Hypomethylation of TL1A and its decoy receptor 3(DR3) expressive level increase have diagnostic value in HBV-associated cirrhosis

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Article

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Abstract

Background: For patients with cirrhosis, early diagnosis is the key to delay the development of liver fibrosis and improve prognosis. TNFSF15, a gene encoding TNF-like ligand 1A (TL1A) as a susceptibility gene for hepatic fibrosis.

Aim: This study aimed to investigate the clinical significance of TL1A and DR3 in the development of cirrhosis and fibrosis.

Methods: All total of 200 patients, including 107 with HBV-associated LC patients, 63 CHB and 30 healthy controls. Then we were analysed the expression of TL1A, DcR3 and other inflammatory cytokines associated with liver fibrosis by using quantify DNA methylation, qPCR and Enzyme-linked immunosorbent assay in serum and PBMCs.

Results: PBMCs TL1A methylation level was significantly lower in patients with HBV-associated LC than the other groups while mRNA level of TL1A was significantly higher in LC group. In addition, serum TL1A and DcR3 expression level was increased in the LC patients.

Conclusions: Hypomethylation of the TL1A promoter is present in HBV-associated LC, and TL1A and DR3 are highly expressed in HBV-associated cirrhosis. These results indicate that TL1A and DR3 may play an important role in the pathogenesis of LC and TL1A methylation levels may serve as a noninvasive biomarker for early diagnosis and progression of LC.

Introduction

Liver cirrhosis (LC), which is a common clinical chronic progressive liver disease, which is caused by the long-term or repeated action of one or more etiologies. Histopathology showed extensive necrosis of liver cells, nodular regeneration of residual liver cells, connective tissue hyperplasia and fibrous septum formation, which led to the destruction of hepatic lobule structure and formation of pseudolobule.[1, 2] In Asia, chronic hepatitis B virus infection is the most important risk factor for the development of cirrhosis and hepatic failure.[3] In the later stage, liver function damage and portal hypertension are the main manifestations, and multiple systems are involved, often resulting in upper gastrointestinal bleeding, hepatic encephalopathy, secondary infection, hypersplenism, ascites, carcinogenesis and other complications. However, in the early stage, due to the strong compensatory function of the liver, no obvious symptoms can be found, and it is difficult to make a clear diagnosis. Imaging examination was more replied, when necessary by liver biopsy pathological examination or laparoscopy to diagnose. Therefore, effective biomarkers are desperately needed for the early diagnosis of LC.[4, 5]

TL1A (Tumor necrosis factor-like cytokine 1A), as a member of the TNF superfamily of proteins (TNFSF) and encoded by TNFSF15, that was located on chromosome 9q32 in human.[6] TL1A protein is induced by inflammatory cytokines such as TNF-α and IL-1 in human umbilical vein endothelial cells and synovial fibroblast-like cells.[6] TL1A has also been shown to be involved in cross-linking activation of Toll-like

receptor (TLR) ligands, enteric bacteria, and Fcγ receptor (FcγR) crosslinking, and was expressed on activated antigen-presenting cells and lymphocytes. In some inflammatory diseases such as human inflammatory bowel disease (IBD)[7, 8], primary biliary cholangitis(PBC)[9] and rheumatoid arthritis (RA) [10], TL1A expressive levels were elevated in serum and related tissues has been reported. In addition, it has been shown in mice that recruitment and secretion of proinflammatory and profibrotic cytokines by macrophages leads to constitutive TL1A expression in myeloid cells, which exacerbates liver fibrosis.[11]

DR3 as one of functional receptor for TL1A, contains a death domain in its cytoplasmic region, which has been reported to be involved in the process of apoptosis processes.[12]It is mainly expressed on NK cells, T cells and other lymphocytes, especially NKT cells, and is enhanced upon their activation.[13] In addition, DcR3 was involved the polarization of native T cells into Th2 cells and the negative regulation of B-cell activation induced by TLR ligands to mediate inflammatory immune responses, thus playing a role in the regulation of various immune cells.[14, 15] The signal induced by TL1A on T cells lead to the secretion of proinflammatory cytokines, and its functions specifically as a T cell co-stimulator was consistent with DR3 being primarily expressed on activated T cells. The interaction between TL1A and DR3 in a recombinant system or in cells that naturally express DR3 results in activation of NF-κB and apoptosis.[16, 17] In consistent of TL1A, elevated serum DcR3 levels has also been shown to be associated with IBD[7, 15], systemic lupus erythematosus (SLE)[18] and rheumatoid arthritis[10], infection and kidney failure.[19]To sum up, TL1A and DR3 play an important role in the pathogenesis of many chronic inflammatory diseases. Therefore, its elevated expression level might play a crucial role in the pathogenesis and early diagnosis of cirrhosis.

In this study, we mainly use Methylation, a quantitative, high-throughput technology for the analysis of DNA methylation, to detect the methylation status of TL1A promoter in patients with HBV-associated LC, chronic hepatitis B (CHB) and healthy controls (HCs). In addition, we examined the expression level of TL1A and DR3 in PBMCs and serum to elucidate the potential clinical value methylation level of TL1A as a non-invasive biomarker for LC diagnosis.

Results

General characteristics of the study populations

In this study, a total of 200 participants were enrolled, including 107 patients with HBV associated LC,63 patients with CHB and 30 HCs. The demographic characteristics, clinical manifestations and laboratory measurements of the subjects were shown in Table 2.

Methylation status of TL1A promoter in LC, CHB and HCs

We analysis the methylation status of TL1A promoter expressed as PMR in with HBV associated LC, CHB and HC groups in PBMC respectively were presented in Fig.2. As shown in the figure, TL1A methylation level was significantly lower in patients with HBV associated LC (median 0.82%, interquartile range 0.45-1.69%) than in those with CHB (median 6.35%, interquartile range 5.05–8.60%, $p < 0.001$) and HC

(median 7.39%, interquartile range 5.91–11.44%, p<0.001), there were no significant differences between the CHB and HC groups(p=0.065).

Association between clinicopathological features and TL1A promoter methylation level

We then determined whether or not TL1A methylation might be a useful biomarker for the detection of HBV-associated LC. Then patients were divided into several subgroups according to basic characteristics and clinicopathological features and shown in Table 3. We found TL1A methylation level was significantly lower in patients>50 years old, in patients with positive HBV-DNA, in patients with positive encephalopathy, in patients with positive ascites, in patients with CTP stage C, and LSM>12.5 group. However, the TL1A promoter methylation level was not significantly correlated with gender and AFP level could be observed. The relationships between TL1A methylation level and LSM, MELD score was further analyzed using spearman rank correlation test. We found PMR value of TL1A was significantly correlated with the LSM and MELD score of LC patients as Fig.3.

