Using Illumina Infinium HumanMethylation 450K BeadChip to explore genome-wide DNA methylation profiles in a human hepatocellular carcinoma cell line

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Abstract. Aberrant DNA methylation is the most common type of epigenetic alteration and is associated with many types of cancer. Although previous studies have provided a few novel DNA methylation markers in hepatocellular carcinoma (HCC), specific DNA methylation patterns and comparisons of the aberrant alterations in methylation between HCC and normal liver cell lines have not yet been reported. Therefore, in the present study the Illumina Infinium HumanMethylation 450K BeadChip was employed to identify the genome-wide aberrant DNA methylation profiles of Huh7 and L02 cells. Following Bonferroni adjustment, 102,254 differentially methylated CpG sites (covering 26,511 genes) were detected between Huh7 and L02 cells. Of those CpG sites, 62,702 (61.3%) sites were hypermethylated (covering 12,665 genes) and 39,552 (38.7%) sites were hypomethylated (covering 13,846 genes). The results of the present study indicated that 40.3% of the CpG sites were in CpG island regions, 20.7% were in CpG shores and 8.8% were in shelf regions. A total of 57.3% hypermethylated CpG sites and 39.4% of the hypomethylated CpG sites had a |β-Differencel ≥50%. Within the significant differentially methylated CpG sites, 490 genes were located within 598 differentially methylated regions. Gene Ontology enrichment analysis revealed that 2,107 differentially methylated genes were associated with 'biological process', 13,351 differentially methylated genes were associated with 'molecular function', and 18,041 differentially methylated genes were associated with 'cellular component'. Kyoto Encyclopedia of Genes and Genomes pathway-based analysis revealed 43 signaling pathways that were associated with 5,195 differentially methylated genes. These results demonstrated that aberrant DNA methylation may be a key and common event underlying the tumorigenesis of Huh7 cells. The present study also identified many subsets of hypo- or hyper-methylated CpG sites, genes and signaling pathways, which have an importance in the occurrence and development of HCC.

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Abbreviations: PLC, primary liver cancer; HCC, hepatocellular carcinoma; DMR, differentially methylated regions; GO, Gene Ontology

Key words: DNA methylation, genome-wide, hepatocellular carcinoma, BeadChip

Introduction

Worldwide, primary liver cancer (PLC) is the second leading cause of cancer-associated mortality in poorly developed countries and the sixth in more developed countries (1). Of the ~782,500 new annual cases of PLC, China accounts for >50% of the associated incidence (2). In 2015, PLC was the fourth most common type of cancer and the third most common cause of cancer-associated mortality in China (3). The majority of PLC cases occurring worldwide are cases of hepatocellular carcinoma (HCC) (1). Many risk factors can induce the development of HCC, including chronic hepatitis B virus or hepatitis C virus infections, chronic alcoholic cirrhosis and high doses of aflatoxin B1 (4). Although a number of studies have identified a few of the molecular alterations associated with the pathogenesis of HCC, the main mechanism underlying HCC is still unclear.

Previous studies have demonstrated that epigenetic alterations are one of the many early events that occur during tumorigenesis (5,6). DNA methylation is the main epigenetic feature in regulating gene transcriptional regulation and preserving genome stability; however, aberrant DNA methylation can lead to the inactivation of tumor suppressor genes or activation of oncogenes, which eventually induces the development of many types of cancer (7,8). A number of studies have also reported alterations in one or several genes at one time; the abnormal methylation of genes, including Ras association domain family member 1 (9), p16, postmeiotic segregation increased 2, MutL homolog 1, MutS homolog 2 (10), Adenomatosis polyposis coli (11) and glutathione S-transferase Pi 1 (12,13), has also been

associated with HCC. Shen *et al* (14) used Illumina Infinium HumanMethylation 27K arrays to analyze 27,578 CpG sites covering 14,495 genes in paired HCC tumor and adjacent non-tumor tissues. The Illumina Infinium HumanMethylation 450K BeadChip represents a significant improvement in the detection of CpG sites (482,421 CpG and 3,091 non-CpG sites), covers 99% of RefSeq genes with multiple sites in annotated promoters (1,500 or 200 bp upstream of the transcription start site), 5'-untranslated regions (UTRs), first exons, gene body, and 3'-UTRs (15). Previously, aberrant DNA hypermethylation of CpG islands was reported to induce the inactivation of tumor suppressor genes (16), which was thought to contribute to tumorigenesis (17). Recently, previous studies have revealed that cancer-associated aberrant DNA methylation not only occurs within CpG islands but may also be detected within CpG shores or CpG shelves (18-20).

To the best of our knowledge, no research analyzing the genome-wide DNA methylation status within a HCC cell line using the Illumina Infinium HumanMethylation 450K BeadChip has been conducted. Therefore, in the present study, the Illumina 450K Methylation BeadChip was employed to screen promoter DNA methylation and the expression profiles of methylated genes in a human hepatocellular carcinoma cell line (Huh7 cells) and in a human normal liver cell line (L02 cells). The results may aid the characterization of differentially methylated CpG sites, regions and genes associated with the pathogenesis of HCC, thereby improving our current understanding of the methylation mechanisms underlying the development and progression of HCC.

