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ORIGINAL

Characterisation of TNF-related apoptosis-inducing ligand in peripheral blood in patients with primary biliary cirrhosis

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Abstract Apoptosis plays a pivotal role in portal tract damage of primary biliary cirrhosis (PBC). Tumour necrosis factor-related apoptosis inducing ligand (TRAIL) is an apoptotic inducer, and it has been reported that the expression of TRAIL receptors is up-regulated by increased bile acid level and the serum level of soluble TRAIL (sTRAIL) is elevated in PBC patients. In the present study, we investigated the association of TRAIL in peripheral blood with the pathogenesis of PBC and chronic hepatitis B. The expression levels of TRAIL mRNA and protein on leukocytes and sTRAIL in plasma from 27 patients with PBC, 25 with CHB and 30 healthy controls were determined respectively by real-time fluorescent quantitative reverse transcription polymerase chain reaction (RT-PCR), flow cytometry (FCM) and ELISA. The expression levels of TRAIL mRNA and protein on leukocytes and plasma sTRAIL were all up-regulated in the patients with PBC and CHB compared to controls. In the two diseased groups, TRAIL mRNA showed significant correlation of both membrane-bound TRAIL (mTRAIL) on monocytes and plasma sTRAIL. So did plasma TNF-α. In PBC patients, mTRAIL and sTRAIL correlated well with γ-glutamyltransferase and alkaline phosphatase, but not with aspartate aminotransferase and alanine aminotransferase. The opposite case was found in CHB patients. These results suggested that both mTRAIL and sTRAIL might be involved in the development and progression of PBC and CHB in humans, but the mechanisms might be different.

Keywords TRAIL · PBC · Chronic hepatitis B · Apoptosis

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Introduction

Primary biliary cirrhosis (PBC) is a slowly progressive autoimmune disease of the liver that primarily affects middle-aged and old women. Its aetiology and pathogenesis are not yet fully understood. The initial histology of PBC is a distinctive pattern of bile duct damage including chronic non-suppurative destructive cholangitis, bile duct loss and chronic cholestasis prominent from the early stages. In comparison, although nonbiliary diseases such as hepatitis B virus (HBV)-related chronic hepatitis (CHB) also show mild bile duct damage, the occurrence of bile duct loss or cholestasis is very rare. There is concrete evidence indicating that apoptosis is possibly the most important mechanism of biliary epithelial cell loss. Apoptotic cells are frequently found on biliary epithelial cells, particularly more cholangitic bile ducts in PBC, but very few occur in control livers [1]. Markers of ongoing apoptosis have been reported within affected portal tracts, including down-regulation of the anti-apoptotic protein bcl-2 [2, 3], increased expression of Fas in BECs from PBC patients, as well as elevated expression of FasL in infiltrating mononuclear cells [4]. Nevertheless, apoptosis-related molecular mechanisms still remain to be understood.

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a transmembrane (type II) glycoprotein, including membrane-bound TRAIL (mTRAIL) and soluble TRAIL (sTRAIL), which triggers apoptosis through interaction with the death receptors DR4 and DR5 [5, 6]. It has been suggested that TRAIL has associations with various autoimmune diseases such as systemic lupus erythematosus [7], neurodegenerative diseases [8] and experimental autoimmune encephalomyelitis [9], etc. A recent study indicated that the gene expression of TRAIL was significantly elevated in biliary epithelial cells from PBC patients, when compared with patients with chronic hepatitis C (CHC) and healthy individuals [10]. Another study reported that the serum sTRAIL level was significantly elevated in PBC and CHC patients compared to healthy controls [11]. However, previous studies failed to clarify the association of the expression of TRAIL gene and protein in peripheral blood leukocytes with PBC.

The aim of this study was to analyse the altered expression of TRAIL gene and protein in peripheral blood leukocytes as well as its association with plasma sTRAIL, TNF-α, alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) from PBC patients and, further, to explore if TRAIL might play a role in the pathogenesis of PBC. For this reason, the expression of TRAIL gene in peripheral blood mononuclear cells (PBMC) from the patients with PBC and CHB was determined by real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). Meanwhile, the expression of mTRAIL on the surface of peripheral blood cells was assayed by flow cytometry (FCM). The sTRAIL levels in plasma were simultaneously determined in these subjects.