TL1A and related cytokines/genes mRNA expression levels change in different groups

Liver cirrhosis is orchestrated by a complex network of cytokine-mediated signaling pathways regulating the activation of HSCs and fibrogenesis. In the process, TNF-α, IL-1 β and IL-6 play a role as multipotent cytokines involved in inflammatory pathway, hematopoietic and immune regulation.[20] Therefore, we examined the mRNA expression level of TL1A and other inflammatory cytokines were measured by using quantitative real-time PCR and the results were shown in Fig.4. TL1A mRNA expression was significantly higher in the PBMCs of LC patients compared with CHB and HCs. However, the altered mRNA level of TL1A was no significant differences in the CHB and HCs patients. The PBMCs mRNA expressive levels of IL-6 and IL-1β increased significantly in LC patients with HBV associated and CHB compared with HCs, whereas the altered mRNA level of TL1A was no significant differences in the HBV-associated LC and CHB patients. In addition, we found the mRNA expressive levels of TGF-α increased significantly in LC patients with HBV-associated LC compared with CHB and HCs, there was also a significant difference between CHB and HC groups(p=0.002). The relationship between TL1A methylation level and mRNA level was analyzed by using spearman rank correlation analysis and found PMR value of TL1A was significantly negatively correlated with mRNA expression level (Fig.7).

We tested the ability of immune complexes and TLR ligands to induce expression of TL1A by PBMCs and lipopolysaccharide (LPS) in higher concentrations induced TL1A at the mRNA level. As shown in Fig.5, the mRNA expression level of TL1A; TGF-α and IL-1β in PBMC was significantly reduced before LPS stimulation, whereas the mRNA expression level of IL-6 was no significant change before and after LPS stimulation.

Serum TL1A and DR3 levels are increased in HBV-associated cirrhosis

The expression level of TL1A and DR3 in serum was detected as shown in Fig.6. As the figure, we found serum TL1A and DR3 levels were significantly higher in HBV-associated LC compared with CHB and

healthy controls. There were no significant differences in TL1A levels among CHB and HC patients. The relationship between TL1A methylation level and serum expressive level was further analyzed by using spearman rank correlation analysis and we found PMR value of TL1A was significantly negatively correlated with serum TL1A expression level as Fig.7.

Diagnostic value of TL1A promoter methylation level

ROC curve was used to assess the diagnostic value of TL1A methylation level, LSM and MELD score. PMR value of TL1A promoter showed an AUC of 0.947 (95%CI 0.911-0.984), higher than that of LSM (AUC 0.897, 95%CI 0.850-0.944) and MELD score (AUC 0.812, 95%CI 0.749 -0.874), as listed in Table 4. The cut-off point was 1.62%, the sensitivity was 72.90%, and the specificity was 87.30%. Then, a model based on binary logistic regression was constructed in Fig.8.

Discussion

In this study, we report for the first time that TL1A methylation levels were significantly reduced in PBMC consistent with the result that mRNA expression level in serum and PBMC were significantly increased in patients with HBV- associated cirrhosis. In LC group, TL1A methylation level markedly reduced in patients more than 50 years old, CTP score more than C stage, LSM more than 12.5Kpa, with positive HBV DNA and symptoms of decompensation compared with the other groups. We further observed that there was a significant positive correlation between TL1A methylation level and MELD score and LSM of patients. Methylation, as a high-throughput quantitative methylation detection method has high sensitivity and specificity. In addition, CpG islands refer to DNA fragments containing large amounts of CpG, which usually leads to gene silencing.[21] In this study, ROC curves of TL1A methylation level showed significantly higher than that of LSM and MELD score in discriminating HBV- associated LC from CHB.

For patients with HBV-associated cirrhosis, early detection and anti-viral treatment is the key to delay the development of liver fibrosis and improve the survival. At present, the gold standard for diagnosis of cirrhosis was liver biopsy. However, due to its invasiveness, clinical diagnosis is mostly based on decompensated symptoms of cirrhosis, combined with imaging examination such as transient elastography (TE) but the early diagnosis of cirrhosis is difficult. TE test liver stiffness measurement (LSM) was use as diagnostic marker for LC is limited owing to image examination is easy affected by patient positions and instrument.[5] In this research, we quantitatively assessed the methylation level of TL1A and ROC curve was performed to further evaluate its diagnostic value. As shown above, TL1A methylation level had a significantly better performance than LSM. These results might indicate that TL1A methylation level have the potential to be one of the non-invasive biomarker for LC diagnosis.

Previous reports have suggested elevated serum TL1A levels in chronic liver inflammatory diseases such as PBC and in mice overexpression of TL1A can accelerate the process of liver fibrosis and worsen liver function.[9, 11] However, the expression and methylation level of TL1A in human HBV-associated cirrhosis has not been clearly defined. Therefore, in this study, we firstly quantitatively assessed TL1A promoter methylation status in HBV -associated LC patients using blood samples and came to the

conclusion that TL1A was hypomethylated in LC. It is also noteworthy that the methylation status of the TL1A promoter was detected only in PBMC, but not in liver tissue, mainly because the present study was designed to evaluate the diagnostic value of TL1A methylation levels as a non-invasive biomarker. In addition, peripheral blood mononuclear cells including monocytes, T cells, B cells and natural killer cells, are considered the first line of the immune system to defend against inflammation, and previous studies have reported that various diseases can influence gene expression in PBMC through host immune or inflammatory responses. [22, 23]

Meanwhile, the result that mRNA level was significantly higher in LC group than CHB and HC groups and PMR value of TL1A was significantly negatively correlated with mRNA expression level in PBMC. However, it is noteworthy that a large reduce in TL1A methylation was observed while mRNA increased not as much as reduced methylation level. The degree of inconsistency may be due to the fact that not only promoter methylation can influence the transcription process, but also microRNAs, histone modifications, and transcription factors.[24, 25] Previous studies have shown that the expression level of serum TL1A and mRNA can also be increased in other liver diseases, such as the late-stage CHC.[9] In addition, elevated serum TL1A levels have been reported in various chronic inflammatory diseases such as IBD [7, 8] and RA [10]. Previous studies were also identified TL1A in intrahepatic small bile ducts as well as infiltrating mononuclear cells. In addition, up-regulation of TL1A and its decoy receptor has been demonstrated in PBMC and plasma of sickle cell anemias.[8, 26] These results suggest that TL1A is involved in the pathogenesis of these chronic inflammatory diseases. In this study, we found that TL1A mRNA expression levels in HBV-associated LC groups and CHB groups were significant increase in PBMC. These results may indicate that TL1A is not only involved in the pathogenesis of LC, but also serves as a common feature of chronic liver inflammation in CHB, although there was no statistically significant difference($p = 0.065$).

