A novel prediction model for diagnosing hepatocellular carcinoma in patients with chronic liver disease based on serological biomarkers

Jun Gao

Nanchang University Second Affiliated Hospital

Chunyan Xie

Nanchang University

Xin Yu

Nanchang University Second Affiliated Hospital

Yingbin Xiao

Nanchang University Second Affiliated Hospital

Xiaogang Peng

Nanchang University

Xiangbao Ying

Nanchang University Second Affiliated Hospital

Daojing Li

Jiangxi Cancer Hospital

Fan Zhou (✉ nczhoufan@sina.cn)

Nanchang University <https://orcid.org/0000-0003-2612-7950>

Research article

Keywords: Hepatocellular carcinoma, 5-hydroxymethylcytosine, AFP, diagnostic model

Posted Date: March 3rd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-15901/v1>

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Abstract

Background: Hepatocellular carcinoma (HCC), the most common type of digestive cancers, is a common complication of chronic liver disease (CLD). Currently, HCC is often diagnosed by radiological examination. 5-Hydroxymethylcytosine (5hmC) in a vital mammalian DNA epigenetic modification that is found in circulating cell-free DNA (cfDNA) and carries tumor-specific epigenetic information. It may also serve as a potential cancer biomarker for HCC diagnosis. Our study aimed to establish a statistical diagnostic model for diagnosing the risk of HCC in individual patients with CLD according objective tests, especially serological tumor markers. **Methods:**Based on the 5hmC-seal technique, the genome-wide-5-hydroxymethylcytosine (5hmC) and gene body 5hmC content in cfDNA samples from 96 patients were analyzed. Patients were recruited from the Second Affiliated Hospital of Nanchang University; 64 patients had HCC and 32 patients had CLD. Peripheral blood samples were prospectively collected, followed by blood preparation and cfDNA extraction. Independent risk factors associated with HCC were identified by logistic regression, and a diagnostic model was developed and evaluated based on sensitivity, specificity, and correct diagnostic results. **Results:**The final model revealed sex, age, 5-Hydroxymethylcytosine (5hmC), and afetoprotein (AFP) as diagnostic indicators in a “discovery” data set. A novel statistical model was formed using the formula: $Z = -18.160 + 0.081 \cdot \text{age} + 1.867 \cdot \text{sex} + 1.066 \cdot (\log \text{AFP}) + 1.964 \cdot (\log 5\text{hmC})$, which had an area under the receiver operating curve of 0.973. With 0.7636 as the cutoff point, the model had an estimated sensitivity of 0.891 and a specificity of 0.969. The novel model provides a reliable and stable capacity to predict HCC in patients with CLD. **Conclusions:** The study findings provide an objective blood serum marker-based model that could enhance the diagnostic efficiency of HCC and form a basis for the early detection of surgically resectable liver cancers in high-risk individuals.

Background

Hepatocellular carcinoma (HCC) is a serious malignant cancer which ranks as the second leading cause of cancer-related deaths worldwide.[1] HCC is characterized by an insidious onset, strong invasiveness, rapid growth, and high mortality.[2] The poor prognosis of HCC is due in great extent to the late stage of diagnosis, as symptoms of early stage disease are often vague. In the advanced stages, there are fewer potentially curative treatment options such as liver resection or transplantation.[3] One of the most common causes of HCC is chronic liver disease (CLD). There are many pathogenic factors of chronic liver disease, such as hepatitis B virus (HBV), hepatitis C virus (HCV), and alcoholism, as well as other causes. Greater than 600,000 liver cancers per year result from chronic liver disease, making this a worldwide public health problem.[4] Therefore, common clinical practice guidelines suggest early and periodic examination of CLD patients who have a high risk of developing HCC.[5–8] Furthermore, early diagnosis and initiation of treatment can improve prognosis and are of great significance in reducing mortality from HCC. According to the guidelines of the American Association for the Study of Liver Disease (AASLD), the utilization of alpha-fetoprotein (AFP) and abdominal ultrasound scans are recommended as the primary monitoring tools for early detection of liver cancer.[5] However, AFP and ultrasound have poor specificity

for the early diagnosis of HCC due to low sensitivity, poor reproducibility, and a high false negative rate, making it an inadequate tool for HCC surveillance.[9–11] Therefore, the identification of novel and more effective diagnostic approaches in HCC is important.

