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Basic Study

Differential expression and significance of 5-hydroxymethylcytosine modification in hepatitis B virus carriers and patients with liver cirrhosis and liver cancer

INTRODUCTION

Primary liver cancer, mainly hepatocellular carcinoma (HCC), is a solid tumor generated from the malignant transformation of hepatocytes or intrahepatic bile duct cells and is one of the most common malignancies in China^[1]. Additionally, the number of hepatitis B virus (HBV) carriers in China is high. If patients develop an infection, chronic HBV infection, liver cirrhosis, and even HCC may occur. The relationship between hepatitis B surface antigen (HBsAg)-positive carriers and liver cancer has been reported in many studies^[2]. There is consensus that liver cirrhosis can progress to liver cancer. Although great progress has been made in the prevention and treatment of hepatitis B in China, including a reduction in the transmission rate of HBV, effective treatments for HBV carriers are still lacking. Drugs such as nucleotide analogs, including entecavir, cannot rapidly reduce HBV titers in the short term. Additionally, administration of these drugs to HBV carriers remains controversial in clinical practice^[3]. At present, the mechanisms underlying the transformation from the HBsAg-positive state or liver cirrhosis to liver cancer is not entirely clear, especially the potential mechanism of epigenetic changes in the occurrence and progression of these diseases. Therefore, the differences in epigenetic modifications between the HBsAg-positive state, liver cirrhosis, and liver cancer must be explored.

DNA hydroxymethylation is a process in which 5-methylcytosine is oxidized to 5-hydroxymethylcytosine (5hmC) by ten-eleven translocation enzymes during DNA

methylation^[4]. Further, 5hmC is an important base in DNA demethylation as well as in epigenetic regulation. Some studies suggest that 5hmC is the sixth genome base and is involved in the assembly of chromosomes and regulation of gene expression^[5]. Studies have shown that the level of hydroxymethylation in tumors and other diseases is significantly different from that in the normal state, and the difference gradually increases with the progression of diseases, indicating that the epigenetic modification of hydroxymethylation is essential in the development of tumors^[6]. However, the mechanism of 5hmC in HBsAg-positive carriers or patients with cirrhosis that develops into liver cancer has not yet been fully elucidated. 5hmC sequencing is a new technology that constructs free nucleic acid fragments containing 5hmC using polymerase chain reaction (PCR) technology and detects the 5hmC sequence using second-generation DNA sequencing technology. Because the number of 5hmC modifications is far less than the number of DNA methylations, 5hmC sequencing has a higher cost performance, efficiency, accuracy, and economy, as well as extensive clinical application value. In this study, we analyzed differentially expressed genes (DEGs) in HBsAg-positive carriers, patients with liver cirrhosis, and patients with liver cancer using 5hmC sequencing technology and further annotated the function of DEGs to explore the similarities and differences in gene hydroxymethylation between HBsAg-positive carriers, patients with liver cirrhosis, and patients with liver cancer and the regulatory role of signaling pathways.

MATERIALS AND METHODS

Participants

Forty HBsAg-positive carriers, forty patients with liver cirrhosis, and forty patients with liver cancer admitted to the First People's Hospital of Yongkang between March 2020 and November 2021 were selected as participants. This study was approved by the medical ethics committee of the hospital. The diagnostic criteria for liver cirrhosis were based on the International Guidelines for the Diagnosis and Treatment of Liver Cirrhosis updated in 2021^[7]. There were no differences in baseline data, such as sex and

age, between the three groups of patients. Inclusion criteria for primary liver cancer were as follows^[8]: body mass index of 20–30 kg/m² in men and 19–34 kg/m² in women; complete results of general biochemical indexes such as liver function; imaging data such as tumor computed tomography/magnetic resonance imaging and serological indexes; a history of hepatitis B; no history of diabetes or other systemic diseases; no history of other infectious diseases; and no pregnancy or lactation.

Detection with 5hmC sequencing

cfDNA extraction: Blood samples from HBsAg-positive carriers, patients with liver cirrhosis, and patients with liver cancer were collected at the time of diagnosis and sent to Zhongke Jinzhen Co., Ltd. for DNA extraction, pyrolysis of specimen precipitate after centrifugation, and extraction of cfDNA from plasma using the Quick-cfDNA Serum & Plasma Kit (Zymo).

