Prediction of Biomarkers for Hepatocellular Carcinoma Through Microarray-Based DNA Methylation Analysis

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Abstract

Background: Aberrant DNA methylation of cytosine guanine dinucleotide sides (CpGs) is one of the earliest and most frequent alterations in cancer. However, there is no complete understanding of the methylome in hepatocellular carcinoma (HCC), and few studies comprehensively evaluated methylation signatures of HCC based on high-throughput platforms.

Objective: Based on the DNA methylation data of HCC, the current study aimed at identifying the specific DNA methylation biomarkers to diagnose HCC.

Methods: The current study used bioinformatics method based on the published microarray data of HCC was implemented in Linyi, Shandong Province, China in 2017. Using GSE57956 data downloaded from the Gene Expression Omnibus database, the differentially methylated genes between HCC and normal groups were identified. Next, hierarchical clustering was conducted to measure whether the differentially methylated genes could distinguish the HCC from the normal samples. Furthermore, functional enrichment analyses were respectively implemented for up- and down-methylated genes to further extract the potential biological processes based on DAVID tool.

Results: According to the cutoff threshold of ≥ 0.2 average beta-values difference, 1340 differentially methylated genes (1660 CpGs) were identified including 978 up-methylated and 682 down-methylated genes. Utilization of the up-methylated and down-methylated genes to enrich gene ontology (GO) terms and biological pathways led to the identification of several important function regions, and the most significant ones were innate immune response, transcription from RNA polymerase II promoter and neuroactive ligand-receptor interaction.

Conclusion: Functional terms (innate immune response, transcription from RNA polymerase II promoter, and neuroactive ligand-receptor interaction) might be as potential targets for HCC diagnosis and treatment.

Keywords: Hepatocellular Carcinoma, DNA Methylation, Pathways, Biomarkers, CpGs

1. Background

Hepatocellular carcinoma (HCC) is the 5th most common cancer and the 3rd most common cause of cancer-related mortality worldwide (1). Unfortunately, in 2012, about 50% of the new cases occurring globally were in China (2). At present, the therapy methods for patient with the HCC are mainly focused on the operative treatments considered as the 1st choice and the most effective treatment method of HCC (3, 4). Although this method has certain curative effects on this disease, the radical treatment of HCC does not appear on the worldwide consensus (5).

Recently, several studies implicated how mRNA-based gene signatures were identified from HCC resection specimens and biopsies improve the prognostic performance

of clinical and pathological variables (6, 7). In the lab, gene silencing or gene knockout was applied to detect the disease-related genes. Unfortunately, the process of selecting pathogenic genes is painful and time consuming; but, computational methods can solve this problem. Many previous studies adopted the comparison of genomics to identify differentially expressed genes (DEGs) to reveal the pathogenic processes of the disease through comparing the affected and control samples (8, 9). However, previous researches exhibited that many of the gene biomarkers detected from different studies on the same disease were frequently inconsistent (10, 11). Moreover, direct translation of these prognostic biomarkers into clinical decision making has not yet occurred. Thus, further understanding of HCC biology is needed to optimize prognostic accuracy

and improve clinical management.

Interestingly, epigenetics is emerging as an attractive candidate for the biological studying of cancers. DNA methylation is the main epigenetic feature of DNA that participates in gene transcriptional regulation, genome stability, cell differentiation, and a wide variety of malignancies (12, 13). Since DNA methylation is stable and easily detected, it is regarded as the most promising diagnostic biomarker for cancer (14), compared with copy number variations (15), or gene/microRNA expression (16). Thus, more and more scholars concentrated on the DNA methylation in this disease. For example, many differentially methylated genes were discovered such as *GABRA5* (17), *APC*, *CDKN2A*, and *AKR1B1* (18), which may act as the potential biomarkers to detect HCC. Despite the fact that some diagnostic panels are identified, there is no complete understanding of the methylome in HCC, and few studies comprehensively evaluated methylation signatures based on high-throughput platforms (19).

