The association between methylation patterns of DNAH17 and clinicopathological factors in hepatocellular carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is a malignancy with poor prognosis. Complex genetic and epigenetic alterations are the two primary causes of HCC. The aim of the study was mainly to explore the correlation between the methylation status of DNAH17 and HCC.

Methods: We evaluated the methylation levels of DNAH17 in 163 HCC samples and their paired normal tissue using Sequenom EpiTYPER assays and performed the TaqMan copy number assay to assess the copy number status of DNAH17 in HCC samples.

Results: The mean methylation levels were significantly decreased in the tumor tissues compared to the paired normal tissues in both selected regions of DNAH17 (amplicon 1: 58.7% vs 84.5%, P < 0.0001; amplicon 2: 69.9% vs 84.5%, P = 0.0060). Contrarily, both RNA-seq and immunohistochemistry indicated the expression of DNAH17 was increased in tumor tissues (P < 0.05). DNMT inhibitor decitabine treatment could increase the expression of DNAH17 in HCC cell lines. DNAH17 gene amplification always companied with hypomethylation status. Moreover, hypomethylation status was associated with several clinical characteristics, such as male patients, higher AFP values, higher age of onset, fibrous capsules, tumor necrosis, liver cirrhosis, and tumor thrombus (P < 0.05). Receiver operator characteristic (ROC) curve analysis demonstrated the methylation levels of DNAH17 could efficiently predict the existence of the fibrous capsule (AUC = 0.695) and tumor thrombus (AUC = 0.806).

Conclusions: These findings suggested that aberrant methylation of DNAH17 was associated with comprehensive HCC clinicopathological factors and could be a promising biomarker for tumor thrombosis in HCC patients.
1 | INTRODUCTION

Hepatocellular carcinoma (HCC), accounting for 70%-90% of all primary liver cancers, is one of the leading causes of cancer death, especially in less-developed countries. Although many new therapeutic methods have been introduced, and surgical techniques are being developed rapidly, the outcomes of HCC treatment have not improved notably. Few patients have an opportunity to receive curative treatment, either primary resection or liver transplantation, because many patients were initially diagnosed at an advanced stage. Tumor thrombosis is one of the common features of the advanced stage of HCC, and it indicates a poor prognosis. Therefore, it is crucial to explore underlying mechanisms of HCC initiation and progression and find more accurate biomarkers, for example, for tumor thrombosis, to help clinicians draw a precise therapeutic strategy.

Currently, aberrant methylation of tumor oncogenes and tumor suppressor genes has been noted in many cancers, leading to expression changes of cancer-related genes that promote tumorigenesis and progression. Solid evidence revealed the significance of methylation deregulation in HCC.

Dynein, which is composed of several light, heavy, and intermediate chains, is an essential protein for the primary cilia. Davey et al. reported that the primary cilia were widely observed in the developing liver. Unlike the motile cilia, the primary cilia are microtubule-based organelles located at the cytomembrane that act as sensors to receive physical and chemical signals. In the past ten years, this largely ignored organelle has been brought to the forefront of cancer research. Loss of cilia was observed in various cancer cells and gradually regarded as a common hallmark of the disease. Restoring the expression of primary cilia appears to be a promising therapeutic method for cancer treatment. However, the detailed mechanism of primary cilia on oncogenesis and tumor progression remains poorly understood. Dynein axonemal heavy chain 1 (DNAH17), which was initially mapped in 1998, is a gene encoding a heavy chain associated with axonemal dynein. Recently, whole-exon sequencing of five paired hepatitis B virus-associated early-stage HCC samples found that DNAH17 was frequently mutated in HCC patients. The data from the Cancer Genome Atlas (TCGA) dataset confirmed the conclusion that genetic alteration of DNAH17 was common. A total of 186 (51%) of 366 sequenced HCC patients had genetic alteration in dynein axonemal heavy chain genes, and DNAH17 was found to be altered in ten percent of all sequenced patients (Figure 1A); it is one of the top two most frequently altered genes among all DNAH genes. This interesting finding encouraged us to explore the potential relationship between DNAH17 and hepatocellular carcinoma.

In this study, we conducted Sequenom EpiTYPER assays to investigate the potential mechanism and the correlation between methylation of DNAH17 and clinicopathological features. We found that overexpression of DNAH17 by down-regulation of methylation levels might contribute to HCC initiation and progression. In addition, the hypomethylation status of the DNAH17 gene, both in tumor tissue and adjacent non-cancerous tissue, could be a promising biomarker for tumor thrombosis in HCC.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

Hepatocellular carcinoma tissues and paired adjacent non-cancerous tissues were collected from 163 patients receiving curative resection at the Shanghai Eastern Hepatobiliary Surgery Hospital from January 2012 to December 2012. All samples were immediately frozen in liquid nitrogen and stored at −80°C until DNA was extracted. According to the TNM staging system of the American Joint Committee on Cancer (AJCC 7th edition), all the patients were diagnosed as HCC, and the pathological features were assessed by two pathologists.

