Whole-genome DNA methylation and hydroxymethylation profiling for HBV-related hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) is a common solid tumor worldwide with a poor prognosis. Accumulating evidence has implicated important regulatory roles of epigenetic modifications in the occurrence and progression of HCC. In the present study, we analyzed 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) levels in the tumor tissues and paired adjacent peritumor tissues (APTs) from four individual HCC patients using a (hydroxy)methylated DNA immunoprecipitation approach combined with deep sequencing [(h)MeDIP-Seq]. Bioinformatics analysis revealed that the 5-mC levels in the promoter regions of 2796 genes and the 5-hmC levels in 507 genes differed significantly between HCC tissues and APTs. These differential genes were grouped into various clusters and pathways and found to be particularly enriched in the 'Metabolic pathways' that include 'Glycolysis/gluconeogenesis', 'Oxidative phosphorylation' and 'Citrate cycle (TCA cycle)', implicating a potential role of metabolic alterations in HCC. Furthermore, 144 genes had both 5-mC and 5-hmC changes in HCC patients, and 10 of them (PCNA, MDM2, STAG1, E2F4, FGF4, FGF19, RHOBTB2, UBE2QL1, DCN and HSP90AA1) were enriched and interconnected in five pathways including the 'Cell cycle', 'Pathway in cancer', 'Ubiquitin mediated proteolysis', 'Melanoma' and 'Prostate cancer' pathways. The genome-wide mapping of 5-mC and 5-hmC in HCC tissues and APTs indicated that both 5-mC and 5-hmC epigenetic modifications play important roles in the regulation of HCC, and there may be some interconnections between them. Taken together, in the present study we conducted the first genome-wide mapping of DNA methylation combined with hydroxymethylation in HBV-related HCC and provided a series of potential novel epigenetic biomarkers for HCC.

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Introduction

Hepatocellular carcinoma (HCC), a common solid tumor, is the third most frequent cause of cancer-related death in the world. Hepatitis B virus (HBV) infection is the main cause of HCC in China (1). Individuals with chronic HBV infection, especially those who have progressed to chronic liver disease and cirrhosis, are at high risk of developing HCC (2,3). Most HCC patients are diagnosed at their advancing stage and refractory to chemotherapy and radiotherapy (4,5). Even if the patients receive liver transplantation, the recurrence rate is still high (6).

Epigenetic modifications are found to play important roles in various biological processes especially in cancer development (7). Methylation of DNA at 5-position of cytosines (5-mC) is a key epigenetic mark that has been extensively studied in many types of malignancies (8). Aberrant DNA methylation of promoter CpG islands has been associated with global hypomethylation and specific loci hypermethylation, which has the potential to become diagnostic markers for the progression of malignant tumors (9). 5-mC can be converted to 5-hydroxymethylcytosine (5-hmC) by the ten-eleven translocation (TET) family proteins. In mammals, 5-hmC is detected in almost all tissues and cell types (10,11). Emerging evidence has shown that 5-hmC and TET family might serve unique biological roles in many biological processes such as gene expression regulation, gene transcription and DNA methylation regulation (12,13). Several studies have found 5-hmC alternations in the epigenetic regulation of various diseases, including cancer (14).

Studies of DNA methylation changes in HCC have led to the identification of several candidate methylated genes as potential tumor biomarkers (15,16), yet, little is known about hydroxymethylation distribution in HCC. In previous studies, DNA methylation was determined using methylation sensitive polymerase chain reaction combined bisulfite restriction

Table I. Clinicopathological features for the four HCC patients included in the study.

Variables	SAM 1	SAM 2	SAM 3	SAM 4
Case number	560	629852	716677	717323
Age (years)	41	53	40	57
Gender	Male	Male	Male	Male
ALT (U/l)	35	30	54	34
AFP(ng/ml)	2	>50000	12628.5	8241
HBV-DNA	10^4	10^3	10^3	10^3
Tumor size (cm)	2x2	7.5x8	3.5x3	8.5x8.5
Tumor number	Single	Multiple	Single	Single
PVTT	No	Yes	Yes	Yes
Grade	Moderate	Poor	Moderate	Moderate

PVTT, portal vein tumor thrombus.

analysis (COBRA) or bisulfite sequencing techniques. With the development of high-throughput sequencing technologies, the whole-genome DNA (hydroxy)methylation profiling in cancer has generated data with significantly higher quantity and quality (17,18). However, most existing studies of DNA methylation in HCC employed Infinium HumanMethylation BeadChip Arrays or Methylation Microarray (19,20), which may have some limitations on resolution and scope. A novel method termed (hydroxy)methylated DNA immunoprecipitation sequencing [(h)MeDIP-Seq], combining DNA immunoprecipitation with high-throughput sequencing, has emerged as an advantageous tool for identifying (hydroxy) methylated CpG-rich sequences in a much faster and more sensitive manner than ever before.

In an attempt to explore the 5-mC/5-hmC changes in HCC, we performed a genome-wide mapping of 5-mC/5-hmC in four paired HCC tissues and adjacent peritumor tissues (APTs) using MeDIP-Seq/hMeDIP-Seq.

Materials and methods

Clinical samples. Total of 4 fresh-frozen primary HCC tissues and paired APTs were included in MeDIP-Seq/ hMeDIP-Seq. The collected cancer tissues were excised within the margins of the cancer lesion, and the APTs were collected from a location at least 3 cm distant from the tumor boundaries. All the collections followed the same protocol. All of the cancerous tissues were diagnosed as primary hepatocellular carcinoma, provided by two independent and experienced pathologists. Fresh-frozen HCC tissues and APTs were collected during the surgical resection.

The four HCC patients were HBV surface antigen-positive without hepatitis C virus (HCV) infection and exhibited the same cirrhosis etiology. Retrospectively data were collected including demographic, preoperative laboratory and pathological parameters from electronic medical records, and are summarized in Table I.

DNA extraction. Genomic DNA was extracted from frozen HCC tissues and paired APTs using the DNeasy Blood and Tissue kit (Qiagen; 69504) according to the manufacturer's protocol. Briefly, tissues were homogenized using a hand-held homogenizer, digested with Proteinase K (Qiagen; 69504) and RNase A (Qiagen; 19101) overnight at 56˚C, precipitated and washed. Concentration and purity of DNA were measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

MeDIP-seq and hMeDIP-seq. As previously described (21,22), the genomic DNA was fragmented using a Covaris sonication system (Covaris, Woburn, MA, USA) according to the parameters. After sonication, the fragments were denatured to produce single stranded DNA (ssDNA). Following denaturation, the ssDNA was incubated with anti-5-mC antibody or anti-5-hmC antibody. The antibody-DNA complexes were captured by protein A/G beads, and the MeDIP and hMeDIP products were collected for sequencing with HiSeq™ 2000 sequencing system (Illumina, Inc., San Diego, CA, USA).

Identification of differential methylation/hydroxymethylation regions (DMR/DHMR). DMR and DHMR identification are based on reads per kilo base of transcript per million mapped reads (RPKM)-normalized to 5-mC and 5-hmC density, used model-based analysis of ChIP-Seq (MACS).

Functional enrichment analysis. Functional enrichment analysis is for the genes associated with DMRs and DHMRs. Gene Ontology (GO) (https://david.ncifcrf.gov/) is a standard classification system inferring gene function and gene products. PANTHER website (http://go.pantherdb.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (Web-based Gene Set Analysis Tool Kit and http://www.kegg.jp/kegg/pathway.html) were also used suggesting physiological functions of these genes.

Ethics statement. All experimental protocols and study methods were approved by the Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University. The written consent was received from each participant in the present study at the time of surgery.

Results

Global DNA (hydroxy)methylation changes in HCC tissues and APTs. We isolated total genomic DNA from the 4 HCC tumor tissues and paired APTs, and employed (h)MeDIP-seq to explore genome-wide 5-mC and 5-hmC profiles for the 8 samples. In total, MACS identified 4.52 million reads and 6.0 million reads of sequencing data for 5-mC and 5-hmC, respectively (Table II). Differential 5-mC and 5-hmC peaks between HCC tumor tissues and paired APTs are shown in Table III. Density distribution of these peaks on the whole genome is shown in Fig. 1. 5-mC and 5-hmC peak enrichment profiles of HCC tumor tissues compared with APTs in genomic areas were shown in Fig. 2. Both CvP and PvC differential peaks of 5-mC were enriched in X5UTR and Exon. 5-hmC peak enrichment seemed average in each genomic features.