Materials and methods

Cell culture. The human HCC cell line, Huh7 and the human normal liver cell line, L02, were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Huh7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), while L02 cells were maintained in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc.). The cell lines were supplemented with 100 U/ml penicillin and 100 g/ml streptomycin in the presence of 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and incubated in a humidified atmosphere containing 5% CO_2 at 37˚C.

DNA preparation and Illumina Infinium HumanMethylation 450K BeadChip assay. DNA was extracted from the two cell lines (Huh7 and L02) using a QIAamp DNA Micro kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Bisulfite modification of 1 μg DNA was conducted using an EZ DNA Methylation kit (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's protocol. The Illumina Infinium HumanMethylation 450K BeadChip assay was performed according to Illumina's standard protocol (Illumina, Inc., San Diego, CA, USA). Experiments with Huh7 and L02 cells were performed in triplicate to avoid false positive and false negative results.

Statistical analysis. The methylation data were processed with the Methylation Module of GenomeStudio software (Methylation v1.9; Illumina, Inc.). The methylation levels of the CpG sites were calculated as β-values: β=intensity of the methylated allele (M)/[intensity of the unmethylated allele (U) + M + 100] (15). A t-test, in addition to analysis of variance with Bonferroni correction for multiple comparisons was used to compare differentially methylated CpG sites between Huh7 and L02 cells. The differentially methylated CpG sites were defined as sites with Adjusted P-values of ≤0.05 and |β-Difference|≥0.2. Methylation measures with a detection P>0.05 and CpG coverage <95% were excluded (14). For the selection of candidate CpG sites that had significant differences between Huh7 and L02 cell methylation levels, the following additional filtering criteria were applied: i) Adjusted P≤0.05, which corresponds to a raw P-value of $\leq 1.06 \times 10^{-7}$; ii) for significantly hypermethylated CpG sites, the |β-Difference| in the methylation levels between Huh7 and L02 cells was >20%, and the mean methylation level for L02 was <25%; and iii) for significantly hypomethylated CpG sites, the methylation level |β-Difference| between L02 and Huh7 cells was >20%, and the mean methylation level for Huh7 cells was <25% (14).

Functional annotation of differentially methylated genes. The genes for which the CpG sites corresponded with differential methylation levels were determined using Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway (www.genome.jp/kegg/) and Gene Ontology (GO; www.geneontology.org/) databases to analyze the pathway and enrichment information of these genes.

Results

Global DNA methylation in Huh7 and LO2 cells. Following a t-test with Bonferroni correction for multiple comparisons, 102,254 differentially methylated CpG sites (covering 26,511 genes) were detected between Huh7 and L02 cells. Within these CpG sites, 62,702 (61.3%) sites were hypermethylated (covering 12,665 genes), and 39,552 (38.7%) sites were hypomethylated (covering 13,846 genes). The results suggested that aberrant DNA methylation may a very common event in Huh7 cells, and alterations in hypermethylation were more frequently observed than hypomethylation. Figs. 1 and 2 present hierarchical cluster analysis of the differentially methylated CpG sites and genes that distinguish Huh7 from L02 cells.

In addition, the results revealed that there were 41,178 (40.3%) CpG sites located in CpG island regions, 21,150 (20.7%) CpG sites were within CpG shores, 9,030 (8.8%) CpG sites were in shelves, and 30,896 (30.2%) CpG sites were in open sea (Table I). Furthermore, the results also demonstrated that within different regions, the distribution of hypo- or hypermethylated CpG sites differed: In CpG island regions, 19,462 (47.3%) CpG sites were hypermethylated and 21,716 (52.7%) CpG sites were hypomethylated. In CpG shore regions, 13,729 (64.9%) CpG sites were hypermethylated and 7,421 (35.1%) CpG sites were hypomethylated. In shelf regions, 7,340 (81.3%) CpG sites were hypermethylated and 1,690 (18.7%) CpG sites were hypomethylated.

Frequency distribution of differentially methylated CpG sites between Huh7 and L02 cells. In the results of the