The important mechanism for macrophages to participate in liver fibrosis is to secrete a series of proinflammatory and profibrotic factor such as IL-1β and TNF-α to perpetuate the proinflammatory pro-fibrotic stimulus, and overexpressed TL1A can up-regulate the expression of TNF-α, and IL-1β in liver tissues and macrophages.[11] In vitro, TL1A is induced in endothelial cells by TNF-α and IL-1β, and in monocytes and dendritic cells by stimulation with TLR ligands.[27] Previous data showed that cultured biliary epithelial cells (BECs) constitutionally express TL1A and various human TLRS and produce inflammatory cytokines and chemokines when stimulated by TLR ligands in vitro.[28, 29] In additional, LPS binding to TLR4 also induces activation of NF-κB and stimulates up-regulation of TNF-α, IL-6 and IL-1β, all of which have been implicated in inflammatory diseases.[30] Therefore, we tested the ability of TLR ligands to induce expression of TL1A in PBMC and found that LPS could in higher concentrations induced TL1A at the mRNA level and detected the expression levels of TNF-α, IL-1β and IL6 in patients with HBV-associated cirrhosis. We found that the expression levels of TNF-α, IL-1β were significantly increased than the other groups, the increase of IL-6 expression level was not statistically significant. These results may indicate that TL1A is involved in the development of HBV-associated cirrhosis inflammation and fibrosis, and that after LPS stimulation can up-regulate the expression of hepatocirrhosis related inflammatory factors such as TNF-α, IL-1β.

Interaction between TL1A and DR3 in-vitro can activate both NF-κB pathways which being pro-inflammatory results in cytokine secretion, cell proliferation and cell activation.[26] In this study, we demonstrated that serum TL1A and DcR3 levels was significant increases in serum TL1A and DcR3 levels were observed in HBV-associated LC than CHB and HC groups. This observation is consistent with previous studies in patients with other cirrhosis[9] and autoimmune diseases such as IBD [7, 8], SLE [18], and RA [10].

There were some limitations to this study. First, the sample size was relatively small and all patients were from a single center, which might lead to selection bias. In addition, the possibility of false positive clinicopathological features were not negligible. Secondly, we only assessed the changes in the expression levels of relevant inflammatory factors before and after LPS stimulation, and the regulatory relationship between them and TL1A has been poor know.

In conclusion, TL1A promoter methylation level was hypomethylated in patients with HBV-associated LC. PMR value of TL1A promoter was showed significantly higher diagnostic value in distinguishing patients with HBV-associated LC from those with CHB than LSM testing, suggesting that TL1A methylation level in PBMCs might serve as a promising non-invasive diagnostic biomarker for HBV-associated LC in the future. In additional we showed that serum and PBMC TL1A and DcR3 levels were increased in HBV-associated LC patients, these changes indicating that TL1A and DcR3 might be involved in the pathogenesis of HBV-associated liver cirrhosis. The exact role of TL1A in the pathogenesis of LC as well as its potential as a therapeutic target and prognosis need to be further studied.

Materials And Methods

Patients

A total of 200 patients were enrolled from the Department of Hepatology, Qilu Hospital, Shandong University, including 107 patients with hepatitis B virus (HBV) associated LC, 63 with chronic hepatitis B (CHB) and 30 healthy controls (HCs). All patients with HBV-associated LC were selected based on the updated 2020 Chinese guidelines on the management of the liver cirrhosis.[5] All patients with HBV associated cirrhosis received standard care. The exclusion criteria and the patient selection process were shown in Fig.1.

Prior to sample collection, informed consent was obtained from all patients. These study and all research methods were approved by the Medical Ethical Committee of Qilu Hospital of Shandong University, with the guidelines of the 1975 Declaration of Helsinki.

Preparation, culture and stimulation of peripheral blood mononuclear cells

Citrate-anticoagulated peripheral blood was obtained from all subjects. After Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) density gradient centrifugation, interface mononuclear cells were collected and washed three times. The cells were resuspended with RPMI1640 containing 10% fetal bovine serum,

100U/mL penicillin and 100lg/mL streptomycin (Gibco, Invitrogen, USA), at 37 °C with 5% CO2.[9] After 12h' incubation for ex vivo stimulation test. The cells from each individual were cultured separately with or without 100 ng/mL LPS (Sigma-aldrich, Saint Louis, MO, USA) at 37 °C with 5% CO2 for another 12h' incubation, the cells were harvested for extraction of total RNA and DNA.

TaqMan probe-based quantitative methylation -specific PCR (MethyLight)

Genomic DNA was extracted from PBMCs by TRIzol Reagent (Invitrogen). EZ DNA Methylation-Gold kit (Zymoresearch, Orange, CA, USA) was used for DNA bi-sulphite modification. MethyLight was performed using EpiTect MethyLight PCR + ROX Vial Kit (QIAGEN, Hilden, Germany). Two sets of primers and probes designed specifically for bisulfite converted DNA were used: a methylated set for the TL1A gene and a reference set for the GAPDH gene to normalize for input DNA. The TL1A and GAPDH gene specific primers and probes sequences were listed in Table 1. We used a total volume of 10μl following the standard protocol provided by manufacturer: 95 ∘C for 15 min, followed by 45 cycles of 95℃ for 15 s and 60℃ for 1 min.[31]

RNA extraction and RT-qPCR

Total RNA of PBMCs was extracted by TRIzol Reagent (Invitrogen) and cDNA was then immediately synthesized using the First-Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. cDNA was either used as template immediately for reverse -transcriptase polymerase chain reaction (RT-qPCR). The 10μl reaction system with condition of denaturation at 95℃ for 30 s, followed by 45 cycles of 95℃ for 5 s, 60℃ for 30 s and 72℃ for 30s.[31] The specific primers of TL1A, TNF-a, IL-6, IL-1β and GAPDH were both described before and the sequences were listed in Table 1.[32-34]

Enzyme-linked immunosorbent assay (ELISA)

Plasma cytokine levels were was measured using the Human Immunoassay Valukine ELISA Kit for TL1A and DR3 (Lengton Bioscience Co, Shanghai, China), which is a competitive method is used to detect the content in samples. According to the manufacturer's protocol, and the absorbance was measured at 450nm. The detection limit of TL1A was 3200ng/l and the detection limit of DcR3 was 8ng/ml. All samples are made in duplicate.

Statistical analysis

Statistical analyses were analyzed using SPSS 26.0 statistical software (SPSS Inc., Chicago, IL, USA). Quantitative variables were expressed as median (centile 25; centile 75). Categorical variables were expressed as number (%). Mann–Whitney U test and Kruska-Wallis H test were used to compare the categorical variables. The Spearman's rank correlation test was used to analyze the relationship between TL1A methylation and quantitative clinical data as well as TL1A methylation, TL1A mRNA expression level and serum TL1A. The student's t test was used to analyze the relationship between TL1A mRNA level before and after LPS stimulation. Receiver operating characteristic (ROC) curve was performed to

assess the diagnostic value of TL1A methylation level and a model based on binary logistic regression was established to estimate the diagnostic value of TL1A methylation level. The optimal cut-off point was defined as the point that maximizes sensitivity plus specificity. The gender HBV-DNA and all other statistical analyses were dichotomous variables. P<0.05 was considered to be statistically significant.[31]

Declarations

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Authorship

Guarantor of the article: Wang K.

