Global DNA 5hmC and CK19 with 5hmC positives cell contents represent a promising biomarker for predicting prognosis in small hepatocellular carcinoma

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Research

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Abstract

**Background:** 5-Hydroxymethylcytosine (5hmC) exists dynamically and exhibits various regulatory functions. It's possibly associated with tumor occurrence and malignant transformation. Nevertheless, the part of 5hmC in small hepatocellular carcinoma (SHCC) is still elusive. The study was directed toward characterizing 5hmC content in SHCC and assessing if global genomic 5hmC content was able to serve as a promising factor for predicting clinical results.

**Methods:** The expression contents of 5mC, 5hmC and 5fC were assessed using ultra-high performance liquid chromatography tandem mass-spectrometry (UHPLC-MS/MS). 5hmC contents and CK19 expression were measured by immunohistochemistry (IHC).

**Results:** The findings in the study displayed that global genomic 5mC, 5hmC, and 5fC contents in SHCC specimens were globally reduced relative to para-tumor tissues \( P<0.001 \). Lymph node metastasis may be found in the small HCC, the non-metastasis group exhibited higher 5mC and 5hmC contents relative to those in metastasis group \( P<0.001 \). HBV DNA was relevant to the decrease in 5mC, 5hmC and 5fC, as evidenced by the measurements in cell lines carrying or not carrying HBV DNA. Moreover, the correlation analysis showed the negative correlation of the content of 5hmC with CK19 expression in SHCC. The decreases of both 5hmC and CK19 with 5hmC positives cell contents in genomic DNA were related to SHCC patients' unfavorable prognosis. Compared with para-tumor tissues, the 5hmC content in SHCC specimens was dramatically reduced.

**Conclusions:** 5hmC and CK19 with 5hmC positives cell contents in genomic DNA possibly serve as a promising biomarker for predicting prognosis in small hepatocellular carcinoma.

**Background**

Hepatocellular carcinoma (HCC) is ranked the 6th most prevalent tumor in the world, and is one of the most prevalent malignant hypervascular tumors characterized by neovascularization[1]. Respond poorly to traditional treatments, recurrence and metastasis are common in HCC and associated with poor prognosis. Risk factors for HCC involve chronic infection with hepatitis B virus (HBV), hepatitis C virus (HCV), smoking, alcohol abuse, dietary aflatoxin, diabetes and obesity[2]. Most HCC are usually detected at terminal and fatal stage, and therefore it's imperative to develop screening methods for early examination. With the continuous improvement of HCC screening methods, the detection rate of SHCC has increased. According to the guidelines of the Asia Pacific Society for the study of liver diseases and the standardized pathological diagnosis guidelines for HCC, single tumor diameter \( \leq 3 \text{cm} \) is defined as SHCC[3, 4]. Due to its special biological behavior and pathological characteristics, it is often considered as a HCC with good prognosis. At present, it mostly depends on AFP, ultrasonography, CT and other diagnostic methods, with certain limitations[5]. DNA methylation is one of the earliest epigenetic modifications, which assumes a critical part in maintaining chromosome structure, X-chromosome silence, gene imprinting and tumor occurrence[6, 7]. Investigating the mechanism, DNA methylation
function and the distinctions in diverse tissues or individuals will profoundly affects human health and disease research; genome DNA methylation, a vital way of epigenetic modifications, performs an essential part in regulating biological processes like gene expression and cell differentiation[8-10]. Therefore, it is suggested that epigenetic mechanism may assume a critical part in SHCC pathogenesis and maintenance.

5-position DNA methylation of cytosine (5mC) is an epigenetic cancer marker. Several studies have shown that the ten eleven translocation (TET) protein family initiates an active DNA demethylation pathway, leading to the transformation of 5mC into 5-hydroxymethylcytosine (5hmc)[11]. 5hmc, a novel epigenetic biomarker, is change the view of tumor epigenetics. It has been reported that the reduction of 5hmc contents is a reflection of unsatisfactory survival of patients with digestive system tumors[12, 13]. However, there is no epigenetic study of 5hmc in SHCC.

