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Research Paper

Correlation of Chitinase 3-Like 1 Single Nucleotide Polymorphisms with Hepatocellular Carcinoma in Taiwan

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Abstract

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death in Taiwan. Multiple risk factors, such as chronic hepatitis B or C virus infection, carcinogen exposure, cirrhosis, and various single-nucleotide polymorphisms (SNPs), are considered to contribute to hepatocarcinogenesis. Chitinase-3-like protein 1 (CHI3L1), a biomarker implicated in inflammation and tissue remodeling, plays a promoting role in angiogenesis, antiapoptosis, and cell proliferation. This study investigated the role of *CHI3L1* SNPs in HCC susceptibility and clinicopathology. Real-time polymerase chain reaction was used to analyze four SNPs of *CHI3L1* in 343 patients with HCC and 686 cancer-free controls. We found associations with HCC susceptibility in *CHI3L1* rs880633 polymorphism carriers with genotypes (TC+CC). We observed that HCC patients had lower frequencies of *CHI3L1* rs6691378 polymorphisms with the variant genotype GA+AA than the wild-type carriers with distant metastasis and positive HBsAg did. In 200 HBsAg negative HCC patients, we observed that the *CHI3L1* rs4950928 polymorphisms carriers with the variant genotype CG+GG had higher frequencies of vascular invasion. Finally, carriers of *CHI3L1* rs6691378 and 10399805 polymorphisms with the variant genotypes GA+AA showed lower levels of alpha-fetoprotein in HCC laboratory status. In conclusion, our results indicate that patients with *CHI3L1* rs880633 variant genotypes TC+CC are at a higher risk of HCC. *CHI3L1* polymorphisms rs880633 or rs4950928 may be potential candidates for predicting poor HCC prognosis and clinical status.

Key words: Single nucleotide polymorphism, CHI3L1, Hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related mortality and the fifth most common cancer worldwide [1, 2]. In Taiwan, HCC is the second leading cause of cancer-related death [3]. Chitinase 3-like 1 (CHI3L1), also known as YKL-40 and human cartilage

glycoprotein 39, is a secreted 40-kD mammalian glycoprotein encoded by the chitinase 3-like 1 gene located on human chromosome 1q32.1 [4]. CHI3L1 is a nonspecific inflammatory biomarker of disease and is secreted by activated macrophages and neutrophils in various tissues exhibiting inflammation, arthritic chondrocytes, cancer cells and vascular smooth muscle cells [5-11]. Because CHI3L1 is expressed and secreted by activated neutrophils and macrophages, previous studies have indicated that CHI3L1 may play an essential role in the inflammatory processes of tumors, such as stimulating angiogenesis and the remodeling of the extracellular matrix [7, 12-14]. High expression of CHI3L1 has been shown to be closely connected with the recurrence and metastasis of various human tumors such as breast cancer, nonsmall cell lung cancer, glioblastoma, and gastric cancer [15-20]. In addition, pretreatment levels of CHI3L1 were reported to be elevated in cervical cancer [21]. Recently, CHI3L1 was shown to correlate positively with p-Akt cell signaling but negatively with E-cadherin expression in HCC [22], and serum CHI3L1 was shown to be an independent prognostic factor for overall and recurrence-free survival in HCC patients receiving curative resection, indicating that elevated serum CHI3L1 levels predict poor prognosis in HCC after surgery [23].

Single-nucleotide polymorphisms (SNPs) are the most common type of DNA sequence variation, and may affect the expression of specific genes [24, 25]. SNPs in the *CHI3L1* promoter region have been associated with elevated serum CHI3L1 levels and a higher risk of schizophrenia [26, 27], differential gene expression [27], and elevated transcript levels [28]. Elevated circulating CHI3L1 levels might be a biomarker for asthma and declining lung function [29]. Moreover, elevated serum CHI3L1 levels and CHI3L1 overexpression are found in and linked to liver injury, advanced liver fibrosis, and poor prognosis in HCC [22, 23, 30]. Zhu et al. also found that serum CHI3L1 level was an independent prognostic biomarker in HCC patients after transcatheter arterial chemoembolization [31]. However, the connection between *CHI3L1* SNP expression and HCC regulation is not well established. Determining the mechanism of *CHI3L1* regulation and expression in HCC requires information on the genetic variant of *CHI3L1* SNPs involved in hepatocarcinogenesis. We performed a case–control study involving four *CHI3L1* SNPs located in the promoter region and exon 5 to analyze the contribution of these polymorphisms of *CHI3L1* to susceptibility to HCC and its pathological development.

