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Hypomethylation of CTCFL promoters as a noninvasive biomarker in plasma from patients with hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is the third deadliest cancer in the world with high morbidity and poor prognosis. CTCFL (CCCTC-binding factor like) is a member of the cancer testis antigen (CTA) family with oncogenic properties. To demonstrate whether the hypomethylation of CTCFL promoters in plasma could be used as a noninvasive biomarker to predict poor prognosis of HCC, we extracted cell-free DNA from the plasma and detected the methylation status of CTCFL in 43 HCC, 5 liver cirrhosis and 6 benign lesion samples using methylation specific PCR (MSP). Our study indicated that the hypomethylation of CTCFL promoters in HCC plasma samples (60.4%) was significantly different from that in benign lesion plasma samples (16.7%) with a p-value of 0.043. Analysis of clinicopathological data showed that the methylation status of CTCFL promoters was significantly correlated with microvascular involvement (MVI) (p=0.001) and postoperative recurrence (p=0.031). Furthermore, clinical prognosis data of 347 HCC patients from The Cancer Genome Atlas (TCGA) database displayed that the hypomethylated group had worse overall survival than the hypermethylated group (p=0.0056). In conclusion, we provide evidence that the hypomethylation of CTCFL promoters in cell-free DNA is a biomarker for monitoring HCC patients, which can be used as a noninvasive prediction index for tumor recurrence and provide the individualized decision-making for clinicians.

Key words: hepatocellular carcinoma, hypomethylation, liquid biopsy, biomarker, recurrence, prognosis

Liver cancer, predominantly hepatocellular carcinoma (HCC), is the third leading cause of cancer death worldwide [1]. Complex etiological factors attribute to the occurrence of liver cancer, such as chronic hepatitis B and C virus (HBV/HCV) infections, alcohol abuse, diabetes, obesity, and some metabolic diseases [2, 3]. Genetic and epigenetic alterations induced by these risk factors play a significant role in the carcinogenesis of hepatocellular carcinoma [4]. Treatments including surgical resection, transplantation, ablation, transarterial chemoembolization, and tyrosine-kinase inhibitors are proven to have survival benefits for HCC patients. Although major progress has been made in the prevention, detection, diagnosis, and treatment of HCC, its prognosis remains poor [5]. Hence, it is urgent to investigate noninvasive biomarkers for monitoring HCC patients.

Recently, liquid biopsies, including the detection of circulating tumor cells, cell-free nucleic acids, and extracellular vesicles, are gradually being implemented in cancer patients [6]. Cell-free DNA (cfDNA) is a small fragment that can be detected in plasma, and the concentration of cfDNA in individuals with cancer can be 50 times higher than normal [7]. Tumor-related genetic and epigenetic alterations, which include single nucleotide mutations, copy number aberrations, and aberrant methylation, have been detected in cfDNA of cancer patients and are associated with tumor burden and malignant progression [8, 9]. Aberrations in DNA methylation, such as hypermethylation of tumor suppressor genes in cfDNA, have been shown as a powerful prognostic factor [10].

Global DNA hypomethylation occurs in most types of cancer [11]. DNA hypomethylation in tumors is accompanied by abnormal activation of "cancer-testis" genes, which contribute to the key process of tumorigenesis and exhibit carcinogenic characteristics [12]. BORIS (Brother of the Regulator of Imprinted Sites), also called CTCFL (CCCTC-binding factor like), is identified as a paralog of CTCF (CCCTC-binding factor) [13]. CTCFL is one of the cancer testis antigen (CTA) family with 11 zinc finger DNA-binding domains [14], and it is predominantly expressed in

spermatocytes during early male germline development. On the contrary, CTCFL is barely expressed in normal tissues other than the testis [15, 16]. But the situation is different in tissues following neoplastic transformation. It's worth noting that CTCFL is abnormally expressed in many primary tumors and cancer cell lines [17–20]. Studies have shown that CTCFL can promote the development, invasion, and metastasis of tumors [21–24]. In addition, epigenetic alterations of CTCFL are considered to be of great value, and the activity of CTCFL promoters is thought to be chiefly regulated by DNA methylation [25, 26]. Hypomethylation of CTCFL promoters results in the universal expression of CTCFL in endometrial cancer, gastric cancer, and hepatocellular carcinoma, which is highly correlated with clinical stage and prognosis [27–29].