Author contributions: Wei X.F. designed the study, performed the research, analyzed the data and wrote the paper. Zhu J.Y. and Liu H.H. revised the paper. Su X. and Li J.H. helped with the statistical analysis. Fan Y.C. contributed to the formal analysis, writing-review and editing. Wang K. contributed to the supervision, design of the study and the critical revision of the paper.

Data Availability Statements

The data that support the findings of this study are available on request from the corresponding author [Kai Wang]. The data are not publicly available due to them containing information that could compromise research participant privacy/consent.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Tables

Table1

Sequences of used primers and probes.

Gene	Forward primer sequence (5'-3')	Primer/probe sequence (5' to 3'
RT-qPCR		
TL1A	GAAATGACAGTATCTGCGGAGTTTA	CAACTAGCTACTGTCTGGCACTGG
IL-6	ATGCAATAACCACCCCTGAC	GAGGTGCCCATGCTACATTT
TNF-α	GCATGATCCGAGATGTGGAACTGG	CGCCACGAGCAGGAATGAGAAG
IL-1β	CCACAGACCTTCCAGGAGAATG	GTGCAGTTCAGTGATCGTACAGG
GAPDH	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAAA
Methylight		
TL1A	ATTTAGTTAGGACGTATATAGGTG	TTTACCAATTTAACAAACCCGAA
GAPDH	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAAA
Probe oligo sequence		
TL1A	CTAAAACCCAAAACAACAAACTCC	
GAPDH	ACCACCACCCAACACACAATAACAAACACA	

Table 2

General clinical characteristics of the patients.

	HCs	CHB	LC
N	30	63	107
Age (years)	22.00(21.00-24.25)	43(35-51)	53(46-61)
Sex (male/female)	17/13	50/13	75/32
ALT (U/L)	10.00(7.00-12.25)	32(18-76)	31(22-61)
AST (U/L)	8(6-10)	25(21-45)	42(29-77)
TBIL (μM)	6.85(4.70-10.20)	12.30(9.10-15.90)	32.40(15.90-62.20)
ALB(g/L)	44.80(43.53-46.80)	48.20(45.50-49.80)	34.30(30.20-40.70)
PLT*10^9	219.50(201.75-238.25)	201(170-241)	66(46-121)
INR	0.93(0.92-0.97)	0.98(0.96-1.07)	1.27(1.12-1.50)
PTA(%)	90(86-94)	102(90-106)	69(54-86)
HBsAg	NA	2900(402-43100)	20050(645.5-931000)
HBV-DNA(+),n(%)	NA	41(65.08)	60(56.07)
AFP(ng/ml)	NA	3.21(2.38-4.60)	8.07(3.56-32.82)
Encephalopathy(%)	0	0	5.61
Ascites (%)	0	0	67.29
CTP stage(%)			
A stage	0	93.65	18.69
B stage	0	4.76	44.86
C stage	0	0	36.45
MELD	NA	57.24(55.10-58.8)	62.74(58.32-66.68)
LSM Kpa	NA	10.40(7.90-12.80)	19.80(14.80-26.50)

Quantitative variables are expressed as the median (centile 25; centile 75). Categorical variables are expressed as number (%). LC=liver cirrhosis, CHB= chronic hepatitis B, HC=healthy control, ALT=alanine aminotransferase, AST=aspartate aminotransferase, TBIL=total bilirubin, ALB=albumin, INR=international normalized ratio, PTA=prothrombin time activity, Cr=creatinine, AFP=alpha fetoprotein, HBsAg=hepatitis B surface antigen, MELD=model for end-stage liver disease, NA=not available, CTP=Child-Turcotte-Pugh, LSM = Liver hardness.

Table 3

Association between clinicopathological features and TL1A promoter methylation level

Parameter	Total number	PMR (%)	P value
Gender			
Male	75	0.80(0.42-1.81)	0.756[a]
Female	32	0.87(0.51-1.57)	
Age			
≤50	40	1.92(0.82-2.73)	<0.001***[a]
>50	67	0.57(0.34-1.06)	
HBV DNA			
Positive	60	0.65(0.35-1.56)	0.012*[a]
Negative	47	1.05(0.62-2.01)	
AFP			
>20	36	0.69(0.43-1.31)	0.064[a]
≤20	71	1.23(0.50-1.93)	
Encephalopathy			
Positive	6	0.48(0.30-0.64)	0.045*[a]
Negative	101	0.91(0.51-1.82)	
Ascites			
Positive	72	0.68(0.35-1.39)	0.001**[a]
Negative	35	1.21(0.64-2.15)	
CTP stage			
A	20	1.21(0.80-2.28)	0.015*[b]
B	48	0.72(0.38-1.62)	
C	39	0.69(0.37-1.54)	
LSM			
>12.5	93	0.75(0.42-1.60)	0.006**[a]
<12.5	14	1.78(0.87-3.82)	

CTP=Child-Turcotte-Pugh, * p<0.05. ** p<0.01. *** p<0.001.

a Mann–Whitney U test. b Kruskal–Wallis H test.

Table 4

Diagnostic values of TL1A methylation level, LSM and MELD for distinguishing HBV-associated LC from CHB.

Parameter	Sensitivity	Specificity	Youden index	AUC	95%CI
PMR	72.90%	87.30%	0.458	0.947	0.911-0.984
LSM	68.2%	92.10%	0.398	0.897	0.850-0.944
MELD	69.20%	81.75%	0.334	0.812	0.749 -0.874