Hence, to better understand the etiology of HCC, it was planned to identify the differentially methylated genes between HCC and normal groups. Next, hierarchical clustering was conducted to measure whether such differentially methylated genes could distinguish the HCC from the normal samples. Furthermore, functional enrichment analyses were respectively implemented on up-methylated and down-methylated genes to further extract the potential biological processes, based on DAVID tool. It can help to elucidate the pathogenesis and identify molecular biomarkers for HCC diagnosis and treatment.

2. Methods

2.1. Collection of DNA Methylation Data

The current pure bioinformatics study based on the published microarray data of HCC was implemented in Linyi, Shandong Province, China in 2017. The HCC samples were downloaded from Gene Expression Omnibus and not made by the authors. There are a total of 4348 datasets in the Gene Expression Omnibus. In the current study, under the filter conditions of “Homo sapiens”, “transcription profiling by array”, “HCC” and the assays, samples were not small, DNA methylation dataset of HCC (accession number: GSE57956) (20) deposited in the GPL8490 platform of Illumina HumanMethylation450 BeadChip, were downloaded from the Gene Expression Omnibus (GEO) in National Center of Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/gds/>). In GSE57956, there were 120 samples including 59 HCC and 61 normal controls. Fifty-nine HCC and 61 adjacent non-tumorous liver tissues were requested from the National Cancer

Centre of Singapore (NCCS)/SingHealth Tissue Repository when the patients' written informed consent was obtained. The specific information about demographical variables and confounding factors are shown in the paper published by Mah et al. (17).

2.2. Data of Pretreating and Detecting Differentially Methylated Genes

The raw microarray data contained 27,578 CpG sites downloaded for further analysis. In order to make the data more accurate and understandable, it is imperative to remove the probes from the dataset when the probes met the following conditions: probes with the distance of single-nucleotide polymorphism (SNP) not greater than 2; probes with minor allele frequency (MAF) < 0.05; cross-hybridization probes; probes located in sex chromosome. After preprocessing, a total of 25,628 CpG sites were obtained for the analysis.

Lumi package (21) was used to pretreat the DNA methylation microarray data. Methylation at individual CpGs is reported as part of methylation changes (beta value), which is a quantitative measure of methylation for each CpGs site range 0 (no methylation) to 1 (complete methylation) (22). Thus, beta-mixture quantile normalization method was utilized to perform data normalization (23). In detail, firstly the beta values of HCC and normal samples were computed, respectively. Subsequently, the absolute value of difference of mean beta values between 2 groups were determined, called D. At the same time, t test was used to extract differentially methylated CpGs between the 2 groups. Differentially methylated CpGs were selected when the criteria was set at $P < 0.05$, and $D > 0.5$.

After the initial processing, further filtering analyses were conducted to promote a more stringent analysis, reduce the number of non-variable sites, and increase the statistical power of following analyses. Specifically, the sites with beta values no more than 0.2, and not less than 0.8 in all samples were deleted, and then, only the CpGs with the absolute β -values the difference between the HCC and normal groups ≥ 0.2 were retained. Finally, a cutoff threshold of ≥ 0.2 average beta-values difference was applied to identify CpGs with considerable methylation differences. The criterion applied in the current study was used by others previously (24-26).

2.3. Clustering Analysis of Differential Methylated Genes

Hierarchical clustering is a common method to determine clusters of similar data in multidimensional spaces (27). The heat map is an effective tool for data visualization, which represents a dataset with 2 dimensions and requires large display spaces when an input dataset contains a large

amount of data or time steps (28, 29). In general, cancers with similar methylation profiles were clustered together. To verify the reliability of the differentially methylated genes and whether such CpGs could distinguish the HCC from the normal groups, hierarchical clustering was performed. The matrix of average beta-value levels of differentially methylated CpGs was generated between HCC and normal samples.