Materials and methods

Study subjects

In this study, we recruited 343 patients with HCC between 2012 and 2016 at the Chung Shan Medical University Hospital, Taiwan. The 686 control groups were recruited at the same hospital without previous cancer history. The diagnoses of HCC were confirmed histologically in all cases. Demographic characteristics and medical information of the patients, including TNM staging, tumor size, lymph-node metastasis, vascular invasion, distant metastasis, presence of HBV surface antigen (HBsAg) and liver cirrhosis, were obtained from their medical records. The blood samples which obtained from the controls and HCC patients were stored in EDTA tubes, centrifuged immediately and stored at −80°C. The Institutional Review Board of Chung Shan Medical University Hospital approved this study (CSMUH No: CS15099), and informed written consent was obtained from each participant.

Selection of chitinase 3-like 1 gene polymorphisms

Three SNPs rs6691378 (-1371, C/T), rs10399805 (-247, C/T), and rs4950928 (-131, G/C) in the promoter region and SNP rs880633 (+2950, T/C) in exon 5 were selected based on the Chinese HapMap (Han Chinese in Beijing, China) data. The SNPs rs6691378, rs10399805 and rs4950928 in the promoter region of the *CHI3L1* gene exhibit strong association of schizophrenia and the SNP rs4950928 G → C transversion impairs the MYC/MAX-regulated transcriptional activity [27]. SNP rs10399805 has been reported to disrupt the C/EBP-AML-1 binding site in the gene promoter and is predicted to increase CHI3L1 expression [32]. The *CHI3L1* SNPs rs6691378 and rs10399805 and *CHI3L1* haplotypes all correlated with the development of cervical pre-cancerous lesions and invasive cancer [33]. The rs880633 was found to modulate age-adjusted lung function in CF patients. The minor allele frequencies (MAFs) of these SNPs were ≧ 5%.

DNA extraction and Single nucleotide polymorphisms genotyping

Genomic DNA was extracted using QIAamp DNA blood mini kits (Qiagen, Valencia, USA) according to the manufacturer's instructions as described previously [34]. The final DNA prepared was stored at -20°C and used as templates for the following experiments. Allelic discrimination of the rs880633 (+2950, T/C), rs6691378 (-1371, G/A), rs4950928 (-131, C/G) and rs10399805 (-247, G/A) polymorphisms was analyzed and asscessed by using

ABI StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The final volume for each reaction mixture was 5 μL, containing 2.5 μL TaqMan genotyping master mix, 0.125 μL TaqMan probe mix, and 10 ng genomic DNA. The reaction conditions included an initial denaturation step at 95 °C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min as described previously[33, 35].

Statistical analysis

The distributions of demographic characteristics and genotype frequencies for different genotypes between the HCC group and control group were analyzed using the chi-square test for categories of variables. The odds ratios (ORs) and their 95% confidence intervals (CIs) of the association between the genotype frequencies and HCC were estimated using multiple logistic regression models by controlling for covariates. A p value of less than 0.05 was considered statistically significant. The data were analyzed using SPSS 12.0 statistical software.

Results

Table 1 shows the statistical analysis of the demographic characteristics and clinical parameters. The 686 controls and 343 patients with HCC showed significant differences in their level of alcohol consumption ($p < 0.001$) (Table 1).

Table 1. The distributions of demographical characteristics and clinical parameters in 686 controls and 343 patients with HCC.