To determine whether CTCFL could be used as a biomarker for liquid biopsies of HCC patients, we extracted cfDNA from the plasma of 76 patients and detected the methylation status of CTCFL promoters. Moreover, we analyzed the correlation between hypomethylation of CTCFL promoters and clinicopathological characteristics. Finally, TCGA data were used to analyze the relationship between methylation and prognosis.

Patients and methods

Cell culture. K562 cells (purchased from the cell bank of the Chinese Academy of Sciences) were cultured in RPMI-1640 media (HyClone, USA), supplemented with 10% fetal bovine serum (Gibco, USA), 100 units/ml penicillin and 100 micrograms/ml streptomycin. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2.

Plasma samples and normal liver tissues. Normal liver tissues (accidental death) and 76 plasma samples were obtained from the West China Hospital of Sichuan University. All plasma samples were stored at –20 °C, and consisted of 43 HCC, 19 liver cirrhosis, and 14 benign lesion plasma samples.

DNA extraction and bisulfite conversion. The genomic DNA was extracted from plasma samples with the Axy Prep Body Fluid Viral DNA/RNA Miniprep Kit (Axy Prep, China). And the genomic DNA from cells and tissues was extracted by using the TIAN amp Genomic DNA Kit (TIANGEN, China). Complete bisulfite conversion of GC-rich DNA was performed by using the EZ DNA Methylation-Gold™Kit (Zymo Research, USA).

Methylation specific PCR (MSP). We used nested PCR to examine the methylation status in the CpG island of CTCFL promoters. The primers were used previously [17]. Primer sequences of the first round of PCR: forward 5'-GTGTTTTTTTTGGGGTTTTTTTTAT-3' and reverse 5'-CCCAAAACAACCCATACTCTTAA-3'; and the following thermal cycle conditions: 95 °C for 6 min; (95 °C for 45 s, 56 °C for 30 s and 72 °C for 45 s) × 36 cycles; 72 °C for 6 min [28]. Primer sequences of the second round of PCR (MSP): unmethylated forward 5'-GTGTATTGTTATTTTTTATTTTTGTGTTAGTTT-3' and unmethylated reverse 5'-ACCCCTCACCACAAAAAACATAACCAA-3'; methylated forward 5'-GTATTGTTATTTTTTATTTTCGCGTTAGTTC-3' and methylated reverse 5'-CCCTCACCGCGAAAAACGTAACCGA-3'. The methylation status was detected by touchdown PCR. The thermal cycle conditions of MSP as follows: 95 °C for 6 min; (95 °C for 30 s, 66 °C for 30 s and decreasing progressively by 1 °C per cycle, 72 °C for 30 s) × 8 cycles; (95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s) × 32 cycles; 72 °C for 6 min.

DNA polyacrylamide gel electrophoresis and silver staining. PCR-amplified products were analyzed by the polyacrylamide gel electrophoresis. The 12% polyacrylamide gel was pre-run for 30 minutes at a constant 80V in the 1 ´TBE buffer, then the gel was run for 2.5 hours with samples. After electrophoresis, the 1% $AgNO_3$ solution was added to a plastic box containing the gel and the box was shaken for 15 minutes. Then the polyacrylamide gel was washed twice with ddH_2O, each time for 3 minutes. Finally, the developer was added, and the reaction was terminated when a clear band appeared. All of the solutions were prepared before use.

Bioinformatics analysis and statistical analysis. We utilized an interactive database (Disease Meth, http://bioinfo.hrbmu.edu.cn/diseasemeth/) that offered calculated methylation value of genes in human cancers [30]. Then we obtained prognostic information from The Cancer Genome Atlas (TCGA, USA) and performed Kaplan-Meier analysis to investigate overall survival (OS). Original methylation data and clinical data derived from TCGA.

To assess the correlation between the methylation status and clinicopathological characteristics, SPSS19.0 was installed. We performed the χ^2 test, Fisher's exact test, and χ^2 test for continuity correction. For all statistical data, $p<0.05$ was statistically significant.

