Distinctive pattern of AHNAK methylation level in peripheral blood mononuclear cells and the association with HBV-related liver diseases

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Abstract
The purpose of this study was to investigate the correlation between AHNAK methylation level in peripheral blood mononuclear cells (PBMC) and the progression of hepatitis B virus (HBV)-related liver disease. Bioinformatics methods were applied to evaluate the AHNAK methylation level in PBMC and T cells at different stages of HBV related liver disease, to investigate the correlation between AHNAK methylation and clinical features, as well as to compare the methylation site of AHNAK in cancer tissues and adjacent tissues. Subsequently, the differentially expressed gene analysis technique was used to analyze the liver disease-related genes and immune-related pathways in hepatitis B patients with different pathological changes. Finally, promoter methylation and mRNA expression of AHNAK gene in liver cancer and adjacent tissues were determined by quantitative polymerase chain reaction (Q-PCR), and the diagnostic value of AHNAK methylation level in hepatopathy was evaluated by receiver operating characteristic (ROC) curve. The promoter methylation level of AHNAK gene in PBMCs decreased with the progression of HBV-related liver disease, and showed significant difference among the patients with various HBV-related liver diseases ($P = 0.0001$). The AHNAK methylation level in PBMCs and T cells was negatively associated with age, white blood cell count, CREA, drinking, and positively associated with APTT and HbsAg. Higher mRNA expression of AHNAK was found in liver cancer tissues than that of adjacent tissues ($P < 0.001$), and the methylation level in PBMC decreased with the progression of hepatitis B-related liver disease. The area under the ROC curve (ROC) was 0.883 ($P < 0.001$) in diagnosis of chronic hepatitis B (CHB), 0.885 ($P < 0.001$) in diagnosis of compensatory liver cirrhosis, 0.955 ($P < 0.001$) in diagnosis of decompensated liver cirrhosis, 0.981 ($P < 0.001$) in diagnosis of hepatocellular carcinoma. Our results revealed that AHNAK methylation level in peripheral blood decreases with the progression of hepatitis B-related liver disease. This provided a potential differential
1 | INTRODUCTION

As one of the commonest malignant tumors, liver cancer ranks the fifth in morbidity and the third in mortality, with about 700 000 deaths worldwide every year.\textsuperscript{1-3} It is reported that liver cancer is associated with a dismal prognosis in most patients, and its five-year survival rate is only about 10%.\textsuperscript{4} However, the five-year survival rate of patients with early liver cancer can be up to 50\%-75\%.\textsuperscript{5} Due to the lack of a singular effective clinical method for early diagnosis, patient with liver cancer is often diagnosed at an advanced stage and the best timing for curative treatment is missed. Therefore, it is of great significance to find effective biomarkers for early diagnosis and prognosis determination of liver cancer.\textsuperscript{6}

Epigenetic modification is an important mechanism for the occurrence and development of many metabolic diseases and cancers.\textsuperscript{7-10} DNA methylation is recognized as one of the first discovered epigenetic modifications, which can regulate gene expression by influencing chromatin structure, DNA conformation, DNA stability, and DNA-protein action mode.\textsuperscript{11} A growing number of studies have shown that DNA methylation is associated with occurrence and prognosis of cancer and might be used as a diagnostic and prognostic molecular marker.\textsuperscript{12,13} For instance, inhibition of HHIP promoter methylation could suppress human gastric cancer cell proliferation and migration\textsuperscript{10}; GSTM2 methylation level can be served as a potential biomarker for breast cancer development\textsuperscript{14}; hypermethylation of EphA5 promoter is an important marker for diagnosis and prognosis of pancreatic cancer.\textsuperscript{15} Our previous study found that the host immune system of liver cancer has unique DNA methylation characteristics, and significant changes of methylation in blood are expected to be noninvasive markers for early diagnosis of liver cancer.\textsuperscript{16}

Our previous study found that the host immune system of liver cancer has unique DNA methylation characteristics, and significant changes of methylation in blood are expected to be noninvasive markers for early diagnosis of liver cancer.\textsuperscript{16} However, there are few studies on the abnormal DNA methylation of single gene in liver cancer.\textsuperscript{17}

AHNAK is a desmosomal connexin, located in chromosome 11: 62433542-62556235, encoding 700 kDa protein, and is expressed in all kinds of cancer cells.\textsuperscript{18,19} It has been reported that low expression of AHNAK can effectively inhibit breast cancer cell invasion and proliferation as a tumor suppressor.\textsuperscript{20,21} In glioma, AHNAK can depress tumor development and serve as a biomarker of tumor prognosis.\textsuperscript{22} Moreover, low expression of AHNAK in melanoma indicates poor prognosis.\textsuperscript{23} Nevertheless, the role of AHNAK in the development and prognosis of liver cancer remains unclear.