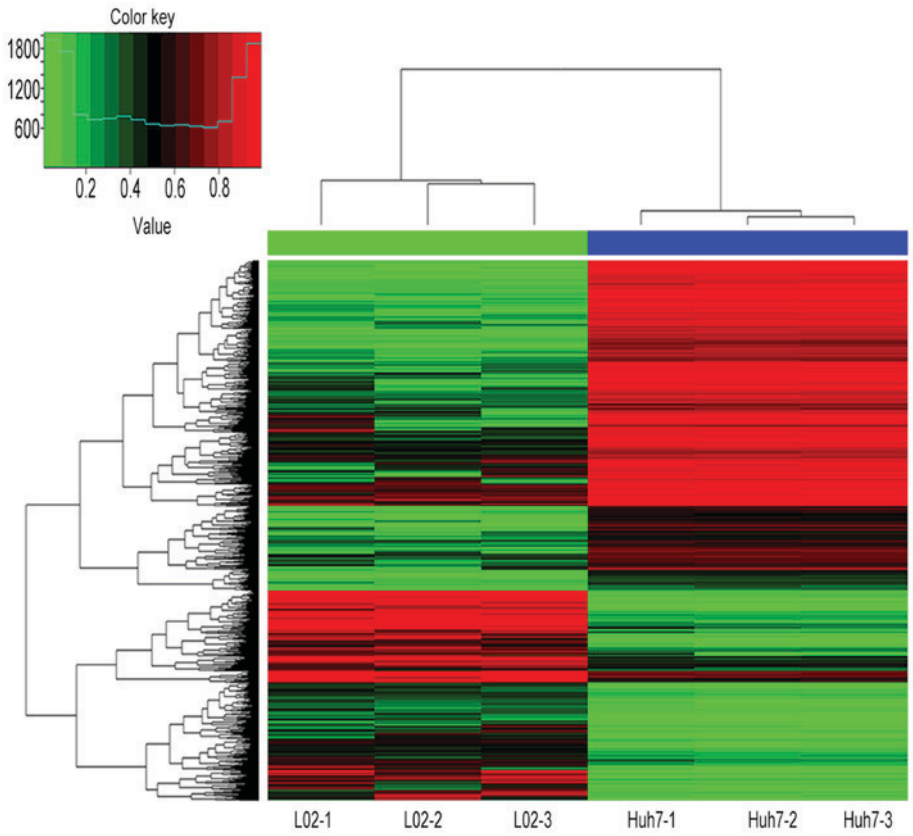

Figure 1. Hierarchical cluster analysis of all of the differentially methylated CpG sites between Huh7 and L02 cells.

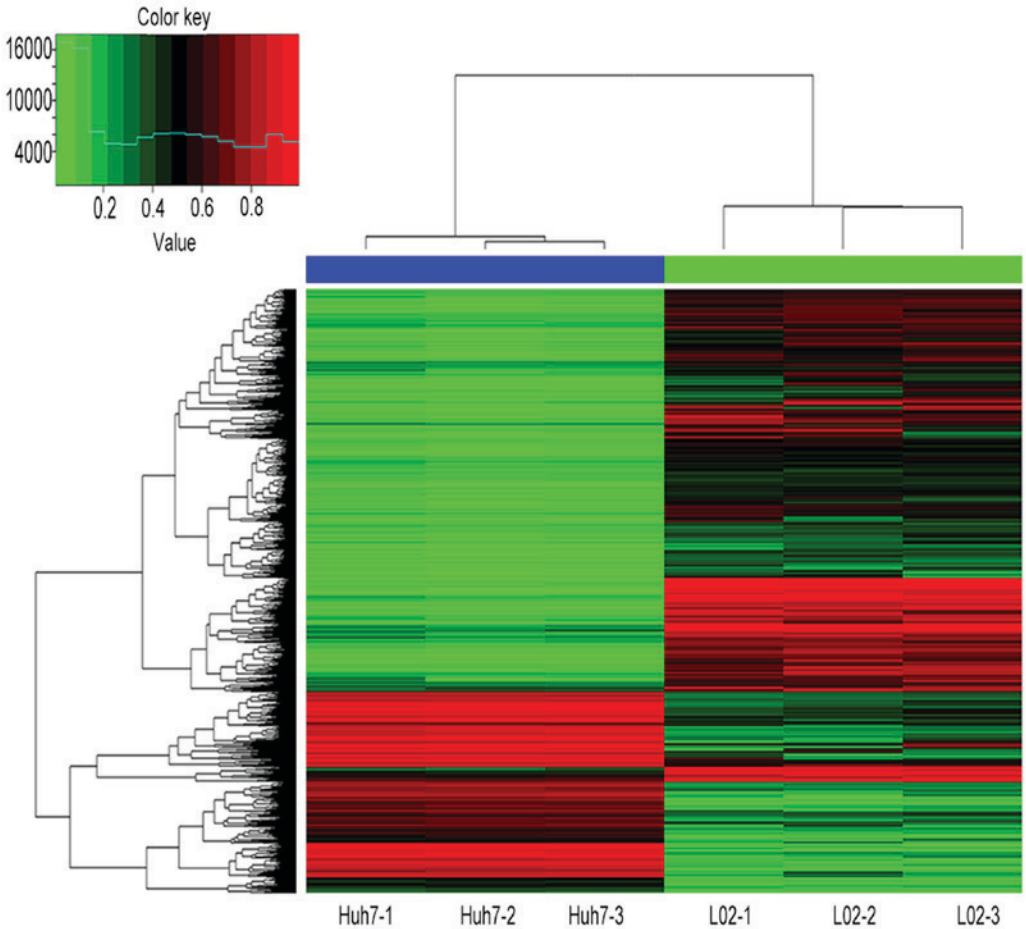

Figure 2. Hierarchical cluster analysis of all of the differentially methylated genes between Huh7 and L02 cells.

Table I. Distribution of all of the differentially methylated CpG sites.

Type	All methylated CpG sites, n (%)	Hypermethylated CpG sites, n (%)	Hypomethylation CpG sites, n (%)
CpG island	41,178 (40.3)	19,462 (47.3)	21,716 (52.7)
CpG shores	21,150 (20.7)	13,729 (64.9)	7,421 (35.1)
CpG shelves	9,030 (8.8)	7,340 (81.3)	1,690 (18.7)
Open sea	30,896 (30.2)	22,171 (71.8)	8,725 (28.2)
Total	102,254	62,702	39,552

Table II. Frequency distribution of all of the differentially methylated CpG sites in Huh7 and L02 cells by methylation status.