Results

The CpG island of CTCFL promoters exhibited demethylation in HCC. DNA methylation occurs mainly in CpG islands, which are preferentially situated in the 5'UTR of genes overlapping promoters [31]. CTCFL has three promoters, defined as A, B, and C promoters, respectively, corresponding to transcription start sites at –1447, –899, and –658 bp upstream of the first ATG (Figure 1A). In addition, the CpG island of CTCFL that has a high frequency of dinucleotide CpG sites is located in promoters B and C (Figure 1A). We used an interactive database named Disease-Meth to analyze the methylation value of the CpG islands in 347 HCC tissues and 50 peritumoral tissues. Interestingly, the CpG island of CTCFL promoters showed significantly lower methylation value in tumor tissues compared with peritumoral tissues (Figure 1B). Therefore, it can be assumed that CTCFL promoters are demethylated in HCC patients.

The methylation status of CTCFL promoters in patients' plasma. We used nested MSP to test the methylation status of CTCFL promoters in HCC, cirrhosis, and

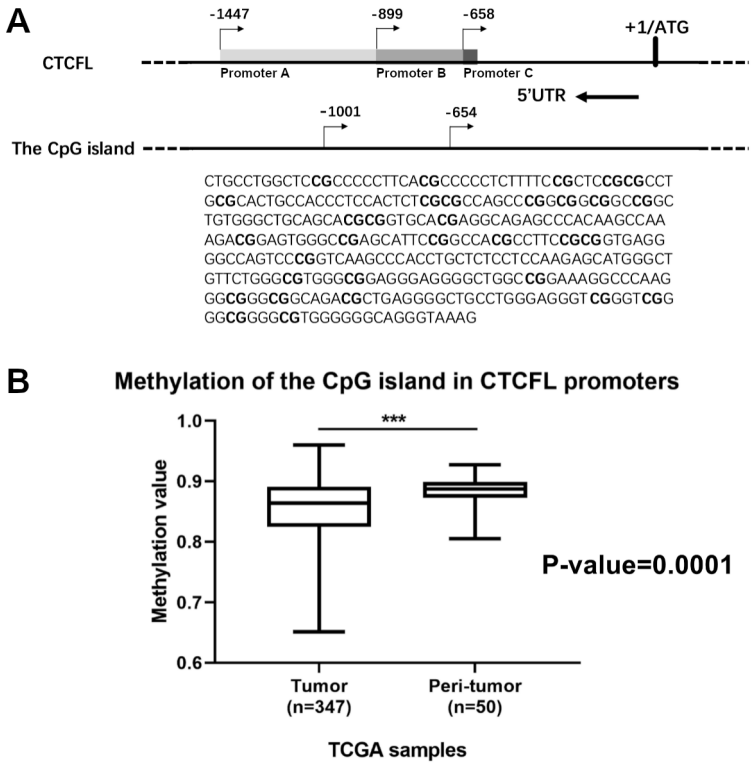

Figure 1. The CpG island of CTCFL promoters exhibited demethylation in HCC. A) A, B, and C promoters of CTCFL correspond to transcription start sites at –1447, –899, and –658 bp upstream of the first ATG; the CpG island of CTCFL promoters has a high frequency of dinucleotide CpG sites. B) Methylation value of the CpG island in 347 HCC tissues and 50 peritumoral tissues (p=0.0001). p<0.05 is considered statistically significant

benign lesion patients' plasma. The sequence amplified by MSP is located in the CpG island of CTCFL promoters (Figure 2A). The methylation status of plasma samples was analyzed by polyacrylamide gel electrophoresis and silver staining (Figure 2B). The results showed that 26 of 43 HCC plasma samples (60.4%) and 10 of 19 cirrhosis plasma samples (52.6%) were demethylated, while 11 of 14 benign lesion plasma samples (78.6%) were methylated (Table 1). The hypomethylation of CTCFL promoters in HCC plasma samples was significantly different from that in benign lesion plasma samples (p=0.026) (Table 1). Besides, unmethylated and methylated products detected by MSP were identified by sequencing (Figure 2C).