A total of 138 male patients and 25 female patients were included in our study. The average age was 51.6 ± 10.3 years old (Mean ± Standard Deviation). Patients’ personal information, family history, clinical testing results, pathological information, and other data were collected in accordance with hospital privacy rules. This study protocol was approved by the Clinical Research Ethics Boards of the Shanghai Eastern Hepatobiliary Surgery Hospital, and informed consents were obtained from all patients.

2.2 | Cell culture and decitabine treatment

Two HCC cell lines, Huh7 and PLC/PRF/5, were selected for decitabine treatment. The cell lines were kindly provided by Cang’s lab (Zhejiang University, Hangzhou, China). They purchased the cell lines from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) (Huh7 Cat# 12800017, PLC/PRF/5 Cat# 41500034). Cell lines were cultured in DMEM (Gibco,
Cat# C11995500BT) with 10% fetal bovine serum (FBS) (Cellmax, Cat# SA102.02) and maintained at 37°C in 5% CO2. Decitabine, a DNMT inhibitor, was purchased from Targetmol (Cat# T1508). For decitabine treatment, cells were pre-cultured to 10%-20% confluence and then cultured with medium containing decitabine for 72 hours at different doses. Two concentrations of decitabine, 5 and 10 μmol/L, were used for treatment. The decitabine was diluted in dimethylsulfoxide (DMSO) (Sigma, Cat# C11995500BT) with 10% fetal bovine serum (Meilunbio, Cat# MB4508). For 5 minutes, goat serum (Meilunbio, Cat# MB4508) was used to block the non-specific binding for 30 minutes. For DNAH17 expression analysis, the sample slides were immunostained with the anti-DNAH17 antibody (R&D System, Cat# MAB9657-SP) overnight at 4°C in 1:200 dilution and then stained with hematoxylin (Meilunbio, MB9897) for 2 minutes. The GTVision III detection system (Gene Tech, Shanghai, China, Cat# GKY00710) was used for detecting the expression of DNAH17, and we assessed the immunohistochemical scores using the upright microscope (Nikon eclipse 80i). The immunostaining was scored according to the German immunoreacted score and evaluated by two pathologists. Intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Scores representing percentage of tumor cells positively stained were 0 (<5%), 1 (5%-25%), 2 (25%-50%), 3 (50%-75%), or 4 (>75%).

2.3 | RNA extraction and real-time quantification PCR
Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the specification, and 1 μg RNA was then reversely transcribed to cDNA with Hifair™ 1st Strand cDNA Synthesis Kit (Yeasen, Shanghai, China, Cat# 11123ES10). Quantitative real-time PCR was performed with Hifair™ qPCR SYBR Green Master Mix (Yeasen, Cat# 11201ES08). qRT-PCR was performed by a 7500 RT-PCR system (Thermo Fisher Scientific, Waltham, MA, USA), and the annealing temperature was 55°C. GAPDH served as a normalizing control. The qPCR primers for DNAH17, forward primer, 5′-TTATACACCAAGTCATCGAAGG-3′ and reverse primer, 5′-AGTCGGCTTGGTCCATCTCTCT-3′; for GAPDH, forward primer, 5′-GTAAGAGCAAGGCTGCGA-3′ and reverse primer, 5′-AGCCCCAGCGTCAAAAAG-3′. The range of the obtained Ct values was 15-30. Each sample was tested in triplicate. The 2−ΔΔCT as a calculation method was performed to analyze the expression of DNAH17 gene in the selected cell lines after decitabine treatment.

2.4 | Immunohistochemistry (IHC)
The 20 pairs of HCC samples and their paired normal liver tissues were selected for immunohistochemistry. The paraffin-embedded tissue blocks were obtained from the department of pathology and sectioned into 3-μm-thick slides. Then, the slides were deparaffinized and rehydrated using xylene (Yonghua Chemical Technology, Jiangsu, China, Cat# 155502104) and ethanol (Yonghua Chemical Technology, Jiangsu, China, Cat# 117902104). The slides were further immersed in 10 mmol/L citrate buffer (pH 6.0, Meilunbio Cat# MA0180) and boiled for 10 minutes in an autoclave. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide (Yonghua Chemical Technology, Cat# 210402104) for 5 minutes. 5% normal goat serum (Meilunbio, Cat# MB4508) was used to block the non-specific binding for 30 minutes. For DNAH17 expression analysis, the sample slides were immunostained with the anti-DNAH17 antibody (R&D System, Cat# MAB9657-SP) overnight at 4°C in 1:200 dilution and then stained with hematoxylin (Meilunbio, MB9897) for 2 minutes. The GTVision III detection system (Gene Tech, Shanghai, China, Cat# GKY00710) was used for detecting the expression of DNAH17, and we assessed the immunohistochemical scores using the upright microscope (Nikon eclipse 80i). The immunostaining was scored according to the German immunoreacted score and evaluated by two pathologists.18 Intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Scores representing percentage of tumor cells positively stained were 0 (<5%), 1 (5%-25%), 2 (25%-50%), 3 (50%-75%), or 4 (>75%).