DNA quality test and results: Qubit accurately quantified the DNA concentration, and Q-sep analyzed the size, distribution, and relative quantification of DNA fragments. After the sample was quantified, the DNA of the sample was first repaired, a tail was added to the 3' end, and the sequencing joint was connected. Biotins were connected by a transglycosylation reaction and click chemistry. The DNA fragment containing 5hmC was captured using streptavidin beads, and PCR amplification was performed to complete the entire library construction. After the library quality inspection, different libraries were sequenced using Nova6000 according to the effective concentration and target data volume. After obtaining the original sequence (sequenced reads), the original gene fragments were returned to the correct human reference genome position to calculate the gene expression of each sample.

Principal component analysis

Principal component analysis (PCA) is an unsupervised multivariate statistical analysis method that can generally reflect the overall differences between samples and the

variation between samples within the group. It is commonly used to evaluate sample differences between groups and consistency within groups. PCA was used for dimensionality reduction of all 5hmC gene sequencing results. After data visualization, outlier samples or sample clusters with high similarity were identified.

Screening of DEGs modified by 5hmC

Through the analysis of the significant difference in the 5hmC modification expression matrix of all samples, functional gene modification differences among HBsAg-positive carriers, patients with liver cirrhosis, and patients with liver cancer were found. In this study, the differences in 5hmC expression were analyzed using normalization, discrete estimation, and significance tests. Normalization and discrete estimation mainly remove the influence of sample sequencing depth differences and reduce the false-positive rate through homogenization. DEGs were further detected using DEseq2 and PoissonDis algorithms. DEGs were screened according to the $\log_2\text{FoldChange} \geq 0.26$, gene 5hmC modified expression value, and adjusted P value ≤ 0.05 . The sample classification of this analysis included three groups: HBsAg-positive carriers, patients with liver cirrhosis, and patients with liver cancer, with 40 patients in each group. In the three types of samples, the differences between the liver cirrhosis group and liver cancer group, HBsAg-positive carrier group, and liver cancer patient group were analyzed. Lists of all differential genes are in the original data.

Functional enrichment analysis

Based on the results of gene ontology (GO) and gene pathway annotation, the DEGs were enriched and classified according to the aforementioned functions and pathways. GO enrichment analysis was used for functional classification, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was used for pathway classification. GO enrichment analysis was performed from three aspects: cellular component (CC), biological process (BP), and molecular function (MF). KEGG is a database used for analyzing gene functions, linking multiple genes with specific

pathways and functions, and classifying genes according to the pathways involved. STRING (<https://string-db.org/>) is currently the largest protein interaction database. DEGs were imported into the STRING database, and the homology, literature, and co-expression relationship of known proteins in the database were used to determine the interaction between the imported encoded proteins to construct a protein-protein interaction (PPI) network analysis.

Statistical analysis

Pearson correlation analysis was performed between each pair of samples using the Cor function in R software; $P < 0.05$ was considered statistically significant. The expression of 5hmC modified genes obeyed a Poisson distribution after standardization. The P -value was calculated using the Wald test and corrected using multiple hypothesis tests. Statistical significance was set at $P < 0.05$.

RESULTS

Analysis of 5hmC sequencing results

The Nova6000 platform was used to measure samples from the three groups of patients. Each group contained 40 patients, and 16455 genes were detected. The percentages of principal components (PC) 1 and 2 in the PCA plot of all genes in HBsAg-positive carriers and patients with liver cancer were 24.933% and 12.06% (Figure 1A), respectively. The percentages of PC1 and PC2 in the PCA plot of differential genes were 46.351% and 14.927% (Figure 1B), respectively, suggesting that differential genes can distinguish HBsAg-positive carriers from patients with liver cancer.

Similarly, when detecting the same number of genes, the percentages of PC1 and PC2 in the PCA plot of all genes in patients with liver cirrhosis and liver cancer were 21.998% and 11.076% (Figure 1C), respectively, and the percentages of PC1 and PC2 in the PCA plot of differential genes were 59.621% and 12.27% (Figure 1D), respectively, suggesting that differential genes have the ability to distinguish between liver cirrhosis and liver cancer.