Demethylation of CTCFL promoters is a potential biomarker for postoperative recurrence. Statistical methods were used to analyze the relationship between the methylation status of CTCFL promoters and clinicopathological characteristics of HCC patients (Table 2). As shown in the Table 2, the demethylation of CTCFL promoters was negatively correlated with tumor nodules (p=0.019) and positively correlated with microvascular involvement (MVI) (p=0.001). It was observed that 36.8% of CTCFL demethylated plasma samples were from patients with postoperative recurrence, instead, there were no patients with recurrence in CTCFL methylated plasma samples (p=0.031). Nevertheless, the methylation status of CTCFL promoters was significantly irrelevant with gender, age, histopathologic grading, cirrhosis, tumor size, metastasis, vessel carcinoma embolus, satellite nodules, invasion of Glisson's capsule, fibrosis grade, HBsAg, and AFP.

Hypomethylation of CTCFL promoters is a potential biomarker for poor prognosis. To determine whether the hypomethylation of CTCFL promoters can be used as a prognostic biomarker, we obtained 347 HCC patients' prognostic information from the TCGA database. Kaplan-Meier analysis was conducted to investigate the overall survival (OS). According to the mean methylation value of CTCFL promoters' CpG island, the data were divided into hypermethylated group and hypomethylated group. There were statistically significant differences in overall survival

Table 1. The methylation status of CTCFL promoters in patients' plasma.

Group	CTCFL status/n (%)		p-value
	Unmethylated	Methylated	
HCC (n=43)	26 (60.5%)	17 (39.5%)	
Liver cirrhosis (n=19)	10 (52.6%)	9 (47.4%)	A=0.564
Benign lesion (n=14)	3 (21.4%)	11 (78.6%)	B=0.026

A: comparison between HCC and liver cirrhosis; B: comparison between HCC and benign lesion. p<0.05 is considered statistically significant

Table 2. Correlation between the methylation status of plasma and clinicopathological characteristics.

Clinicopathological parameters	CTCFL status/n (%)		p-value
	Unmethylated	Methylated	
Gender			
Male	17 (39.5)	14 (32.6)	0.387
Female	9 (20.9)	3 (7.0)	
Age (years)			
<55	13 (30.2)	9 (21.0)	1.000
≥55	13 (30.2)	8 (18.6)	
Recurrence			
Present	7 (16.3)	0 (0.0)	**0.031**
Absent	19 (44.2)	17 (39.5)	
Histopathological grading			
Moderately and poorly	15 (34.8)	11 (25.6)	0.755
Well	11 (25.6)	6 (14.0)	
Cirrhosis			
With	15 (34.8)	10 (23.3)	1.000
Without	11 (25.6)	7 (16.3)	
Tumor size (cm)			
<5cm	18 (41.9)	9 (20.9)	0.343
≥5cm	8 (18.6)	8 (18.6)	
Tumor nodules			
Single	26 (60.5)	13 (30.2)	**0.019**
Multiple	0 (0.0)	4 (9.3)	
Metastasis			
With	1 (2.3)	1 (2.3)	1.000
Without	25 (58.2)	16 (37.2)	
Vessel carcinoma embolus and satellite nodules			
With	3 (7.0)	1 (2.3)	0.930
Without	23 (53.5)	16 (37.2)	
MVI			
With	17 (39.5)	2 (4.7)	**0.001**
Without	9 (20.9)	15 (34.9)	
Invasion of Glisson's capsule			
With	12 (27.9)	9 (20.9)	0.760
Without	14 (32.6)	8 (18.6)	
Fibrosis grade			
1–3	10 (23.3)	7 (16.2)	1.000
4–6	16(37.2)	10 (23.3)	
HBsAg			
+	20(46.5)	15 (34.8)	0.595
–	6 (14.0)	2 (4.7)	
AFP (ng/ml)			
<25	9 (20.9)	9 (20.9)	0.344
≥25	17 (39.5)	8 (18.6)	

MVI: Microvascular involvement; HBsAg: Hepatitis B surface antigen; AFP: α-fetoprotein. p<0.05 is considered statistically significant

between the two groups (p=0.0056) and the hypomethylated group showed a worse prognosis (Figure 3A). Moreover, 347 samples were divided into two groups according to the quartile of methylation level, and the hypomethylated group had significantly worse overall survival than the hypermethylated group (p=0.0038) (Figure 3B). As a result, the hypomethylation of CTCFL promoters may be a biomarker for poor prognosis.