2.5 | DNA preparation and bisulfite conversion
Following the manufacturer’s instructions, genomic DNA was extracted from HCC tissues and matched normal tissues using the QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany). The concentrations of extracted DNA were measured by a NanoDrop 2000 (Thermo, Wilmington, USA). Genomic DNA (500 ng-1 μg) was converted by sodium bisulfite according to the manufacturer’s protocol of the Epitext Fast DNA Bisulfite Kit (QIAGEN).

2.6 | Gene bioinformatics and primer design
We obtained the information of DNAH17 from the UCSC genome database (http://genome.ucsc.edu/). To identify the possible CpG sites, the CpG Island Finder of DBCAT software (http://dbscat.cgm.ntu.edu.tw/) was used to scan the sequences from chr17:76414778 to chr17:76573476. The primers used for quantitative methylation analysis of this gene were as follows: amplicon 1, forward primer aggaagaga-gGGTTTTTTTTGAGTTTTTGTATTTT and reverse primer cagtaatacgactcactatagggagaaggctATTTATAACAAC-CTACAATTTCCCA; amplicon 2, forward primer ag-gaaagagGGTTATTATAGGTGGTAGGGAGTGG and reverse primer 5′-AGCCCCAGCGTCAAAAAG-3′. The range of the obtained Ct values was 15-30. Each sample was tested in triplicate. The 2−ΔΔCT as a calculation method was performed to analyze the expression of DNAH17 gene in the selected cell lines after decitabine treatment.
2.7 Mass array quantitative methylation analysis

We used the Sequenom EpiTYPER assay to detect the methylation levels of the converted genomic DNA. This process includes three main steps: PCR amplification, SAP cleanup, and T cleavage. To verify the efficiency of PCR amplification, gel electrophoresis was performed after SAP cleanup. T cleavage was processed after confirming high efficiency of amplification. The products were then transferred to a SpectroCHIP® array and analyzed on the MassARRAY® Analyzer 4 instrument. We randomly picked 12 pairs of HCC and adjacent liver tissue samples for repeated experiments to verify the consistency of the experiment. The results of 24 DNA samples yielded a highly consistent result ($R^2 = 0.95$).

2.8 Gene copy number assay

$DNAH17$ gene (Applied Biosystems, Cat #4400326) was used as a standard reference gene. The copy number of target gene was analyzed and calculated by CopyCaller™ Software 2.1 (Applied Biosystems, Waltham, MA, USA). Predicted Ct values were used for the further analysis.

2.9 Statistical analysis

SPSS 20.0 version was used to perform the statistical analysis (IBM, Armonk, NY, USA). Given the abnormal distribution of the methylation levels in each CpG sites and the expression data, nonparametric Wilcoxon signed rank test was conducted to compare the methylation and expression differences and between HCC tissues and matched normal tissues. Paired two-tailed Student’s $t$ test was conducted to compare the IHC score differences between HCC tissues and matched normal tissues. Also, paired two-tailed Student’s $t$ test was used in decitabine treatment assay. Correlation between methylation-level differences and clinicopathological characteristics were evaluated by linear regression analysis, adjusted with age and gender. Correlations between the methylation levels of each CpG sites were assessed by Pearson correlation analysis. Linear regression analysis was
**TABLE 1** Association between methylation levels (%) of *DNAH17* gene and clinicopathological parameters