In this study, we carried out UHPLC-MS/MS and immunochemistry (IHC) staining to analyze the 5mC, 5hmc and 5fC contents in the whole genome DNA, so as to link these information with the clinical characteristics and survival results of SHCC.

Results

Clinical characteristics

Matched pairs of primary SHCC and para-tumor tissues were collected from 63 patients that received surgical excision from 2010 to 2012 at the Renji Hospital in Shanghai, The patients’ maximum tumor diameters were ≤3cm and all of them were at stages A or B (BCLC staging). The majority of patients developed HBV infection. The specific clinicopathological data were displayed in Table 1. The study has been agreed by the Renji Hospital Ethics Committee. All patients signed informed consent before surgery.

Table 1. Clinical characteristics of small hepatocellular carcinoma
| Characteristics                        | Value |
|---------------------------------------|-------|
| Demographics                          |       |
| No. of patients                       | 63    |
| Age, years                            |       |
| ≥5                                   | 40    |
| <5                                   | 23    |
| Gender, (female/male)                 | 7/56  |
| Tumor size, cm                        |       |
| ≤2                                   | 26    |
| 2-3                                  | 37    |
| AFP(mg/ml)                            |       |
| ≤25                                  | 45    |
| >25                                  | 18    |
| CK19 index                           |       |
| < 20%                                | 20    |
| ≥ 20%                                | 26    |
| HBV DNA(IU/ml)                        |       |
| <10^3                                | 36    |
| ≥10^3                                | 27    |
| Cirrhosis                             |       |
| Yes                                  | 42    |
| NO                                   | 21    |
| BCLC staging                          |       |
| 0                                    | 18    |
| A                                    | 35    |
| B                                    | 10    |
| Recurrence                            | 29    |

Abbreviations: AFP, alpha-fetoprotein; HBeAg, HBV e antigen; HBsAg, HBV surface antigen; 5hmC, 5-hydroxymethylcytosine, 5mC, 5-methylcytosine, CK19, Cytokeratin 19, SHCC small hepatocellular carcinoma, BCLC staging, Barcelona Clinic Liver Cancer staging

Global genomic 5mC, 5hmC and 5fC contents were reduced in SHCC metastasis
To assess the global changes in 5mC, 5hmC and 5fC contents in SHCC, UHPLC-MS/MS was conducted to determine global 5mC,5hmC and 5fC contents in 63 SHCC and para-tumor tissues. The difference of global 5mC,5hmC and 5fC contents were observed between SHCC and para-tumor tissues. We found the 5mC, 5hmC and 5fC contents in tumor specimens were dramatically reduced relative to those in para-tumor tissues (P<0.001) (Fig. 1A.B.D.E.G.H).

To investigate whether a notable change in the global contents of 5mC, 5hmC and 5fC in SHCC metastasis, we analyzed the contents in different metastasis of SHCC. The results showed that the contents in SHCC tissue metastasis were notably reduced relative to para-tumor tissues. There was a visible change in 5mC and 5hmC contents in metastasis of SHCC and para-tumor tissues (Fig. 1C.F). However, except that the 5fC content in metastasis of SHCC para-tumor tissues continued to reduce dramatically relative to that in non-metastasis, while no noticeable distinction was detected in SHCC tissues (Fig. 1I).

**The reduction in 5mC, 5hmC and 5fC contents in SHCC genomic DNA was relevant to HBV DNA level**

HBV DNA level in the blood is regarded as a more exact reflection of an immediate HBV infection status. The patients were classified to two groups based on HBV DNA level. We observed that 5mC, 5hmC and 5fC contents in HBV DNA-high group were reduced more dramatically (Fig. 2A and 2B).

To demonstrate whether HBV infection caused a reduction in 5mC, 5hmC and 5fC contents in HCC cells, we investigated two HCC cell lines that had been integrated with HBV DNA namely HepG2.2.15 integrated with complete HBV DNA. We observed that the global 5mC,5hmC and 5fC contents were dramatically reduced in the genome of these two integrated cell lines, relative to their original cells (Fig. 2C and 2D), and the decreases seemed even more notable over that in HCC tissues (Fig. 2C and 2D with Fig. 2A and 2B). Overall, these findings revealed that the 5hmC and 5fC contents of HCC tumor cells have reduced in genomic DNA and HBV infection could result in severer abnormality. Then, we preliminarily investigated the relevant mechanisms.