Materials and methods

Subjects

This study was approved by the research ethics committee of our institution. All subjects gave their informed consent prior to commencement of the study. Twenty-seven PBC patients (6 males, 21 females; mean age 51 ± 10), 25 patients with chronic hepatitis B (CHB) (15 males, 10 females; mean age 50 ± 11) and 30 healthy volunteers (10 males, 20 females; mean age 49 ± 8) were recruited. The diagnosis of PBC was based on internationally accepted criteria [12], i.e., on the basis of clinical immunological test (anti-mitochondrial antibodies (AMA) positively at titre > 1:40) and manifestation and unexplained elevation of ALP and GGT showing a cholestatic pattern of liver biochemistry in the absence of an alternative explanation. AMA-negative suspected patients had a liver biopsy performed. The diagnosis of CHB was based on clinical, laboratory and imaging findings. Fresh heparinised peripheral blood samples were obtained from the 82 subjects.

Flow cytometry analysis of expression of mTRAIL on peripheral blood leukocytes

FCM was performed using PE-labelled monoclonal antibody for human TRAIL (eBioscience, USA), according to the manufacturer's instructions. In brief, 100 μl of whole blood was incubated with 20 μl PE-conjugated mouse antihuman TRAIL antibody for 30 min at room temperature in the dark. After incubation with the RBC lysis buffer, the mixture was washed in stain buffer containing bovine serum albumin and sodium azide. Cells were resuspended in 500 μl staining buffer and analysed by flow cytometer (BeckmanCoult, USA) as follows. Firstly, gating was employed effectively by forward and side light scatter signals of different intensities for the selection of lymphocytes, monocytes and neutrophils within a mixed population of cells from peripheral blood according to their heterogeneous characteristics. Secondly, the percentages of TRAIL-positive cells were analysed in every gate. Negative control was prepared by incubating with an isotype-matched control antibody (IgG1, λ).

PBMC preparation and RNA extraction

PBMCs were isolated from peripheral venous blood of the subjects by Ficoll-Hypaque density gradient centrifugation. Total cellular RNA was extracted using the Trizol RNA extraction kit in accordance with the manufacturer's instructions. RNA yield and purity were determined spectrophotometrically at 260/280 nm.

Standard plasmid construction

For real-time quantitative RT-PCR, primers and Taqman-MGB probes were designed according to Beacon Designer 2.1 software and synthesised by Shanghai Genecore Biotechnologies (Shanghai, China). The sequences are shown in Table 1. cDNA was synthesised by RT-PCR reactions using a SuperScript‘ III Platinum“ two-step quantitative RT-PCR kit (Invitrogen, USA) in accordance with the manufacturer's instructions. PCR products were purified through gel extraction. Ligation of the pGM-T plasmid vector and purified PCR fragment was performed with T4 DNA ligase (Promega, USA). Plasmids were purified on columns with a Qiagen kit and quantified by A260 measurement. To control the validity of our primers, the amplified products were sequenced. Recombinant plasmid

Table 1 Primer and probe sequences of TRAIL and β-actin

Name		Sequence	Amplicon size (bp)
TRAIL	Sense primer	5'-TCCTCAGAGAGTAGCAGCTCACA-3'	133
	Antisense primer	5'-TGAATGCCCACTCCTTGATG-3'	
	Probe	5'-FAM-TCTTCTCCAAACTCCAAGAA-MGB -3'	
β-Actin	Sense primer	5'-CATTGCCGACAGGATGCA-3'	142
	Antisense primer	5'-CATCTGCTGGAAGGTGGACAG-3'	
	Probe	5'-FAM-TGAAGATCAAGATCATTGCT-MGB-3'	

pGM-T-TRAIL was used as positive control and pGM-T-β-actin was used as endogenous control.

Real-time quantitative RT-PCR

To prepare the standard curve, the recombinant plasmids were gradient diluted with sterilised water to $1x10^9$, 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 copies/μl. We synthesised cDNA from the extracted total RNA by SuperScript‘ III Platinum“ two-step quantitative RT-PCR kit. Real-time quantitative PCR reactions were performed in the ABI-Prism 7000 sequence detector under the following cycling conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Each reaction mixture (50 μl total volume) contained 4 μl cDNA template, 25 μl Platinum“ Quantitative PCR SuperMix-UDG, 1 μl ROX, 1 μl sense primer, 1 μl antisense primer, 1 μl Taqman-MGB probe and 17 μl sterilised water. The results were analysed by Sequence Detection Software (Applied Biosystems). The level of gene expression was determined by interpolation with the standard curve.