Analysis of DMR and hDMR-associated genes in promoter regions. Promoter region is an important gene regulation

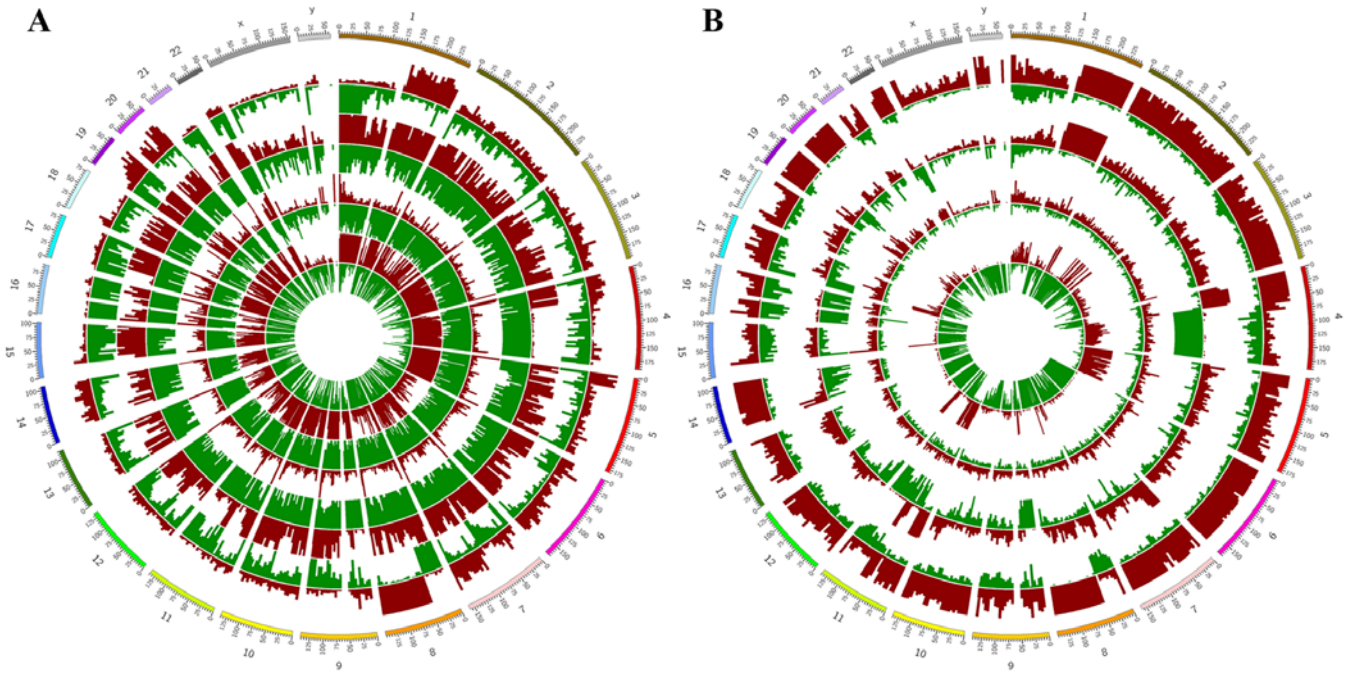

Figure 1. (A) Density distribution of 5-mC peaks on each chromosome. (B) Density distribution of 5-hmC peaks on each chromosome. From the outside to the inside, SAM-716677, SAM-717323, SAM-629852, SAM-560, respectively. Red, peaks upregulated in HCC tumor tissues compared with paired APTs. Green, peaks downregulated in HCC tumor tissues compared with paired APTs (calculated the peaks number in each five mb).

Table II. Number of reads generated by (h)MeDIP-Seq for each sample.

		560 (P/T)	629852 (P/T)	716677 (P/T)	717323 (P/T)
MeDIP-Seq					
Total number of reads	P	178,832,47	288,179,93	22939802	20338428
	T	176,540,07	204,678,83	21452236	31407738
Total number of mapped read	P	129,391,26	222,400,94	21408527	16080416
	T	152,462,39	160,719,42	19448683	28980186
Mapping rate (%)	P	72.35%	77.17%	93.32%	79.06%
	T	86.36%	78.52%	90.66%	92.27%
hMeDIP-Seq					
Total number of reads	P	251,088,30	299,964,13	340,096,74	316,801,82
	T	340,606,22	314,499,77	265,629,54	269,660,39
Total number of mapped reads	P	191,931,19	248,284,70	265,222,32	239,597,42
	T	266,231,98	247,343,23	213,428,28	214,664,23
Mapping rate (%)	P	76.44%	82.77%	77.98%	75.63%
	T	78.16%	78.65%	80.35%	79.60%

Table III. Differentially expressed peaks of 5-mC and 5-hmC MACS of each paired samples.

Samples	MeDIP-CvP	MeDIP-PvC	hMeDIP-CvP	hMeDIP-PvC
560	51475	44772	14853	53739
629852	9137	35823	9061	5748
717323	70363	87400	16464	17036
716677	17408	23586	42497	9332

CvP, upregulated peaks in HCC tumor tissues compared with paired APTs; PvC, downregulated peaks in HCC tumor tissues compared with paired APTs.

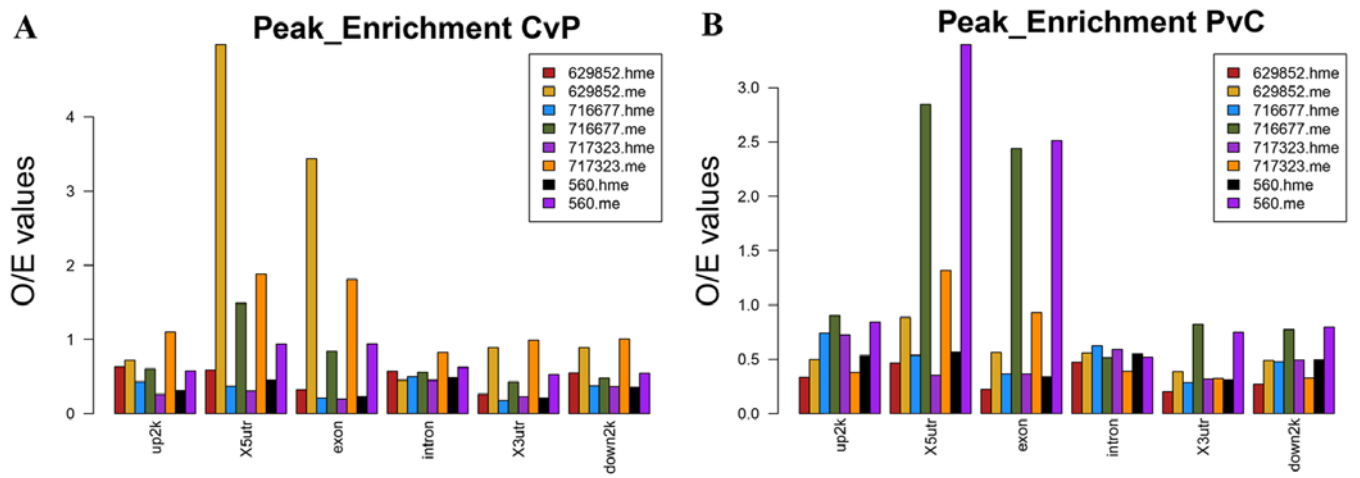

Figure 2. Enrichment of 5-mC and 5-hmC peaks of HCC tissues compared with APTs in genomic areas. (A) Upregulated peaks of 5-mC and 5-hmC in HCC tissues compared with APTs (CvP), (B) downregulated peaks of 5-mC and 5-hmC in HCC tissues compared with APTs (PvC). O/E, obtain/expect. O/E>1 means peaks relatively significant enriched area.