| Parameters          | Number | Mean methylation level of amplicon 1 (%) | Mean methylation level of amplicon 2 (%) |
|---------------------|--------|---------------------------------------|---------------------------------------|
|                     | Normal | Tumor | P          | Normal | Tumor | P          |
| Gender              |        |       |            |        |       |            |
| Female              | 25     | 83.8  | 71.9       | 0.0076** | 86.0  | 79.4       |
| Male                | 139    | 84.8  | 55.9       | 0.0120*  | 83.8  | 68.2       |
| Age                 |        |       |            |        |       |            |
| ≤55                 | 105    | 84.2  | 62.4       | 0.0406*  | 84.3  | 71.6       |
| >55                 | 58     | 85.2  | 52.7       | 0.1632   | 83.8  | 67         |
| Diabetes            |        |       |            |        |       |            |
| Yes                 | 14     | 87.9  | 58.1       | 0.3685   | 82.1  | 66.3       |
| No                  | 144    | 84.3  | 62.3       | 0.6084   | 84.5  | 70.5       |
| AFP                 |        |       |            |        |       |            |
| <20                 | 70     | 84.2  | 49.9       | 0.0010** | 84.4  | 65.8       |
| ≥20                 | 93     | 84.8  | 65.5       | 0.0334*  | 84.0  | 73.1       |
| CEA                 |        |       |            |        |       |            |
| ≤5                  | 146    | 84.2  | 59.4       | 0.7844   | 84.1  | 69.4       |
| >5                  | 17     | 87.4  | 52.6       | 0.1173   | 84.5  | 74.4       |
| HBV                 |        |       |            |        |       |            |
| Yes                 | 137    | 84.6  | 57.9       | 0.2032   | 84.1  | 69.5       |
| No                  | 25     | 85.9  | 61.7       | 0.0769   | 84.5  | 71.5       |
| Alcohol habit       |        |       |            |        |       |            |
| Yes                 | 34     | 85.4  | 55.6       | 0.8151   | 85.6  | 69         |
| No                  | 129    | 82.8  | 60.1       | 0.8239   | 83.6  | 70.3       |
| Tumor number        |        |       |            |        |       |            |
| 1                   | 145    | 84.3  | 59.8       | 0.0862   | 84.3  | 70.4       |
| 2                   | 12     | 88.2  | 43.5       | 0.4471   | 82.1  | 64.8       |
| 3                   | 5      | 83.8  | 43.2       | 0.2284   | 86.3  | 64         |
| Liver cirrhosis degree | 102  | 84.2  | 60.7       | 0.0648   | 85.2  | 73.1       |
| No                  | 30     | 86.3  | 46.9       | 0.0185*  | 84.6  | 62.5       |
| Mild                | 24     | 84.2  | 61.8       | 0.8396   | 81.9  | 66.7       |
| Moderate            | 2      | 80.6  | 81.1       | 0.316    | 55.0  | 52.0       |
| Liver cirrhosis degree | 2    | 84.5  | 53.6       | 0.0008** | 85.1  | 68.6       |
| No                  | 56     | 84.8  | 70.7       | 0.3316   | 82.4  | 72.4       |
| Tumor necrosis      |        |       |            |        |       |            |
| Yes                 | 124    | 83.8  | 56.1       | 0.0370*  | 83.7  | 69.3       |
| No                  | 39     | 87.3  | 67.0       | 0.6477   | 85.7  | 71.7       |
| Satellite tumor     |        |       |            |        |       |            |
| Yes                 | 44     | 84.5  | 65.8       | 0.1202   | 82.0  | 73.5       |
| No                  | 117    | 84.6  | 56.5       | 0.2284   | 85.2  | 68.6       |
| Microvascular invasion | 57   | 83.1  | 60.7       | 0.6549   | 81.7  | 70.0       |
| No                  | 106    | 85.3  | 58.0       | 0.8396   | 85.6  | 69.8       |

(Continues)
used to explore the correlation between copy number status and methylation levels, and the methylation differences among different CNV groups were calculated by nonparametric Mann-Whitney test. We also conducted ROC curve to assess the methylation level of DNAH17 as a predictive biomarker, and the discriminatory capacity was evaluated by calculating the area under curve (AUC). In general, a useless test has an AUC of 0.5, while an ideal test (one that has zero

| Parameters       | Number | Mean methylation level of amplicon 1 (%) | Mean methylation level of amplicon 2 (%) |
|------------------|--------|-----------------------------------------|-----------------------------------------|
|                  |        | Normal | Tumor | P     | Normal | Tumor | P     |
| Tumor thrombus   |        |        |       |       |        |       |       |
| Yes              | 21     | 81.9   | 53.3  | 0.4415| 77.5   | 58.5  | 0.0063**|
| No               | 142    | 84.8   | 59.0  |        | 85.4   | 71.5  |        |
| TNM Stage        |        |        |       |       |        |       |       |
| I–II             | 17     | 84.0   | 59.1  | 0.2744| 86.5   | 69.5  | 0.7876|
| III–IV           | 146    | 84.7   | 58.7  |        | 83.9   | 69.9  |        |

*P < 0.05;
**P < 0.01.