Analysis of DEG expression level

Differences between patients with liver cirrhosis and those with liver cancer and differences between HBsAg-positive carriers and patients with liver cancer were analyzed. According to the levels of 5hmC modified genes in each sample, DEGs between HBsAg-positive carriers and patients with liver cirrhosis or liver cancer were screened. The results showed 32 DEGs between HBsAg-positive carriers and patients with liver cancer, of which 30 were upregulated and 2 genes were downregulated by 5hmC modification. The distribution of DEGs is shown by a volcanic and thermal map in Figure 2A and C. All DEGs upregulated and downregulated in HBsAg-positive carriers and liver cancer groups are shown in Table 1. There were 20 DEGs between patients with liver cirrhosis and those with liver cancer, of which 17 were upregulated and 3 genes were downregulated by 5hmC modification. The distribution of the DEGs is shown by a volcanic and thermal map (Figure 2B and D). All DEGs that were upregulated and downregulated in patients with liver cirrhosis and liver cancer are shown in Table 2.

GO and KEGG enrichment analysis of DEGs in HBsAg carriers and patients with liver cancer

According to the results of DEG detection, GO enrichment analysis was used for the functional annotation of the 5hmC sequences. After the difference analysis between HBsAg carrier and liver cancer groups, the top 10 enriched GO terms related to BP, CC, and MF were used as the main functions of differential hydroxymethylation genes between HBsAg carriers and patients with liver cancer. The main pathways involved are shown in Figure 3A. The numbers of genes involved in the GO BP of TOP3 were 4, 4, and 4, respectively, which were mainly related to digestion, homophilic cell adhesion *via* plasma membrane adhesion membrane, and cellular lipid catabolic processes. The numbers of genes included in the MF of TOP3 were 3, 2, and 2, respectively, which were related to the activities of secondary active transmembrane transporter activity,

alcohol binding, and solute:cation symporter activity. The numbers of genes in TOP3 cell components were 5, 4, and 2, respectively, which were mainly related to the apical part of the cell, apical plasma membrane, and cell cortex region. These three kinds of gene function together suggest that the main differences between HBsAg-positive carriers and patients with liver cancer may be digestive function and cell information transmission.

From the perspective of pathway enrichment, 92 pathways were found to be closely related to KEGG enrichment in HBsAg carriers and patients with liver cancer. The main pathways are shown in **Figure 3B**. Among them, the number of pathways related to digestive secretion, such as carbohydrate digestion and absorption, insulin and bile secretion, galactose metabolism, and the glucagon signaling pathway, were most significant. The peroxisome proliferator-activated receptor and mitogen-activated protein kinase (MAPK) signaling pathways were also significantly enriched in differentially hydroxymethylated genes. These results indicate that the main difference between HBsAg carriers and patients with liver cancer lies in their digestive function. Soluble carrier family 2 member 2 (*SLC2A2*) and Fos proto-oncogene, AP-1 transcription factor subunit (*FOS*) were involved in the most enriched pathways. *SLC2A2* is mainly enriched in insulin and bile secretion, the prolactin signaling pathway, and central carbon metabolism in cancer. *FOS* is mainly enriched in the prolactin, MAPK, and B-cell receptor signaling pathways and the PD-L1/PD-1 pathway, suggesting that *SLC2A2* is related to liver-digestive function and *FOS* is related to immune signal transduction.

GO and KEGG enrichment analysis of DEGs in the liver cirrhosis and liver cancer groups

After the difference analysis between the liver cirrhosis and liver cancer groups, the main pathways involved in the GO enrichment analysis were determined, as shown in Figure 4A. Among them, the numbers of genes involved in the GO BP of TOP3 were 2, 2, and 2, respectively, which were mainly related to the bile acid biosynthetic process, ectodermal placode development, and ventricular cardiac muscle tissue development.

The numbers of genes included in the MF of TOP3 were 3, 3 and 3, respectively, which were related to monooxygenase activity, oxidoreductase activity, acting on paired donors, and cofactor binding. The numbers of genes contained in the TOP3 cell components were 1, 1, and 1, respectively, which were mainly related to the hydrolysis of short peptides, ubiquitination and protein synthesis. These three types of gene functions jointly suggest that the main differential functions between liver cirrhosis and liver cancer may be bile acid metabolism and oxidoreductase activity.