Variable	Controls (N=686)	Patients (N=343)	p value
Age (yrs)	**Mean ± S.D.**	**Mean ± S.D.**	
	62.11 ± 4.33	62.92 ± 11.67	p=0.109
Gender	**n (%)**	**n (%)**	
Male	490 (71.4%)	245 (71.4%)	p=1.000
Female	196 (28.6%)	98 (28.6%)	
Alcohol consumption			
No	579 (84.4%)	218 (63.6%)	p<0.001
Yes	107 (15.6%)	125 (36.4%)	
Tobacco consumption			
No	425 (62.0%)	205 (59.8%)	p =0.497
Yes	261 (38.0%)	138 (40.2%)	
Stage			
I+II		224 (65.3%)	
III+IV		119 (34.7%)	
Tumor T status			
≤T2		228 (66.5%)	
>T2		115 (33.5%)	
Lymph node status			
N0		331 (96.5%)	
N1+N2		12 (3.5%)	
Metastasis			
M0		325 (94.8%)	
M1		18 (5.2%)	
vascular invasion			
No		282 (82.2%)	
Yes		61 (17.8%)	

Mann-Whitney U test was used between controls and patients with HCC.

Genotype distributions and associations between HCC and *CHI3L1* gene polymorphisms are shown in Table 2. The alleles with the highest distribution frequency for rs6691378, rs10399805, rs4950928, and rs880633 genes of *CHI3L1* in both the controls and HCC patients were homozygous for G/G, G/G and C/C and heterozygous for T/C. After the adjustment of several variables, we found that people with the rs6691378, rs10399805, and rs4950928 polymorphisms of the *CHI3L1* gene did not have a significantly increased risk of HCC relative to wild-type (WT) carriers (Table 2). However, subjects with the *CHI3L1* polymorphic rs880633 TC+CC genotypes exhibited significantly ($p < 0.05$) higher risks (odds ratio [OR] = 1.329 95% CI = 1.009-1.750) of HCC than did their corresponding WT homozygous subjects (Table 2).

Table 2. Distribution frequency of *CHI3L1* genotypes in 686 controls and 343 patients with HCC.

Variable	Controls (N=686) n (%)	Patients (N=343) n (%)	OR (95% CI)	AOR (95% CI)
rs6691378				
GG	321 (46.8%)	159 (46.4%)	1.00	1.00
GA	296 (43.1%)	151 (44.0%)	1.030 (0.784-1.353)	1.039 (0.784-1.376)
AA	69 (10.1%)	33 (9.6%)	0.966 (0.612-1.524)	1.008 (0.631-1.610)
GA+AA	365 (53.2%)	184 (53.6%)	1.018 (0.785-1.320)	1.033 (0.790-1.350)
rs10399805				
GG	335 (48.8%)	165 (48.1%)	1.00	1.00
GA	289 (42.1%)	153 (44.6%)	1.075 (0.820-1.409)	1.111 (0.841-1.467)
AA	62 (9.0%)	25 (7.3%)	0.819 (0.496-1.350)	0.899 (0.538-1.501)
GA+AA	351 (51.2%)	178 (51.9%)	1.030 (0.794-1.334)	1.074 (0.822-1.403)
rs4950928				
CC	509 (74.2%)	245 (71.4%)	1.00	1.00
CG	160 (23.3%)	92 (26.8%)	1.195 (0.886-1.610)	1.182 (0.870-1.607)
GG	17 (2.5%)	6 (1.8%)	0.733 (0.286-1.883)	0.724 (0.275-1.908)
CC+CG	177 (25.8%)	98 (28.6%)	1.150 (0.861-1.537)	1.138 (0.845-1.534)
rs880633				
TT	290 (42.3%)	122 (35.6%)	1.00	1.00
TC	315 (45.9%)	174 (50.7%)	1.313 (0.991-1.739)	1.303 (0.976-1.740)
CC	81 (11.8%)	47 (13.7%)	1.379 (0.909-2.093)	1.432 (0.933-2.198)
TC+CC	396 (57.7%)	221 (64.4%)	1.327 (1.015-1.734)*	1.329 (1.009-1.750)*

The odds ratios (ORs) and with their 95% confidence intervals (CIs) were estimated by logistic regression models. The adjusted odds ratios (AORs) with their 95% confidence intervals (CIs) were estimated by multiple logistic regression models after controlling for alcohol consumption. * p value < 0.05 as statistically significant.