### TABLE 2
Methylation status (%) of DNAH17 in amplicons in HCC patients

| CpGs       | Group | Mean (%) | △Mean (%) | P value | CpGs       | Group | Mean (%) | △Mean (%) | P value |
|------------|-------|----------|-----------|---------|------------|-------|----------|-----------|---------|
| CpG_3.4.5.6| Normal | 93.9     | 24.5      | <0.0001 | CpG_1      | Normal | 46.5     | −4.0      | 0.0925  |
|            | Tumor  | 69.4     |           |         |            | Tumor  | 50.5     |           |         |
| CpG_8.9    | Normal | 94.9     | 27.2      | <0.0001 | CpG_2      | Normal | 87.8     | 14.3      | <0.0001 |
|            | Tumor  | 67.7     |           |         |            | Tumor  | 73.4     |           |         |
| CpG_10.11  | Normal | 90.5     | 35.4      | <0.0001 | CpG_3.4    | Normal | 94.6     | 23.3      | <0.0001 |
|            | Tumor  | 55.1     |           |         |            | Tumor  | 71.3     |           |         |
| CpG_12     | Normal | 67.5     | 27.2      | <0.0001 | CpG_5      | Normal | 79.7     | 21.4      | <0.0001 |
|            | Tumor  | 40.2     |           |         |            | Tumor  | 58.3     |           |         |
| CpG_13     | Normal | 83.2     | 28.7      | <0.0001 | CpG_7      | Normal | 92.7     | 18.6      | <0.0001 |
|            | Tumor  | 54.5     |           |         |            | Tumor  | 74.0     |           |         |
| CpG_14     | Normal | 82.4     | 28.1      | <0.0001 | CpG_10     | Normal | 96.5     | 8.6       | <0.0001 |
|            | Tumor  | 54.3     |           |         |            | Tumor  | 87.9     |           |         |
| CpG_15     | Normal | 81.0     | 27.8      | <0.0001 | CpG_11.12  | Normal | 85.7     | 14.3      | <0.0001 |
|            | Tumor  | 53.2     |           |         |            | Tumor  | 71.4     |           |         |

| CpG_13     | Normal | 87.8     | 14.3      | <0.0001 |
|            | Tumor  | 73.4     |           |         |
| CpG_14     | Normal | 83.0     | 15.0      | <0.0001 |
|            | Tumor  | 68.0     |           |         |
| CpG_15     | Normal | 91.1     | 25.1      | <0.0001 |
|            | Tumor  | 66.0     |           |         |
| CpG_16     | Normal | 85.2     | 20.0      | <0.0001 |
|            | Tumor  | 65.2     |           |         |
| CpG_17     | Normal | 99.7     | 2.7       | <0.0001 |
|            | Tumor  | 96.9     |           |         |
| CpG_18     | Normal | 43.5     | −0.4      | 0.7864  |
|            | Tumor  | 43.9     |           |         |
false negatives and zero false positives) has an AUC of 1.0. A $P$ value $<0.05$ was considered as statistically significant.

3 | RESULTS

3.1 | Patient characteristics

The clinical and pathological characteristics of the patients are listed in Table 1. A total of 163 patients were included in this study. The male-to-female ratio was 138:25, and 137 patients had HBV-related HCC. Thirty-four patients consumed more than 50 grams per day (g/d) of alcohol. Ninety-three patients were AFP positive, and 17 patients were CEA positive. For the clinicopathological characteristics, 21 patients presented with tumor thrombus and 17 patients had multiple liver tumors. Liver cirrhosis was found in 56 patients, and satellite tumor was detected in 44 patients. Most of the patients (89.6%) in this study were classified as stage III according to the staging system of AJCC.

![Figure 2](image)

**FIGURE 2** The methylation alternations of DNAH17 in HCC. The mean methylation levels and standard deviation are shown as bar-and-whiskers plots (center line, mean; error bars, SD). A, The mean methylation levels were significantly decreased in 147 HCC tumor tissues in amplicon 1 and amplicon 2 of DNAH17 (Wilcoxon signed rank test). B, The methylation of the CpG sites close to the two studied amplicons decreased in 50 HCC samples from TCGA data set (Wilcoxon signed rank test). C, The correlation between mean methylation levels of amplicon 1 and amplicon 2 (Pearson correlation analysis). D, Correlation of the methylation status between cg00577144 site and cg10217661 site from TCGA data set (Pearson correlation analysis).
Expression of analyses of DNAH17 mRNA and protein in HCC. A, The up-regulated of DNAH17 mRNA expression in HCC tissues from our previous RNA-seq data set (n = 11, Wilcoxon signed rank test). B, Increased DNAH17 mRNA expression in HCC from TCGA database (n = 50, Wilcoxon signed rank test). C, Representative IHC image of DNAH17 in HCC samples and ANT (P1: patient 1, P2: patient 2, P3: patient 3). D, Heatmap showed the difference for the IHC scores of 20 HCC patients (P = 0.0058, Paired two-tailed Student’s t test). E, The expression of DNAH17 predicts a significantly better RFS rate (HR = 1.5, P = 0.0051). F, The expression of DNAH17 was increased after decitabine treatment in HepG2 and PLC/PRF/5 cell lines (center line, mean; error bars, SD; Paired two-tailed Student’s t test).