Discussion

Hepatocellular carcinoma (HCC) is one of the most common malignancies with high morbidity, high mortality, and poor prognosis [32]. Until now, biopsies have been one of the gold standards for clinical decision-making [33]. However, because of the invasive characteristic of tumor biopsies, it is difficult to dynamically monitor tumor development [34]. Therefore, as a noninvasive method, liquid biopsies can provide important prognostic information for patients with HCC.

As is known to all, aberrations in DNA methylation are hallmarks of human cancers, including hypomethylation of oncogenes and hypermethylation of tumor suppressor genes [35]. On the one hand, abnormal hypermethylation of tumor suppressor genes in circulating DNA can predict HCC development and serve as a noninvasive biomarker for the prognosis of HCC patients with partial resection [36, 37]. On the other hand, hypomethylation of oncogenes in liquid biopsies is mainly used for early noninvasive detection of the tumor, such as pancreatic ductal adenocarcinoma (PDAC) and bladder cancer [38, 39]. Nonetheless, few studies have identified hypomethylation of oncogenes in cfDNA as a biomarker for tumor prognosis and recurrence.

Previous studies in our laboratory have found that CTCFL is an oncogene with high expression in liver cancer tissues and cells, which is closely related to invasion and recurrence [21, 40]. And hypomethylation of CTCFL promoters in HCC tissues is strongly associated with patients' clinical stage and prognosis [28]. So far, the methylation status of CTCFL promoters in cfDNA of tumor patients has not yet been reported. Here, we first investigated the methylation status of CTCFL promoters in 43 HCC, 19 liver cirrhosis, and 14 benign lesion plasma samples. Our study demonstrated that the CpG island of CTCFL promoters was demethylated in 60.5% (26/43) of HCC patients, but only in 21.4% (3/14) of benign lesion patients (p=0.026). Unexpectedly, there was no statistical difference in hypomethylation rates between HCC samples and cirrhosis samples (p=0.564). Considering that the expression of CTCFL in HCC tissues is significantly higher than that in cirrhosis tissues (p<0.0001) [41], we speculate this paradox may be due to the small sample size and complex molecular mechanisms. Among various prognostic factors, the presence of microvascular invasion (MVI) is increasingly considered as a reflection of enhanced local invasion abilities of HCC [42]. Our data showed that the hypomethylation of CTCFL promoters was significantly correlated with microvascular involvement (MVI) (p=0.001), suggesting that CTCFL demethylation may be involved in tumor invasion. Furthermore, in HCC patients, 36.8% of CTCFL demethylated group were from patients with postop-