By estimating the distribution frequency of clinical statuses and *CHI3L1* genotype frequencies in the HCC patients, we clarified the role of *CHI3L1* gene polymorphisms in HCC clinicopathologic statuses such as clinical stage (tumor, node, metastasis), tumor size, lymph node metastasis, distant metastasis, vascular invasion, Child-Pugh score, HBsAg, antihepatitis C virus (anti-HCV), and liver cirrhosis. No significant associations of rs10399805, rs4950928, and rs880633 gene polymorphisms with clinicopathologic statuses were observed (Data not shown). However, among the 343 HCC patients, those with the GA+AA polymorphic rs6691378 gene had a lower risk of distant metastasis (OR = 0.314; 95% CI =

0.109–0.900, p = 0.024) and a lower level of positive HBsAg (OR = 0.567; 95% CI = 0.368–0.875, p = 0.010) than the patients with rs6691378 WT did; however, no difference was found regarding the clinical stage, tumor size, lymph node metastasis, vascular invasion, Child–Pugh score, anti-HCV, and liver cirrhosis (Table 3). Moreover, in 200 HBsAg-negative HCC patients, we observed that those showing the *CHI3L1* rs4950928 genetic variant CG+GG had a higher risk of vascular invasion (OR = 2.710; 95% CI = 1.267–5.796, p = 0.009) relative to those showing the rs4950928 WT; no difference was observed regarding the clinical stage, tumor size, lymph node metastasis, Child–Pugh score, anti-HCV, and liver cirrhosis (Table 4).

Table 3. Odds ratio (OR) and 95% confidence interval (CI) of clinical status and *CHI3L1* rs6691378 genotypic frequencies in 343 HCC patients.

Variable	Genotypic frequencies			
	GG (N=159)	GA+AA (N=184)	OR (95% CI)	p value
Clinical Stage				
Stage I/II	100 (62.9%)	124 (67.4%)	1.00	p=0.383
Stage III/IV	59 (37.1%)	60 (32.6%)	0.820 (0.525-1.281)	
Tumor size				
≦ T2	104 (65.4%)	124 (67.4%)	1.00	p=0.698
> T2	55 (34.6%)	60 (32.6%)	0.915 (0.584-1.434)	
Lymph node metastasis				
No	154 (96.9%)	177 (96.2%)	1.00	p=0.740
Yes	5 (3.1%)	7 (3.8%)	1.218 (0.379-3.916)	
Distant metastasis				
No	146 (91.8%)	71 (97.3%)	1.00	p=0.024*
Yes	13 (8.2%)	5 (2.7%)	0.314 (0.109-0.900)	
Vascular invasion				
No	132 (83.0%)	150 (81.5%)	1.00	p=0.718
Yes	27 (17.0%)	34 (18.5%)	1.108 (0.635-1.934)	
Child-Pugh grade				
A	123 (77.4%)	138 (75.0%)	1.00	p=0.610
B or C	36 (22.6%)	46 (25.0%)	1.139 (0.691-1.876)	
HBsAg				
Negative	81 (50.9%)	119 (64.7%)	1.00	p=0.010*
Positive	78 (49.1%)	65 (35.3%)	0.567 (0.368-0.875)	
Anti-HCV				
Negative	81 (50.9%)	98 (53.3%)	1.00	p=0.668
Positive	78 (49.1%)	86 (46.7%)	0.911 (0.596-1.394)	
Liver cirrhosis				
Negative	33 (20.8%)	35 (19.0%)	1.00	p=0.688
Positive	126 (79.2%)	149 (81.0%)	1.115 (0.655-1.897)	

The ORs with analyzed by their 95% CIs were estimated by logistic regression models.
> T2: multiple tumor more than 5 cm or tumor involving a major branch of the portal or hepatic vein(s). * p value < 0.05 as statistically significant.

Alpha-fetoprotein (AFP), aspartate aminotransferase, and alanine aminotransferase are common clinical pathological markers of HCC. We analyzed the levels of these pathological markers, which are associated with *CHI3L1* genotypic frequencies, to clarify the relationship between the progression of clinical status and level of clinical pathological markers in HCC patients. Table 5 shows the association between the *CHI3L1* genotypic frequencies and HCC laboratory status; no significant association with the rs4950928 and rs880633 gene polymorphisms was observed. However, patients with the rs6691378 GA+AA (p = 0.026) or rs10399805 GA+AA (p = 0.047) genetic variant exhibited higher levels of AFP (Table 5) compared with those with the wild-type gene polymorphisms.