A total of 18 CpG sites were identified in amplicon 1 and amplicon 2, and 13 CpG sites (CpG1, CpG2, CpG3.4, CpG5, CpG7, CpG8, CpG11.12, CpG13, CpG14, CpG15, CpG16, CpG17, and CpG18) were successfully genotyped (Figure 1D). Pearson correlation analysis showed that the methylation levels of each site were significantly correlated in amplicon 1 and amplicon 2, respectively (Tables S1 and S2). In amplicon 1, the methylation levels of the 12 CpG sites were significantly decreased in HCC samples compared to paired normal tissues (Table 2). In amplicon 2, we also observed the hypomethylation status of 11 CpG sites in HCC tissues (Table 2). The differences in methylation levels between tumor tissues and matched normal tissues ranged from 24.5% to 35.4% in amplicon 1, and from 2.4% to 25.1% in amplicon 2 (Table 2). The methylation levels of all CpG sites in amplicon 1 were found to be decreased more than 20%. The methylation levels of five CpG sites (CpG3.4, CpG5, CpG15, and CpG16) were found to be down-regulated more than 20% in amplicon 2. In amplicon 1, the mean methylation level in the tumor tissues (58.7%) was significantly lower than that in the paired normal tissues (84.6%, P < 0.0001, Figure 2A). In amplicon 2, the mean methylation level was also decreased in the tumor tissues (69.9%) compared to the paired normal tissues (84.5%, P < 0.0001, Figure 2A). Moreover, our results were consistent with the data from the TCGA data set. Three CpG sites (cg09577144, cg07255197, and cg05414903) were located very close to amplicon 1. One CpG site (cg10217661) was close to amplicon 2. All four CpG sites were found to exhibit hypomethylation status in HCC tissue (Figure 2B).

We further analyzed the correlation of the methylation status between amplicon 1 and amplicon 2 using Pearson correlation analysis. Interestingly, the methylation status of the two amplicons presented a powerfully positive correlation (r = 0.625, P < 0.0001, Figure 2C). We also found significant correlation between the three CpG sites close to amplicon 1 and the CpG site close to amplicon 2 (Figures 3D, S1 and S2).

Expression levels of DNAH17 in HCC tissues and their paired adjacent non-cancerous tissues

A transcriptome data set that contained 11 paired HCC samples was established in our previous study. In our RNA-seq data set, the expression levels of DNAH17 showed a trend to increase in the tumor tissues (Figure 3A). We further proceeded to analyze the RNA-seq data of 50 paired HCC samples in the TCGA data set. The expression levels were significantly higher in tumor tissues than in normal tissues (P = 0.0002, Figure 3B). We further used IHC to detect the protein changes in 20 paired HCC samples. We found 11 of 20 pairs of HCC samples (55%) presented high expression of DNAH17 in the tumor tissues compared with ANT, and DNAH17 expression in HCC tissues was significantly higher than ANTs in most of HCC samples (P = 0.0058, Figure 3C,D).

In addition, the survival data from the GEPIA website demonstrate that the high DNAH17 expression group predicts a worse disease-free survival (HR = 1.5, P = 0.0051, Figure 3E). For overall survival, there was no significant difference between low-expression patients and high-expression patients (Figure S3).

To verify the expression of DNAH17 was regulated by the methylation status, we selected two HCC cell lines, HepG2 and PLC/PRF/5, for decitabine treatment. The expression levels of DNAH17 in both two HCC cell lines were increased after decitabine treatment for three days (Figure 3F).