|β-difference|, %	Hypermethylated CpG sites, n (%)	Cumulative, %	Hypomethylated CpG sites, n (%)	Cumulative, %	Total CpG sites, n (%)	Cumulative, %
≥60	25,585 (40.8)	40.8	10,796 (27.3)	27.3	36,381 (35.6)	35.6
50≤x<60	10,352 (16.5)	57.3	4,791 (12.1)	39.4	15,134 (14.8)	50.4
40≤x<50	9,681 (15.4)	72.7	6,159 (15.6)	54.0	15,840 (15.5)	65.9
30≤x<40	8,848 (14.1)	86.8	8,018 (20.3)	75.3	16,866 (16.5)	82.4
20≤x<30	8,236 (13.1)	100.0	9,788 (24.7)	100.0	18,024 (17.6)	100.0
Total	62,702	-	39,552	-	102,254	-

present study, 35,937 (57.3%) hypermethylated CpG sites and 15,587 (39.4%) hypomethylated CpG sites were observed to have an |β-Difference|≥50%. A total of 18,529 (29.5%) of hypermethylated CpG sites and 14,177 (35.9%) hypomethylated CpG sites had an |β-Difference|≥30% but <50%. A total of 8,236 (13.1%) hypermethylated CpG sites and 9,788 (24.7%) of hypomethylated CpG sites had an |β-Difference|<30% but ≥20% (Table II). Collectively, these results revealed that DNA aberrant hypermethylation in Huh7 cells was more frequent than in L02 cells, which may serve a potential role in genomic instability.

Significant differentially methylated CpG sites and genes. To reduce the potential impact of an extreme β value on methylation differences, the present study applied stringent criteria to select potentially biologically important CpG sites (14). A total of 5,285 significantly hypermethylated CpG sites (covering 3,222 genes) and 2,659 significantly hypomethylated CpG sites (covering 2,204 genes) were observed. For the significantly hypermethylated CpG sites, there were 1,544 sites in CpG islands, 1,137 sites in CpG shores, 655 sites within CpG shelves and 1,949 sites in open sea regions. By contrast, for the significantly hypomethylated CpG sites, there were 1,201 sites in CpG islands, 632 sites in CpG shores, 133 sites in CpG shelves and 693 sites in open sea regions (Table III). The top 20 differentially hypermethylated and hypomethylated sites and genes are presented in the Tables IV and V.

Significant differentially methylated regions (DMRs). The results of the present study revealed that 390 significantly hypermethylated CpG sites (covering 287 genes) and 208 significantly hypomethylated CpG sites (covering 203 genes) were in DMRs. For the significantly hypermethylated CpG sites, 64 sites were in cancer-specific (c)-DMRs, 125 sites were in reprogramming-specific (r)-DMRs and 201 sites were in DMRs; for the significantly hypomethylated CpG sites, 30 were located within cDMRs, 74 were located within rDMRs and 104 were located within DMRs (Table VI; Fig. 3).

Table III. Distribution of genomic regions for significant differentially methylated CpG sites in Huh7 cells when compared with L02 cells.

Type	Hypermethylated CpG sites, n	Hypomethylated CpG sites, n
CpG island	1,544	1,201
CpG shores	1,137	632
CpG shelves	655	133
Open sea	1,949	693
Total	5,285	2,659

GO enrichment and KEGG pathway analysis. GO enrichment and the KEGG Pathway database were employed to analyze information regarding the differentially methylated genes. The results of GO enrichment revealed that there were 2,107 differentially methylated genes associated with 'biological process', and the most enriched groups included negative regulators of cell proliferation, negative regulators of transcription such as RNA polymerase II promoter, and synaptic transmission. A total of 13,351 differentially methylated genes were associated with 'molecular function', and the most enriched groups included protein binding, DNA binding and metal ion binding. A total of 18,041 differentially methylated genes were

Table IV. Top 20 significant hypermethylated CpG sites and genes within differentially methylated regions in Huh7 cells when compared with L02 cells.

CpG sites	Adjust P-value	|β-difference|	Mean Huh	Mean L02	Hypermethylated genes
cg11058366	$2.09x10^{-6}$	0.924	0.934	0.010	ERBB4
cg13245152	$1.69x10^{-6}$	0.863	0.876	0.013	PAX6
cg25758545	$1.69x10^{-6}$	0.863	0.876	0.013	SALL4
cg14950829	$1.69x10^{-6}$	0.925	0.940	0.015	PCDH8
cg09260089	$1.69x10^{-6}$	0.952	0.968	0.016	NKX6-2
cg11459773	$1.69x10^{-6}$	0.876	0.891	0.015	BCL3
cg12989574	$1.69x10^{-6}$	0.965	0.983	0.018	GPC6
cg03129384	$1.69x10^{-6}$	0.956	0.974	0.018	FAM196A; DOCK1
cg03396151	$1.69x10^{-6}$	0.929	0.947	0.018	MEIS2
cg04556126	$1.69x10^{-6}$	0.920	0.938	0.018	ZIC4
cg20317123	$1.69x10^{-6}$	0.947	0.966	0.019	TCF21
cg21062760	$1.69x10^{-6}$	0.876	0.894	0.018	ZBTB32
cg09454560	$1.99x10^{-6}$	0.624	0.636	0.013	LRFN2
cg12090740	$1.69x10^{-6}$	0.892	0.911	0.020	BCL2
cg24249411	$1.69x10^{-6}$	0.887	0.907	0.020	BDNF
cg00057722	$1.69x10^{-6}$	0.929	0.950	0.021	-
cg08640046	$1.69x10^{-6}$	0.810	0.828	0.018	-
cg03283124	$2.03x10^{-6}$	0.898	0.920	0.021	PCDH9
cg13087076	$1.69x10^{-6}$	0.890	0.912	0.021	DYDC2
cg25453154	$2.03x10^{-6}$	0.820	0.839	0.020	ZCCHC24

Data are presented to 3 decimal places.

