High expression of 5-hydroxymethylcytosine is associated with a more favorable hepatoblastoma prognosis in Chinese patients

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Abstract

Background

To analyze associations between the strength of expression of 5-hydroxymethylcytosine (5hmC) and survival outcomes of Chinese hepatoblastoma patients.

Methods

We collected and analyzed clinical data of hepatoblastoma patients aged <15 years treated at the Sun Yat-Sen University Cancer Center or the First Affiliated Hospital of Sun Yat-sen University from Feb 2010 to Sept 2018. Patients were treated according to the Children's Oncology Group protocol. Specimens for pathology were collected by biopsy or surgical resection before initiation of chemotherapy. The level of expression of 5hmC was analyzed in tissue samples from 100 patients by immunohistochemistry. The prognostic value of 5hmC was evaluated by Cox regression and Kaplan-Meier analyses.

Results

We enrolled 100 patients with hepatoblastoma (median follow-up, 43.0 months; range, 18.4–131.6). The total recurrence rate was 30.0%. Three-year overall survival (OS) rates of high and low 5hmC expressors was $92.6\% \pm 3.6\%$ and $80.3\% \pm 5.9\%$, respectively. Three-year event-free survival (EFS) of high and low expressors was $84.4\% \pm 5.1\%$ and $49.8\% \pm 7.7\%$, respectively. Thus, high levels of 5hmC expression are associated with more favorable OS and EFS. Multivariate analysis indicated that 5hmC expression level was an independent prognostic indicator for OS and EFS.

Conclusions

Our findings showed that strong hepatoblastoma 5hmC expression was associated with lower recurrence rates and longer EFS and OS. Thus, 5hmC expression may have prognostic relevance in hepatoblastoma.

Background

Hepatoblastoma (HB) is the most common pediatric malignant tumor of the liver, accounting for nearly 80% of primary liver tumors in children [1]. With the rapid development of medical science and technology, therapeutic options for HB have been greatly improved. In the past 40 years, the overall survival (OS) rate of HB has increased from 30% to about 80% [2]. Accurate pathological diagnosis, classification and clinical staging are the main bases for formulating individual treatment plans and predicting the prognosis of children with Hb [3]. However, the prognosis of high-risk hepatoblastoma is still very poor, and 5-year OS is 54% ~ 58% [4, 5]. Therefore, new biomarkers are urgently needed to identify patients with a high risk of recurrence and/or poor survival.

Epigenetics is a promising new field in cancer research, and DNA methylation is an important model for epigenetic characterization [6]. The detection of 5hmC represented a breakthrough in the field of epigenetics [7]. The conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) is catalyzed by proteins encoded by the ten-eleven translocation (TET) gene family, which consists of TET1, -2, and -3 [8, 9]. 5hmC is an intermediate residue in the process of active demethylation, which seems to be related to pluripotency maintenance and transcriptional activation of stem cells [10]. At present, the biological mechanism of action of 5hmC in cancer is still unclear. Liu et al. reported that 5hmC levels correlate positively with less aggressive tumor behavior in hepatocellular carcinoma [11].

In the present study, we applied immunohistochemical methods to retrospectively establish a correlation between 5hmC protein expression levels and clinical outcomes of hepatoblastoma. We studied the relationship between 5hmC and tumor history, clinicopathological characteristics (including age, gender, stage, OS and event-free survival (EFS)). We found that strong 5hmC protein expression was an independent favorable prognostic factor in children with hepatoblastoma.

Patients And Methods

Ethics statement

This research was approved by the Institutional Review Board (IRB) of Sun Yat-sen University Cancer Center (SYSUCC; Guangzhou, China), IRB number B2021-324-01. Written informed consent was obtained from each patient's parents involved in the study. All the original data are deposited at <http://www.researchdata.org.cn>.

Patients and Specimens

The patients included in this study were newly diagnosed as having HB at the SYSUCC and the First Affiliated Hospital of Sun Yat-sen University, between Feb 2010 and Sept 2018. The criteria for enrolling patients were as follows: 1) pathologically diagnosed as HB; 2) age less than 18 years; 3) tissue specimens obtained before initial treatment; 4) complete and detailed treatment process and follow-up data available. The specimens were obtained from HB patients by needle biopsy or open surgery. Chemotherapy regimens of different risk groups are shown in Supplementary Tables 1 and 2.