3.4 | The copy number variants of DNAH17 in HCC samples

DNAH17 copy number data and methylation-level data were downloaded from Oncomine, a website analyzing TCGA data online. We observed that the methylation levels of DNAH17 was significantly correlated with the CNV alternations, and the methylation values were down-regulated in amplification patients compared to diploid patients (P = 0.0208, Figure 4A). To confirm the connection between copy number variants and the methylation of DNAH17, we used the TaqMan copy number assays to assess the copy number status of DNAH17 in our cohort and found the copy number was increased in 30.8% HCC samples when compared to ANT (Figure 4B). In the amplicon 2, the copy number amplification samples always companied with hypomethylation status (Figure 4C). In the amplicon 1, although no significant association between methylation status and CNVs, the methylation levels in patients with no <4 copy numbers showed marginal difference to other patients with 2 or 3 DNAH17 copy number (Figure 4D). No significant correlation was found between copy number and clinicopathological features.
3.5 | Correlation between the methylation of DNAH17 and clinicopathological features

To determine the role of DNAH17 as a biomarker in HCC, the correlation between the methylation status of DNAH17 and comprehensive clinicopathological features was analyzed (Table 1). Because most of the CpG sites were significantly correlated with each other, we calculated the mean values of the methylation level of all CpG sites to investigate the integrated effect of the selected amplicons. For amplicon 1, the hypomethylation status of DNAH17 in HCC tissues was detected in patients with fibrous capsules (53.6% vs 70.7%, *P* = 0.0008, Figure 5A), tumor necrosis patients (56.1% vs 67.0%, *P* = 0.0370, Figure 5A), older patients (52.7% vs 62.4%, *P* = 0.0406, Figure 5A), AFP-negative patients (49.9% vs 65.5%, *P* = 0.0010, Figure 5A), and male patients (56.1% vs 67.0%, *P* = 0.0076, Figure 5A). For amplicon 2, we observed that the mean methylation level of DNAH17 in tumor tissues was significantly down-regulated in patients with tumor thrombus (58.5% vs 78.5%, *P* = 0.0063, Figure 5B) and negative AFP (64.5% vs 73.2%, *P* = 0.0334, Figure 5B). The HCC samples with liver cirrhosis had lower DNAH17 methylation levels than those without liver cirrhosis (non-cirrhosis vs cirrhosis, 73% vs 64%, *P* = 0.0047, Figure 5B). A similar result was also found in paired normal tissue; the methylation level was significantly decreased (Figure S4). However, the methylation difference was obviously higher in tumor tissues (9%) than in paired normal tissues (2%). Hypomethylation status of DNAH17 in HCC tissues was detected in male HCC patients (68.2% vs 79.1%, *P* = 0.0120, Figure 5B). As previously mentioned, the methylation levels of DNAH17 in both amplicon 1 and amplicon 2 were significantly associated with age. We further analyzed the data from TCGA data set to verify the correlation. The same trends were detected in TCGA data set, even though the differences were marginally significant (Table S3).

3.6 | ROC curve analysis of methylation levels in HCC and adjacent non-cancerous tissues

To evaluate whether methylation levels of DNAH17 can serve as a useful biomarker in HCC, ROC curves were plotted. The capacity of discrimination was assessed by calculating the AUC. We found that the methylation level of CpG12 in amplicon 1 (AUC = 0.714, *P* < 0.0001, Figure 6A) and the mean methylation level of amplicon...
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1 (AUC = 0.697, P = 0.0003, Figure 6B) discriminated HCC patients without fibrous capsules. ROC curve analysis showed that the mean methylation level of amplicon 2 in adjacent non-cancerous tissues could efficiently discriminate HCC cases with tumor thrombus from those without tumor thrombus (AUC = 0.806, P < 0.0001, Figure 6C). However, the ability of discrimination of the mean methylation level in tumor tissues was lower than in adjacent non-cancerous tissues (AUC = 0.695, P = 0.0093, Figure 6D).

4 | DISCUSSION

DNA methylation, as one of the most widely studied epigenetic modifications, is crucial in various cancers. In the present study, hypomethylation status and overexpression of DNAH17 were detected in HCC samples compared to paired normal tissues, suggesting that this epigenetic change of DNAH17 might participate in HCC progression. Importantly, we found that DNAH17 methylation was associated with gender, age, serum AFP values, liver cirrhosis, tumor fibrous capsule, tumor necrosis, and tumor thrombus. ROC curve analysis showed that hypomethylation status of DNAH17 in HCC patients could be a biomarker to predict the existence of fibrous capsule and tumor thrombus. In addition, we investigated the promoter region of two different isoforms in our experiment, which provide an intact view of this gene methylation status.

The protein encoded by DNAH17 is a subunit of axonemal dynein, which is a basic structure for primary cilia. Many studies indicated that the primary cilia played an important role in embryonic development, cell differentiation, cell division, and tumor progression through Hedgehog (Hh)
As we described above, loss of primary cilia is a common feature of malignant cells, whereas our results showed that the expression of DNAH17 was up-regulated in HCC tissues by hypomethylation. The paradox might observed be because the primary cilia are both positive and negative effectors of Hh signaling. Moreover, Wang et al reported that dynein axonemal heavy chain 8, a DNAH family protein, was overexpressed in prostate cancer tissues compared to normal prostate tissues and could promote cancer metastasis.