**Global genomic 5hmC contents positively correlate with cell proliferation**

To test our hypothesis that 5hmC is decrease in SHCC,and significantly decreased in metastasis cancers,we measured 5hmC contents and CK19 expression across the full spectrum od cervical lesions,by immunohistochemistry(IHC). The results shown 5hmC conten and CK19 with 5hmC positives cell content was decreased in metastasis of SHCC tissues compared to the non-metastasis of SHCC tissues,the CK19 expression was upregulated in metastasis of SHCC,and downregulated in non-metastasis of SHCC(Fig.3A).

The Pearson correlation analysis displayed that a notably positive relation existed between 5hmC contents and 5hmC positive cells ratio (r=0.528, P<0.001) (Fig. 3B). We subsequently measured the correlation between SHCC cell proliferation and 5hmC level through CK19 staining and observed that
5hmC positive cells were closely correlated with CK19 positive cells (r=0.444, P=0.002) (Fig. 3C). These observations also displayed that 5hmC contents exhibited a positive correlation with CK19 positive cells (r=0.428, P= 0.003) (Fig. 3D).

**Decreased 5hmC and CK19 with 5hmC positives cell contents in SHCC genomic DNA were related to patients’ unfavorable prognosis**

To assess the latent prognosis value of 5mC, 5hmC, 5fC, and CK19 with 5hmC positives cell contents, we used time to disease-free survival (DFS) and overall survival (OS) as the clinical endpoint. We observed that besides 5hmC content, CK19 with 5hmC positives cell content was also related to prognosis. Patients with low 5hmC content or CK19 with 5hmC positives cell content displayed worse DFS and OS rates over those with high 5hmC content or CK19 with 5hmC positives cell content, respectively (Fig. 4).

**Discussion**

Epigenetic modifications are of significance to natural development and frequently change in the course of tumor occurrence[9]. Several studies have revealed that the 5hmC deprivation in various cancers might assume a critical part in pathogenesis[14-19]. Recent studies also showed focused on the important function of 5hmC in HCC proliferation and displayed a pathophysiological part and clinical significance for HCC patients[20-22], but only a friction in this group was SHCC. Nonetheless, whether 5hmC content in pediatric SHCC is changed remains unknown. As well known to us, the study firstly elaborated the change of 5hmC contents in SHCC. We revealed that global genomic 5hmC contents in SHCC tumor tissues were dramatically reduced relative to para-tumor tissues. These findings further confirmed the previous results of the decreased global genomic 5hmC content that was observed in other cancers. Further, we observed that 5hmC score according to IHC staining with 5hmC antibody was closely associated with 5hmC contents, indicating that IHC staining was effective in determining 5hmC level.

The study indicated that HBV infection was involved in the reduction of 5mC, 5hmC, or 5fC contents, as evidenced by the observations of HBV DNA-integrated cell lines. HBV largely affected 5mC, 5hmC, and 5fC contents (more significant in integrated cell lines), which may cause reduced 5mC, 5hmC, and 5fC contents. Therefore, the mechanism underlying HBV influencing 5mC, 5hmC, and 5fC contents gained wide attention. HBV has been found to influence DNMTs and TETs expression or activity[23, 24], nevertheless, the conclusions obtained were not exact. The relevant correlation and mechanisms between them remained to be thoroughly investigated.