Immunohistochemistry And Evaluation Criteria

The tissue specimens were fixed in formalin and embedded in paraffin blocks, and then sectioned at a thickness of 4 μ m. Paraffin-embedded sections were deparaffinized in xylene and rehydrated with decreasing concentrations of alcohol. Next, slides were soaked in 3% H₂O₂ water for 30 minutes to block endogenous peroxidase activity and then blocked with 10% FBS (Gibco; USA) for 1 hour to reduce non-specific staining. Incubation with anti-5hmC rabbit mAb (1:10000, Active Motif, USA) followed at 4°C overnight. The sections were finally incubated with goat anti-rabbit secondary antibody (1:10000; ZSGB BIO, China) for 2 hours, dehydrated and sealed after visualization with DAB (ZSGB BIO).

The immunohistochemical staining results were evaluated according to the intensity of staining and the proportion of tumor cells with an unequivocal positive reaction. The intensity was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. The frequency of positive cells was defined as follows: 0, less than 5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; and 4, greater than 75%. The composite score is the product of these two scores. Composite scores of 0 to 5 were considered to indicate low or negative expression, and 6 to 12 were taken as strong expression. A fluorescence microscope (Olympus BX61, Japan) was employed for image acquisition. Scoring was done independently by two pathologists.

Follow Up

After treatment, patients were reviewed and followed up regularly. Monitoring included alpha-fetoprotein (AFP), whole blood routine, biochemical routine, electrocardiogram, echocardiogram, chest and abdomen CT. The last follow-up time was on 23rd Dec, 2020 and the median observation time was 43 months. OS was defined at the endpoint of the study as the period from the date of initial diagnosis to death or the last follow-up. EFS was defined as the period from the date of initial diagnosis to the occurrence of an event or the last follow-up. The occurrence of an event included disease recurrence, progressive disease, death, or diagnosis of a second malignant neoplasm.

Statistical analysis

All data analyses were performed using SPSS software (version 25.0, SPSS Company, Chicago, Illinois, USA). OS and EFS were calculated by the Kaplan-Meier method and compared using the log-rank test. Prognostic factors predicting OS and EFS were assessed by multivariate Cox proportional hazards regression analyses. All covariates that had clinical significance were included in a multivariate Cox proportional hazards model. Results are given as mean $\pm$ S.D. All statistical tests were two-sided, and $P < 0.05$ was taken to indicate a significant difference.

Results

Immunohistochemical Characteristics

Typical examples of immunohistochemical staining are shown in Figure 1. We observed 5hmC staining mainly of the nuclei of tumor cells and hepatocytes, with very little cytoplasmic staining.

Baseline Characteristics

A total of 157 patients diagnosed with HB was identified during the study period. Based on our inclusion criteria, 57 patients were excluded because no tissue specimens before initial treatment were available for 37 (23.6%), complete and detailed treatment process data were not available for 14 (8.9%), and the remaining 6 were lost to follow-up (3.8%). The patients' baseline characteristics are summarized in Table 1. A total of 100 patients was finally included in the study, 63% being male and 37% female, with a median age

at diagnosis of 20.0 months (range, 0.9–107.0 months). AFP was elevated (>1000 ng/mL) in 92% of patients. Based on the results of the imaging studies, patients were classified using the PRETEXT staging system. Twenty patients had distant metastasis, 17 had vascular invasion and 6 had tumor rupture at diagnosis. Fetal histology (n=32) was the most common pathological subtype in our patients according to postoperative pathology results. All patients were classified using the The Children's Oncology Group (COG) risk group scale. Using the COG risk stratification system, zero patients were considered as being very low-risk, 9 as low-risk, 52 as intermediate-risk, and 39 as high-risk. There were 54 5hmC^{High} and 46 5hmC^{Low} patients. Correlations of 5hmC expression with clinicopathologic characteristics are shown in Table 1.

Table 1
Characteristics of patients included in the study

Clinicopathological indexes		No. of patients		
		5-hmC ^{low}	5-hmC ^{high}	P
Gender	Male	29	34	0.993
	Female	17	20	
Age(months)	≤36	30	40	0.335
	≥36	16	14	
AFP at diagnosis (ng/mL)	≤1000	2	6	0.214
	>1000	44	48	
PRETEXT staging	I	4	5	0.842
	II	23	30	
	III	9	7	
	IV	10	12	
Metastasis at diagnosis	No	37	43	0.920
	Yes	9	11	
Vascular Invasion	Absent	42	41	0.041
	Present	4	13	
Tumor Rupture	No	44	50	0.521
	Yes	2	4	
Histological subtype	Epithelial	2	4	0.420
	Fetal(Pure Fetal excluded)	11	19	
	Pure Fetal	0	2	
	Embryonal	4	2	
	Mixed embryonal–fetal	9	5	
	Mixed epithelial–mesenchymal	7	11	
	macrotrabecular	1	1	
	HB (unkown)	12	10	
COG risk group	Very low risk	0	0	0.380
	Low risk	6	3	