Therefore, this study utilizes bioinformatics to analyze the sequencing data from previous studies to identify the relationship of AHNAK methylation with the progression and immune-related signaling pathways of HBV-related liver diseases. The study results provided evidence for establishment of potential early diagnostic and prognostic biomarkers for liver cancer.

2 | MATERIALS AND METHODS

2.1 | Tissue(Blood) samples

In total, 242 Hepatitis B patients who were admitted into Beijing You’an Hospital, Capital Medical University during 1 June 2013 to 30 December 2016 were recruited. The study sample included 34 chronic hepatitis B (CHB), 32 compensatory liver cirrhosis (CLC), 35 decompensated liver cirrhosis (DCLC), 141 hepatocellular carcinoma (HCC) (stage 0 = 32; stage A = 38; stage B = 14; stage C = 36; stage D = 21) patients. As control, eighteen healthy volunteers were involved in this study. The method of obtaining PBMC and T cells refers to our previous study, which has been published.\textsuperscript{16} All participants and their family signed informed consents, and the study was approved by the medical research ethics committee of Beijing You’an Hospital, Capital Medical University (EC-B-031-A02-V9.0).

2.2 | Data acquisition

Methylated pyrosequencing data were collected from previous studies.\textsuperscript{16} We obtained methylation data of hepatocellular carcinoma (HCC) from Cancer Genome Atlas (TCGA, https://gdc.cancer.gov/) platform. The gene expression dataset (GSE89377/E-MTA-950) was collected from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) database and array express (E-MTAB-950, https://www.ebi.ac.uk/arrayexpress) database. GEO dataset included 107 samples covering nine stages of HCC. We included 13 normal, 20 chronic Hepatitis (choronic), 12 cirrhosis (CS), five early hepatocellular carcinoma (eHCC) samples for analyses. E-MTAB-950 dataset included three chronic hepatitis with HBV (CHB) and 10 normal samples.
2.3 Gene expression and pathway analysis

The gene expression profile data were normalized by the Linear Models for Microarray Data (LIMMA, http://www.bioconductor.org/packages/release/bioc/html/lmma.html) package in R. Also we used LIMMA to identify DEGs by comparing expression value between cancer and normal group samples. A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed to evaluate the different expression of proteins on the regulation of TCR between different groups.

2.4 Network analysis

The Search Tool for the Retrieval of Interacting Genes (STRING), which provides information for experimental and predicted interactions, is an online database. STRING was applied to search and to determine an interaction network between AHNAK and TCR pathway.

2.5 DNA extraction, bisulfite modification, and methylation-specific PCR (MSP)

Genomic DNA was extracted from peripheral blood mononuclear cells which derived from patients and controls using the QiAamp DNA mini kit (Qiagen NV, Venlo, Netherlands) in line with the manufacturer’s protocol. The quality and quantity of the isolated DNA were measured by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). Bisulfite treatment of genomic DNA was processed using an EpiTect Fast DNA Bisulfite Kit (Qiagen NV). Bisulfite-treated DNA samples were kept at −20°C for further use. After analyzing 2000-bp region upstream of the transcription start site (AHNAK promoter region), one CpG island was identified (Figure 1A). With bisulfite-treated DNA as the template, the methylation pattern in the CpG island within AHNAK promoter was measured by MSP, and MSP primers were designed as follows: [for methylated DNA F 5′- GTGGAAATTTAGTTTTGTGC- 3′ and R 5′- TTACTTAATTCCAAAATCCGT- 3′ and for unmethylated DNA F 5′- GTGGAAATTTAAGTTAGTTTTGTGC- 3′ and R 5′- GTGGAAATTTAAGTTAGTTTTGTGC- 3′].