2.4. GO Analysis of Differentially Methylated Genes

As reported, GO analysis is broadly used as functional enrichment researches for large-scale genes. Database for Annotation, Visualization, and Integrated Discovery (DAVID) is a soft tool that can provide the researchers a comprehensive set of functional annotation to reveal the biological meaning of a large number of genes (30). In the current study, in order to find out enrichment function classification, GO enrichment analyses were performed for up- and down-methylated genes, respectively. Concretely, the Fisher exact test was firstly employed to classify the GO terms. Afterwards, Benjamini-Hochberg procedure was used to correct P values into false discovery rate (FDR) (31). Significant GO terms were identified when the threshold was set as $FDR < 0.01$.

2.5. Pathway Analysis of Differentially Methylated Genes

Pathway analysis is the 1st choice to gain insight into the underlying biology of differential expression genes and proteins, as it reduces complexity and increases explanatory power (32). In the current paper, the employed pathway database was the KEGG (<http://www.genome.jp/kegg/>) that is a database of biological systems and provides a knowledge based reference to link genomes to life through the process of pathway mapping, which is to map a genomic or transcriptomic content of genes to KEGG reference pathways to infer systemic behaviors of the cell or the organism (33). In the current study, the significant pathways enriched by the differentially methylated genes were identified based on KEGG database and DAVID tool utilizing the Fisher test, and the cutoff criteria of significance was determined by FDR. Herein, FDR was set to 0.01. Similar to the GO analysis, the pathways were performed for up- and down-methylated genes.

3. Results

3.1. Differentially Methylated Genes

After quality control and normalization to remove 4 types of probes, overall 25,628 methylated sites were reserved from the chip dataset of 120 samples. A volcano

plot showing the distribution of the 25,628 methylated CpGs was constructed, as described in Figure 1. After a preliminary screening, there were 7507 differentially methylated CpGs (comprising 5202 genes), including 4615 up-methylated and 2892 down-methylated sites. The volcano plot in Figure 1 helped to observe the distribution of the up-methylated and down-methylated genes.

Subsequently, those 7507 methylated CpGs initially identified as differentially methylated sites were subjected to further filtering. Based on the cut off threshold of ≥ 0.2 mean beta-values difference, a total of 1660 unique CpGs (involved in 1340 genes) were extracted to be differentially methylated between the 2 groups. Among these 1340 genes, 978 were differentially up-methylated and 362 were differentially down-methylated.

3.2. Hierarchical Clustering Analysis

In the current paper, hierarchical clustering analysis was applied to verify if the differentially methylated genes could distinguish the HCC samples from the controls. The heat map of the 1340 differentially methylated genes is exhibited in Figure 2; accordingly, the up-methylated and down-methylated distribution of the differentially methylated regions is shown in 120 samples. As expected, through the heat map, it was pretty clear that the HCC samples were separated from the normal controls.

3.3. GO Analysis

Overall, 25 GO functions were significantly enriched by differentially up-methylated genes, as shown in Table 1. These GO terms were ranked in ascending order on the basis of FDR values, and the top 3 significant functions were the innate immune response ($FDR = 5.05E - 06$), cell-cell signaling ($FDR = 6.45E - 06$), and neutrophil chemotaxis ($FDR = 4.15E - 05$). For down-methylated genes, GO functions mainly included transcription from RNA polymerase II promotor ($FDR = 2.19E - 06$), energy reserve metabolic process ($FDR = 2.82E - 06$), etc., as described in Table 2.

3.4. Pathway Analysis

Based on $FDR < 0.01$, a total of 11 significant pathways were enriched with up-methylated genes including pancreatic secretion ($FDR = 2.45E - 07$), fat digestion, and adsorption ($FDR = 9.42E - 06$), linoleic acid metabolism ($FDR = 7.01E - 05$), neuroactive ligand-receptor interaction ($FDR = 2.35E - 04$), etc. Specific information is shown in Table 3.

It is noteworthy that down-methylated genes were significantly involved in 8 pathways presented in Table 4. Among these 8 pathways, the top 3 were neuroactive ligand-receptor interaction ($FDR = 1.98E - 05$), pancreatic secretion ($FDR = 3.85E - 04$), and pathways in cancer ($FDR = 5.17E - 04$).