Figures

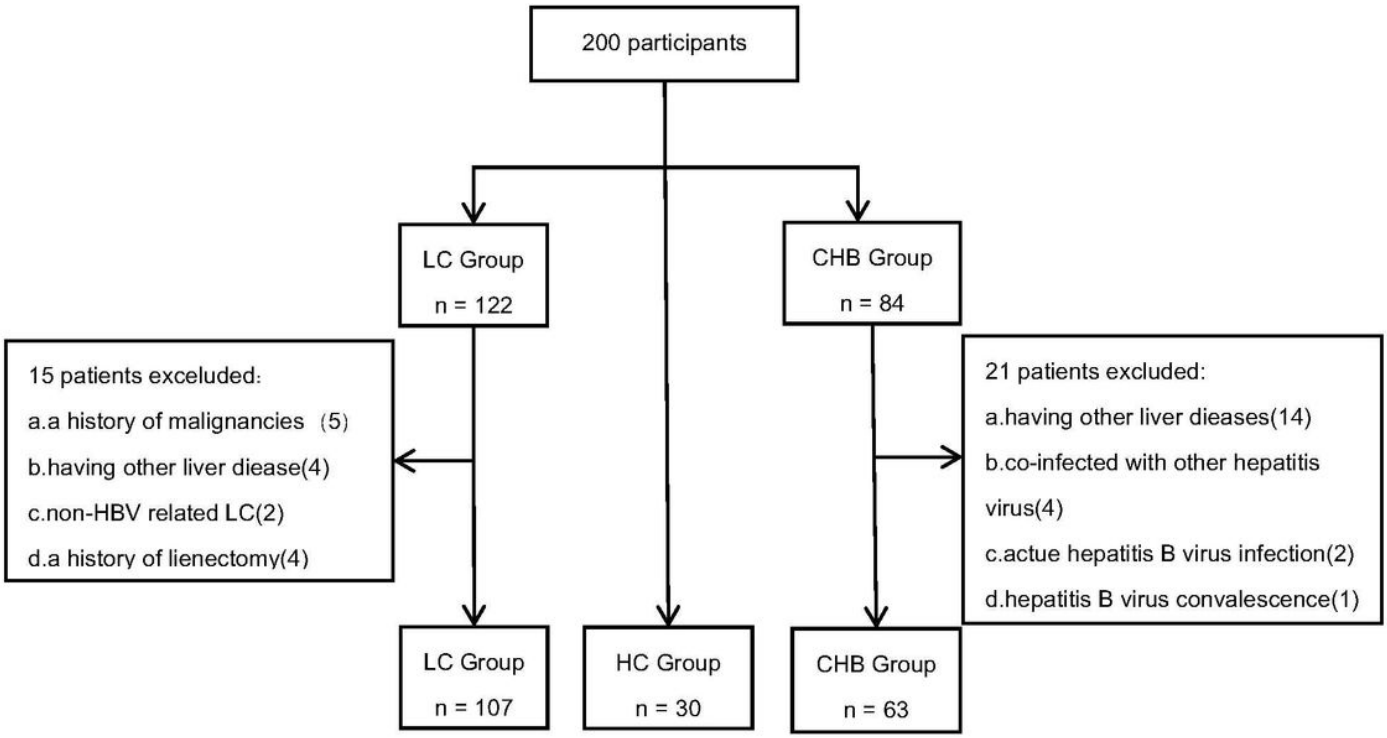


Figure 1

Patient selection process

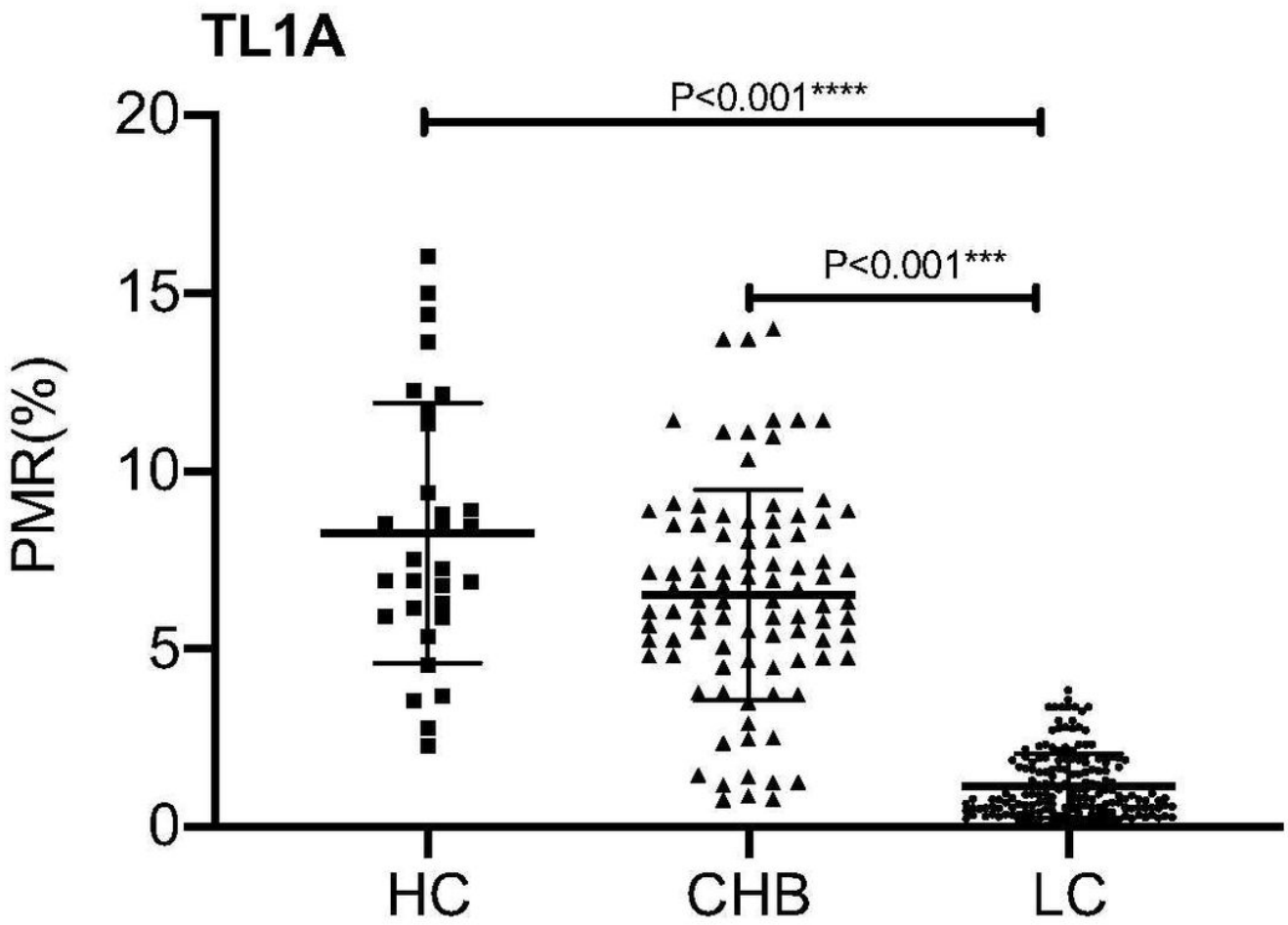


Figure 2

TL1A methylation level in PBMCs form HBV-associated LC, CHB and HCs

TL1A methylation level was significantly lower in HBV-associated LC(n=107) than in those with CHB(n=63) and HC patients(n=30) ($p < 0.001$), respectively, by using the Mann–Whitney U-test.