Determination of sTRAIL, TNF-α, ALP, GGT, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma

Plasma samples were prepared immediately by centrifugation of peripheral venous blood. The levels of plasma sTRAIL and TNF-α were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol. TNF-α and sTRAIL kits were respectively purchased from Bender (USA) and DIACLONE (France). Sensitivities of these two assays were as follows: TNF-α, 8 pg/ml and sTRAIL, less than 64 pg/ml. Their concentrations were determined by the optical densities obtained and the standard curves. The plasma levels of ALP, GGT, AST and ALT were automatically measured on a Roche P800 biochemical analyser.

Statistical analysis

Data were expressed as the mean ± SD. Student's *t*-test was made to test for differences in means between groups. The correlation of two different parameters was calculated with Pearson's correlation coefficient. p values less than 0.05 were considered significant.

Results

Expression of mTRAIL on peripheral blood leukocytes in PBC and CHB patients

As leukocytes are well suited to flow cytometric analysis for the reason that the normal suspension of blood leukocytes contains a mixture of cell types that happen to give off forward and side light scatter signals of different intensities, thereby allowing them to be distinguished from each other by flow cytometric light scatter parameters, mTRAIL expression on peripheral blood leukocytes was determined by FCM in this study. The results indicated that almost no TRAIL was expressed on peripheral blood leukocytes from healthy individuals, while the positive rates of TRAIL on monocytes, lymphocytes and neutrophils were significantly higher in patients with PBC and CHB than healthy controls ($p < 0.01$ for each). When we compared the positive rates of TRAIL on the three types of cells between two diseased groups, no significant difference was found (Fig. 1).

Expression of TRAIL mRNA in PBMCs in PBC and CHB patients

Amplified products were visualised on 1.5% agarose gels stained with ethidium bromide and photographed under UV light (Fig. 2). The successfully constructed plasmids and correctly targeted fragments were respectively confirmed by sequencing (Fig. 3). To create a standard curve with the plasmid DNA templates, the constructed plasmids were diluted to $1x10^9$, 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 copies/μl. By such a serial dilution, the TRAIL gene was quantified in the unknown samples with known amounts of input copy numbers, by which cycle threshold (Ct) values were plotted and the standard curve was generated. The results indicated that the levels of TRAIL mRNA in PBMC were significantly higher in patients with PBC and CHB than in healthy controls (Fig. 4).

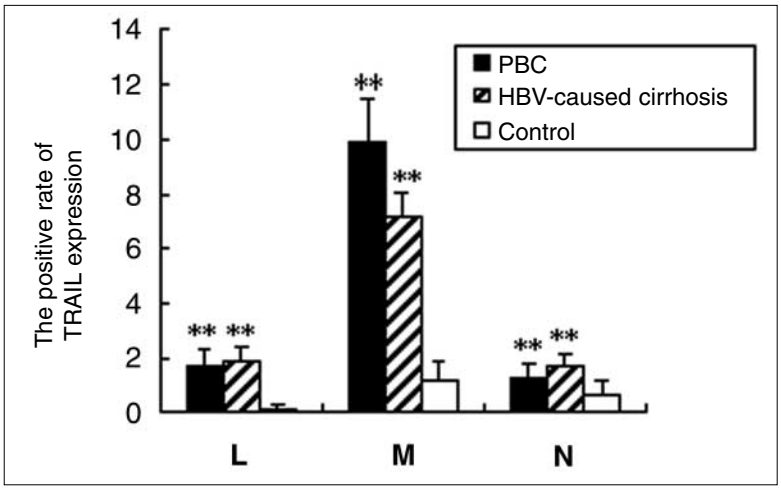

Fig. 1 The positive rates of TRAIL on peripheral blood cells. *L* lymphocytes, *M* monocytes, *N* neutrophils, $^{**}p < 0.01$ *vs*. control

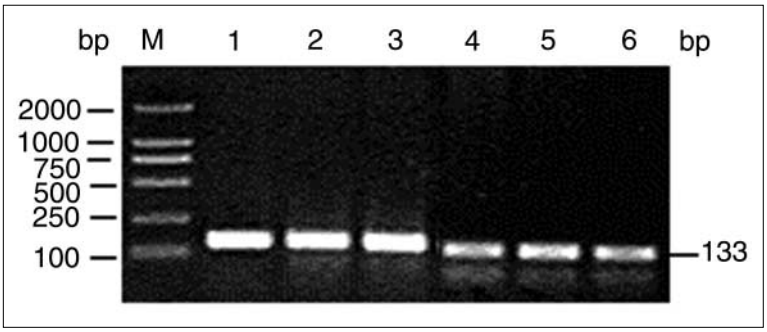

Fig. 2 Agarose gel electrophoresis of TRAIL and β-actin RT-PCR products. M for DL 2000 DNA Ladder marker, 1–3 and 4–6 respectively for β-actin and TRAIL RT-PCR products