The limited biomarkers effectively predicting prognosis in SHCC reveals that it’s necessary to develop more reliable prognosis markers [3]. Several studies displayed that 5hmC contents were relevant to clinical results in different cancers[13, 14, 20, 25]. Analysis of multiple SHCC cohorts displayed a great distinction between the utility in SHCC metastasis and non-metastasis as a prognosis marker. Nonetheless, applying SHCC histological grading to risk stratification has been unconvinced and it was inconsistent with tumor grading with patients’ results[3, 26]. Currently, DNA copy number profiles and DNA methylation patterns are able to predict clinical results of HCC patients[3, 27]. The study displayed
that high 5hmC content is an independent prognosis factor of unfavorable PFS and OS in pediatric SHCC. Low 5hmC contents in most solid tumors usually means higher tumor grade and worse results[3, 11, 13, 14, 27, 28]. Nevertheless, our findings displayed that high 5hmC contents were independently relevant to inferior OS in SHCC and 5hmC was possibly correlated with tumor occurrence and malignant transformation. These observations needed to be further demonstrated.

The improvement of early diagnosis and treatment of small HCC has become one of the bottlenecks restricting the further development of liver surgery, New theories and concepts of small HCC are urgently needed in clinical practice to guide medical practice. Modern tumor molecular biology studies have shown that tumor is a genetic disease caused by multi-gene mutation and long-term accumulation[5]. HCC also exhibited higher CpGi methylation, Besides CpGi hypermethylation, global H3K27me3 and DNA hypomethylation decreases without recurrent genetic alterations in HCC displays that epigenetic mechanisms are the core of HCC occurrence[29-35]. Further, some studies indicated that decreased H3K27me3 is not deregulated by genes but epigenetics[36, 37]. This work first found the distinction in 5hmC contents between metastasis and non-metastasis SHCC, indicating that 5hmC may be involved in the aberrant DNA methylation. The epigenetic alternation mechanism should be paid more attention in future studies.

The 5hmC contents in SHCC were positively related to ck19 expression. Some studies revealed that higher ck19 expression was possibly relevant to unfavorable prognosis[38-42]. These findings validated previous conclusions and further indicated that high 5hmC contents were relevant to inferior results. Nonetheless, several studies found that 5hmC contents were negatively correlated with cell proliferation of various cancers[13, 14, 21, 27]. These distinctions may result from the different tumor occurrence in benign tumor relative to other malignant tumors. Nonetheless, the mechanism underlying 5hmC affecting SHCC cell proliferation remains to be thoroughly investigated.

There exist some limitations in the study. Firstly, the sample size is small and the follow-up period is comparatively short, which restricted our ability to find out powerful survival predictors. Further studies are required to validate the conclusions of our study. Secondly, IHC was applied to categorize the molecular subgroup. Therefore, fresh frozen tumor specimens should be used in future studies and methylation arrays are also warranted.

Conclusions

The study indicated that 5hmC acts as a promising prognosis predictor which may ameliorate clinical risk stratification for SHCC and 5hmC contents are relevant to cell proliferation and molecular subgrouping. These observations displayed that the mechanisms underlying 5hmC modulation may provide new thoughts for future treatments.

Methods
**Study design and specimens**

The study was mainly directed toward evaluating the clinical features of DNA hydroxy methylcytosine in SHCC. UHPLC-MS/MS was applied to study 5hmC abundance. Additionally, we took advantage of IHC to perform molecular classification. Sixty-three patients all together that were diagnosed with SHCC in Shanghai Renji Hospital from January 2010 to December 2012 participated in the study. Clinical data, involving age at diagnosis, gender, tumor size, etc., were acquired through retrospective chart review. Follow-up assessments were conducted on all patients through either a telephone interview or an outpatient consultation. The study has been agreed by the ethics committee of Shanghai Renji Hospital, Shanghai Jiaotong University. All patients signed the informed consent.

Tumor specimens were collected during first surgery prior to adjuvant treatments. All specimens were snap-frozen at ~80 °C or fixed with formalin buffer (4%) and embedded with paraffin. As control specimens, all healthy cerebellums were obtained from the School of Life Sciences of Shanghai Jiaotong University.