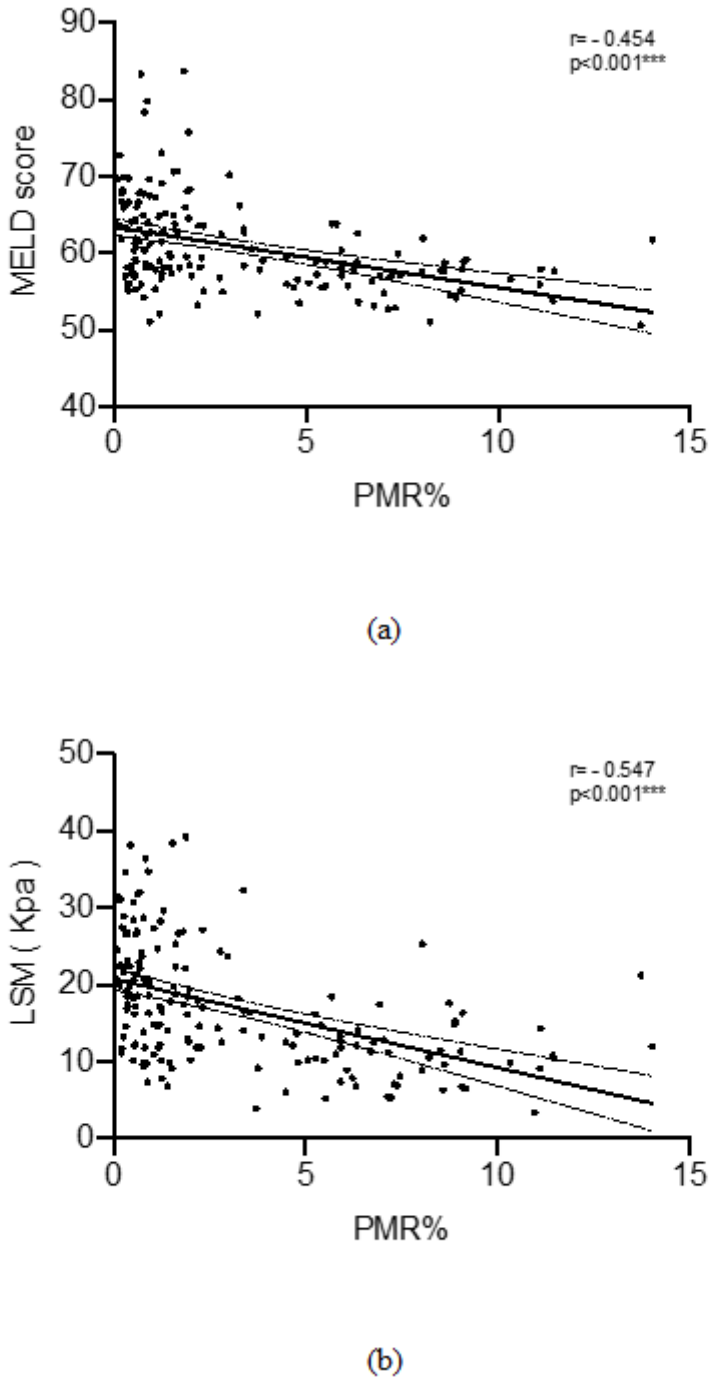


Figure 3

Relationships between TL1A methylation level, MELD score and LSM

PMR value of TL1A promoter was significantly correlated with MELD and LSM (Spearman's r= -0.454, -0.547, p<0.001, respectively).