Table V. Top 20 significant hypomethylated CpG sites and genes within differentially methylated regions in Huh7 cells when compared with L02 cells.

CpG sites	Adjust P-value	|β-difference|	Mean Huh	Mean L02	Hypomethylated gene
cg10739344	$1.70x10^{-6}$	0.894	0.019	0.913	WDR76
cg00618865	$1.69x10^{-6}$	0.945	0.023	0.967	PLXND1
cg16267343	$1.69x10^{-6}$	0.898	0.024	0.922	NPR3
cg00138041	$1.69x10^{-6}$	0.943	0.029	0.972	PRDM8
cg01529365	$1.69x10^{-6}$	0.930	0.031	0.961	-
cg09564253	$1.69x10^{-6}$	0.902	0.031	0.933	LASP1
cg08176368	$1.69x10^{-6}$	0.926	0.033	0.959	MMP9
cg08812555	$2.05x10^{-6}$	0.784	0.029	0.813	DKK1
cg25612391	$1.77x10^{-6}$	0.741	0.028	0.769	SLC25A42
cg22417879	$1.70x10^{-6}$	0.908	0.035	0.943	SDCBP2
cg15019790	$1.69x10^{-6}$	0.924	0.036	0.960	SIX2
cg07407787	$1.69x10^{-6}$	0.922	0.036	0.958	ARSG; SLC16A6
cg08361684	$1.69x10^{-6}$	0.911	0.038	0.949	FJX1
cg16195157	$1.69x10^{-6}$	0.900	0.038	0.938	DNAJB1
cg15842502	$1.69x10^{-6}$	0.913	0.040	0.953	RB1
cg13848566	$1.95x10^{-6}$	0.934	0.041	0.975	GAS1
cg27454412	$1.81x10^{-6}$	0.781	0.034	0.815	C7orf50
cg02152578	$1.69x10^{-6}$	0.931	0.041	0.972	AHCYL1
cg13355248	$1.69x10^{-6}$	0.792	0.038	0.829	NPTX1
cg16443866	$1.69x10^{-6}$	0.878	0.042	0.920	STC2

Data are presented to 3 decimal places.

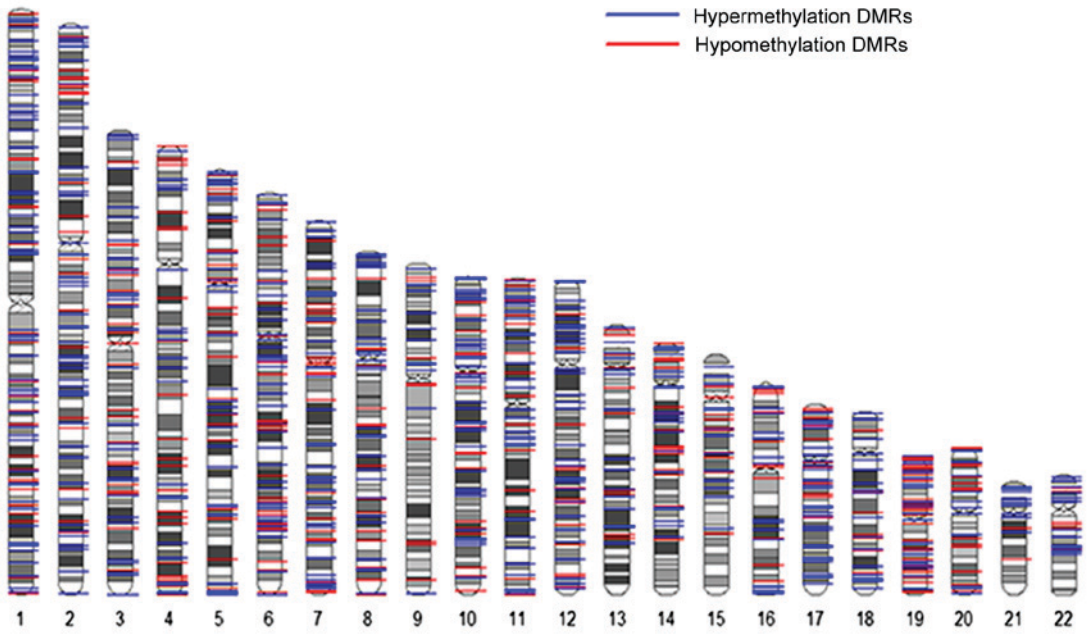

Figure 3. Significantly hypermethylated and hypomethylated genes located in DMRs. DMRs, differentially methylated regions.