Tumor “liquid biopsy technology” based on cell-free DNA (cfDNA) detection has been a hot spot in cancer research in recent years. Cell-free DNA (cfDNA), which is formed by tumor cells releasing nucleic acid fragments into the bloodstream, is considered a novel tumor molecular marker.[12] CfDNA carries mutations from the original tumor, such as single nucleotide variations, insertion or deletion changes, and epigenetic aberrations, which can be detected in the early stages of tumor development.[13] Therefore, tumor liquid biopsy technology, which is based on cell-free DNA (cfDNA) detection, has emerged as a non-invasive, real-time and comprehensive dynamic method of monitoring tumors while overcoming the limitations of traditional tissue puncture.[14–15]

Epigenetic changes, particularly due to abnormal DNA methylation processes, are closely related to the occurrence and development of tumors. 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) are the fifth and sixth bases on DNA and play a vital role in the epigenetic modification of genes.[16] 5hmC is an important epigenetic modification which is produced by an enzyme of the TET (ten-eleven translocation) family via oxidation of 5mC. Additionally, the gene distribution of 5hmC precisely corresponds to the regulation of gene activity, and is more sensitive than 5mC in reflecting gene expression status.[17] By detecting the distribution of 5hmC in the genome of cfDNA, gene expression and regulation information can be obtained.[18–19] Therefore, 5hmC is considered a potentially reliable diagnostic biomarker in human cancers, including HCC. This is due to its tissue and cancer specificity, and more importantly, the rapid development of liquid biopsy technology.[20–22]

In the present study, we utilized a cell-free 5hmC sequencing method based on selective and sensitive chemical labeling (hMe-seal), to analyze blood samples from a cohort of 64 HCC patients and 32 CLD patients. Our results demonstrated that 5hmC modifications in the cfDNA of HCC patients exhibited distinct features, with 5hmC gains significantly enriched compared to those in the cfDNA of CLD controls. In addition, our study showed that the area under the receiver operating characteristic curve (AuROCs) for AFP and 5hmC were 0.816 (95% CI 0.731–0.902) and 0.891 (95% CI 0.811–0.972) respectively, showing the ability to differentiate between HCC and CLD. A recent study showed that the combination of AFP and 5hmC may improve the efficiency of tumor detection, especially for the early detection of tumors, by increasing the AuROCs.[23]

Based on this evidence, we determined that an early screening tool for the diagnosis of liver cancer could be developed using two serological measurements, AFP and 5hmC, via establishment of a statistical model. Because diagnostic models require favorable, effective functions for the diagnosis of disease, we carefully collected clinical data to accurately classify the patients according to disease type.

Methods

Study design and blood samples

A case–control study was conducted to measure the biomarkers AFP and 5hmC in blood samples collected between January 2018 and January 2019 at the Second Affiliated Hospital of Nanchang University, Nanchang, China. Samples were taken from patients with HCC or chronic liver disease (CLD) (Table 1). For all patients with liver cancer, the diagnoses were made based on clinical manifestations, pathological examination, imaging examination, and other related examinations according to international guidelines.[24] Additionally, the cancer patients did not undergo surgery, radiofrequency ablation, intervention, radiotherapy, chemotherapy or any other anti-tumor treatments. Patients with incomplete clinical data or other primary tumors were excluded. Blood samples from subjects were collected before any radical treatment according standard operating procedures.[21] The control CLD samples were obtained from patients who were treated for CLD in the infectious diseases department at the same institution. Pathogenic factors of CLD were related to HBV, HCV, alcoholism, or “other” causes. The diagnosis of CLD was based on the analysis of clinical manifestations, liver biopsy and imaging examination. The study protocol was approved by the Medical Ethics committee of the Second Affiliated Hospital of Nanchang University, and all study subjects were provided with written, informed consent. For each subject, the following clinical data were collected: age, gender, race, etiology of liver disease, white blood cell counts (WBC), hemoglobin, total bilirubin (TBil), albumin (ALB), alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), and 5-Hydroxymethylcytosine (5hmC). The derivation cohort was developed based on these blood samples.