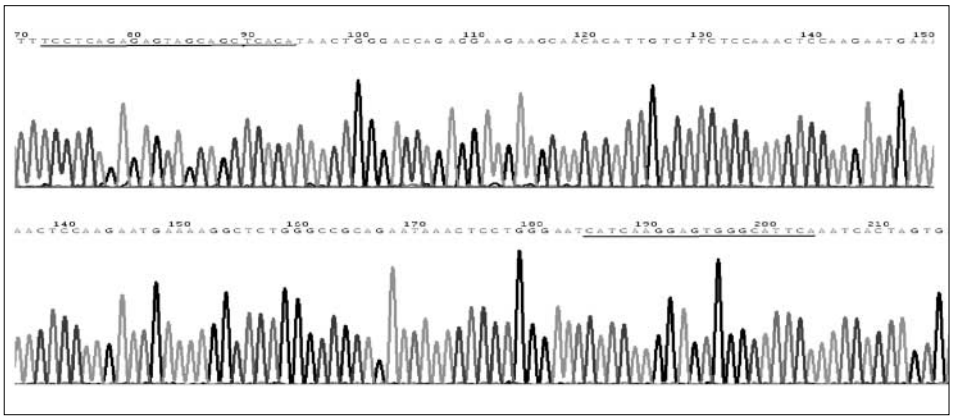

Fig. 3 Sequencing of TRAIL gene fragment

However, there was no significant difference between the two diseased groups.

Expression of plasma sTRAIL, TNF-α, GGT, ALP, AST and ALT in PBC and CHB patients

The plasma concentrations of sTRAIL and TNF-α were 1448.4 ± 179.5 and 52.3 ± 10.6 (pg/ml) in PBC patients and 1624.1 ± 415.9 and 1406.8 ± 497.9 (pg/ml) in CHB patients. Both of them were significantly elevated compared to healthy controls [1098.5 ± 264.7 and 16.9 ± 3.8 (pg/ml)]. The TNF-α level was significantly higher in CHB patients than in PBC ones. For sTRAIL, no significant difference was found between the two diseased groups. Both the PBC patients and the CHB ones showed higher plasma levels of GGT and ALP compared to healthy controls, especially the PBC ones. The ALT and AST levels were also elevated in either PBC patients or CHB ones, especially in CHB ones.

Correlations between the levels of mTRAIL on monocytes or sTRAIL and TRAIL mRNA or laboratory parameters of disease activity in PBC or CHB patients

In our study, it was observed that TRAIL mRNA showed a close association with either mTRAIL or sTRAIL in

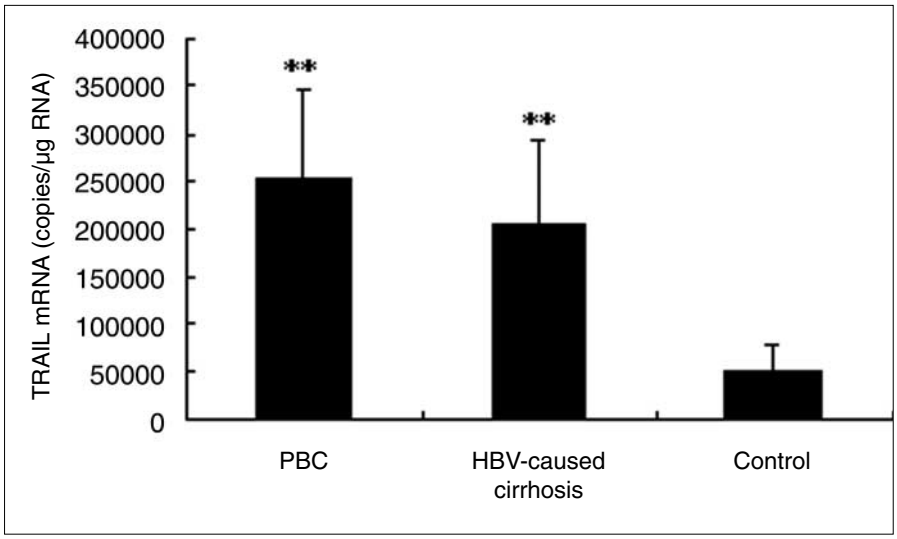

Fig. 4 Expression of TRAIL mRNA in PBMCs in various groups. $^{**}p < 0.01$ *vs.* control