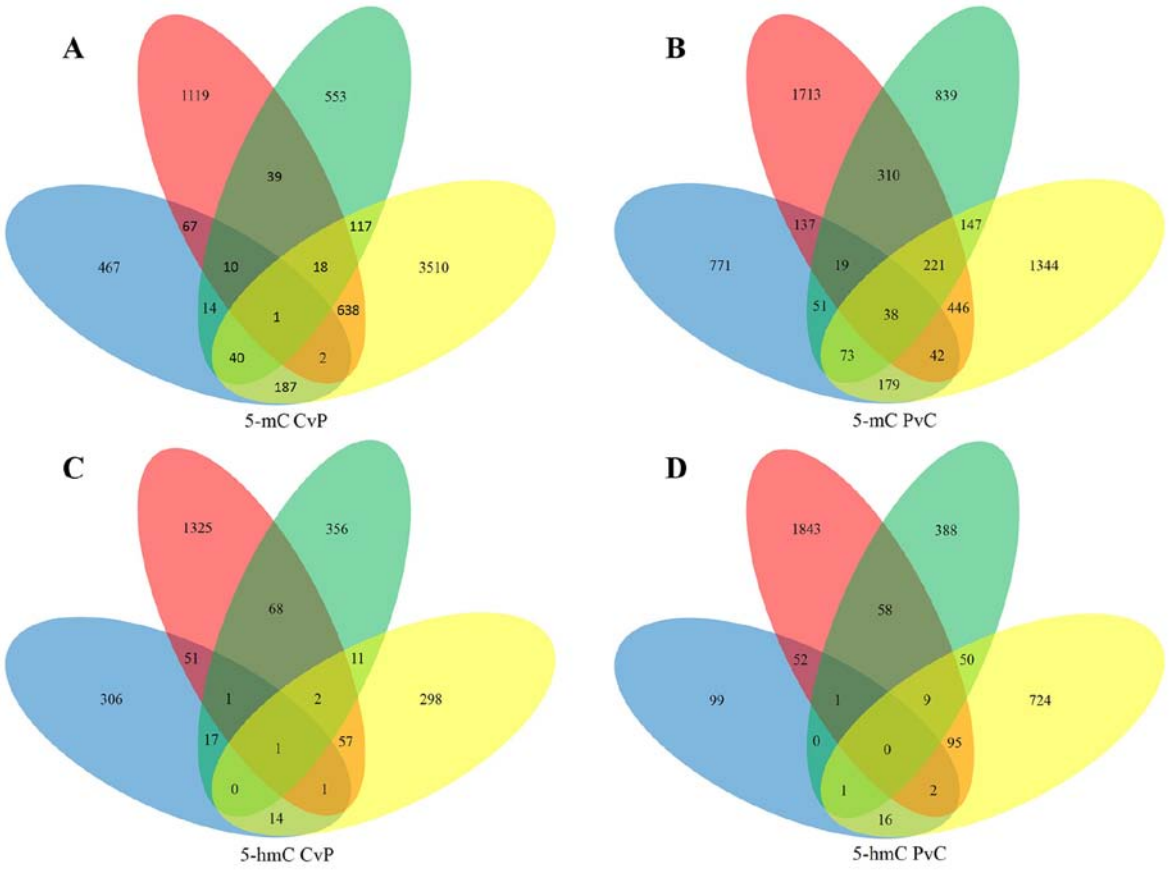

Figure 3. Composition of DMR and DHMR-associated genes in four paired samples. (A) A total of 1133 hypermethylated genes were found in at least two samples (5-mC CvP); (B) 1663 hypomethylated genes were found in at least two (5-mC PvC); (C) 223 upregulated 5-hmC genes were found in at least two samples (5-hmC CvP); (D) 284 downregulated 5-hmC genes in HCC tissues compared to APTs were found in at least two samples (5-hmC PvC). Blue, SAM-560; red, SAM-629852; green, SAM-716677; yellow, SAM-717323.

region, and the methylation or demethylation at this region plays a key regulatory role in gene expression. In the present study, we carried out further bioinformatics analysis to identify locus-specific DMRs and hDMRs between HCC tumor tissues and APTs in the promoter region (-3.5K to +1.5K of TSS). The total differential 5-mC peaks (DMRs) that exhibited significant difference between the two groups (>2-fold, $P<0.05$) were associated with nearly 4097 genes in terms of RefSeq ID. Of the four samples, 1924, 788, 4521 and 734 genes had hypermethylation (5-mC-CvP) at their promoters while 2956, 1667, 2490 and 1310 genes had hypomethylation (5-mC-PvC), respectively (Table IV).

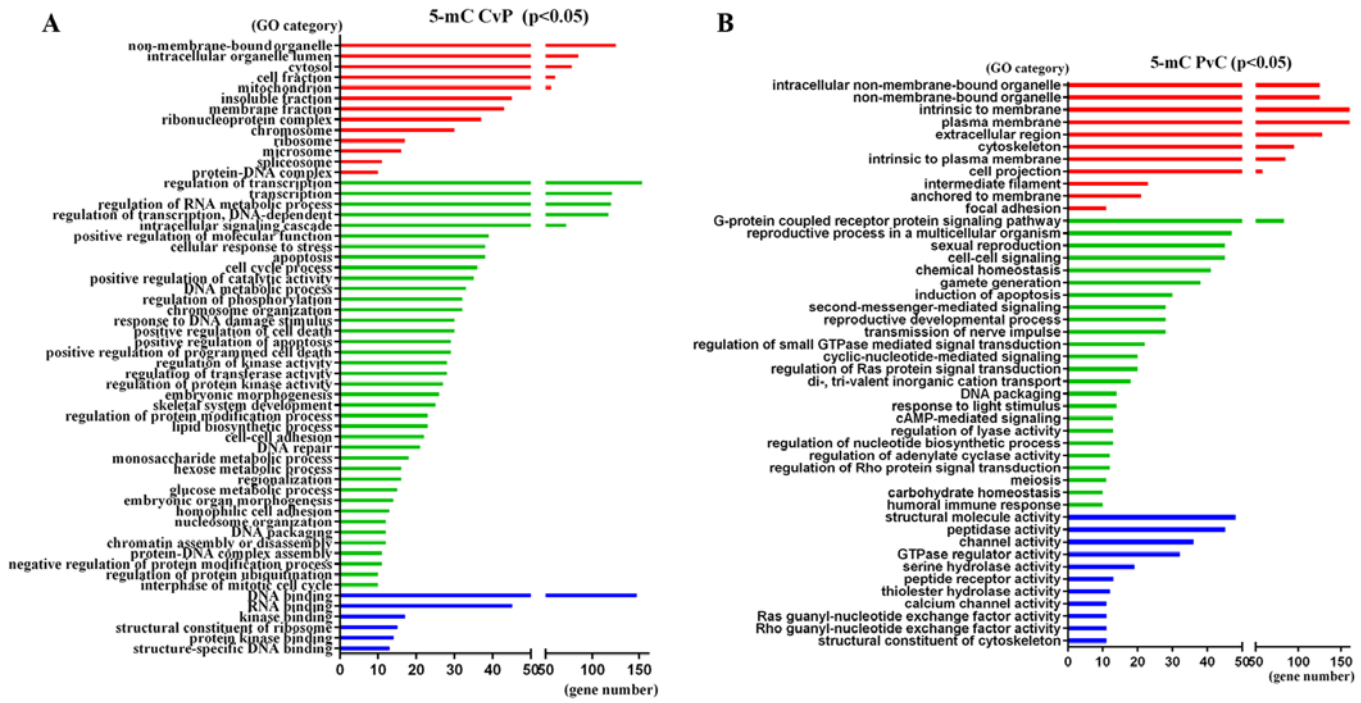

Figure 4. (A) The significant GO categories of hypermethylated genes (5-mC CvP) in HCC tissues compared with APTs; (P<0.05). (B) The significant GO categories of hypomethylated genes (5-mC PvC) in HCC tissues compared with APTs (P<0.05). (Red, cellular component; green, biological process; blue, molecular function).

Table IV. Numbers of DMRs and hDMRs associated genes.