Clinicopathological indexes	No. of patients	
Intermediate risk	24	28
High risk	16	23

Survival

The median follow-up period was 43.2 months (range, 18.4–131.6 months). The 1- and 3-year OS of the 100 investigated patients was 94.0 ± 0.24 and $86.9 \pm 0.34\%$, respectively. The median OS was 40.4 months. The 1- and 3-year DFS was $84.0 \pm 0.50\%$ and $67.9 \pm 0.37\%$, respectively. The median DFS was 33.3 months. The difference between patients with 5hmC^{High} and 5hmC^{Low} was statistically significant for both OS ($P=0.044$, Figure 2a) and DFS ($P < 0.001$, Figure 2b).

Univariate And Multivariate Analysis Of Prognostic Factors

All factors listed in Table 1 were included in univariate analyses (Table 2), of which age at diagnosis (OS 92.7% vs 73.3% $P=0.010$, DFS 75.2% vs 50.1% $P=0.011$), PRETEXT staging (OS 95.0% vs 73.3% $P=0.005$, DFS 80.5% vs 47.8% $P=0.001$), metastasis at diagnosis (OS 92.3% vs 65.0% $P=0.001$, DFS 73.2% vs 48.1% $P=0.032$), COG risk group (OS 98.2% vs 69.2% $P < 0.001$, DFS 82.2% vs 45.2% $P < 0.001$) and 5hmC expression level (OS 92.6% vs 80.3% $P=0.044$, DFS 84.7% vs 49.8% $P < 0.001$) were identified as significant prognostic factors for clinical outcome. Multivariate analyses showed that of these factors, age at diagnosis (HR 3.293, 95% CI 0.966-11.224, $P=0.057$), COG risk group (HR 8.661, 95% CI 1.332-56.299, $P < 0.001$) and 5hmC expression level (HR 0.230, 95% CI 0.068-0.781, $P=0.044$) were independent prognostic factors for OS (Table 2). For DFS, multivariate analysis showed that only COG risk group (HR 4.387, 95% CI 1.577-12.202, $P < 0.001$) and 5hmC expression (HR 0.197, 95% CI 0.085-0.458, $P < 0.001$) were independent prognostic factors (Table 2).

Table 2

Univariate analysis and multivariate analyses of 5-hmC expression associated with survival and recurrence

	Overall survival				Event-free survival			
	Univariate P	Hazard ratio	95% CI	P	Univariate P	Hazard ratio	95% CI	P
Gender	0.645			NA	0.918			NA
Age at diagnosis($\leq$ 36 months vs. $\geq$ 36 months)	0.010	3.293	0.966-11.224	0.057	0.011			NS
AFP ($\leq$ 1000 vs. $>$ 1000)	0.268			NA	0.298			NA
PRETEXT staging(I/II vs. III/IV)	0.005			NS	0.001			NS
Metastasis(Yes vs. No)	0.001			NS	0.032			NS
Vascular Invasion(Absent vs. Present)	0.032			NS	0.259			NA
Tumor Rupture(Yes vs. No)	0.338			NA	0.605			NA
COG risk group(Low/Intermediate vs. high)	$\leq$ 0.001	8.661	1.332-56.299	0.024	$\leq$ 0.001	4.387	1.577-12.202	$\leq$ 0.001
5-hmC expression (high vs. low)	0.044	0.230	0.068-0.781	0.018	$\leq$ 0.001	0.197	0.085-0.458	$\leq$ 0.001

Discussion

At present, there are few data on the relationship between 5hmC and hepatoblastoma prognosis. In this study, we explored the relationship between the level of expression of 5hmC by immunohistochemistry and the clinical features and prognosis of 100 cases of hepatoblastoma. We investigated recognized clinical prognostic factors (diagnostic age, gender, AFP level, etc.) and factors used for risk classification (PRETEXT stage, metastasis, vascular invasion, tumor rupture, etc.) [12]. Other variables have low prognostic value and were therefore ignored. This study is limited to patients in a single center, which may introduce bias. We found that the level of expression of tumor 5hmC was not correlated with gender, age, AFP level at diagnosis, PRETEXT stage, metastasis, histological subtype or COG risk group. However, in univariate and multivariate analysis, the expression of 5hmC was identified as an independent prognostic factor. This suggests that the expression of 5hmC may be a powerful prognostic factor in hepatoblastoma.