The KEGG enrichment results of the liver cirrhosis and liver cancer groups differed from those of the above enrichment pathways. The results showed that 22 pathways were closely related; the main pathways involved are shown in Figure 4B. Among them, pathways related to the metabolism of various biological components were enriched, but specific pathways were different, such as primary bile acid biosynthesis, cholesterol metabolism, retinol metabolism, and drug metabolism (cytochrome P450), as well as cell death-related signaling pathways such as cytotoxicity, apoptosis, and necrotizing apoptosis mediated by natural killer cells, which were also significantly enriched in differentially hydroxymethylated genes. These results indicate that the main difference between liver cirrhosis and liver cancer is first reflected in the metabolic disorders of various active substances, and that the death behavior of tumor cells is different from that of non-tumor cells.

PPI analysis of proteins encoded by DEGs

Proteins usually perform biological functions by binding to one another. According to the <https://string-db.org/> STRING protein interaction database, DEGs were analyzed using PPI, and the results are shown in Figure 5A, which shows the PPI network of DEGs in HBsAg carriers and patients with liver cancer. *PCK1* and *SLC2A2* were most closely related to each protein. Protein interactions were mainly concentrated between genes such as *FABP1*, *APOC3*, *SI*, *KRT20*, *SLC5A1*, *SLC10A2*, *RBP2*, and *AKR1B10*; Figure 5B displays the PPI network of patients with liver cirrhosis and those with liver

cancer. It shows that only *PROX1* and *TBX3* have potential protein interactions, and the relationship between other proteins is unclear.

DISCUSSION

China has the most frequent occurrences of liver cancer, accounting for 47% of cases globally. Liver cancer ranks third among malignant tumors in China. In 2018, the number of newly diagnosed patients with liver cancer in China was 390000, and the number of deaths was 360000. Therefore, it remains a major threat to the health of the Chinese population^[9]. Although many basic and clinical studies have explored the mechanisms of liver cancer formation and development, it remains unclear, and there are limited studies on epigenetic modifications in the occurrence and development of liver cancer, especially on the regulation of hydroxymethylation. To further define the potential relationship between HBsAg carrier status, liver cirrhosis, and liver cancer in the epigenetic modification of hydroxymethylation, serum samples from 40 HBsAg carriers, 40 patients with liver cirrhosis, and 40 patients with liver cancer were collected in our hospital. The bioinformatics method of 5hmC sequencing was used to conduct an in-depth analysis of these datasets, and DEGs were screened for 5hmC; the genes and pathways closely related to them were identified through bioinformatic analysis.

In this study, 16455 hydroxymethylated genes were identified. Sequencing showed that 32 genes had significant differences in 5hmC modifications between HBsAg carriers and patients with liver cancer, and 20 genes had significant differences in 5hmC modifications between patients with liver cirrhosis and liver cancer. These genes may have potential loci that have not been discovered or clearly studied, leading to the occurrence and development of liver cancer, which is congruent with the findings of previous studies on epigenetic modifications^[10]. Studies have shown that multiple DEGs of 5hmC are upregulated and downregulated in HBsAg carriers, patients with liver cirrhosis, and patients with liver cancer, involving multiple cellular signaling pathways and biological processes^[11].

GO enrichment analysis showed that 32 DEGs were enriched in both HBsAg carriers and patients with liver cancer, indicating that liver cancer progression is the result of multiple pathways. For example, in GO BP analysis, the digestion and cellular lipid catabolic processes are enriched; digestion is one of the most important functions of the liver, and lipid catabolism is part of the digestive link and is responsible for one of the most basic biological processes^[12]. In GO MF, the enrichment of secondary active transmembrane transporters and cation symporter activity was the most obvious, indicating that transmembrane transporters and ion transporters are involved in the digestive function of liver cells and may affect the progression of liver cancer^[13]. In GO CC analysis, ²the apical part of the cell and apical plasma membrane were enriched, suggesting that the interaction between cells mainly occurs on the cell membrane^[14]. Interestingly, in the GO BP analysis of the liver cirrhosis and liver cancer groups, the most obvious enrichment was bile acid biosynthesis and metabolic processes. Bile acid can increase the contact area of lipase by emulsification of fat, improve the activity of pancreatic and lipoprotein lipases, and promote intestinal fat transport to promote fat metabolism^[15]. In the GO MF analysis, oxidation-related enzymes were enriched, indicating that the redox function of liver cirrhosis was further affected after liver cancer progression, which is consistent with the results of Chen *et al*^[16]. In GO CC analysis, the enrichment of the proteasome complex further supports the results of these molecular functions. Minor *et al*^[17] also pointed out that HBV proteins can promote the occurrence and development of HCC by forming a ubiquitinated proteasome complex.