Table 4. Odds ratio (OR) and 95% confidence interval (CI) of clinical status and *CHI3L1* rs4950928 genotypic frequencies in 200 HCC patients with HBsAg negative status.

Variable	Genotypic frequencies			
	CC (N=143)	CG+GG (N=57)	OR (95% CI)	p value
Clinical Stage				
Stage I/II	96 (67.1%)	40 (70.2%)	1.00	p=0.677
Stage III/IV	47 (32.9%)	17 (29.8%)	0.868 (0.446-1.690)	
Tumor size				
≦ T2	97 (67.8%)	41 (71.9%)	1.00	p=0.572
> T2	46 (32.2%)	16 (28.1%)	0.823 (0.419-1.618)	
Lymph node metastasis				
No	137 (95.8%)	57 (100%)	1.00	p=0.116
Yes	6 (4.2%)	0 (0.0%)	---	
Distant metastasis				
No	138 (96.5%)	55 (96.5%)	1.00	p=0.997
Yes	5 (3.5%)	2 (3.5%)	1.004 (0.189-5.328)	
Vascular invasion				
No	125 (87.4%)	41 (71.9%)	1.00	p=0.009*
Yes	18 (12.6%)	16 (28.1%)	2.710 (1.267-5.796)	
Child-Pugh grade				
A	113 (79.0%)	41 (71.9%)	1.00	p=0.282
B or C	30 (21.0%)	16 (28.1%)	1.470 (0.727-2.972)	
Anti-HCV				
Negative	40 (28.0%)	17 (29.8%)	1.00	p=0.793
Positive	103 (72.0%)	40 (70.2%)	0.914 (0.465-1.794)	
Liver cirrhosis				
Negative	36 (25.2%)	9 (15.8%)	1.00	p=0.151
Positive	107 (74.8%)	48 (84.2%)	1.794 (0.802-4.017)	

The ORs with analyzed by their 95% CIs were estimated by logistic regression models.
> T2: multiple tumor more than 5 cm or tumor involving a major branch of the portal or hepatic vein(s)
* p value < 0.05 as statistically significant.

Table 5. Association of *CHI3L1* genotypic frequencies with HCC laboratory status.

Characteristic	α-Fetoprotein [a] (ng/mL)	AST [a] (IU/L)	ALT [a] (IU/L)	AST/ALT ratio [a]
rs6691378				
GG	5597.8 ± 1780.4	143.0 ± 23.8	124.6 ± 19.6	1.38 ± 0.07
GA+AA	1658.8 ± 565.3	130.9 ± 20.7	107.7 ± 15.5	1.57 ± 0.13
p value	**0.026***	0.700	0.495	0.218
rs10399805				
GG	5307.3 ± 1716.9	140.6 ± 23.0	121.8 ± 18.8	1.38 ± 0.07
GA+AA	1795.3 ± 588.1	132.7 ± 21.3	109.7 ± 16.1	1.58 ± 0.14
p value	0.047*	0.800	0.625	0.211
rs4950928				
CC	3833.9 ± 1089.8	145.3 ± 20.1	114.4 ± 13.4	1.52 ± 0.10
CG+GG	2611.8 ± 1472.4	114.6 ± 21.3	118.2 ± 27.2	1.37 ± 0.09
p value	0.533	0.374	0.889	0.374
rs880633				
TT	1254.1 ± 417.2	101.2 ± 14.7	84.6 ± 10.4	1.48 ± 0.10
TC+CC	4716.2 ± 1346.9	156.0 ± 22.8	132.6 ± 18.1	1.49 ± 0.11
p value	0.061	0.093	0.061	0.938

Mann-Whitney U test was used between two groups.
[a] Mean ± S.E.
* p value < 0.05 as statistically significant.

Discussion

This study provides novel information regarding the effects of *CHI3L1* SNPs on HCC susceptibility and clinicopathology. Alcohol can cause progressive perivenous injury, impaired hepatocyte function, and endothelial cell pore loss [36, 37]. CHI3L1 serum levels have been shown to potentially provide prognostic information because they are elevated in alcoholic patients and are related to the presence of liver fibrosis [38]. We observed that alcohol consumption was more common in the HCC patients than in the controls (Table 1). CHI3L1 might secrete by hepatic stellate cells [39], which are believed to be the main effector cell in liver fibrogenesis [36, 37]. Elevated CHI3L1 levels have been found to be associated with an increased risk of liver fibrosis and to be involved in the activation of the innate immune system [40]. Thus, alcohol-consuming HCC patients may sustain high levels of baseline serum CHI3L1, thereby triggering liver fibrosis and cirrhosis.