Additional samples from the validation cohort were used to validate the diagnostic model derived from the development cohort. These were obtained between January 2019 and June 2019 at the Tumor Hospital of Jiangxi Province, Nanchang, China. The validation study included 40 subjects; of these, 25 HCC patients and 15 controls with CLD were included in this analysis and the same screening criteria were used to validate the performance of the model (Table 1). For each subject, these clinical data were collected: age, gender, race, etiology of liver disease, white blood cell counts (WBC), hemoglobin, total bilirubin (TBil), albumin (ALB), alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), and 5-Hydroxymethylcytosine (5hmC). This study was approved by the Medical Ethics committee of the Tumor Hospital of Jiangxi Province. The validation cohort was developed based on these blood samples.

Assays For 5hmc And Afp

To access the genome-wide 5hmC profiles and gains in cfDNA, we used the 5hmC-Seal, a highly sensitive and selective chemical labelling-based sequencing technology developed and optimized by YiBien Technology Co.,Ltd (Shanghai, China). Detailed information about preparation of cfDNA, 5hmC-Seal library construction, sequencing and data processing has been reported in the literature.[21] As AFP is a routine examination for patients with HCC or CLD, measurements were collected via clinical data. No adverse events occurred during this study.

Statistical analysis

Model development

Continuous variables that confirm a normal distribution are represented as mean $\pm$ standard deviation, and categorical variables are represented as frequencies. For univariate and multivariate analyses, logistic regression was employed to calculate the odds ratios (ORs) to evaluate the strength of the correlation with liver cancer. In the multivariable models, age, sex, albumin, bilirubin, 5hmC, AFP, were weighed as risk factors. For statistical analysis, patients with missing data were removed; more than 96% of cases had complete data on diagnostic model evaluations. Log transformation was performed due to the extreme skew of the data. Logistic regression was based on a complete case study applying a stepwise backward selection method. When the model was established, the Hosmer-Lemeshow test was used to evaluate the goodness of fit for the diagnostic model, and the area under the curve of 5hmC and AFP was calculated to evaluate the sensitivity, specificity, and optimal threshold of the diagnosis of liver cancer. VIF (variance inflation factor) was used to evaluate whether the research factors had collinearity in the model. Data were analyzed by GraphPad Prism 7.0 (San Diego, CA) and SPSS 22.0 (SPSS, Inc., Chicago, IL). Measurement data are represented as mean $\pm$ standard deviation.

Model Validation

Model validation was performed in a data set of 40 patients diagnosed with HCC or CLD. Using the model derived from the development cohort, the predictive risk score was obtained to identify patients with and without HCC. This was then compared with the actual diagnosis value.

Table 1. Characteristics of patients with hepatocellular carcinoma and CLD

Factors	Derivation		Validation	
	HCC (n=64)	CLD (n=32)	HCC (n=25)	CLD (n=15)
Demographic data				
Age (years)	56.31±12.14	50.35±11.56	56.40±13.02	48.00±10.13
Male (no.)	85.8%(55)	68.8%(22)	84.0%(21)	73.0%(12)
Etiology				
HBV	42	20	17	11
Alcohol	11	5	4	2
HCV	2	1	1	0
HBV+HCV	1	2	0	0
Other				
PBC	2	1	1	0
NASH/NAFLD	3	1	0	1
Unknown	3	2	2	1
LFTs				
WBC (10 ⁹ /L)	7.19±5.24	4.83±1.73	6.33±2.67	4.11±1.27
Hemoglobin (g/L)	125.22±24.49	117.48±23.50	114.16±19.45	116.40±22.40
TBIL (umol/L)	32.54±31.37	35.25±33.19	33.61±32.27	36.06±34.55
Albumin	31.60±2.86	32.76±3.27	30.88±2.47	31.54±3.01
HCC biomarkers				
AFP (ng/ml)	284.29±162.24	12.45±8.08	269.36±150.44	11.30±7.03
Log ₁₀ AFP (ng/mL)	1.82±0.92	0.93±0.46	1.81±0.90	0.74±0.35
CEA	1.92±1.08	1.76±1.04	1.86±1.17	1.69±1.01
5hmC	641.56±278.73	134.71±122.30	599.84±218.84	126.20±118.55
Log ₁₀ 5hmC	2.70±0.51	2.04±0.29	2.67±0.57	1.69±0.34

NOTE: For all continuous variables, values are presented either as mean (±SD).