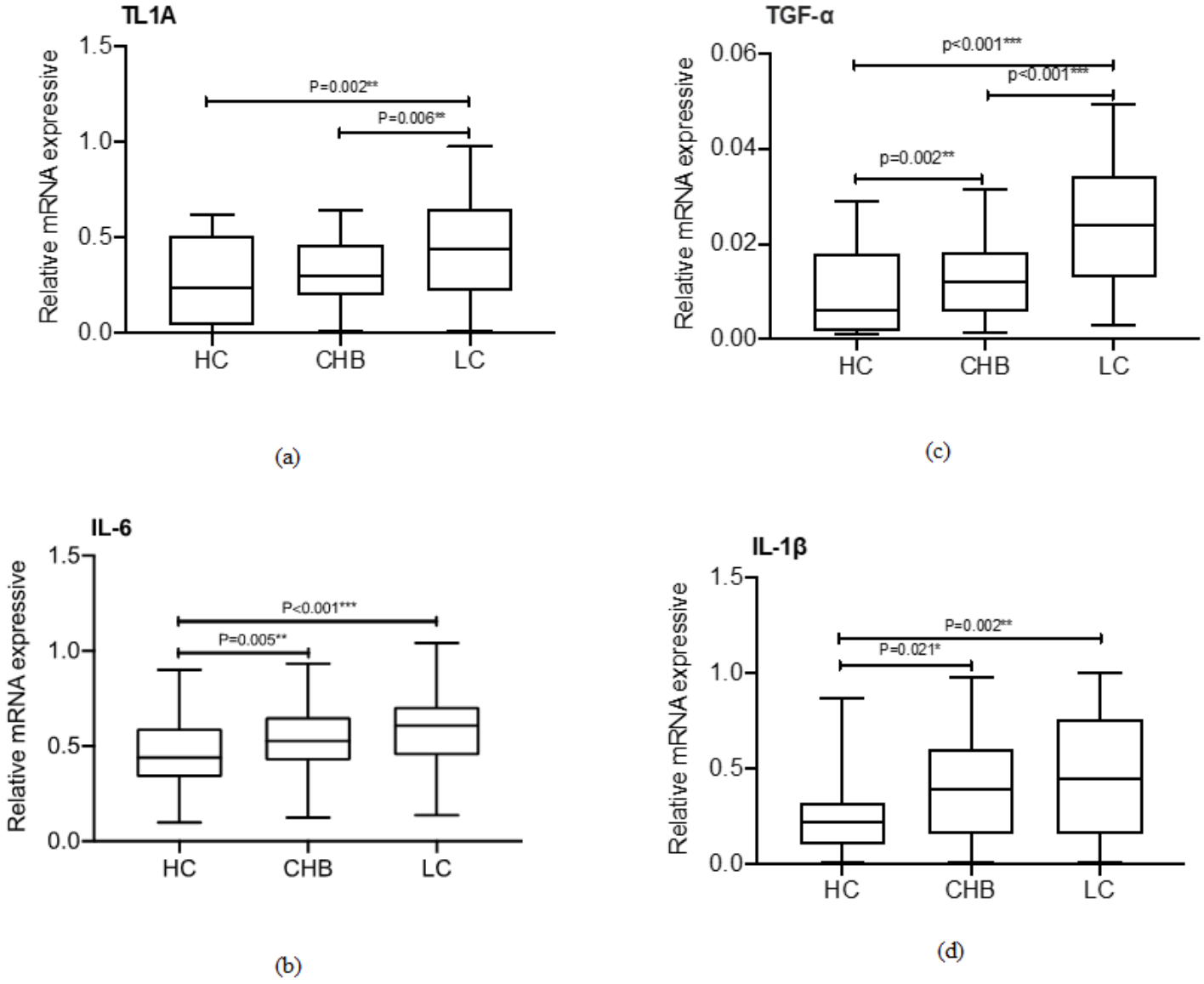


Figure 4

TL1A mRNA level in patients with HBV-associated LC, CHB and HCs

(a) TL1A mRNA level was significantly higher in LC group (median 0.441, interquartile range 0.223–0.653) than CHB (median 0.303, interquartile range 0.200–0.467, p =0.006) and HC group ((median 0.240, interquartile range 0.042–0.510, p =0.002). (b) IL-6 mRNA level was significantly higher in LC (median 0.607, interquartile range 0.450-0.707, p<0.001) and CHB (median 0.526, interquartile range 0.423-0.653, p=0.005) than HC group (median 0.440, interquartile range 0.335-0.593). (c) TGF-αmRNA level was significantly increase in LC(median 0.015, interquartile range 0.004-0.028) than CHB (median 0.011, interquartile range 0.003-0.019, p<0.001) and HCs (median 0.006, interquartile range 0.002-0.018, p<0.001) ,and CHB higher than HC group (p =0.002).(d) IL-1βmRNA level was significantly higher in LC(median 0.444, interquartile range 0.151-0.747,p=0.002) and CHB(median 0.390, interquartile range 0.156-0.604, p=0.021) group than HC group (median 0.220, interquartile range 0.101- 0.317).

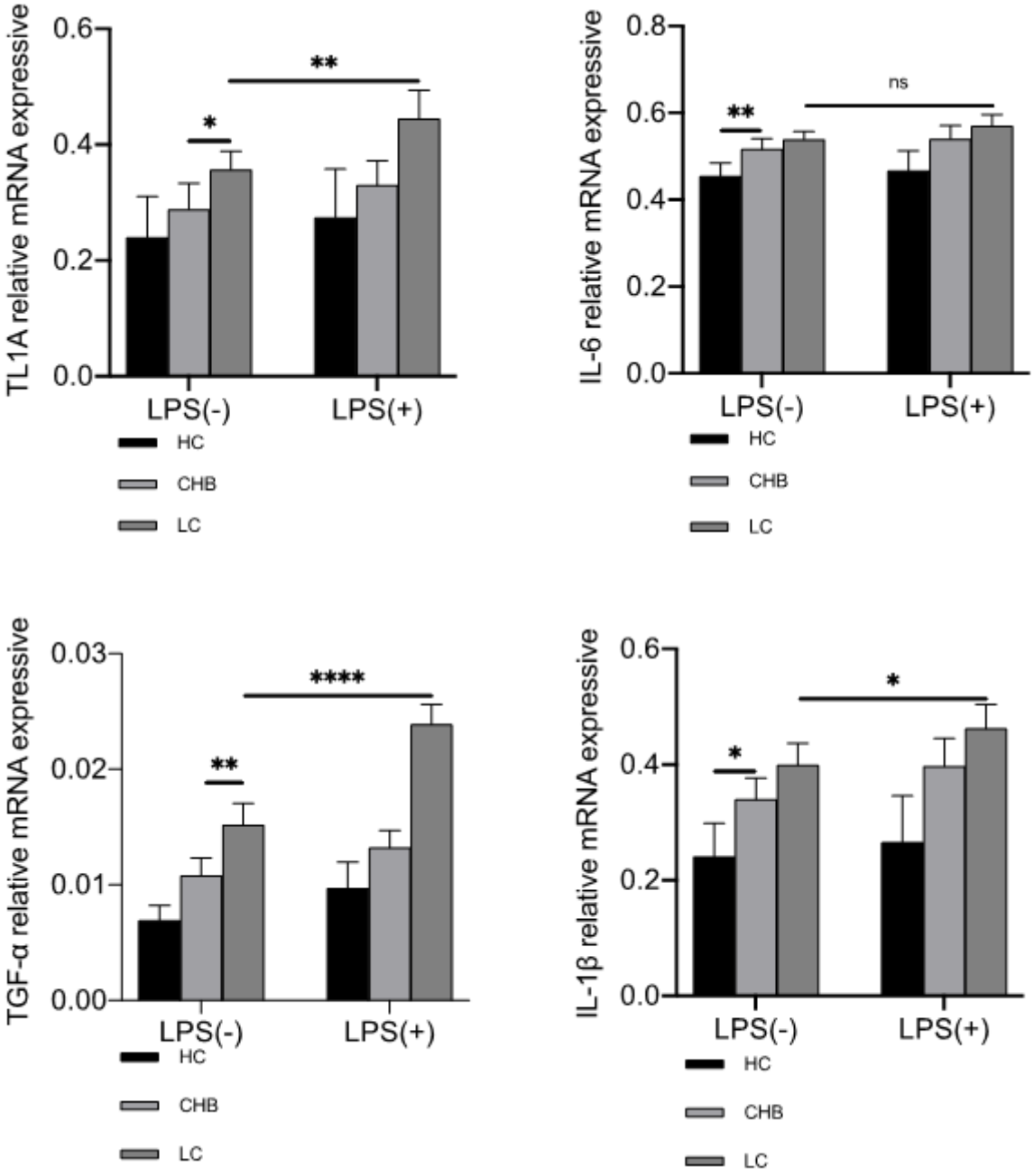


Figure 5

Relative mRNA expressive level in peripheral blood mononuclear cells (PBMC) were compared before and after LPS stimulation in patients with HBV-associated LC, CHB and HCs

The expression levels of TL1A, TGF-α and IL-1β were significantly increased in LPS stimulated group than the non-LPS stimulated group, respectively. Data are expressed as median (centile 25; centile 75). ∗p < 0 05, ∗∗p<0.01 and ∗∗∗p < 0 001, ∗∗∗∗p < 0 0001.