**Figure 1** Methylation level of AHNAK promoter in PBMC and T cells in different liver diseases. A, Human AHNAK promoter was mapped to a base 337-bp upstream of the gene, mainly containing one CpG island. B, distribution map of AHNAK promoter methylation level in PBMC and T cells at different disease stages; C, methylation level of AHNAK promoter in PBMC at different disease stages; D, methylation level of AHNAK promoter in T cells at different disease stages.
DNA F 5′- GGAAATTTAAGTTAGTTTTGTGTGG - 3′ and R 5′- CCTTACTTAATTCCCAAACTCCATA- 3′], according to the previously described principle. The reaction condition for MSP was 10 minutes at 95°C, followed by 40 cycles of 30 seconds at 94°C and 30 seconds at 72°C, and then 5-min extension at 72°C. After separated on 1.5% agarose gels, the MSP products were then stained by ethidium bromide and visualized under UV spectrophotometer. Water blanks were served as negative control.

2.6 | Sample preparation, RNA isolation, reverse transcription, and Q-PCR

Total RNA was isolated using Trizol reagent (Invitrogen Carlsbad, CA, USA). cDNA was obtained from 2 μg total RNA by reverse transcription in accordance with the kit (Takara Biotechnology Ltd., Dalian, China). The cDNA was stored at −20°C. Real-time quantitative PCR (RQ-PCR) was performed using a GoTaq 2-Step RT-qPCR System (Promega, Madison, WI, USA) on ABI PRISM 7900 system (Applied Biosystems, Grand Island, NY, USA). The abundances of AHNAK mRNA were estimated by housekeeping gene β-actin. Gene relative expression is analyzed by the 2^−∆∆Ct method. The experiments were performed in triplicate.

2.7 | Statistical analysis

Student’s t test and one-way analysis of variance (ANOVA) were used to compare the level of AHNAK methylation between two and more groups, respectively. Spearman’s correlation was used to assess the correlations between AHNAK methylation and continuous variables. Linear regression was utilized to evaluating the correlations between AHNAK methylation and discrete variables. Statistical significance was defined as a two-tailed P < 0.05 for all analyses. The ROC curve analysis was performed using SPSS19.0 (IBM Corporation, Chicago, IL, USA). Other analyses were conducted using R3.1.1 or GraphPad Prism version 6.0 (GraphPad software, San Diego California USA).

3 | RESULTS

3.1 | Promoter methylation level of AHNAK in PBMC was associated with the progression of HBV-related liver disease

In order to understand the relationship between AHNAK methylation level and the progression of HBV related liver disease, we analyzed the methylation level in PBMC and T cells.
cells in different course samples. The results demonstrated that AHNAK methylation level showed no certain regularity in different samples (Figure 1B). In PBMC, the methylation level decreased as the severity of the disease increased, but this change was not obvious in T cells (Figure 1C,D). The statistical analysis suggested that AHNAK methylation level in PBMC showed significant difference among different groups ($F$ value = 4.09, $P < 0.001$), while no significant difference was found in T cells ($F$ value = 0.197, $P > 0.05$) (Table 1). There was significant difference in AHNAK methylation level in PBMC between control (NC) and DCLC groups, NC and HCC (stage 0, A, B, C, D) groups, CHB and HCC (stage 0, A, B, C, D) groups, CLC and HCC (stage 0, A, B, C, D) groups, respectively (Table 2).

3.2 | AHNAK methylation level in PBMC and T cells was pertinent to age, WBC count, CREA, APTT, drinking, and HBsAg

To further explore the correlation between AHNAK methylation level in PBMC and T cells and clinicopathological features of liver disease, Spearman’s correlation and linear regression were applied. As shown in Tables S1 and S2, AHNAK methylation level in PBMC was correlated with age, WBC count, CREA, APTT, smoking, drinking, sex, and HBsAg; AHNAK methylation level in T cells was correlated with age, WBC count, CREA, APTT, drinking, and HBsAg.