Table 1. List of Significant GO Terms Enriched by the Up-Methylated Genes

GO Terms	FDR-Value
Innate immune response	5.05E-06
Cell-cell signaling	6.45E-06
Neutrophil chemotaxis	4.15E-05
Humoral immune response	5.87E-05
Cell adhesion	1.11E-04
Intermediate filament cytoskeleton organization	1.86E-04
Arachidonic acid secretion	2.43E-04
Negative regulation of endopeptidase activity	2.44E-04
Lipid catabolic process	4.24E-04
Phototransduction	4.85E-04
Regulation of immune response	5.60E-04
Visual perception	8.38E-04
Regulation of heart contraction	1.06E-03
Cytoskeleton organization	1.49E-03
Defense response to bacterium	1.51E-03
Cardiac muscle contraction	1.59E-03
Inflammatory response	1.79E-03
Adaptive immune response	1.83E-03
Phosphatidylethanolamine acyl-chain remodeling	1.97E-03
Calcium ion transmembrane transport	2.22E-03
Positive regulation of cytokine secretion	2.39E-03
Potassium ion transmembrane transport	2.55E-03
Cellular defense response	2.58E-03
Intermediate filament organization	2.79E-03
Phosphatidylinositol acyl-chain remodeling	2.79E-03

4. Discussion

HCC, as one of the leading causes of cancer-related death, is the main event leading to death in patients with cirrhosis (34). Due to the high mortality and the high recurrence rate, the prognosis of patients with HCC is still frustrating (35). In addition, the molecular pathogenesis of HCC studied by different scholars was equivocal and inconsistent. Significantly, analyzing DNA methylation data were widely used to explore the abnormally methylated genes associated with HCC and enabled the identification of targets for therapeutic strategies. Therefore, to elucidate the pathogenesis and identify molecular biomarkers for HCC, bioinformatics analysis was conducted based on methylated data. Specifically, differential methylation genes were identified, and cluster analysis was, then, implemented. Finally, GO and pathway analyses for differ-

Table 2. List of Significant GO Terms Involved in the Down-Methylated Genes

GO Terms	FDR-Value
Transcription from RNA polymerase II promoter	2.19E-06
Energy reserve metabolic process	2.82E-06
Cell fate commitment	5.95E-05
Palate development	8.76E-05
Adenylate cyclase-activating adrenergic receptor signaling pathway	1.96E-04
Cellular response to BMP stimulus	2.28E-04
Nephric duct morphogenesis	2.57E-04
Embryonic forelimb morphogenesis	3.31E-04
Aortic valve morphogenesis	5.02E-04
Anterior/posterior pattern specification	6.44E-04
Negative regulation of transcription from RNA polymerase II promoter	1.05E-03
Negative regulation of cell proliferation	1.99E-03
Negative regulation of neuron apoptotic process	2.11E-03
Dorsal/ventral pattern formation	2.46E-03
Positive regulation of transcription, DNA-templated	3.69E-03
Cyclic nucleotide biosynthetic process	4.83E-03
Synaptic transmission, dopaminergic	6.14E-03
Post-embryonic development	6.22E-03

Table 3. List of the Significant Pathways Enriched With the Up-methylated Genes, According to FDR < 0.01

Pathways	FDR Value
Pancreatic secretion	2.45E-07
Fat digestion and absorption	9.42E-06
Linoleic acid metabolism	7.01E-05
Neuroactive ligand-receptor interaction significant pathway	2.35E-04
Alpha-Linolenic acid metabolism	2.43E-04
Arachidonic acid metabolism	4.27E-04
Ether lipid metabolism	1.23E-03
Cytokine-cytokine receptor interaction	2.20E-03
Glutamatergic synapse	3.29E-03
Amoebiasis	5.86E-03
Adrenergic signaling in cardiomyocytes	7.98E-03

ential methylation genes were performed. As expected, through the heat map, it was quite clear that the disease samples were separated from the controls. This fully demonstrated that these differentially methylated genes

Table 4. Significant Pathways Enriched by the Down-methylated Genes, Based on FDR < 0.01