KEGG analysis showed that the DEGs between HBsAg carriers and liver cancer groups had the largest number of genes enriched in the series of digestive and metabolic pathways, and the genes enriched in liver digestive function-related pathways accounted for the largest proportion of DEGs. Most genes are involved in digestive processes such as insulin and bile secretion, galactose metabolism, and the glucagon signaling pathway. In addition to these pathways, DEGs in the liver cirrhosis and liver cancer groups were also related to death processes, such as apoptosis and

necrotizing apoptosis. Normal liver cells often develop abnormal metabolic behavior during their gradual development into tumor cells. This abnormal behavior affects the normal function of the liver, which can be considered a progressive feature of liver cancer. However, most previous studies concluded that no obvious pathological changes appeared in the liver of HBV carriers without disease. A few studies pointed out that the secretion of digestive juice in HBsAg-positive carriers might be related to the occurrence and development of liver cancer. An increasing number of researchers believe that the normal transaminase levels of HBsAg-positive carriers do not indicate fibrosis or inflammatory necrosis in the liver, which may also show different degrees of hepatocyte degeneration, necrosis, and even fibrosis^[18]. In the liver tissue of HBV carriers with normal alanine aminotransferase (ALT) levels and without treatment, the proportion of liver inflammation grade G2-3 was 25.0%, which was higher than that in the group with a slight increase in ALT, and HBV carriers were accompanied by different degrees of liver fibrosis^[18]. Studies have shown that insulin inhibits HBsAg expression in hepatocytes^[19]. Moreover, the presence of insulin resistance-related diseases during entecavir treatment is associated with a slower decline in HBsAg expression^[20]. Researchers have found that, while lovastatin is used to block the p21Ras signaling pathway of insulin, it also inhibits the secretion of HBsAg in Hep3B cells, which may be caused by the instability of lipid rafts due to the depletion of cholesterol from the membrane^[21]. Therefore, it is reasonable to believe that changes in liver digestive function-related pathways are closely related to the progression of liver cancer.

PPI analysis showed that protein interactions were mainly concentrated among genes with *PCK1* and *SLC2A2* as the central nodes, and protein interactions were mainly concentrated among genes such as *FABP1*, *APOC3*, *SI*, *KRT20*, *SLC5A1*, *SLC10A2*, *RBP2*, and *AKR1B10*. Most of them are involved in the regulation of cellular insulin, glucocorticoids, glucagon, and other signaling pathways and play a key role in glucose metabolism and gluconeogenesis. The *PCK1* gene, encoding cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C), functions as a glucoamylase in the

liver and kidneys^[22]. Studies have found that the type 2 diabetes phenotypes, such as obesity, fat malnutrition, fatty liver, and even death in serious circumstances, appear in mice after knockout of systemic and tissue-specific *PCK1* genes^[23]. In this study, *PCK1* was highly expressed in liver cancer tissues ($\log_2FC = 0.519299173$). Considering that the 5hmC modification of *PCK1* may affect the transcription and translation of the PEPCK-C isoenzyme, it is necessary to verify the effect of 5hmC on its protein expression and explore the relationship between *PCK1* and the occurrence and development of liver cancer. *SLC2A2* is a membrane glycoprotein that encodes liver cells, pancreatic β cells, and intestinal and renal epithelial cells and mediates easy bidirectional glucose transport. Owing to its low affinity for glucose, it is a glucose sensor rather than the main transporter of glucose. This gene mutation is reportedly related to susceptibility to Fanconi-Bickel syndrome and non-insulin-dependent diabetes^[24]. It participates in many processes, such as cell proliferation, differentiation, and apoptosis, by regulating glucose metabolism^[25,26]. This study found that the 5hmC modification level of *SLC2A2* was slightly increased ($\log_2FC = 0.297032232$); however, whether the 5hmC modification affects the function of *SLC2A2* requires further confirmation. The valuable information obtained in the PPI network construction of liver cirrhosis and liver cancer groups is limited, and we can only infer that PROX1-TBX3 may have molecular interactions and a role in the progression of liver cirrhosis to liver cancer.