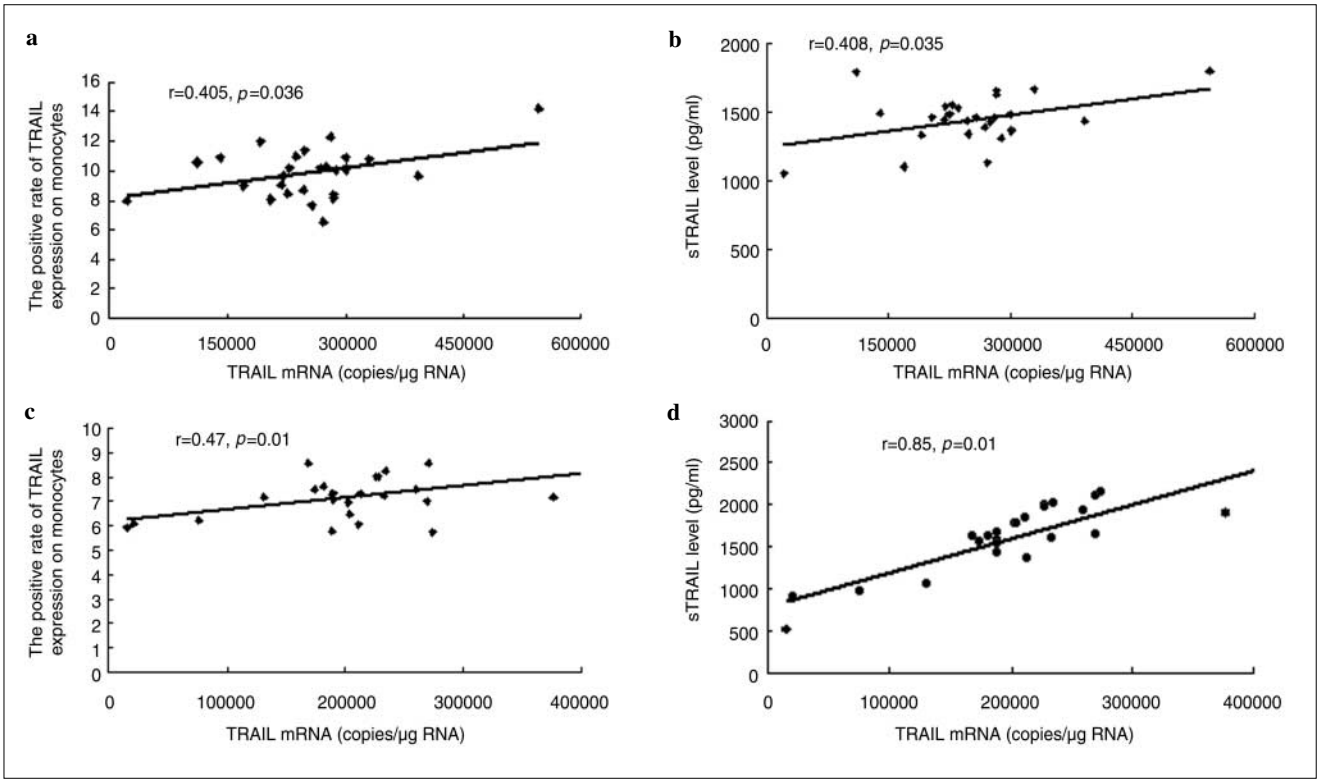

Fig. 5a-d Associations of TRAIL mRNA with mTRAIL and sTRAIL in patients with PBC and CHB (**a** and **b** for PBC, **c** and **d** for CHB)

Table 2 The levels of plasma sTRAIL, TNF-α, GGT, ALP, ALT and AST in populations

	Healthy controls (n = 30)	PBC patients (n = 27)	HBV-caused cirrhosis patients (n = 25)
sTRAIL (pg/ml)	1098.5 ± 264.7	1448.4 ± 179.5**	1624.1 ± 415.9*
TNF-α (pg/ml)	16.9 ± 3.8	52.3 ± 10.6**	1406.8 ± 497.9**
GGT (U/l)	19.1 ± 5.4	376.7 ± 85.7**	102.5 ± 55.4**
ALP (U/l)	38.5± 8.6	359.1 ± 89.8**	120.7 ± 36.4**
ALT (U/l)	22.6 ± 6.8	69.9 ± 32.5**	217.3 ± 96.5**
AST (U/l)	16.4 ± 5.6	83.0 ± 29.6**	229.2 ± 97.3**