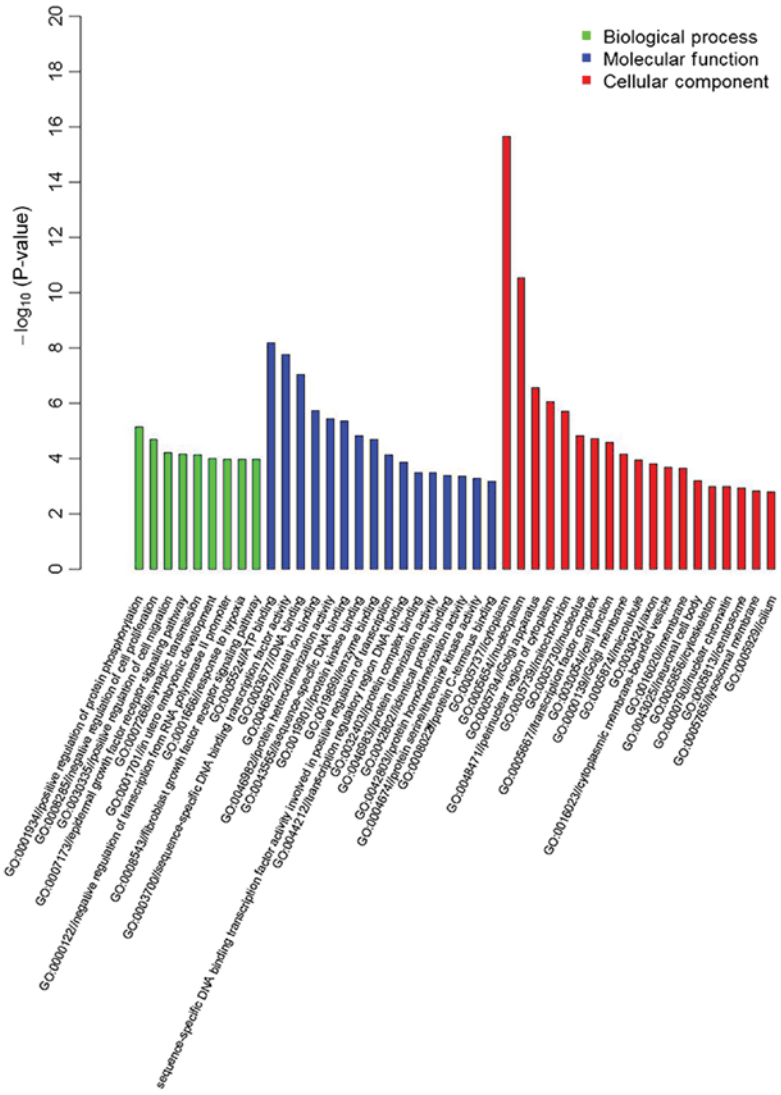

Figure 4. GO enrichment analysis of all of the differentially methylated genes. Green bars indicate 'biological process, blue bars indicate 'molecular function', and red bars indicate 'cellular component'. GO, Gene Ontology.

Table VI. Significant differentially methylated regions.

DMRs	Significantly hypermethylated sites (n)	Significantly hypermethylated sites (n)
cDMRs	64	30
Island	16	4
Shores	36	18
Shelves	5	3
Open sea	7	5
rDMRs	125	74
Island	38	13
Shores	70	50
Shelves	7	3
Open sea	10	8
DMRs	201	104
Island	179	70
Shores	13	21
Shelves	3	0
Open sea	6	13
Total	390	208

DMRs, differentially methylated regions; cDMRs, cancer-specific-DMRs; rDMRs, reprogramming-specific-DMRs.

associated with 'cellular component', and the most enriched groups included the nucleus, cytoplasm and cytosol (Fig. 4). The top 20 significant differentially methylated genes obtained from GO enrichment analysis are listed in Table VII.

KEGG Pathway-based analyses revealed that 43 signaling pathways involved 5,195 differentially methylated genes, and these genes were significantly enriched in specific pathways, including the cancer, metabolic, mitogen-activated protein kinase (MAPK), calcium, Wnt, hepatitis C, Erb-B2 receptor tyrosine kinase (ErbB), transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), p53 and Notch signaling pathways (Table VIII).

Discussion

DNA methylation is the main epigenetic modification and regulator of gene expression in humans. Aberrant changes in genomic methylation patterns have been observed in many cancer cell lines; these are regarded as the major type of molecular aberration in malignancies (7,8). Previous studies have evaluated aberrant DNA methylation in HCC by analyzing tumor tissues and adjacent non-tumor tissues (14,21-23). Their main aims were to identify novel potential biomarkers for the diagnosis of HCC or to study the associations between methylation and cirrhosis-associated HCC; the study design and enrolment criteria differed when selecting various patients for study. In addition, variations in ethnicity and the effects of the cutting edge of adjacent non-tumor tissues may lead to differing results observed across the different studies. Although previous studies have indicated a few novel DNA methylation markers that are associated with HCC, specific DNA methylation patterns associated with the progression of HCC and alterations in methylation between HCC and normal liver cells have yet to be identified. In the present study, Illumina Infinium HumanMethylation 450K BeadChip was used to identify global DNA methylation profiles in Huh7 and L02 cells.