The *CHI3L1* SNP rs880633 is located in the promoter region exon 5 of the *CHI3L1* gene. We found that *CHI3L1* SNP rs880633 TC+CC genetic variants occur more frequently in HCC patients than in controls (Table 2). The rs880633 polymorphism has been shown to modulate age-adjusted lung function in cystic fibrosis lung disease, and *CHI3L1* might be a potential biomarker for this condition [41]. However, the detailed mechanism and influence of *CHI3L1* rs880633 genetic variants have not been thoroughly investigated. The promoter SNP -131C → G (rs4950928) in the *CHI3L1* gene was found to be involved in *CHI3L1* serum level modulation and asthmatic lung disease [29]; it is located in the core promoter of *CHI3L1* within a binding site for MYC and MAX transcription factors. The minor allele (-131G on the forward strand) was found to disrupt binding and to be associated with lower messenger RNA levels in peripheral blood cells, reduced levels of circulating *CHI3L1* protein, and, on the basis of a luciferase assay, reduced transcription [27]. However, in contrast to the findings of Ober et al., James et al. found that the rs4950928 CC genotype is associated with greater levels of circulating *CHI3L1* [42]. Moreover, in a previous report, Abd El-Fattah et al. shown that CHI3L1 rs4950928 SNPs have no significant association with colorectal cancer [43]. In the present study, we found that the *CHI3L1* rs4950928 CG+GG genotype carriers among the 200 HBsAg-negative HCC patients indicate a greater risk of vascular invasion (p = 0.009) (Table 4). CHI3L1 has been identified as a promoter of angiogenesis in neoplasms and is involved in activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway in endothelial cells [44-46]. CHI3L1 has been shown to modulate vascular endothelial cell morphology and stimulate migration of endothelial cells by promoting the formation of branching tubules [47, 48]. CHI3L1 may be involved in tumor angiogenesis. An in vivo study by Shao et al. found that CHI3L1 contributes to the growth of primary and metastatic tumors [49]. Our results are consistent with these findings in correlating *CHI3L1* rs4950928 SNPs with malignancy and poor prognosis in HCC.

AFP, desgamma-carboxy prothrombin time, and AFP-leptin 3, have been identified as potential candidates for evaluating the prognosis of HCC patients [50]. However, these candidates are not always sufficient for the prediction of prognosis and recurrence in HCC [51]. In the present study, we found that HCC patients with rs6691378 GA+AA (p = 0.026) or rs10399805 GA+AA (p = 0.047) genetic variant have lower levels of AFP (Table 5). CHI3L1 levels in both healthy adults and in diseased patients have been found to be affected by CHI3L1 genetic variations [26, 29, 52, 53]. The rs10399805 SNP is located at position -247 and disrupts the C/EBP-AML-1 binding site in the gene promoter; it has been speculated that it increases CHI3L1 expression [32]. However, the rs10399805 polymorphism has been found to be associated with diseases such as atopy and schizophrenia [32, 54]. Regarding rs6691378 and rs10399805, the genetic contribution of *CHI3L1* to schizophrenia has been shown to vary, despite it being mechanistically involved in the disease process [55]. In addition, our previous study demonstrated that the *CHI3L1* SNPs rs6691378 and rs10399805 and the *CHI3L1* haplotype correlate with the development of cervical precancerous lesions and invasive cancer [33]. The precise roles of rs6691378 and rs10399805 in CHI3L1 expression and regulation in HCC remain unclear. This may be a result of the variation and coordination of the regulation of different *CHI3L1* polymorphisms in CHI3L1 serum levels.

In conclusion, our study shows that the SNPs of the *CHI3L1* gene are potential tumor markers for HCC. Carriers of *CHI3L1* genetic variants of rs880633 polymorphisms show various correlations with CHI3L1 modulation, HCC severity, and HCC prognosis. *CHI3L1* may provide a marker for evaluating HCC prognosis.

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Competing Interests

The authors have declared that no competing interests exist.

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