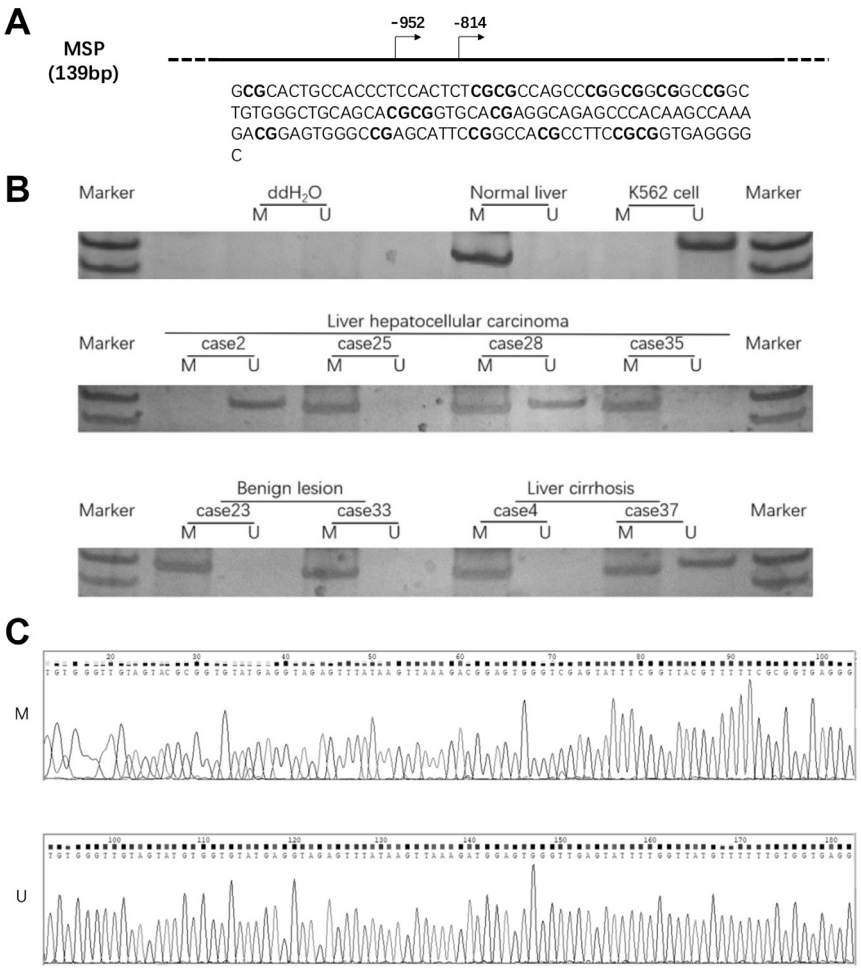

Figure 2. The methylation status of CTCFL promoters in HCC patients' plasma. A) The sequence detected by MSP is 139 bp. B) The methylation status of samples was shown by polyacrylamide gel electrophoresis and silver staining, ddH_2O is blank control, normal liver tissue is a negative control, K562 cells are positive control. C) Sequencing results of unmethylated and methylated products detected by MSP. M = Methylated, U = Unmethylated.

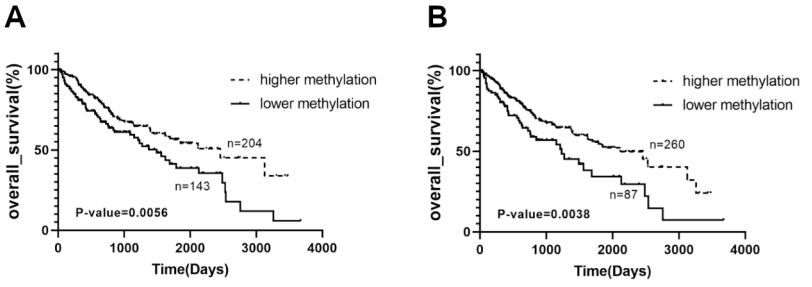

Figure 3. Hypomethylation of CTCFL a potential biomarker for poor prognosis. A) According to the mean methylation value of CTCFL promoters' CpG island, the 347 data were divided into hypermethylated group and hypomethylated group (p=0.0056). B) According to the quartile of methylation level, the 347 data were divided into hypermethylated group and hypomethylated group (p=0.0038). Kaplan-Meier analyses were conducted to investigate overall survival (OS). p<0.05 is considered statistically significant

erative recurrence, while the methylated group had no recurrence (p=0.031). Consistent with this, clinical prognosis data from the TCGA database indicated that HCC patients with CTCFL demethylation showed worse prognosis than those with methylation (p=0.0056). Since all plasma samples were obtained from the West China Hospital of Sichuan University only two months ago, we were unable to get patients' prognosis information. Therefore, we tentatively put forward that the hypomethylation of CTCFL promoters could be used to predict the malignant degree and poor prognosis of HCC.

In conclusion, we provide evidence that the CpG islands of CTCFL promoters are largely demethylated in HCC patients' plasma. The methylation status of CTCFL promoters in cell-free DNA may be an effective biomarker for monitoring postoperative recurrence in patients with HCC. Hypomethylation of CTCFL promoters could be used as a noninvasive biomarker to predict poor prognosis of HCC. Of course, the small sample size is a deficiency of our study. In a future study, the sample size should be expanded and patients should be followed up to provide powerful evidence for the view that the methylation status of CTCFL promoters could be a molecular marker of liquid biopsies in hepatocellular carcinoma.

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