DNAH17 is located at 17q25.3, which resides in an amplicon that is significantly associated with many cancers, including HCC. It was reported that 17q25.1-3 copy number gain was a prognostic marker for poor patient survival in HCC. In liver cancer, the methylation-correlated expression genes and DNA copy number-correlated expression genes are significantly co-regulated. We then explored the methylation status and copy number of DNAH17 in our cohort and noted that a lower methylation level of HCC samples was observed in DNAH17 amplification patients compared with the rest patients, which consisted with the data from TCGA data set. This finding indicated that the DNAH17 overexpression in HCC tissue was possibly regulated by the synergy of gene amplification and hypomethylation. However, we should realize that the qPCR method is not an ideal method to assess the copy number variant in tumor tissues because of the high heterogeneity of tumor. The different proportion of cancer cells in different tumor samples could also bring bias into our study.

It is well known that male patients are markedly predominant in morbidity and mortality in HCC. Shen et al explored genome-wide DNA methylation profile changes in HCC and found that some CpG sites were significantly associated with gender. A similar phenomenon was also detected in our study. Lower methylation levels of DNAH17 promoter in HCC samples were found in male patients. In AFP-negative HCC patients, we found that the methylation level of DNAH17 in HCC samples was lower than that in AFP-positive HCC patients. Several studies also indicated that the methylation status of some HCC-related genes in HCC tissues was associated with abnormal serum AFP level. Furthermore, this finding encouraged us to explore whether the methylation detection of DNAH17 gene in circulating tumor DNA (ctDNA) could be a sensitive biomarker for AFP-negative HCC patients which would be useful for HCC early diagnosis. We also found the hypomethylation status of the DNAH17 isoform 1 promoter was significantly associated with tumor necrosis and the existence of fibrous capsule. In both ANT and HCC tissues, the methylation differences of the DNAH17 isoform 2

![FIGURE 6 ROC curves analysis of the methylation levels in DNAH17 for clinical features. A, ROC curve of the CpG12 methylation in amplicon 1 in tumor tissues for prediction the fibrous capsule. B, ROC curve of mean methylation level of all CpGs in amplicon 1 for prediction the fibrous capsule. C, ROC curve of mean methylation level of amplicon 2 in adjacent non-cancerous tissues for prediction the tumor thrombus. D, ROC curve of mean methylation level of amplicon 2 in tumor tissues for prediction the tumor thrombus.](image-url)
promoter between liver cirrhosis patients and non-cirrhosis patients were significant. The hypomethylation status of DNAH17 isoform 2 was detected in liver cirrhosis tissues. However, the methylation difference between cirrhosis patients and non-cirrhosis patients was 4.5 times higher in HCC tissues than in ANT. Therefore, we speculated that DNAH17 is not only associated with liver cirrhosis, but also plays a more important role in oncogenesis under the liver cirrhosis background. The underlying mechanism needs to be confirmed in future studies.

Macrovascular invasion, including tumor thrombosis in the portal vein and hepatic vein, is a poor prognosis factor. However, not all tumor thrombosis in HCC patients could be detected preoperatively, which often leads to a dilemma during surgery. Here, we found that HCC patients with tumor thrombus were always accompanied by hypomethylation of DNAH17 in the promoter of isoform 2, which implied that down-regulated methylation of DNAH17 might promote HCC metastasis. We further evaluated the potential values of DNAH17 methylation in our study for clinical diagnosis purposes. The discriminatory capacity of DNAH17 methylation levels was evaluated to distinguish HCC patients with tumor thrombus through ROC curve analysis. Our findings showed that the hypomethylation status of DNAH17 in both tumor tissues and adjacent non-cancerous tissues could help discriminate HCC patients with tumor thrombus from those without tumor thrombus. More importantly, the AUC of the mean methylation level in paired normal tissues was more than 0.8. Our result indicated that the methylation status of DNAH17 was a promising biomarker for tumor thrombosis, which could provide more information about HCC features in the preoperative biopsy and help clinicians generate an individual treatment strategy. Moreover, the significantly aberrant methylation of DNAH17 in HCC tissues suggested that this gene could be a promising candidate for liquid biopsy in future research.

In summary, hypomethylation status of the DNAH17 gene was detected in HCC. The DNAH17 overexpression in HCC tissue was possibly regulated by the synergy of gene amplification and hypomethylation. We revealed that methylation alterations in DNAH17 have a correlation with age, gender, serum AFP level, liver cirrhosis, tumor necrosis, fibrous capsule, and tumor thrombus. Importantly, ROC analysis demonstrated that hypomethylation status could be a promising biomarker to predict the existence of the tumor thrombosis. The underlying mechanism needs to be further confirmed.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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