Abbreviations: PBC, primary biliary cirrhosis; NASH, nonalcoholic steatohepatitis; NAFLD, nonalcoholic fatty liver disease; WBC, white blood cell counts; TBIL, total bilirubin; AFP, alpha fetoprotein; CEA, carcinoembryonic antigen; 5hmC, 5-Hydroxymethylcytosine.

Results

Patient characteristics

Two phases were included in our study (Fig. 1). Characteristics of the patients in two cohorts are summarized in Table 1. The development cohort included blood samples from 64 patients with hepatocellular carcinoma; 61 had tangible evidence of relevant CLD and the presence or absence of underlying CLD could not be confirmed in the remaining 3. The corresponding data for the non-malignant liver disease (CLD) group were 93.8% and 6.2%, respectively. Patient mean age in the HCC group was 56.3 years, while the CLD group had a mean age of 50.3. Both groups had a greater proportion of males. Among all the data included in the derivation cohort, data integrity was higher than 98%.

The Validation cohort included 40 patient samples, including 25 patients with HCC and 15 patients with CLD. Among the liver cancer patients, 23 had underlying disease with CLD, and the presence or absence of CLD could not be determined in the other 2. The corresponding data for the non-malignant liver disease (CLD) group were 93.3%, and 6.7%, respectively. Patient mean age in the HCC group was 56.4 years, while the CLD group had a mean age of 48.0. Both groups had a greater proportion of males. The

development and validation cohorts had similar demographic characteristics in terms of mean age and male to female ratio, with most of the patients in the two cohorts exhibiting CLD etiology.

The median values of log (AFP), log (5hmC) in patients with HCC was higher than in patients with CLD (Fig. 2A-B). Moreover, the two biomarkers revealed effective discriminatory capacity for distinguishing between liver cancer and CLD patients (auROCs: log (AFP) 0.816, log (5hmC) 0.891; Fig. 3; Supplementary Table 3).

Model Performance In The Development Cohort

Based on the discovery data set, 4 variables were included in the statistical diagnostic model: log (AFP), log (5hmC), and age and sex, which were the most important factors for identifying patients with HCC (Supplementary Table 2). The diagnostic model for identifying patients with HCC used the following formula: $Z = -18.160 + 0.081 \cdot \text{age} + 1.867 \cdot \text{sex} + 1.066 \cdot (\log \text{AFP}) + 1.964 \cdot (\log 5\text{hmC})$

Correlation Between Risk Factors

Because four variables were contained in the diagnostic model, the occurrence of multiple collinearity was possible, which can affect the fit of the diagnostic model. Therefore, the variance inflation factor was used to test for multicollinearity in the regression model. The obtained coefficient of variance inflation was 1.164 for age, 1.034 for sex, 1.187 for AFP, and 1.351 for 5hmC, indicating that there was no multicollinearity between variables. Therefore, it was considered reasonable to keep these risk factors in the model

Goodness-of-fit Test And The Auroc

The Hosmer-Lemeshow goodness-of-fit statistical analysis was used to assess the goodness-of-fit of the diagnostic model. The results showed that $P = 0.778$, $\chi^2 = 4.805$, indicating that there was no significant difference between the predicted diagnosis and actual diagnosis. A receiver operating characteristic curve analysis was performed on the diagnostic model, and the results showed that the area under the receiver operating characteristic (auROCs) of the model for predicting the diagnosis of patients with HCC in the CLD individuals was 0.973, 95% (0.945–1.000), (Fig. 3; Supplementary Table 3), indicating that the new model had sufficient discrimination ability.

Model Performance Of The External Validation Cohort

Validation cohort

In the validation cohort, data from 40 patients were collected to assess the discriminating power of the diagnostic model using the above formula. P values were calculated for each individual patient. Using 0.7636 as the cutoff point, when the p value was > 0.7636 , patients were considered to have HCC and when the p value was < 0.7636 , they were considered to have a normal CLD. The p values of 21 patients were greater than 0.7636 and 19 patients had p values below 0.7636. Among the 21 patients who were considered to have HCC according to the diagnostic model, 19 were confirmed to have HCC. Among the 19 patients who were considered to have no HCC according to the diagnostic model, 13 were confirmed to have a normal CLD. Therefore, the sensitivity and specificity of the model in the validation cohort were 76.00% (19/25) and 86.67% (13/15), respectively (Supplementary Table 4).