$^{*}p < 0.05$ *vs.* healthy controls, $^{**}p < 0.01$ *vs.* healthy controls

Table 3 The correlations of TRAIL protein with TNF-α, GGT, ALP, ALT and AST in diseased groups

	PBC patients				CHB patients			
	TRAIL on monocytes		sTRAIL		TRAIL on monocytes		sTRAIL	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
TNF-α (pg/ml)	0.423	0.028	0.796	0.000	0.467	0.019	0.823	0.000
GGT(U/l)	0.406	0.036	0.508	0.007	0.227	0.275	0.012	0.956
ALP(U/l)	0.520	0.005	0.423	0.028	0.111	0.596	0.375	0.064
ALT (U/l)	0.025	0.900	0.235	0.238	0.423	0.035	0.471	0.017
AST (U/l)	0.192	0.338	0.242	0.224	0.403	0.046	0.728	0.001

PBC and CHB patients, as did TNF-α. In PBC patients, there were significant correlations between mTRAIL or sTRAIL and GGT or ALP, but no significant correlation was observed between mTRAIL or sTRAIL and ALT or AST. The case was the opposite in CHB patients (Fig. 5 and Table 3).

Discussion

mTRAIL has been confirmed to be a potent apoptosis inducer when engaged with the receptors DR4/DR5, expressed on a variety of cell types. The soluble form of TRAIL is believed to be shed from cellular membrane compartments by proteolytic cleavages or realised in association with vesicles. It has been reported that purified recombinant human sTRAIL was able to induce apoptosis of normal human hepatocytes [13], and injection of TRAIL (60 μg/mouse i.v.) into bile duct ligated mice promoted hepatocyte apoptosis and serum ALT values [14]. In the current study, we compared the expression of TRAIL protein and gene in peripheral blood leukocytes and plasma sTRAIL and TNF-α in PBC and CHB patients with healthy individuals. The results demonstrated that although TRAIL existed mainly in the form of soluble sTRAIL in peripheral blood in populations, the expression levels of TRAIL gene and protein in peripheral blood leukocytes and plasma sTRAIL and TNF-α were all significantly up-regulated in patients with PBC and CHB, compared with healthy controls. In the diseased groups, both plasma sTRAIL and mTRAIL on monocytes correlated well with plasma TNF-α. These results indicated that the up-regulation of TRAIL in peripheral blood as well as plasma TNF-α was not specific for PBC. TRAIL and TNF-α might interact and play a role in the pathogenesis of PBC and CHB. It is well known that in PBC patients the areas around injured intrahepatic biliary epithelial cells are frequently filtrated with various immune cells such as T lymphocytes, monocytes, neutrophils, NK cells, etc. In our study, it is observed that the expression of TRAIL on peripheral blood monocytes, lymphocytes and neutrophils is elevated compared to PBC patients. Therefore, but for sTRAIL, mTRAIL might also play a pivotal role in the pathogenesis of PBC.

As we know, ALP and GGT are two important biochemical markers of cholestasis, indicating the development and progress of cholestasis [15], while ALT and AST are key indicators for damage of hepatocytes. The present study showed that in PBC, the expression of TRAIL was closely associated to ALP and GGT, but in CHB, with ALT and AST. As a result, it could be concluded that TRAIL might be closely associated with hepatocyte damage in CHB, as HBV sensitises hepatocytes to TRAIL-induced apoptosis [16, 17]. On the other hand, TRAIL might be closely related to cholestasis in PBC; the possible mechanism of this is that bile acids could induce expression of TRAIL-R2/DR5 in liver cells and biliary epithelial cells, rendering them susceptible to TRAIL-induced apoptosis [18, 19].

Conclusions

Unfortunately, our study does not include enough histological features to classify the disease, thus the exact correlation of TRAIL and the disease activity couldn't really be assessed. Despite this limitation, through our investigations, it could be concluded that the abnormal expression of TRAIL in peripheral blood is not specific for PBC or CHB. This is the first description, to our knowledge, of the altered expressions of TRAIL mRNA and membrane-bound protein in peripheral blood leukocytes as well as sTRAIL and TNF-α in PBC patients and their role in the development and progression of PBC. However, the molecular mechanisms of overexpression of TRAIL and detailed role of TRAIL in pathogenesis of PBC still remain to be clarified.

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Conflict of interest The authors declare that they have no conflict of interest related to the publication of this manuscript.

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