Samples	5-mC-CvP	5-mC-PvC	5-hmC-CvP	5-hmC-PvC
560	1924	2957	385	2061
629852	734	1310	457	171
717323	4521	2490	391	897
716677	788	1667	1506	507

CvP, upregulated genes in HCC tumor tissues compared with paired APTs; PvC, downregulated genes in HCC tumor tissues compared with paired APTs.

The same analysis was carried out to search for differential 5-hmC peaks (DHMRs) between HCC tumor tissues and APTs in the promoter. An average of 1593 genes were associated. Genes (385, 1506, 391 and 457) showed higher 5-hmC levels (5-hmC-CvP) in the promoter in HCC tissues compared with APTs of the four samples, while 2061, 507, 897 and 171 genes showed lower 5-hmC levels (5-hmC-PvC), respectively. (Table IV).

Functional enrichment analysis of 5-mC and 5-hmC associated genes. A total of 1133 5-mC-CvP genes and 1663 5-mC-PvC genes were found in at least two samples (Fig. 3A and B). The significant GO categories of these genes are shown in Fig. 4 (P<0.05). The most enriched term of 5-mC-CvP genes is 'Transition metal ion binding' (GO:0046914, P=7.20E-06), 'Regulation of RNA metabolic process' (GO:0051252, P=4.10E-05) and 'DNA binding' (GO:0003677, P=4.60E-05), while the 5-mC-PvC genes were enriched in 'Plasma membrane' (GO:0005886, P=2.80E-05), 'Keratin filament' (GO:0045095, P=6.50E-05) and 'Cyclic-nucleotide-mediated signaling' (GO:0019935, P=8.70E-05).

The KEGG pathway analysis showed that the significantly differential hypermethylated genes were enriched in several pathways such as 'Metabolic pathways' (adjP=0.0021) (such as GBE1, GALNT6, NDUFS6, HEXB, RRM1 and CKMT2) and 'Pathways in cancer' (adjP=0.0027) (such as CDKN2A, CDKN2B, APC, GSTP1, DAPK3, FADD, FGF4 and FGF19), while differential hypomethylated genes were enriched in 'Neuroactive ligand-receptor interaction' (adjP=0.0011) (such as P2RY4, SSTR5, AVPR2, MAS1, NTSR1 PRSS3, GHSR and CALCRL) and 'Calcium signaling pathway' (adjP=0.0140) (such as ATP2B3, RYR1, NTSR1, NOS1, HTR5A, SLC25A31, GNAS and DRD5). In Table V all significant KEGG pathways (adjp<0.05) are listed. For protein class by PANTHER website, both hypermethylated and hypomethylated genes were mainly associated with 'Nucleic acid binding', 'Hydrolase' and 'Receptor' (Fig. 5).

Next, DHMRs between HCC tumor tissues and APTs in the promoter were subjected to the same analysis. A total of 223 5-hmC-CvP genes and 284 5-hmC-PvC genes were found in at least two samples (Fig. 3C and D). The most significant GO categories of 5-hmC-CvP genes were 'Plasma membrane' (GO:0005886, p=3.10E-05), 'G-protein coupled receptor protein signaling pathway' (GO:0007186, P=3.60E-03) and 'Intrinsic component of membrane' (GO:0031224, P=1.10E-02). 5-hmC-PvC genes were enriched in 'Cytosol' (GO:0005829, P=3.10E-02), 'Nucleoplasm part' (GO:0044451, P=1.90E-02) and 'Transcription factor complex' (GO:0005667,

Table V. KEGG pathway analysis of hypermethylated and hypomethylated genes.

A, KEGG pathway analysis of hypermethylated genes

Pathway name	ID	Gene	EntrezGene	Statistic
Regulation of actin cytoskeleton	04810	20	WASF2, GNG12, NRAS, FGF4, APC, CYFIP2, PDGFB, FGF19, MAP2K2, TIAM2, ARHGAP35, GNA12, PIK3R5, TMSB4Y, ARHGEF1, PPP1CA, ITGB4, RRAS2, ACTN3, MYL12B	C=213;O=20;E=5.37;R=3.72; rawP=5.64e-07;adjP=6.26e-05
Systemic lupus erythematosus	05322	15	HIST1H4F, HIST1H4K, HIST1H2BM, H2AFY, HIST1H2BI, ELANE, HIST1H2AL, HIST3H2BB, HIST1H3G, ACTN3, TROVE2, HIST3H2A, HIST1H3J, HIST1H2AJ, HIST1H4H	C=136;O=15;E=3.43;R=4.37; rawP=1.96e-06;adjP=0.0001
Metabolic pathways	01100	51	GBE1, GALNT6, NDUFS6, HEXB, RRM1, CKMT2, GDA, BCAT1, RPE, GLUL, SDHA, HYAL2, CYP51A1, NDUFV1, HMGCS1, NDUFA2, CYP4F11, CTPS1, SUCLG2, POLR3C, DGAT1, LDHB, HMGCR, B3GALT6, ALOX12, MGAT4B, SMPD1, TBXAS1, AK4, BST1, POLG2, HYAL4, DGKE, POLR3G, GALNT3, AK1, ATP6V1D, SGSH, TCIRG1, B3GAT2, PC, DCXR, DHRS9, CEPT1, PLCB4, SQLE, ACADM, GPI, PTDSS1, UGT2B7, ALOX15B	C=1130;O=51;E=28.51;R=1.79; rawP=5.73e05;adjP=0.0021
Pathways in cancer	05200	21	CDKN2B, NRAS, LAMC1, FGF4, DVL3, CTBP2, APC, PDGFB, GSTP1, FGF19, MAP2K2, DAPK3, FADD, FZD7, CSF3R, BMP2, LEF1, PIK3R5, MSH3, BMP4, CDKN2A	C=326;O=21;E=8.22;R=2.55; rawP=9.84e05;adjP=0.0027
Gastric acid secretion	04971	8	ADCY6, PLCB4, SLC4A2, CFTR, ADCY5, KCNJ1, KCNJ15, CALML6	C=74;O=8;E=1.87;R=4.29; rawP=0.0006;adjP=0.0133
Purine metabolism	00230	12	AK1, PDE4A, ADCY5, PDE7A, RRM1, AK4, ADCY6, GDA, PDE4D, POLR3C, PDE3B, POLR3G	C=162;O=12;E=4.09;R=2.94; rawP=0.0009;adjP=0.0148
Pancreatic secretion	04972	9	ADCY6, PLCB4, CELA2A, SLC4A2, CFTR, CELA3B, BST1, ADCY5, ATP2B1	C=101;O=9;E=2.55;R=3.53; rawP=0.0011;adjP=0.0148
Melanoma	05218	7	NRAS, FGF4, PDGFB, PIK3R5, FGF19, MAP2K2, CDKN2A	C=71;O=7;E=1.79;R=3.91; rawP=0.0021;adjP=0.0194
Tight junction	04530	10	NRAS, PRKCZ, MPP5, CTTN, RRAS2, ACTN3, CLDN14, MYL12B, TJAP1, CLDN11	C=132;O=10;E=3.33;R=3.00; rawP=0.0020;adjP=0.0194
Basal cell carcinoma	05217	6	BMP2, DVL3, APC, LEF1, BMP4, FZD7	C=55;O=6;E=1.39;R=4.32; rawP=0.0026;adjP=0.0222
Cell cycle	04110	9	CDKN2B, PCNA, STAG1, YWHAZ, ORC1, TFDP2, CDC23, CDKN2A, ORC6	C=124;O=9;E=3.13;R=2.88; rawP=0.0043;adjP=0.0341
Glioma	05214	6	NRAS, PDGFB, PIK3R5, MAP2K2, CDKN2A, CALML6	C=65;O=6;E=1.64;R=3.66; rawP=0.0059;adjP=0.0409
Vascular smooth muscle contraction	04270	8	GNA12, ADCY5, ARHGEF1, CALML6, PLCB4, ADCY6, PPP1CA, MAP2K2	C=116;O=8;E=2.93;R=2.73; rawP=0.0093;adjP=0.0492
Glutathione metabolism	00480	5	OPLAH, GPX7, GSTP1, GSTM4, RRM1	C=50;O=5;E=1.26;R=3.96; rawP=0.0084;adjP=0.0492
Insulin signaling pathway	04910	9	PRKAG2, NRAS, PIK3R5, PRKCZ, CALML6, PPP1CA, PDE3B, MAP2K2, PRKAR1A	C=138;O=9;E=3.48;R=2.59; rawP=0.0085;adjP=0.0492
Oocyte meiosis	04114	8	SPDYA, ADCY5, SLK, CALML6, ADCY6, YWHAZ, PPP1CA, CDC23	C=112;O=8;E=2.83;R=2.83; rawP=0.0076;adjP=0.0492

Table V. Continued.