Discussions

HCC is a severe malignancy with high mortality worldwide.[25] Although advanced surgical techniques and radiation therapy have recently been proposed, the survival of patients with HCC remains poor.[26–27] The above clinical characteristics highlight the need for more efforts in the prevention and early detection of HCC.[5–7] In the current study, we developed a model based on simple serological biomarkers which had high-efficiency for diagnosing the presence or absence of HCC in CLD patients. This method provides a novel and simple way to diagnose HCC, allowing for early intervention in these high-risk patients.

Measurement of serum AFP and abdominal ultrasound examination are widely used as screening tools for the early detection of HCC in chronic liver disease patients.[28] However, the diagnostic efficiency of these methods remains poor.[5] Studies have confirmed that some patients with liver injury and embryogenic tumors also have elevated serum AFP levels, while ultrasound detection has different degrees of missed diagnosis and misdiagnosis.[29] Therefore, finding better tumor markers is necessary for the diagnosis of early liver cancer.

Recent studies have shown that 5hmC, an intermediate product in the process of cytosine demethylation, exerts a significant influence in the regulation of gene expression and is considered to be an effective epigenetic biomarker for tumor diagnosis or prognosis.[21] For example, Zhang et al., detected the genome wide distribution of 5hmC in cfDNA from 66 patients with NSCLC and 67 healthy individuals, identifying the 5hmC signatures of cfDNA with high sensitivity and specificity for NSCLC.[30] In a prospective study of 1204 patients with HCC and 392 high-risk Chinese patients with chronic hepatitis or liver cirrhosis, Cai et al., demonstrated the 5hmC-Seal approach to be clinically useful, outperforming AFP, with the potential to be a novel class of epigenetic biomarkers.[23] In addition, the development of highly sensitive 5hmC markers in cfDNA could serve as a promising noninvasive approach for HCC diagnosis for the general population.[21] In this study, based on a sensitive 5hmC-Seal method, the profiles of cell-free 5hmC in the blood of HCC patients and CLD subjects were obtained. This method exhibited higher sensitivity and specificity compared with AFP. Even at low or negative AFP values, there remained significant discriminating capacity.

Ideal tumor marker detection requires high sensitivity, early detection of tumor patients, high specificity, and organ specificity.[31] The detection of single tumor markers could not satisfy these factors, therefore, a combination of multiple serum tumor and risk factors associated with cancer would be more clinically useful for the early diagnosis of tumors.[31–32] Using logistic regression, we identified the independent factors associated with hepatocellular carcinoma and elucidated the relationship between HCC and these factors. The results were a good combination of these independent clinical indicators and provide a model established and evaluated based on sensitivity, specificity, and correct diagnostic proportions.

The current study findings have not fully confirmed or elucidated the specific mechanisms of liver cancer. However, epidemiological studies have confirmed that CLD is an important risk factor for liver cancer, and more than 70% of liver cancer is caused by CLD.[33] CLD patients are a high-risk group for the occurrence of liver cancer, and this has been reported to be more common among elderly patients.[4] This finding is consistent with the results of our study, which indicated that age was an important factor for identifying patients with HCC. Further, there is a significant gender difference in HCC, and the incidence of male HCC is more than twice that of female HCC. This study finding was also consistent with many reports in the literature.[34–36] According to the logistic regression analysis, AFP and 5hmC also indicated the presence of HCC. Therefore, the model is based on four objective variables: age, sex, AFP, 5hmC. In the model development, the variance inflation factor test confirmed that there was no multicollinearity in the regression model, and the goodness-of-fit testing confirmed the rationality of the diagnostic model based on the four objective variables.

The optimal way to validate a diagnostic model is to assess its performance in an independent patient cohort. We used a cohort of patients diagnosed with HCC or CLD who were treated at a single hospital from January 2019 to June 2019. The model performed well in the validation cohort compared with the derivation cohort (sensitivity: 76.00%, specificity: 86.67% vs sensitivity: 89.10%, specificity: 96.90%). The model had comparable discrimination ability in the validation cohort, demonstrating that this model had an acceptable and stable discrimination ability for diagnosing liver cancer in patients with CLD, and indicating that this model is valuable for identifying HCC.