Thus far, a large number of studies has reported a relationship between 5hmC and several different tumors (malignant melanoma, cervical squamous cell carcinoma, non-small cell lung cancer, etc.) [13–16]. Usually, 5hmC is mainly present in embryonic stem cells and adult neural cells. In hepatocellular carcinoma, the expression of 5hmC was reported to be lower than in the surrounding normal tissues [17, 18]. It was also reported that in adult cancer, lower TET expression can reduce the level of 5hmC in solid and hematopoietic tumors [9, 13, 19, 20]. However, the activity of these enzymes in pediatric tumors has rarely been established. Studies have shown that 5hmC profiles are prognostic for the outcome of neuroblastoma [21, 22]. Rivas et al. reported increased expression of the TET gene in hepatoblastoma [23]. Animal models indicated that overexpression of TET inhibited differentiation during embryonic development [9, 20, 24, 25]. It is speculated that the up regulation of TET in hepatoblastoma indicates that liver differentiation is blocked, which is consistent with the long-suggested hypothesis that inhibiting the differentiation pathway of organ stem cells leads to embryonic tumorigenesis. The increase of 5hmC content in these tumors confirms the functional role of the observed TET overexpression. Although the previous study of Rivas et al. assessed the expression of 5hmC in archived hepatoblastoma tissues, the number of cases was limited and lacked further validation [23]. Our study is the largest analysis of 5hmC protein expression in hepatoblastoma.

The main limitation of this work at present is its retrospective nature. However, it is worth noting that this is a sample of children with hepatoblastoma in China. Hepatoma is a rare tumor with a low incidence rate, and to the best of our knowledge the present study is the first to report the significance of 5hmC expression for hepatoma prognosis. Our results show that high expression of 5hmC predicts relatively less invasive tumor behavior. Importantly, 5hmC expression enables us to more accurately predict the true prognosis of patients with hepatoblastoma. It is worth noting that 5hmC can be measured using a simple technique (immunohistochemistry), so it can be carried out in any reasonably equipped clinical pathology laboratory; also, the antibody is a commercially-available monoclonal, so it should produce robust and reproducible results. Additionally, the scoring system is simple and the consistency between observers is good. Histopathologists can easily use the same sections for diagnostic evaluation. All these features increase the opportunity to translate this biomarker into clinical application. A scoring system based on image analysis is possible and can make scoring easier, but it is not easy for histopathologists because they review cases under a microscope and increase the complexity of biomarker translation.

Future work will need to accurately determine how 5hmC should be used. In particular, it will be important to assess whether combination with other biomarkers and with clinicopathological features can increase accuracy of prediction. However, this requires a much larger study. Ideally, thousands of cases will eventually form a statistical prediction model, at which time the prognostic scoring system of new cases will need to be externally verified. The present study provides strong support for exploring this approach. In addition, the epigenetics of 5hmC may help to classify hepatoblastoma risk and guide clinical targeted treatment strategies in the future.

Conclusion

In this cohort of Chinese children with hepatoblastoma, higher 5hmC levels correlated with less aggressive tumor behavior. Low 5hmC expression was associated with lower OS rates and higher cumulative recurrence rates. 5hmC expression may have prognostic relevance in hepatoblastoma.

Abbreviations

Abbreviation	meaning
5hmC	5-hydroxymethylcytosine
OS	overall survival
EFS	event-free survival
HB	Hepatoblastoma
TET	ten-eleven translocation
IRB	Institutional Review Board
SYSUCC	Sun Yat-sen University Cancer Center
AFP	alpha-fetoprotein
COG	The Children's Oncology Group

Declarations

Funding

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Conflicts of interest/Competing interests

The authors declare no conflict of interest.

Availability of data and material

All original data were deposited online <http://www.researchdata.org.cn>.

Consent for publication

Patients' legal guardians have signed informed consent regarding publishing their data and photographs.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Junting Huang, Yang Hu, Huadong Chen. The first draft of the manuscript was

written by Juntong Huang and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

The study was approved by the Review Board (IRB) of Sun Yat-sen University Cancer Center.

Consent to participate

Informed consent was obtained from legal guardians.

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Figures

Figure 1

Typical picture of IHC analysis in HB tissue with different 5hmC expression levels. (scale bar, 50μm)

Figure 2

Kaplan-Meier survival curves about OS(a) and DFS(b) in 100 HB patients expressing different 5hmC levels

Supplementary Files

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