In the present study, a total of 102,254 differentially methylated CpG sites were detected across the whole-genome of Huh7 and L02 cells; more hypermethylated CpG sites (62,702; 61.3%) were observed than hypomethylated (39,552; 38.7%) CpG sites. The results indicated that within Huh7 cells, aberrant DNA methylation was a very common event and that hypermethylation of CpG sites occurred more frequently than hypomethylation. In addition, stringent criteria were employed to select the significantly differentially methylated CpG sites, genes and DMRs. Finally, 5,285 (66.5%) significantly hypermethylated and 2,659 (33.5%) hypomethylated CpG sites were identified. It has been reported that, in many types of diseases including cancers, aberrant DNA methylation is a common event, particularly aberrant DNA methylation of CpG islands or within promoter regions, which are associated with tumor suppressor gene inactivation or oncogene activation (16). The results of the present study indicated that within a CpG island, a greater number of significantly hypermethylated CpG sites (1,544) were observed than significantly hypomethylated CpG sites (1,201). This result is consistent with previous HCC genome-wide methylation studies (14,24-27). Yates *et al* (18) and Dudziec *et al* (19) demonstrated that aberrant DNA methylation occurs in CpG islands, but can also be detected in the regions adjacent to CpG islands, CpG shores and CpG shelves (15), and may lead to tumorigenesis (20,28,29). The present study also demonstrated these points; significantly hypermethylated CpG sites in the CpG shores regions were more abundant than significantly hypomethylated CpG sites (1,137 to 632). In addition, in the CpG shelf regions, the significantly hypermethylated CpG sites were more frequent than significantly hypomethylated CpG sites (655 to 133).

DMRs are stretches of DNA in the genome. Varied DNA methylation patterns are seen between different organisms, and adjacent sites or a group of sites in proximity to each other tend to have different methylation patterns between different diseases (30). DMRs are associated with many diseases including several types of cancer (31). There are also many types of DMRs: Tissue-specific DMRs, cDMRs, rDMRs, imprinting-specific DMRs and aging-specific DMRs (20). In the present study, there were 390 differentially hypermethylated CpG sites located within DMRs, 233 (59.7%) were in island regions, 119 (30.5%) were in shore regions, and 15 (3.8%) were in shelf regions. In addition, there were 208 differentially hypomethylated CpG sites located within DMRs, 87 (41.8%) were in CpG island regions, 89 (42.8%) were in shore regions and 6 (2.9%) were in shelf regions. These results indicated that within HCC cells, aberrant DNA methylation may occur within CpG shore regions, which can also cause DNA transcriptional silencing and inactivation of gene function. Hepatocarcinogenesis was also associated with genomic instability and inactivation of gene function; the results of the present study concerning DMRs suggests that aberrant methylation within these sites may be an important epigenetic mechanism associated with

Table VII. Top 20 significant differentially methylated genes in Gene Ontology enrichment.

GO enrichment	Top 20 significantly hypermethylated genes in GO enrichment	Top 20 significantly hypomethylated genes in GO enrichment
Biological process		
Positive regulation of protein phosphorylation	ERBB4	-
Negative regulation of cell proliferation	ERBB4	-
Epidermal growth factor receptor signaling pathway	ERBB4	-
Synaptic transmission	PCDH8	-
Negative regulation of transcription from RNA polymerase II promoter	SALL4; NKX6-2; MEIS2; TCF21; ZBTB32;	-
Fibroblast growth factor receptor signaling pathway	ERBB4	-
Molecular function		
Protein binding	ERBB4; PAX6; BCL3; DOCK1; ZBTB32; BCL2	PLXND1; LASP1; MMP9; DKK1; DNAJB1; RB1; GAS1; AHCYL1
ATP binding	ERBB4	-
Sequence-specific DNA binding transcription factor activity	PAX6; NKX6-2; BCL3	SIX2; RB1
DNA binding	PAX6; SALL4; BCL3; ZIC4; ZBTB32	PRDM8; RB1
Metal ion binding	SALL4; ZIC4; ZBTB3	PRDM8; ARSG; NPTX1
Protein heterodimerization activity	BCL2	NPR3; SDCBP2
Sequence-specific DNA binding	BCL2	SIX2
Protein kinase binding	PAX6	-
Transcription regulatory region DNA binding	ERBB4; TCF21	-
Protein complex binding	-	SIX2
Protein dimerization activity	TCF21	-
Identical protein binding	BCL2	MMP9; RB1
Protein homodimerization activity	ERBB4; BCL2	SDCBP2
Protein C-terminus binding	-	SDCBP2
Cellular component		
Nucleus	ERBB4; PAX6; SALL4; NKX6-2; BCL3; DOCK1; MEIS2; ZIC4; TCF21; ZBTB32; BCL2	PRDM8; SIX2; RB1
Cytosol	ERBB4	-
Cytoplasm	ERBB4; PAX6; SALL4; BCL3; DOCK1; BCL2; BDNF	SDCBP2; DNAJB1
Nucleoplasm	ERBB4; ZBTB32	RB1
Golgi apparatus	-	STC2
Perinuclear region of cytoplasm	BCL3; BDNF	NPTX1
Mitochondrion	ERBB4; BCL2	SLC25A42
Nucleolus	ERBB4; PAX6	DNAJB1; RB1
Transcription factor complex	LRFN2	-
Cell junction	PCDH	-
Membrane	ERBB4; DOCK1; BCL2	SLC16A6
Nuclear chromatin	PAX6	-

GO, Gene Ontology.

hepatocarcinogenesis. These results may provide more information regarding the associations between HCC and aberrant DNA methylation.

Furthermore, the present study listed the top 20 significantly hyper- and hypo-methylated CpG sites, and genes in DMRs within Huh7 cells compared with L02 cells. The top 20

Table VIII. Kyoto encyclopedia of genes and genomes pathway analysis of differentially methylated genes.