Early diagnosis of and intervention in high-risk populations is regarded an effective treatment for HCC. [37–38] Various techniques have been used clinically to detect tumor information, including methods based on liquid biopsy such as early liquid biopsy assays targeting exosomes or noncoding RNAs.[39–40] Though promising, these approaches do not offer satisfactory performance in terms of specificity and sensitivity suitable for a clinical scale. Based on a cell-free 5hmC sequencing method, we detected an increased 5hmC gain in blood samples from HCC patients compared with CLD controls. Combined with AFP levels, sex, and age, we developed a diagnostic model for distinguishing HCC in high risk individuals with CLD, showing improved performance compared with normal diagnostic markers. Additionally, given its non-invasiveness and the tissue-specificity of 5hmC, the model may also serve as a convenient tool for detection in the general population.

Because this study had a limited number of cases and was based on a Chinese patient population with a high CLD background, further validation in prospective studies will be necessary to demonstrate the generalizability of the results to other populations and geographical regions. With further research, we believe this model will have superior performance and universality for HCC diagnosis.

Conclusions

In summary, we have identified important factors for HCC in CLD patients, and developed and validated a novel, non-invasive diagnostic model for surgically resectable HCC using the highly sensitive 5hmC-Seal assay. Compared with the pathological examination, preoperative blood testing is convenient, widely available, and routinely performed in clinical practice. This model may provide a useful tool for the preoperative diagnosis of and early intervention in HCC, which could lay the foundation for developing a future pan-cancer, non-invasive screening tool.

Abbreviations

HCC: Hepatocellular carcinoma; CLD: Chronic liver disease; 5hmC: 5-Hydroxymethylcytosine; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AASLD: American Association for the Study of Liver Disease; AFP: Alpha-fetoprotein; cfDNA: Cell-free DNA; 5mC: 5-methylcytosine; TET: Ten-eleven translocation; hMe-seal: Hydroxymethyl selective chemical labelling; AuROCs: Area under the receiver operating characteristic curve; WBC: White blood cell counts; Tbil: Total bilirubin; ALB: Albumin; AFP: Alpha-fetoprotein; CEA: Carcinoembryonic antigen; ORs: Odds ratios.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Medical Ethics committee of the Second Affiliated Hospital of Nanchang University, and all study subjects were provided with written, informed consent.

Consent for publication

Not applicable.

Availability of data and materials

The datasets obtained in the study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no conflict of interests.

Funding

This investigation was funded the Key Foundation of Jiangxi Provincial Science and Technology Department (No. 20171ACB21064), the Youth Science Foundation of Jiangxi Provincial Science and Technology Department (No. 20151BAB205105). The funders had no role in the design of the study and collection, analysis, and interpretation of data and in producing the manuscript.

Author contributions

JG, C-YX, XY, Y-BX, X-GP, X-BY, D-JL and FZ designed the study. JG and C-YX collated the data, conducted data analyses, and produced the initial draft of the manuscript. XY, Y-BX, X-GP, X-BY, D-JL and FZ contributed to drafting the manuscript. All authors have read and approved the final submitted manuscript.

Acknowledgements

We thank YiBien Technology Company for the technical assistance with 5hmC-seal sequencing and the members of our research team for their contributions. We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

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Supplementary Table Legends

supplementary Table2 Parameter estimates (SE) and OR (95% CI) of variables based on the model.

supplementary Table3 Diagnostic performance of biomarkers alone and the model performance.

supplementary Table4 Sensitivity and specificity analysis in the validation cohort.

Figures

Figure 1. Study design. HCC, Hepatocellular carcinoma; CLD, chronic liver disease; AFP, alpha-fetoprotein; 5hmC, 5-hydroxymethylcytosine; ROC, receiver operating characteristics.

Figure 1

Study design. HCC, Hepatocellular carcinoma; CLD, chronic liver disease; AFP, alpha-fetoprotein; 5hmC, 5-hydroxymethylcytosine; ROC, receiver operating characteristics.

Figure 2

(A) Log AFP values in the HCC and CLD patients. (B) Log 5hmC values in the HCC and CLD patients. **P < 0.01

Figure 3

The ROC curves for Log AFP, log 5hmC and model for predicting the diagnosis of patients with HCC in the CLD individuals.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable3.docx](#)
- [SupplementaryTable4.docx](#)
- [SupplementaryTable2.docx](#)