B, KEGG pathway analysis of hypomethylated genes

Pathway name	ID	Gene	EntrezGene	Statistic
Neuroactive ligand-receptor interaction	04080	26	P2RY4, SSTR5, AVPR2, MAS1, NTSR1, PRSS3, GHSR, CALCRL, CHRM4, F2RL3, HTR5A, GRM8, HTR1D, SSTR3, DRD5, GABRB3, P2RX6, CNR1, GRM4, LEP, UTS2R, SSTR4, MC5R, ADRA2B, PARD3, HRH1	C=272;O=26;E=10.05;R=2.59; rawP=1.10e05;adjP=0.0011
Dilated cardiomyopathy	05414	11	GNAS, SGCA, TPM2, CACNA1C, ADCY5, CACNB2, ADCY9, TPM4, ADCY6, CACNA2D4, ADCY7	C=90;O=11;E=3.33;R=3.31; rawP=0.0005;adjP=0.0098
Bile secretion	04976	10	GNAS, SLC4A5, KCNN2, ADCY5, ATP1A4, ADCY9, ADCY6, HMGCR, ADCY7, AQP8	C=71;O=10;E=2.62;R=3.81; rawP=0.0003;adjP=0.0098
Calcium signaling pathway	04020	16	ATP2B3, RYR1, NTSR1, NOS1, HTR5A, SLC25A31, GNAS, DRD5, CACNA1C, CALML5, P2RX6, ADCY9, CACNA1B, CALML3, ADCY7, HRH1	C=177;O=16;E=6.54;R=2.45; rawP=0.0009;adjP=0.0140
Pathogenic *Escherichia coli* infection	05130	8	FYN, TUBA3C, ARPC1B, NCK2, ARPC2, TUBB8, TUBA3E, TUBB3	C=56;O=8;E=2.07;R=3.87; rawP=0.0010;adjP=0.0140
Chemokine signaling pathway	04062	16	CXCR5, CX3CR1, BCAR1, TIAM2, SHC1, GNGT2, ADCY5, ADCY9, IL8, ADCY6, GRK1, ARRB2, TIAM1, PARD3, ADCY7, GRK7	C=189;O=16;E=6.99;R=2.29; rawP=0.0019;adjP=0.0169
Gap junction	04540	10	GNAS, TUBA3C, ADCY5, ADCY9, GJD2, ADCY6, TUBB8, TUBA3E, TUBB3, ADCY7	C=90;O=10;E=3.33;R=3.01; rawP=0.0018;adjP=0.0169
Gastric acid secretion	04971	9	GNAS, CALML5, ADCY5, ATP1A4, ADCY9, ADCY6, ATP4B, CALML3, ADCY7	C=74;O=9;E=2.74;R=3.29; rawP=0.0016;adjP=0.0169
Melanogenesis	04916	10	GNAS, CALML5, ADCY5, FZD9, ADCY9, POMC, ADCY6, CALML3, TCF7L2, ADCY7	C=101;O=10;E=3.73;R=2.68; rawP=0.0042;adjP=0.0317
Pancreatic secretion	04972	10	GNAS, CTRB1, CELA3A, ATP2B3, ADCY5, PRSS3, ATP1A, ADCY9, ADCY6, ADCY7	C=101;O=10;E=3.73;R=2.68; rawP=0.0042;adjP=0.0317

C, the number of reference genes in the category; O is the number of genes in the gene set and also in the category; E, the expected number in the category; R, ratio of enrichment; rawP, P-value from hypergeometric test; adjP, P-value adjusted by the multiple test adjustment.

P=1.60E-02). The significant GO categories of these genes are shown in Fig. 6.

KEGG pathway analysis revealed that 5-hmC-CvP genes were enriched in 'MAPK signaling' (such as FGF4, FGF19, MEF2C and FGF3) and 'Pathway in cancer' (such as MMP9, SMAD4, FGF19 and FGF3), while 5-hmC-PvC genes were enriched in 'Cell cycle' (such as MDM2, STAG1 and E2F4) and 'Metabolic pathways' (such as ALG9, FLAD1, ST3GAL4, NDUFC2, POLR2J3, DHRS9 and G6PD). All significant pathways are listed in Table VI. The PANTHER classification system identified that DHMRs associated genes were mainly enriched in 'Nucleic acid binding' and 'Hydrolase', the same as the DMRs (Fig.5).

KEGG pathway analysis of '*metabolic pathway*'*-associated genes*. There were several 5-mC and 5-hmC changed genes enriched in 'Metabolic pathways' further KEGG pathway analysis for these genes revealed that they were gathered in glucose metabolism [including 'Glycolysis/gluconeogenesis' (00010), 'Pentose and glucuronate interconversions' (00030), 'Starch and sucrose metabolism' (00500), 'Glycosaminoglycan degradation' (00531)], energy metabolism [including 'Oxidative phosphorylation' (00190), 'Citrate cycle (TCA cycle)' (00020), 'Carbon metabolism' (01200)], and amino acid metabolism, [including 'Biosynthesis of amino acids' (01230), 'Cysteine and methionine metabolism' (00270), 'Arginine and proline metabolism' (00330), 'Arachidonic acid metabolism' (00590)] 'Purine metabolism' and 'Pyrimidine metabolism' (Fig. 7).

Both DMR and DHMR-associated genes. A total of 141 genes were found with both 5-mC and 5-hmC changes in at least two patients. KEGG pathway analysis for these 141 genes identified

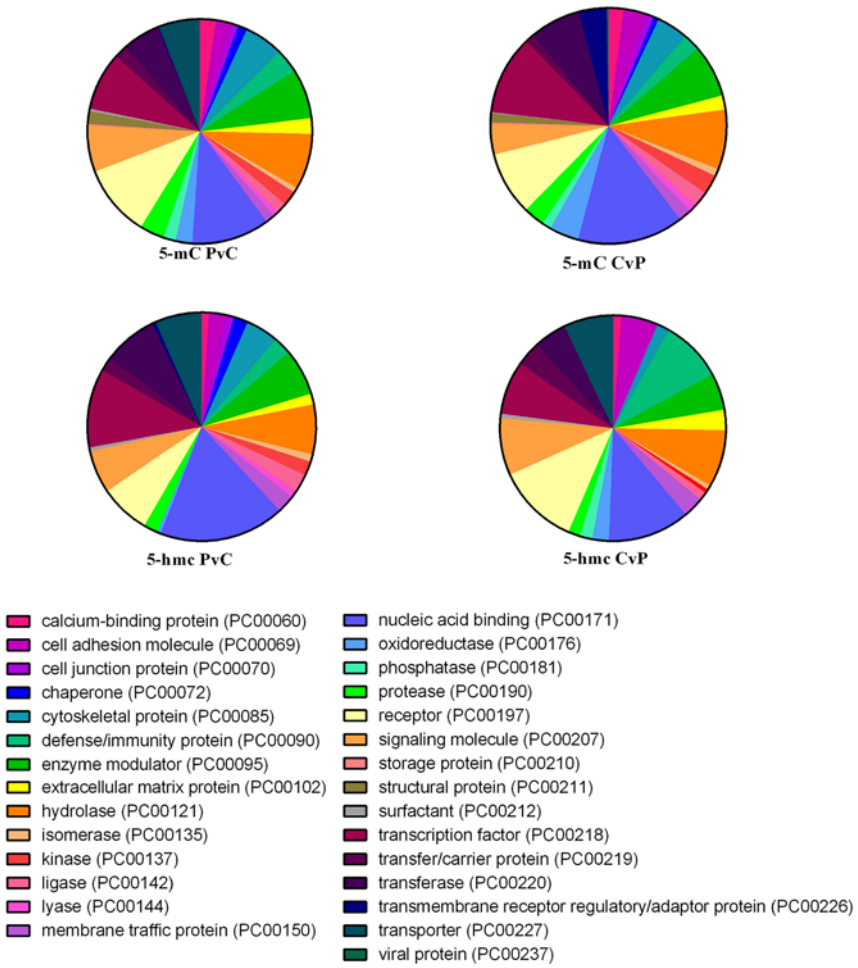

Figure 5. PANTHER protein class categories of hypermethylated (5-mC CvP)/hypomethylated (5-mC-PvC)/upregulated 5-hmC (5-hmC-CvP)/downregulated 5-hmC (5-hmC-PvC) genes in HCC tissues compared with APTs. (The chart legends show each detailed category).