CONCLUSION

This study showed that the 5hmC modification of genes in the liver digestion-related pathway may be closely related to the occurrence and development of liver cancer. Abnormal bile and insulin secretion by tumor cells may promote or symbolize the occurrence and development of liver cancer. *SLC2A2* and *PCK1* are the central nodes of the DEGs, which may be most closely related to the occurrence of liver cancer. *FABP1*, *APOC3*, *SI*, *KRT20*, *SLC5A1*, *SLC10A2*, *RBP2*, and *AKR1B10* may be the key genes in the protein regulatory network. 5hmC modification of these genes alters their transcription

and other functions and plays a regulatory role. These differences in the 5hmC modification levels of DEGs provide further insights into the development and progression of liver cancer. We plan to verify the 5hmC modification levels of these genes to further explore the mechanisms of liver cancer.

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Figure Legends

Figure 1 Principal component analysis after 5-hydroxymethylcytosine sequencing of hepatitis B surface antigen-positive carriers, patients with liver cirrhosis, and patients with liver cancer (40 cases per group). A: Principal component analysis (PCA) plot of all genes in hepatitis B surface antigen (HBsAg)-positive carriers and patients with liver cancer; B: PCA plot of differential genes between HBsAg-positive carriers and patients with liver cancer; C: PCA plot of all genes in patients with liver cirrhosis and those with liver cancer; D: PCA plot of differential genes between patients with liver cirrhosis and those with liver cancer. PCA: Principal component analysis; HBV: Hepatitis B virus.

Figure 2 Analysis of 5-hydroxymethylcytosine differences after sequencing. A, B: The gene volcano maps of hepatitis B surface antigen (HBsAg)-positive carriers and patients with liver cancer (A) and of patients with liver cirrhosis and those with liver cancer (B). The blue point indicates that the change of gene hydroxymethylation log2 fold is less than -0.26, and the red point indicates that the change of gene hydroxymethylation log2 fold is more than 0.26; C, D: Gene hot maps for HBsAg-positive carriers and patients with liver cancer (C) and for patients with liver cirrhosis and those with liver cancer (D). The blue block colors indicate a lower level of gene hydroxymethylation, and the red block colors indicate a higher level of gene hydroxymethylation. HBV: Hepatitis B virus.

Figure 3 Results of 5-hydroxymethylcytosine sequencing differential gene enrichment analysis between hepatitis B surface antigen carriers and patients with liver cancer. A: Gene ontology enrichment analysis of 5-hydroxymethylcytosine (5hmC) sequencing differentially expressed genes (DEGs); B: Kyoto Encyclopedia of Genes and Genomes enrichment analysis of 5hmC sequencing DEGs. GO: Gene ontology; CC: Cellular component; BP: Biological process; MF: Molecular function.

Figure 4 Enrichment analysis results of 5-hydroxymethylcytosine sequencing differential genes between patients with liver cirrhosis and those with liver cancer. A: Gene ontology enrichment analysis of 5-hydroxymethylcytosine (5hmC) sequencing differentially expressed genes (DEGs); B: Kyoto Encyclopedia of Genes and Genomes enrichment analysis of 5hmC sequencing DEGs. GO: Gene ontology; CC: Cellular component; BP: Biological process; MF: Molecular function.

Figure 5 Protein-protein interaction network analysis of differentially expressed genes. A: Protein-protein interaction (PPI) network of differentially expressed genes (DEGs) in hepatitis B surface antigen carriers and patients with liver cancer; B: PPI network of DEGs in patients with liver cirrhosis and those with liver cancer.