**Definition of CK19 and 5hmC via immunohistochemistry**

Briefly, tissue sections were cut into 5 μm, and then deparaffinized and rehydrated with ethanol and xylene. Next, the slides were exposed to 3% H2O2 for 10 min in phosphate buffer to weaken endogenous peroxidase activity. Slides were cultivated overnight with rabbit monoclonal anti-H3K27me3 antibody (1:150, C36B11, Cell Signaling, Danvers, MA, USA) through the standardized Leica Bond protocol IHC-F. The Leica Bond Polymer Refine DAB detection kit was utilized as the relevant guidance. All IHC slides were assessed by two different neuropathologists; the scoring methods were conducted as depicted in the previous reports [9]. In line with H3K27me3 positive staining, when over 80% cells exhibited nuclear positivity, it means scored positive, or else scored negative.

**Assessment of global genomic 5mC, 5hmC and 5fC contents through UHPLC-MS/MS**

The absolute amount of 5mC, 5hmC and 5fC in SHCC was determined as depicted before. In brief, DNA was separated through the Wizard® Genomic DNA Purification Kit (A1620, Promega, Madison, WI, USA) as the relevant guidance. DNA (1 μg/specimen) was heated at 100 °C for 180 s and subsequently incubated with nuclease P1 (2U, Sigma, N8630, Darmstadt, Germany) at 42 °C for 6 h. Next, alkaline phosphatase (1 U, Sigma, M183A) was supplemented and incubated at 37 °C for 6 h more. In the end, the specimen was diluted to 0.06 mL and filtered (0.45 μm, PALL). Nucleosides were extracted through UHPLC on T3 column (WATERS, 186003538, MA, USA) and monitored via triple-4 quadrupole mass spectrometer (WATERS, ACQUITY UPLC XEVO TQ-S). The mass transitions of m/z 228.4 to 112.2 (C), m/z 242.3 to 126.1 (mC), m/z 258.2 to 124.2 (hmC) were detected and recorded. The contents were obtained through comparing the standardized curve of pure nucleoside standards running on the same batch of specimens.

**IHC analysis of 5hmC and CK19**
The primary antibodies used involved 5hmC (1:800, ab214728), 5mC (1:200,ab10805) and CK19 (1:1500, ab15580), and all of them were purchased from Abcam. Immunohistochemical detection of 5hmC and CK19 was conducted as depicted above except for DNA denaturation. The staining and score methods were conducted as depicted before. Briefly, dark brown staining pattern was taken as positive staining, which was limited to the nuclear region. Negative represented scant or fine granular background staining or no staining. The average value of the five snapshots was obtained to reflect the ratio of positive cells.

Data analysis

Data were analyzed through SPSS 23 (IBM Corp., New York, NY, USA) and two-sided P < 0.05 meant data distinction. The variable normality was evaluated. Data were presented as average ±SD or median (minimum to maximum). The Mann–Whitney U test and t test were performed to assess distinctions in median and average values, respectively. Fisher’s exact test was applied to evaluate the correlations between categorical variables. To explain the part of 5hmC content in a more clinic-related manner, 5hmC content was divided to two groups through Cut off Finder. The cutoff values (0.102%) were referred to as the points with the most notable split between groups, involving DFS and OS.

OS from first surgery that conducted the pathological diagnosis to death was calculated. DFS from first diagnosis (first treatment beginning) to the first disease recurrence was calculated. Kaplan–Meier curves of OS and DFS were acquired and log-rank tests were used for clinically and demographically analyzing the distinctions in OS and DFS.

Abbreviations

5mC: 5-methylcytosine; 5hmC: 5-hydroxymethylcytosine; 5fC: 5-formylcytosine; 5caC: 5-carboxylcytosine; DNMTs: DNA methyltransferases; TET: ten-eleven translocation; CK19: Cytokeratin 19, SHCC: small hepatocellular carcinoma, BCLC staging: Barcelona Clinic Liver Cancer staging; TNM: tumor, node and metastasis; DFS: disease-free survival, OS: overall survival.

Declarations

Authors’ contributions

JJ contributed in funding and carried out the experiments, performed statistical analyses and drafted the manuscript. YT performed bioinformatic analyses and drafted the manuscript. CD participated in data analysis. WJ carried out tissue sample collection. GF conceived of the study and participated in its design and coordination, data analysis, and helped to draft the manuscript. SJ conceived of the study and participated in its coordination. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Not applicable.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study was approved by the Renji Hospital Ethics Committee.

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