Pathways	FDR Value
Neuroactive ligand-receptor interaction	1.98E-05
Pancreatic secretion	3.85E-04
Pathways in cancer	5.17E-04
Alcoholism	7.06E-04
Dopaminergic synapse	4.43E-03
Hippo signaling pathway	4.92E-03
Gastric acid secretion	5.52E-03
cAMP signaling pathway	5.61E-03

Figure 1. Volcano Plot of the Methylation Data of 120 Samples Including 59 Hepatocellular Carcinoma and 61 Normal Controls

This figure shows mean methylation differences between HCC and normal (x axis) versus log-transformed p-values (y axis). The blue points demonstrated the differentially methylated genes.

could serve as a distinguishing label, which could be applied to diagnosis biomarkers.

After conducting GO analysis for up-methylated genes, several GO terms considered as the highly related to HCC were screened out. Among these, innate immune response was the most significant GO term. In detail, immune response refers to the defense responses when human are faced with the variation of themselves or xenogenous components. In the midst of this, the innate immune response is the 1st line of defense against infectious diseases, and the principal challenge for the host is to detect the pathogen

and mount a rapid defensive response (36). Up to now, many scholars studying HCC proved that when human is infected with the hepatitis B virus (HBV), the immune response is triggered starting at the antiviral T-cells quickly (37). In addition, other evidence accumulated that precise evaluation of local immune responses could be useful to predict prognosis for HCC (38). Moreover, a former study exhibited that the prognosis of HCC was predicted based on the unique immune response signature (39). Accordingly, the researches on the immune response in HCC collectively proved that it may act as a good revelation of the development of this disease.

For down-methylated genes, the most important GO term was transcription from RNA polymerase II promoter. In detail, the RNA polymerase II promoter is a key component in the regulation of gene expression (40). A previous study demonstrated that the strongest promoter precedes the surface antigen gene composed of the mature HBV genome coding sequence (41). Notably, the HBV is the major etiology of HCC (42). In addition, a large noncoding RNA is implemented as a marker for murine HCC, which is usually produced by RNA polymerase II (43). Therefore, it was inferred that the GO term of RNA polymerase II promoter plays an important role in the development of HCC.

According to pathway analysis, neuroactive ligand-receptor interaction was the most abnormal pathway for the down-methylated genes in the current study. As reported, neuroactive steroid could influence the modulation of GABA receptor, and GABA receptors are capable of controlling cell proliferation (44). Cell proliferation is the hallmark of cancer (45), and suppressing cell proliferation has anti-cancer effect on HCC (46). Therefore, the current study findings indicated that the pathways of neuroactive ligand-receptor interaction might be closely related to the initiation and progression of HCC.

5. Conclusion

Currently, many studies identified the CpG sites differentially methylated between the HCC and adjacent non-tumorous tissues (47). Nevertheless, the role of epigenetic changes such as aberrant DNA methylation in HCC remains largely unclear. Based on DNA methylation data, it was tried to extract the candidate gene and pathway biomarkers for HCC. It was the main strong point in the current study. However, several limitations should be considered in the current study analysis. Firstly, the data utilized in the current study were downloaded from the GEO database, not made by the authors. Secondly, the sample size was small. Moreover, the results were bioinformatics-based, but these findings were not proved using animal experiments, which was the main weak point. Thus, further

Figure 2. The Hierarchical Clustering Was Performed as the Heat Map, Which Showed the Differential CpGs Methylation Level Distribution of the High and Low Methylation in Samples

Column and row respectively represented the samples and the CpG sites, the color shows a quantitative measure of methylation for each CpGs site range 0 (no methylation) to 1 (complete methylation). In detail, green represents the low methylation and red represents the high methylation. Healthy controls and HCC samples are shown by blue and red bars, respectively.

experimental studies are still urgently needed. Although, there were some drawbacks, it was believed that the differential methylation genes and functions might provide a clue to understand the potential etiology of HCC. Additionally, this DND-methylation method might provide analysis on other corresponding studies. In conclusion, functional

terms (innate immune response, RNA polymerase II promoter, and neuroactive ligand-receptor interaction) might act as potential therapeutic targets for HCC.

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