**Figure 2** The heat map analysis for liver disease-related genes. A, comparisons of liver disease-related genes in eHCC, CS, Normal, and Chronic groups; B, comparisons of liver disease-related genes in CHB and Normal groups. Note: Red indicates high expression, and green indicates low expression; the darker the color, the higher the expression. All the comparisons are compared to the Normal group.
Promoter methylation level of AHNAK was decreased in liver cancer tissues

To verify the reliability of the previous analysis, methylation data of hepatocellular carcinoma (HCC) collected from the cancer genome atlas (TCGA) database were processed by t test or Wilcoxon rank-sum test. The findings showed that liver tissues displayed lower methylation level of AHNAK promoter than adjacent tissues (Tables S3 and S4).

| TABLE 3 | Differential gene expression in different groups of immune-related signaling pathways |
|---------|-----------------------------------------------------------------------------------|
| **Group** | **High expression** | **Low expression** |
| eHCC vs normal | Rho/Cdc42; PLC-γ1; Ras; PI3K; IKKγ | CD45; CD4/8; FγN; CD3σ; PAK; GRB2; AP1; COT; IκB |
| Chronic vs normal | CTLA4; FγN; Rho/Cdc42; NFAT; PI3K; MKK7 | CBL; PAK; ZAP70; ErK; AP1; BCL-10; PDK1; GSK-3β; IκB |
| CS vs normal | CTLA4; SHP1; CD38/CD3e/CD3γ/NCK; p38; ITK; ICOS; PI3K | CBL; PAK; LAT; GRB2; SOS; BCL-10; AP1; IκB; NIK; IKKα |
| CHB vs normal | GRB2; Rho/Cdc42; NFAT; PDK1; Raf | CTLA4; CD45; CD4/8; LCK; FγN; CD36; SLP-76; NCK; PLC-γ1; Ras; GRP1; Ras; p38; MEK1/2; PI3K; AKT; PKCB; MALT1; ICOS |
3.4 | Changes of related genes and signaling pathways in hepatitis B patients with different pathological changes

Subsequently, analysis for related genes and signaling pathways was carried out. As shown in Figure 2, MAP2K7 and MAPK14 were highly expressed in chronic hepatitis; LAT, SOS2, and BCL-10 were highly expressed in normal tissues; PTPN6, LCK, and CTLA4 were highly expressed in CS. As shown in Figure 3, higher expressions of RHOA, MAP2K1, NFKBIA, and FOS were found in normal and HBV tissues. Compared with the normal group, there were higher expressions of Rho/Cdc42, PLC-γ1, Ras, and PI3K, while lower expressions of CD45, CD4/8, FγN, PAK, GRB2, and AP1 in the CHB group (Figure 3B, Table 3); higher expressions of Rho/Cdc42 and PI3K, while lower expressions of AP1, BCL-10, and PDK1 in the Chronic group (Figure 3C, Table 3); higher expressions of CTLA4, SHP1, p38, and PI3K, while lower expressions of CBL, PAK, LAT, GRB2, and SOS in the CS group (Figure 3D, Table 3).

3.5 | AHNAK mRNA expression was elevated in liver cancer tissues and AHNAK methylation level in PBMC decreased with increasing severity of disease

Next, an investigation of AHNAK mRNA expression was conducted in 60 cases of liver cancer tissues and adjacent tissues, and the methylation level of AHNAK promoter in PBMC was determined in 260 cases with different liver diseases. The results revealed that liver cancer tissues had significantly higher AHNAK mRNA expression than the adjacent tissues ($P < 0.001$, Figure 4A). There were 8 cases of methylation in NC group (44.44%), 13 cases in CHB group (38.23%), 11 cases in CLC group (34.38%), 11 cases in DCLC group (31.43%), and 141 cases in HCC group (27.66%).
(38.23%), 11 in CLC group (34.38%), 11 cases in DCLC group (31.43%), 141 cases in HCC group ($P < 27.66$%). It shows that the methylation level of AHNAK decreases with the increase of disease severity (Figure 1B,C). Although the diagnosis and treatment of liver cancer has been better developed, such as surgical resection and liver transplantation, the survival rate is still disappointing.4,31 On cellular molecular level, both down-regulated expression of mir-33a and up-regulated expression of TRIM14 suggest poor prognosis in patients with liver cancer.37,38 In the current study, significantly decreased promoter methylation level and increased mRNA expression of AHNAK were shown in liver cancer tissues (all $P < 0.05$), indicating that AHNAK methylation level can be served as a marker for the diagnosis of liver cancer.

Collectively, highly expression of AHNAK is associated with the occurrence of liver cancer, which is helpful to the early detection and diagnosis. However, the function and mechanism of AHNAK in liver cancer require further study.

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### CONFLICTS OF INTEREST

The authors have declared that they have no conflict of interest.

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SUPPORTING INFORMATION
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