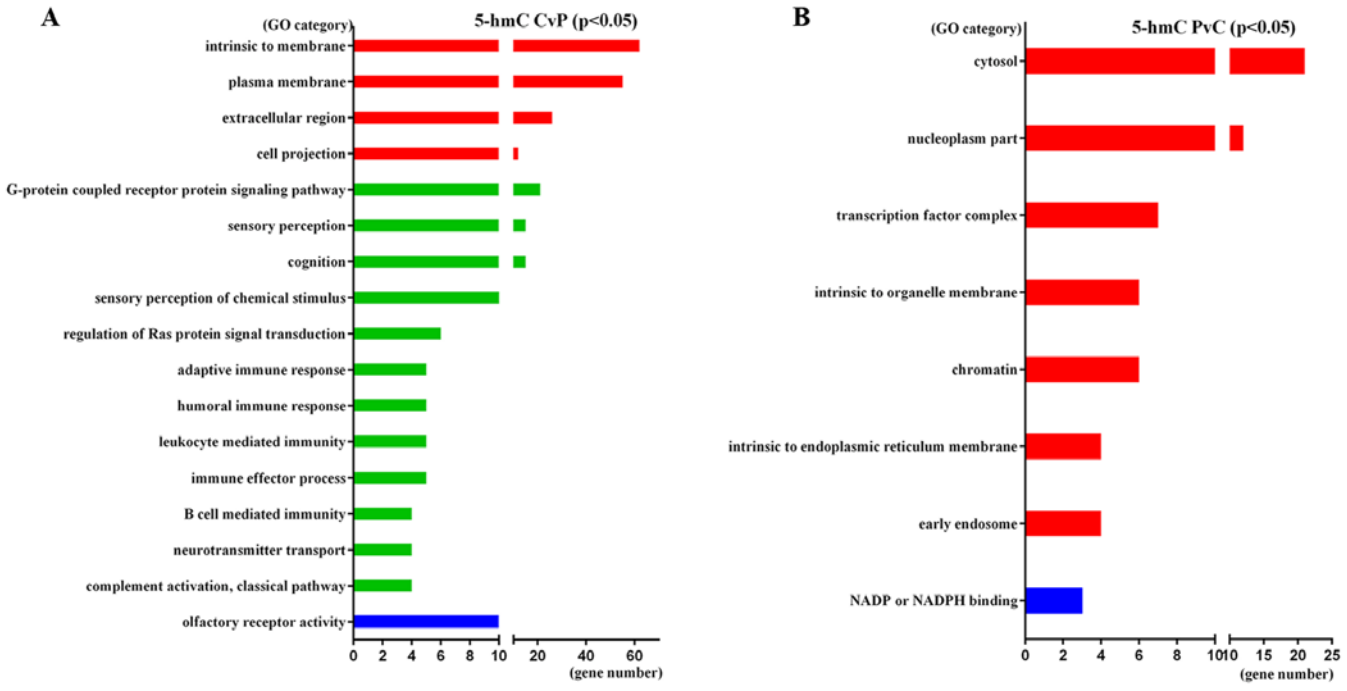

Figure 6. (A) The significant GO categories of upregulated 5-hmC genes (5-hmC-CvP) in HCC tissues compared with APTs (P<0.05). (B) The significant GO categories of downregulated 5-hmC genes (5-hmC-PvC) in HCC tissues compared with APTs (P<0.05) (Red, cellular component; green, biological process; blue, molecular function).

Table VI. KEGG pathway analysis of upregulated and downregulated 5-hmC related genes.

A, KEGG pathway analysis of upregulated 5-hmC related genes

Pathway name	ID	Gene	EntrezGene	Statistic
Olfactory transduction	04740	9	OR2T3, OR51V1, OR2L3, OR51F2, OR56A1 OR2M3, OR52N5, OR4M2, OR4N4	C=388;O=9;E=1.65;R=5.47; rawP=4.63e-05;adjP=0.0005
Antigen processing and presentation	04612	3	KIR3DL1, KIR3DL2, KIR3DL3	C=76;O=3;E=0.32;R=9.30; rawP=0.0042;adjP=0.0092
Melanoma	05218	3	FGF4, FGF19, FGF3	C=71;O=3;E=0.30;R=9.96; rawP=0.0035;adjP=0.0092
Natural killer cell mediated cytotoxicity	04650	4	KIR3DL1, ICAM2, KIR3DL2, KIR3DL3	C=136;O=4;E=0.58;R=6.93; rawP=0.0028;adjP=0.0092
Pathways in cancer	05200	5	MMP9, FGF4, SMAD4, FGF19, FGF3	C=326;O=5;E=1.38;R=3.61; rawP=0.0130;adjP=0.0238
MAPK signaling pathway	04010	4	FGF4, FGF19, MEF2C, FGF3	C=268;O=4;E=1.14;R=3.52; rawP=0.0278;adjP=0.0382
RNA transport	03013	3	NUP62, GEMIN4, NXT2	C=151;O=3;E=0.64;R=4.68; rawP=0.0268;adjP=0.0382

B, KEGG pathway analysis of downregulated 5-hmC related genes

Pathway name	ID	Gene	EntrezGene	Statistics
Cell cycle	04110	5	MDM2, STAG1, E2F4, CDK4, TFDP1	C=124;O=5;E=0.78;R=6.37; rawP=0.0012;adjP=0.0140
TGF-β signaling pathway	04350	4	DCN, GDF6, E2F4, TFDP1	C=84;O=4;E=0.53;R=7.52; rawP=0.0020;adjP=0.0140
Staphylococcus aureus infection	05150	3	C1QB, FCGR3B, C3AR1	C=55;O=3;E=0.35;R=8.62; rawP=0.0052;adjP=0.0243
Epithelial cell signaling in *Helicobacter pylori* infection	05120	3	F11R, ATP6V1G2, ATP6V0A4	C=68;O=3;E=0.43;R=6.97; rawP=0.0093;adjP=0.0254
Ubiquitin mediated proteolysis	04120	4	RHOBTB2, UBE2Q1, UBE3B, MDM2	C=135;O=4;E=0.85;R=4.68; raw P=0.0109;adjP=0.0254
Protein processing in endoplasmic reticulum	04141	4	HSP90AA1, ERP29, DNAJA2, TXNDC5	C=165;O=4;E=1.04;R=3.83; rawP=0.0212;adjP=0.0371
Metabolic pathways	01100	13	ALG9, FLAD1, ST3GAL4, NDUFC2, POLR2J3, DHRS9, G6PD, ATP6V1G2, SMPD1, ASMT, ATP6V0A4, NOS1, AKR1A1	C=1130;O=13;E=7.15;R=1.82; rawP=0.0290;adjP=0.0451

C, the number of reference genes in the category; O, the number of genes in the gene set and also in the category; E, the expected number in the category; R, ratio of enrichment; rawP, P-value from hypergeometric test; adjP, P-value adjusted by the multiple test adjustment.

five major pathways involved ('Cell cycle', 'Pathway in cancer', 'Ubiquitin mediated proteolysis', 'Melanoma' and 'Prostate cancer') were enriched (adjp<0.05). Ten interconnected and enriched genes (PCNA, MDM2, STAG1, E2F4, FGF4, FGF19, RHOBTB2, UBE2QL1, DCN and HSP90AA1) were revealed (Fig. 8).