Table 1 Differential genes between hepatitis B surface antigen-positive carriers and patients with liver cancer after 5-hydroxymethylcytosine sequencing

Symbol	Ensembl	log2FoldChange	padj
<i>SI</i>	ENSG00000090402	0.849003917	0.000172596
<i>FAM83B</i>	ENSG00000168143	0.532487323	0.00425853
<i>PCK1</i>	ENSG00000124253	0.519299173	0.006848676
<i>KCNN2</i>	ENSG00000080709	-0.510730422	0.011078186
<i>FAT1</i>	ENSG00000083857	0.47499449	0.02542581
<i>FGF19</i>	ENSG00000162344	0.428605733	0.036854977
<i>FABP1</i>	ENSG00000163586	0.427372957	0.025268146
<i>SLC10A2</i>	ENSG00000125255	0.417559665	0.005504835
<i>ISX</i>	ENSG00000175329	0.391889896	0.005936182
<i>APOC3</i>	ENSG00000110245	0.374978715	0.017711634
<i>NOVA1</i>	ENSG00000139910	0.367147827	0.006848676
<i>KRT20</i>	ENSG00000171431	0.361001798	0.049694505
<i>DUSP1</i>	ENSG00000120129	0.341097702	0.001523335
<i>RBP2</i>	ENSG00000114113	0.330491105	0.049694505
<i>PCLO</i>	ENSG00000186472	0.307272826	0.000520372
<i>BRINP3</i>	ENSG00000162670	0.297534011	0.001107995
<i>SLC2A2</i>	ENSG00000163581	0.297032232	0.006630228
<i>AKR1B10</i>	ENSG00000198074	0.296134756	0.012660193
<i>SEMA3A</i>	ENSG00000075213	0.295498456	7.92E-05
<i>SLC5A1</i>	ENSG00000100170	0.295343232	0.027331533
<i>ROBO1</i>	ENSG00000169855	0.294408057	0.026805139
<i>ROPN1B</i>	ENSG00000114547	-0.292505301	0.002291301
<i>PRR16</i>	ENSG00000184838	0.291799543	0.049694505
<i>TNFSF15</i>	ENSG00000181634	0.286998557	0.01533013
<i>CDH17</i>	ENSG00000079112	0.284940571	0.001204498

<i>MUC17</i>	ENSG00000169876	0.284637524	0.022168549
<i>PCDH7</i>	ENSG00000169851	0.284173648	0.00131498
<i>FOS</i>	ENSG00000170345	0.28075648	0.008754834
<i>GABRA2</i>	ENSG00000151834	0.27973066	0.049694505
<i>NRCAM</i>	ENSG00000091129	0.270536808	0.047702973
<i>DPP10</i>	ENSG00000175497	0.268657512	0.005504835

Table 2 Differential genes between patients with liver cirrhosis and those with liver cancer after 5-hydroxymethylcytosine sequencing

Symbol	Ensembl	log2FoldChange	padj
<i>MUC8</i>	NA	-0.61023	0.048721
<i>PROX1</i>	ENSG00000117707	0.510971	0.047662
<i>TBX3</i>	ENSG00000135111	0.380857	0.04644
<i>DSC2</i>	ENSG00000134755	0.366222	0.021397
<i>PSME1</i>	ENSG00000092010	0.340336	0.036054
<i>C2orf72</i>	ENSG00000204128	0.339959	0.015473
<i>PTP4A1</i>	ENSG00000112245	0.33768	0.043273
<i>KCTD12</i>	E0NSG00000178695	0.325745	0.016818
<i>FMO2</i>	ENSG00000094963	0.320229	0.040782
<i>LGALS9C</i>	ENSG00000171916	-0.29776	0.040782
<i>CYP3A5</i>	ENSG00000106258	0.292488	0.048557
<i>SNAPC5</i>	ENSG00000174446	0.290892	0.020989
<i>TMEM176A</i>	ENSG00000002933	0.285556	0.04535
<i>SMPDL3A</i>	ENSG00000172594	0.283335	0.036054
<i>TNFSF10</i>	ENSG00000121858	0.281624	0.009552
<i>CYP27A1</i>	ENSG00000135929	0.275649	0.038131
<i>FAM20A</i>	ENSG00000108950	0.275255	0.040338
<i>CCDC67</i>	NA	0.268828	0.036054
<i>IL37</i>	ENSG00000125571	-0.26789	0.021397
<i>RBM47</i>	ENSG00000163694	0.267243	0.038605

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