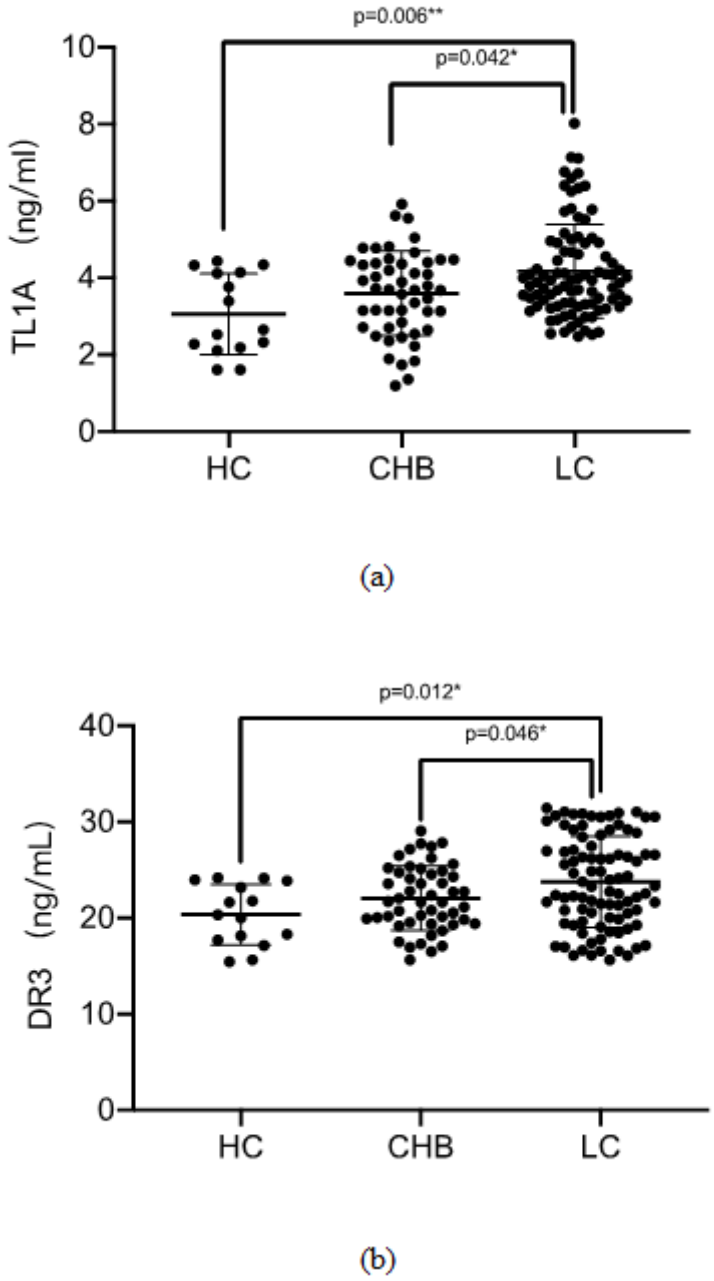
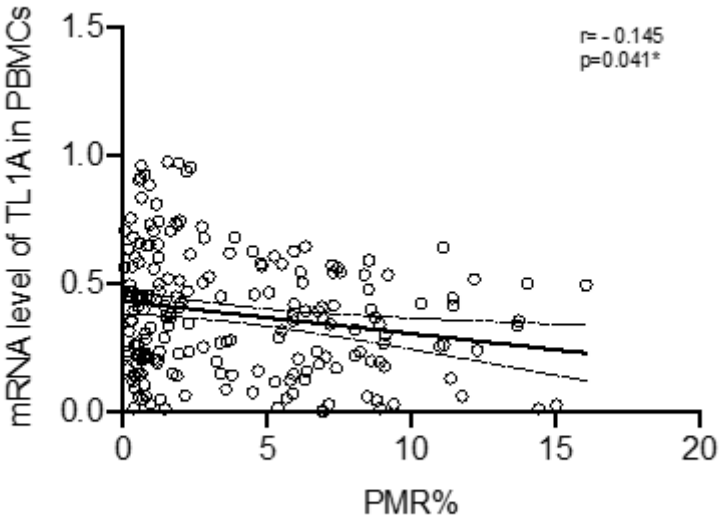


Figure 6

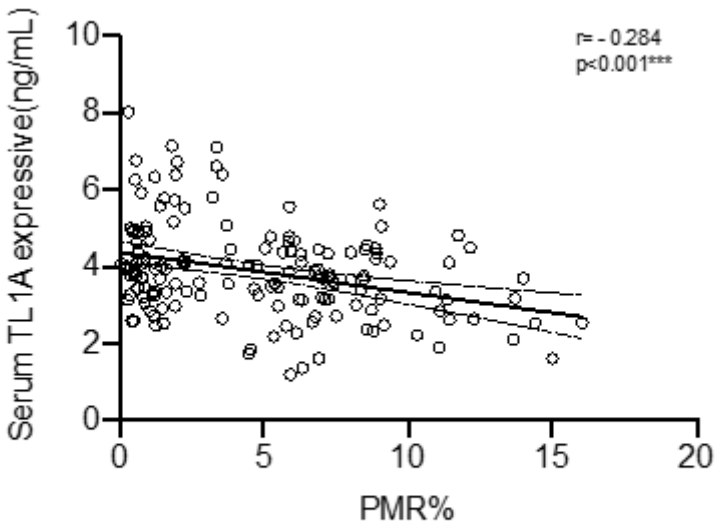
Serum TL1A levels in LC, CHB and HCs patients

(a) Serum TL1A levels were significantly higher in patients with LC (n=90, median=3.92ng/ml, interquartile range 3.30–4.91ng/ml) as compared with CHB (n=50, median 3.71ng/ml, interquartile range 2.92–4.44ng/ml, P=0.019) and healthy controls ((n=15, median 2.65ng/ml, interquartile range 2.19–4.14ng/ml, P=0.006). (b) Serum DR3 levels were significantly higher in LC patients (n=90, median 23.31ng/ml, interquartile range 19.84–27.73ng/ml), as compared with CHB (n=50, median 21.70ng/ml, interquartile range 19.50–24.80ng/ml, p=0.046) and healthy controls ((n=15, median 20.37ng/ml,

interquartile range 17.72–23.90ng/ml, p=0.012). Horizontal lines represent mean values for each group. Statistical differences were analysed using a two-tailed Mann-Whitney U-test.



(a)



(b)

Figure 7

Relationships between TL1A methylation level and mRNA level in PBMCs, and TL1A expressive in serum

Significant correlation was observed between PMR value of TL1A promoter and mRNA level in PBMCs (Spearman's r = -0.145, p= 0.041), and TL1A expressive in serum (Spearman's r = -0.228, p=0.004).

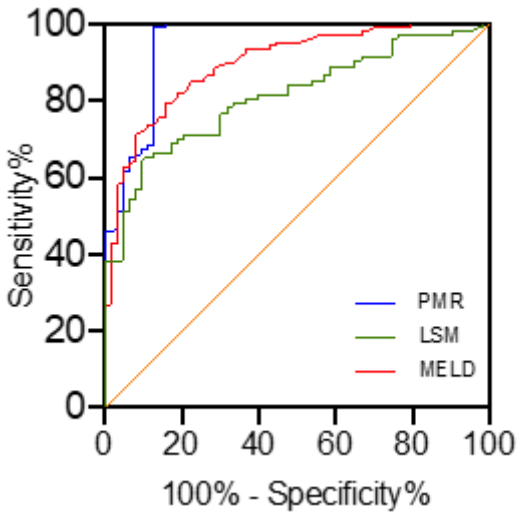


Figure 8

ROC curves of TL1A methylation level, LSM level and MELD in discriminating HBV-associated LC from CHB

PMR values of TL1A promoter showed an AUC of 0.987, higher than that of AFP (AUC=0.914) and MELD(AUC=0.812). The optimal cut-off point of 1.07% was selected. (cut-off PMR=1.62%).