Discussion

DNA methylation is one of the major epigenetic mechanisms that regulate gene expression in humans, and the alterations of methylation profiles are regarded as one of the major molecular aberrations in malignancies (23,24). Several

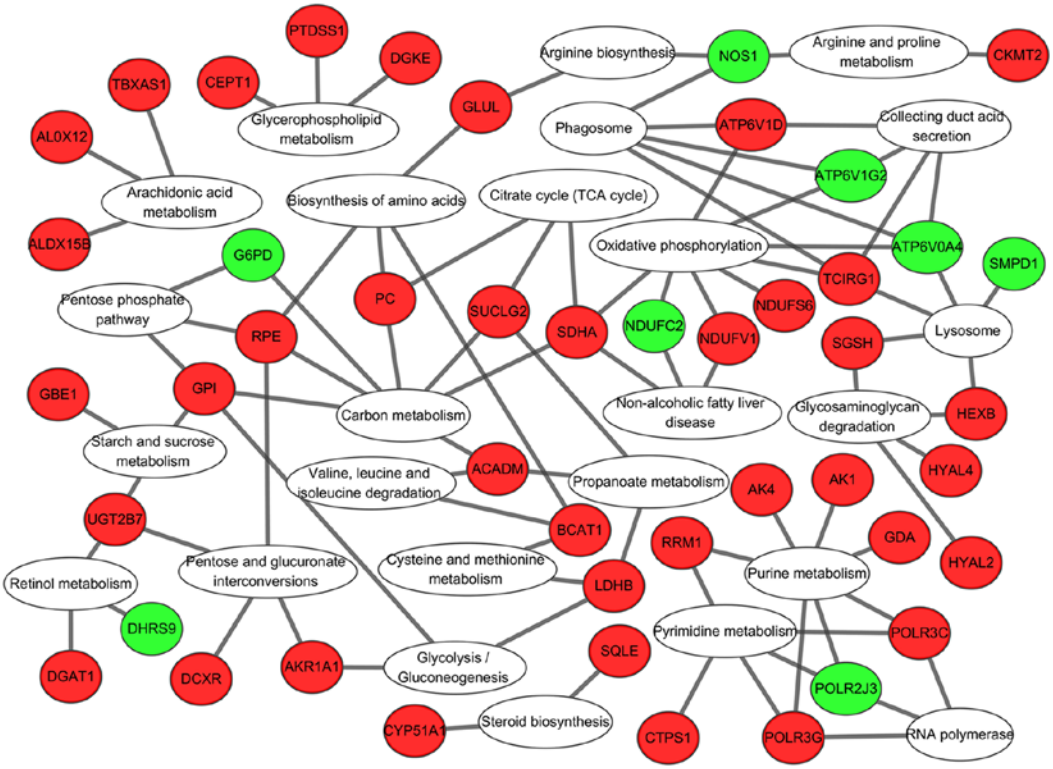

Figure 7. KEGG pathway analysis of 'Metabolic pathway' involved genes (Red, 5-mC changed genes; green, 5-hmC changed genes; white, KEGG pathways).

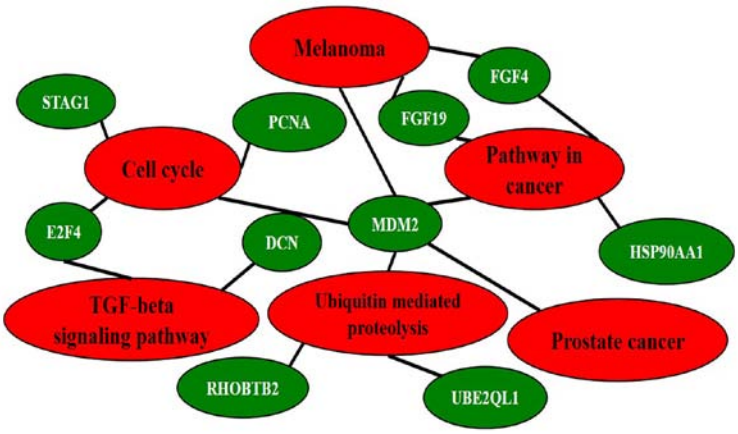

Figure 8. KEGG pathway analysis of both 5-mC and 5-hmC changed genes. (adjp<0.05) (adjp, P-value adjusted by the multiple test adjustment; red, KEGG pathways; green, genes).

studies of genome-wide DNA methylation have shown that the characteristic features of CpGs and certain microRNAs had differences in methylation levels between HCC and non-cancerous livers (25,26). Thus far, only a few studies have reported that DNA hydroxymethylation is associated with several human cancers (14,27), yet, the biological significance of 5-hmC in tumorigenesis remains unclear. In the present study we showed widespread alterations in DNA methylation and hydroxymethylation in HCC tumor tissues and paired APTs. 5-mC and 5-hmC levels exhibited no significant differences between the groups, which might be due to the huge variation between HCC individuals. The strong features of this study include the highly sensitive method and high-throughput sequencing used, which enabled the non-biased mapping of aberrant (hydroxy) methylation sites between the tumor tissues and APTs, and distinguished the alternation of 5-mC from that of 5-hmC. To the best of our knowledge, this is the first report on the genome-wide profiling of 5-mC and 5-hmC in HCC using this technique.

As is known, in the mammalian genome, methylation takes place only at cytosine bases that are located 5' to a guanosine in a CpG dinucleotide, known as CpG islands. Most CpG islands are found in the proximal promoter regions (28). Methylation and demethylation in promoter region may regulate gene expression, playing important roles in biology process, especially in the development of tumors (25,29,30). The

identification of genes that are specifically hypermethylated (which results in gene silencing) or hypomethylated (which results in increased transcription) might lead to the discovery of new factors that are important for tumor initiation and progression. In this study, we identified 1133 hypermethylated genes and 1663 hypomethylated genes in the promoter regions, many more than reported in a previous study (19), proving that MeDIP-seq has a better sensitivity than microarray, and more methylated sites can be found by using this novel technology. GO analysis showed they were enriched in various biological processes. Hypermethylation mostly gathered in 'Regulation'-related biology processes, including 'Regulation of RNA metabolic process' (P=4.10E-05), 'Regulation of kinase activity' (P=8.80E-03) and 'Regulation of transcription' (P=9.40E-04). Although it is widely accepted that 'DNA methylation suppresses gene expression', this statement is an over-simplification. Methylation at the promoter regions can change the interactions between proteins and DNA, which can lead to the alterations in chromatin structure and either a decrease or an increase in the rate of transcription (31,32). We indeed found hypermethylation genes enriched in 'protein-DNA complex assembly' (P=9.40E-03). Furthermore, the position of the methylation change relative to the transcription start site is critical to the outcome (23). 'Binding' is another category where the hypermethylated genes are enriched, and it includes 'DNA binding' (P=4.60E-05), 'Ion binding' (P=1.20E-03) and 'RNA binding' (P=3.60E-02), which are accordant with the results of Zhai *et al* (33) In contrast, hypomethylation genes are enriched in totally different categories, such as 'Plasma membrane' (p=2.80E-05), 'Cytoskeleton' (P=9.70E-03) and 'G-protein coupled receptor protein signaling pathway' (P=4.60E-03).

