has been well established as a biomarker for identifying patients more likely to benefit from immunotherapy. Advanced technologies for cell-free DNA methylation detection further make it possible to develop non-invasive blood tests to pre-select immunotherapy-responsive patients. According to our results, hypomethylation of the NFATc1 gene body was observed in bladder cancer cells, indicating an activating state in NFATc1 transcription, and this state could be used to predict PD-L1 expression. The nuclear factor of activated T cells 1 (NFATc1) is one of the main isoforms of NFAT expressed in T cells and plays an essential role in regulating gene transcription in response to T-cell receptor (TCR)-mediated signals (29–31). NFATc1 can also be activated by the B cell receptor (BCR) and upregulate the IL-10 chemokine to activate the JAK2/STAT3 pathway in B cell lymphoma cells, ultimately inducing PD-L1 expression (32). The correlation between NFATc1 and PD-L1 expression is similar in bladder cancer. According to Kawahara et al., PD-L1 is more highly expressed in high-grade bladder cancer than in low-grade bladder cancer and is positively correlated with the expression of NFATc1 genes (33). Therefore, the STAT1-PD-L1-NFATc1 pathway was proposed to reveal the immunosuppressive mechanism of PD-L1, which may increase the potential for PD-L1-based antitumor immunotherapy in bladder cancer (33). In this study, we built a machine learning-based model to directly predict PD-L1-high bladder cancers through the methylation level of NFATc1 and achieved an AUROC score of 0.884. Future studies should focus on whether this precise prediction can be achieved using cell-free DNA.

In the present study, we employed improved methods of genome-wide methylation and hydroxymethylation mapping by combining oxidative-reduced representation bisulfite sequencing (oxRRBS) with traditional RRBS. Standard RRBS cannot distinguish between 5mC and 5hmC, thus rendering previous studies to report the sum of 5mC and 5hmC. In oxRRBS, selective oxidation of 5hmC to fC can be achieved using potassium perruthenate. The hydroxymethylation amounts can be determined by subtracting the bisulfite sequencing signals obtained from oxRRBS from RRBS(18, 34, 35). Therefore, an accurate 5mC and 5hmC fraction could be separately calculated using RRBS + oxRRBS, thus providing more detailed epigenetic information at a higher resolution and being more suitable for use as a biomarker for bladder cancer diagnosis and prognosis.
Declarations

Competing interest

The authors declare no competing financial interest.

Ethics approval and consent to participate

All the protocols were approved by the Ethical Committee of the Xuzhou Central Hospital (EC. XZXY-LI-20200708-024). All the subjects provided a written consent form.

Consent for publication

Not applicable.

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Authors' Contributions

Zhen-Duo Shi, Xiao-Xiao Han and Yang Dong designed the research and analyzed the data. Kun Pang, Bing-Zheng Dong, Lin Hao, and Qing Liang performed research and analyzed data. Xiao-Xiao Han and Yang Dong wrote the paper. Zhen-Duo Shi and Cong-Hui Han revised the manuscript critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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Data Availability
Data was deposited in the NCBI GEO database. We will provide the accession number when it is approved by the GEO official.

References


**Figures**
Figure 1

5mC and 5hmC profiling of bladder cancer samples at single-base resolution. (A) Number of significant 5mC and 5hmC DMRs identified in bladder cancer samples. (B) The genomic annotations of 5mC and 5hmC DMRs. Pathway enrichment of 5mC hyper (C) and hypo DMRs (D) in bladder cancers. Pathway enrichment of 5hmC hyper (E) and hypo DMRs (F) in bladder cancers.
Figure 2

The rewiring 5mC and 5hmC landscape in recurrent bladder cancer. (A) Number of 5mC and 5hmC DMRs identified in recurrent bladder cancer samples (left). The genomic annotations of 5mC and 5hmC DMRs (right). (B) RNA-seq normalized count of fatty acid metabolism related genes with 5hmC DMRs. (C) 5mC and 5hmC specific qPCR for TUGB1 and EZHIP DMRs. (D and E) Correlation between differential
methylation profiles in recurrent bladder cancer and five patients who developed recurrent UBC after tissue collection.

**Figure 3**

Methylation alternations in PD-L1 highly expressed bladder cancer. (A) Number of 5mC and 5hmC DMRs identified in PD-L1 highly expressed bladder cancer samples (left). The genomic annotations of 5mC and 5hmC DMRs (right). (B) Heatmap showing the methylation alternations of 5mC DMR biomarkers in PD-L1 highly expressed bladder cancer samples. (C) Scatter plot showing the correlation between differential 5hmC level and differential 5mC level in PD-L1 highly expressed bladder cancers.
Figure 4

Utilizing 5mC biomarkers for predicting immunotherapy response. (A) 5mC, 5hmC and RRBS methylation level alternations of TET gene. (B) The prediction AUC score of TET 5mC, 5hmC and RRBS methylation in predicting PD-L1 highly expressed bladder cancers. (C) Track plot showing the 5mC DMRs annotated to NFATC1 (left). The prediction AUC score of one NFATC1 DMR for predicting PD-L1 highly expressed bladder cancers (right).

Supplementary Files
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