Pathway	Number of differentially methylated genes (n)
Pathways in cancer	309
Focal adhesion	186
MAPK signaling pathway	251
Wnt signaling pathway	143
Axon guidance	120
TGF-β signaling pathway	81
Basal cell carcinoma	55
Regulation of actin cytoskeleton	191
Colorectal cancer	61
Adherens junction	71
Chronic myeloid leukemia	71
ECM-receptor interaction	81
Endocytosis	186
Pyrimidine metabolism	97
Non-small cell lung cancer	57
Hedgehog signaling pathway	54
Neurotrophin signaling pathway	116
Glioma	63
Endometrial cancer	52
VEGF signaling pathway	71
ErbB signaling pathway	80
Small cell lung cancer	80
Lysosome	110
Metabolic pathways	964
Calcium signaling pathway	159
Purine metabolism	150
Ubiquitin mediated proteolysis	128
Insulin signaling pathway	128
Notch signaling pathway	45
Protein processing in endoplasmic reticulum	156
RNA polymerase	32
Hepatitis C	116
Renal cell carcinoma	61
Aminoacyl-tRNA biosynthesis	42
B cell receptor signaling pathway	69
Thyroid cancer	29
Melanoma	65
Oocyte meiosis	103
Adipocytokine signaling pathway	64
Melanogenesis	94
Vascular smooth muscle contraction	115
Selenocompound metabolism	26
p53 signaling pathway	63

significantly hypermethylated genes, which were high-ranking with notable differences in the absolute value of β-difference, included the following: ERBB4, paired box 6, splat like transcription factor 4, protocadherin (PCDH)-8, NK2 homeobox 6, B-cell lymphoma (BCL)-3, glypican 6, family with sequence similarity 196 member A, dedicator of cytokinesis 1, Meis homeobox 2, Zic family member 4, transcription factor 21, zinc finger and BTB domain containing 32, leucine rich repeat and fibronectin type III domain containing 2, BCL2, PCDH9, DPY30 domain-containing protein 2, zinc finger CCHC-type containing 24, brain-derived neurotrophic factor, cg00057722 and cg08640046. The functional role of these genes in HCC requires further study. The top 20 significant differentially hyper- and hypo-methylated genes from GO enrichment were also listed. These genes, which were located within DMRs, were mainly associated with 'cell differentiation development', 'transcription factor activity', 'sequence-specific DNA binding', 'cellular development process' and 'cell junction'.

Additionally, through GO enrichment analysis, the present study revealed that aberrant DNA methylation in HCC was associated with cell differentiation and proliferation, and through KEGG pathway analysis, 43 signaling pathways associated with HCC were identified, including pathways in cancer, MAPK signaling, Wnt signaling, VEGF signaling and p53 signaling pathways. Previous studies have demonstrated that aberrant DNA hypermethylation can downregulate the expression of cell cycle inhibitors, $p16^{INK4A}$, p53 and factors involved in TGF-β/mothers against decapentaplegic signaling (32,33). Thus far, researchers have revealed that the inactivation of Wnt pathway-associated antagonists is linked to the aberrant DNA hypermethylation of some genes (34,35). Activation of the ERB receptor and MAPK signaling pathways, as well as the regulation of epigenetic proteins that were previously demonstrated to promote cancer growth and metastasis, have been reported to be possible candidate targets for anticancer treatment in multiple types of cancer, including HCC (32,36).

In addition, HCC cells may escape or become tolerant to chemotherapy via various mechanisms, therefore, identifying novel drugs is very important for the future therapy of HCC. The application of inhibitors of DNA methylated drugs in the treatment of cancer has gradually attracted the attention of researchers (37), including 5-azacytidine (5-aza-C), decitabine (5-aza-2'-deoxycytidine, 5-aza-dC), 1-β-D-arabinofuranosyl-5-azacytosine, dihydro-5-azacytosine (38), SGI-110 (previously known as S110), a dinucleotide of 5-aza-2'-deoxycytidine and deoxyguanosine, containing 5-azaCdR moiety, which has been revealed to be very effective in inhibiting DNA methylation, though its stability and cytotoxicity are comparable to that of decitabine (39), and a non-nucleoside DNA methyltransferase inhibitor, SGI-1027 (40,41). To the best of our knowledge, there have been only a few studies investigating the effects of demethylation agents on HCC *in vitro*.

In conclusion, the present study detected genome-wide DNA methylation patterns occurring in Huh7 cells, and identified numerous differentially hypo- and hypermethylated CpG sites, genes, DMRs and signaling pathways associated with HCC. Additionally, the diversity in methylation within Huh7 cells was also observed. The results of the present study may provide important information regarding the molecular mechanisms underlying methylation in Huh7 cells, which may be useful in future research into the underlying mechanisms associated with HCC. In addition, HCC cells may escape or develop tolerance to chemotherapy via various mechanisms, therefore, identifying novel drugs is very important for future therapies of HCC. The application of inhibitors of DNA methylation for the treatment of cancer has gradually attracted more attention within the field (37),

and there have been a few studies investigating the effects of demethylation agents in HCC *in vitro*. The results of the present study may provide a useful basis for future research into effective HCC therapies.

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Availability of data and materials

The analyzed datasets generated during this study are available from the corresponding author on reasonable request.

Authors' contributions

JZ conceived and designed the study. NS, CZ, YS, BZ and BC performed the experiments. NS and AJ analyzed the data. NS wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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