Our KEGG pathway analysis identified some interesting pathways for hypermethylation. 'Pathway in cancer' contained genes such as CDKN2A and CDKN2B (cyclin-dependent kinase inhibitor 2A/2B) that are recognized as a tumor suppressor genes. The inactivation of CDKN2A/2B have been reported in several primary tumors (34-36). There might be three different molecular mechanisms resulting in the loss of the CDKN2A/2B gene functions, namely homozygous deletions, point mutations, and transcriptional silencing by methylation at CpG islands. Methylation of CDKN2 has been observed in cell lines and cancer specimens derived from glioma, breast, colonic, head and neck cancers, hepatoblastoma, and in transitional cell carcinomas of the bladder (37-39). Shen *et al* (40) used Illumina Methylated Arrays and pyrosequencing technique and indicated that CDKN2A may be a potential biomarker for early HCC diagnosis. Another important gene in this pathway is APC (adenomatous polyposis coli) which also is an important tumor associated gene. The profiling of gene promoter hypermethylation across human tumor types showed that APC promoter hypermethylation occurred in tumors including colon, breast, kidney, bladder, esophagus, stomach, pancreas and liver tumors (41). Furthermore, studies showed that high-level APC promoter methylation is a useful biomarker and predictor in esophageal adenocarcinoma, breast and prostate cancer (42-44). Methylation of APC in HCC is frequent and occurs in a gene-specific and disease-specific manner. It was detected more frequently in hepatitis C virus-positive HCC (45,46). Other genes such as GSTP1 (Glutathione S-transferase P1) have also been found to be epigenetically silenced by promoter methylation, and associated with increased risk and shortened survival in patients with various tumors, including HCC, breast and prostate cancer (47-49). Promoter methylation and epigenetic silencing of DAPK3 (death-associated protein kinase 3) and FADD [Fas (TNFRSF6)-associated via death domain] have not been studied in depth to the extent of those genes, and only a few studies implicated their participation in cancers such as oral squamous cell carcinoma and non-muscle invasive bladder carcinoma (50-52). Their promoter methylation could also be a potential marker for HCC, and further studies are needed to confirm this. Other identified pathways such as 'Cell cycle' (PCNA, STAG1, YWHAZ, ORC1, TFDP2 and CDC23) and 'Chemokine signaling pathway' (GNG12, NRAS, ELMO1, PIK3R5, ADCY5, PRKCZ and PLCB4) are also considered to be important for the development and progression of malignant carcinoma, and the involved genes with their methylation status may provide potential novel biomarkers for HCC.

5-hmC is usually found in human embryonic stem (ES) cells and particularly abundant in certain genomic regions such as enhancers associated with histone modifications and other protein-DNA interaction sites based both on the information of gene expression and sequence composition (53). However, in human HCC tumor tissues and paired APTs, the locations of DHMRs seemed not to be significantly different among the whole genomic region. Although there is substantial evidence indicating that hydroxymethylation may be associated with actively transcribed genes, the exact biochemical mechanisms still remain enigmatic (54,55). Our GO analysis showed that compared with APTs, both high-level hydroxymethylated genes and hypomethylated genes in HCC tissues were enriched in the same pathways, namely the 'Plasma membrane' (P=3.10E-05) and 'G-protein coupled receptor protein signaling pathway' (P=3.60E-03), indicating that hydroxymethylation as a kind of demethylation may play similar or related roles with hypomethylation. In contrast, the downregulated hydroxymethylated genes are found mainly gathered in the 'Cytosol' (P=3.10E-02).

Although the KEGG pathway analysis for 5-hmC did not come up with as many enriched genes as that for 5-mC, they can still be categorized into a number of meaningful pathways. For instance, high level hydroxymethylated genes such as MMP9, SMAD4, FGF19, FGF3 and MEF2C were enriched in 'Pathway in cancer' and 'MAPK signaling pathway', while low level hydroxymethylated genes were mostly enriched in 'Metabolic pathways' and 'Cell cycle'. 'TGF-β signaling pathway' related genes DCN, E2F4, TFDP1 also have strong correlations with tumors (56-58). Since there have been few studies on the hydroxymethylation of these genes, more work is needed to fully elucidate the potential roles of hydroxymethylation. Protein class analysis by PANTHER website showed that both DMR and DHMR-associated genes were in similar category, indicating that genes with hypermethylation or demethylation epigenetic changes tend to be coordinately regulated to participate in similar or related biology processes. Further work is warranted to test this hypothesis.

Over half a century ago, Warburg linked metabolism and cancer through enhanced aerobic glycolysis (59). This metabolic switch places the emphasis on producing intermediates

for cell growth and division. The most rapidly growing tumor cell lines obtain up to 50% of their total ATP production from glycolytic metabolism, with a corresponding decrease in oxidative phosphorylation and in cell mitochondrial content (60,61). With numerous in-depth studies, the multi-faceted links between metabolism and cancer have now been revealed. Cellular metabolism is regulated by both oncogenes and tumor suppressor genes in a number of key signaling pathways. Metabolism generates oxygen radicals, which contribute to oncogenic mutations. Activated oncogenes and loss of tumor suppressors in turn alter metabolism and induce aerobics (62). In the present study, we found several 5-mC and 5-hmC changed genes enriched in 'Metabolic pathways', and further analysis showed they were specifically clustered in 'Glycolysis/gluconeogenesis', 'Oxidative phosphorylation' and 'Citrate cycle (TCA cycle)' (Fig. 7), metabolic pathways were proven to be critical in controlling cancer cell survival and proliferation. Although the regulatory mechanisms underlying aerobic and glycolytic metabolic pathways are complex, our findings indicate that (hydroxy)methylation-based epigenetic modifications may affect the development of HCC through the regulation of cellular metabolism.

DNA methylation as a characterized epigenetic mechanism, its relationship with other biochemical pathways represents a critical stage in the elucidation of biological information processing. Some amino acid metabolism has been related to DNA methylation in tumors, such as homocysteine metabolism and the dynamics of methionine cycle (63,64). Accordingly, this study also found several 5-mC and 5-hmC changed genes that are associated with amino acid metabolism, including 'Arginine biosynthesis', 'Cysteine and methionine metabolism', 'Valine, leucine and isoleucine degradation' and 'Arginine and proline metabolism' which may provide new clues for studying the relationship between (hydroxy)methylation and metabolism in HCC.

The present study found that a total of 141 genes have both 5-mC and 5-hmC changes in at least two of the HCC patients. KEGG pathway analysis showed five pathways ('Cell cycle', 'Pathway in cancer', 'Ubiquitin mediated proteolysis', 'Melanoma' and 'Prostate cancer') including ten genes (PCNA, MDM2, SAG1, E2F4, FGF4, FGF19, RHOBTB2, UBE2QL1, DCN and HSP90AA1) are enriched (Fig. 8). It is known for decades that, PCNA (proliferating cell nuclear antigen) acts as a central coordinator of DNA transactions by providing a multivalent interaction surface for factors involved in DNA replication, repair, chromatin dynamics, and cell cycle regulation (65), and is involved in the progression of tumors and highly altered in some tumors (66). Furthermore, studies have shown that the p21 protein negatively regulates targeting of DNA-MTase to the replication associated PCNA. They proposed that the presence of p21 prevents DNA-MTase access to replicating DNA, thereby impeding hypermethylation in normal cells (67). The present study indicated that (hydroxy) methylation of PCNA might be associated with HCC, which warrants further study. The 90-kDa heat shock protein HSP90AA1, another p21 regulator, has been found highly expressed in many cancers. Its mechanism in the tumorigenesis is varied (68,69). Here we provided evidence that methylation or hydroxymethylation of HSP90AA1 may play a crucial role in HCC. The epigenetic alterations of other identified genes such as MDM2, SAG, FGF4, FGF19, RHOBTB2 and DCN in HCC and other cancers also deserve further research.

One of the potential limitations of the present study is the sample size, which may not be sufficiently large. This is mainly due to the high cost of (h)MeDIP-seq, which precludes its application in a large scale. Nevertheless, we performed a rather comprehensive methylation and hydroxymethylation profiling of human HCC tumor tissues and paired APTs, and correlated multiple (hydroxy)methylation-altered genes with a number of important biological pathways. 'Metabolic pathways' are found to contain the largest number of (hydroxy) methylation-altered genes, indicating the crucial roles of metabolic processes (such as glycolysis/gluconeogenesis, oxidative phosphorylation and citrate cycle) in the occurrence and progression of HCC. Some of the identified (hydroxy) methylation-altered genes may serve as biomarkers for the diagnosis and prognosis of HCC. Future studies with a larger sample size combined with a series of biochemical approaches hold the promise of elucidating the specific roles of epigenetic modifications in the pathogenesis of HCC.

Acknowledgements

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