European Review for Medical and Pharmacological Sciences 2015; 19: 467-476

Association of ulcerative colitis with TNF-related apoptosis inducing ligand (TRAIL) gene polymorphisms and plasma soluble TRAIL levels in Chinese Han population

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Abstract. – OBJECTIVE: The precise etiology of inflammatory bowel diseases (IBDs) is still unknown although dysregulation of apoptosis likely plays an important role in this pathogenesis. However, the significance of mucosal T-cell apoptosis in ulcerative colitis (UC) is unclear. In the present work we investigated the role of TNF-related apoptosis-inducing ligand (*TRAIL*), which is implicated in various human disorders.

PATIENTS AND METHODS: Results from a total of 393 UC patients and 1292 healthy individuals were analyzed in this study. We determined the three single nucleotide polymorphisms of *TRAIL* in 3' untranslated regions (UTR), and examined the plasma soluble TRAIL (sTRAIL) levels by enzyme-linked immunosorbent assay.

RESULTS: We found that the mutant genotypes of TRAIL (G1525A/G1588A/C1595T and G1525A and G1588A) were much lower in UC patients compared to the controls. Furthermore, mutant allele and genotype of *TRAIL* C1595T were more prevalent in severe UC patients than in other patients ($p < 0.001$; $p = 0.005$, respectively). The three polymorphic sites in 3'UTR were in a perfect linkage disequilibrium in our study. In contrast to controls, the GAT haplotype was increased ($p < 0.001$), while the AAT haplotype was decreased in UC patients ($p < 0.001$). Besides, the plasma levels of sTRAIL were significantly higher in UC patients than in controls ($p < 0.001$).

CONCLUSIONS: Our findings suggested that increased occurrence of the genetic mutations of *TRAIL* in 3'UTR and possibly decreased plasma levels of sTRAIL might lead to a lower risk of UC attack in Chinese patients.

Key Words:
TNF-related apoptosis-inducing ligand, Ulcerative colitis, Genes, Polymorphism.

Abbreviations

CI = confidence interval; CD = Crohn's disease; IBDs = Inflammatory bowel diseases; LP = lamina propria; OR = odds ratio; SNPs = single-nucleotide polymorphisms; TNF = Tumor-necrosis factor; TRAIL = TNF-related apoptosis-inducing ligand; UC = ulcerative colitis; 3'UTR = 3' untranslated regions.

Introduction

Inflammatory bowel diseases (IBDs) are chronic intestinal disorders that comprise Crohn's disease (CD) and ulcerative colitis (UC). Although the specific etiology of IBD is unknown, a combination of environmental, genetic, and immunological factors likely play a role involving an uncontrolled immune response within the intestinal lumen leads to inflammation in genetically predisposed individuals[1]. Numerous clinical studies have revealed that the colonic mucosa of IBD are infiltrated with activated T lymphocytes, neutrophils, and plasma cells[2]. These findings corroborate the notion that IBD is characterized by a highly activated state of the mucosal immune system and excessive mucosal damage[3]. The underlying mechanisms for the uncontrolled immune response in IBD patients have not been fully unveiled.

Apoptosis, or programmed cell death, is recognized as a common regulatory mechanism for normal development and homeostasis of the immune system. Dysregulation of apoptosis has been demonstrated to be involved in the pathogenesis of various diseases, including UC and CD. Recent development, furthermore, indicates

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that T-cell resistance against apoptosis contributes to the inappropriate T-cell accumulation and perpetuation of chronic mucosal inflammation in IBD. It has been shown that mucosal T cells are highly resistant to apoptosis in CD[4-7]. However, the data from UC are less consistent, as T-cell apoptosis has been reported to be reduced[4,7,8] or increased[5,6,9]. Thus, the significance of altered apoptosis in the pathogenesis of UC still needs to be ascertained.

Tumor-necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL), or Apo-2L, was initially identified by its sequence homology with other TNF family members, such as TNF-α and FasL[10]. TRAIL is primarily expressed as a type II membrane protein and its C-terminus can be processed proteolytically to form a soluble ligand[16]. Both full length, membrane-expressed TRAIL and the soluble version, sTRAIL, rapidly induce apoptosis in a wide variety of cell types through interaction with the death receptors DR4 (TRAIL-R1) and/or DR5 (TRAIL-R2) on transformed or infected cells[16,17]. Contrary to TNF-α and FasL, membrane-bound TRAIL is conditionally expressed in immune cells such as natural killer cells, B cells, monocytes and dendritic cells following cytokine stimulation[18-21]. Moreover, intracellular stores of TRAIL have also been found in polymorphonuclear neutrophils[22-26] that are released after a variety of stimuli[23,27,28].

Although the biological and immunological roles of TRAIL have not been completely elucidated, there is increasing evidence that its function is influenced not only by the genetic polymorphisms, but also the receptor system of TRAIL. The *TRAIL* gene is located on chromosome 3q26, and its five exons encode an approximately 1.77 kb mRNA[29]. Previous studies have implicated that the 3' untranslated regions (3'UTR) of TRAIL have wide ranging influences on gene regulation. Five single-nucleotide polymorphisms (SNPs) have been identified in *TRAIL* exons: three in the 3' UTR at 1525, 1588 and 1595 sites, while the other two in exon 1 at position 192 and 912 do not alter the encoded amino acid sequence[29,30]. In Japanese population, the polymorphism at position 1595 of *TRAIL* gene was reported to be associated with the susceptibility to multiple sclerosis, suggesting that this site might be a functional variant[31]. Yan et al[30] evaluated four SNPs in the promoter region of *TRAIL* in Chinese population as well, and discovered that no SNPs at –707, –665, and –597 sites were detected.

So far, much progress has been made in the understanding of TRAIL contributing to the development of many human diseases, especially for tumors and autoimmune disorders. Nevertheless, it is uncertain whether or not the functional SNPs of *TRAIL* gene have an impact on the predisposition to UC. Therefore, on the basis of the findings mentioned above, we examined the association of the three polymorphic sites (G1525A/G1588A/C1595T) in 3' UTR of *TRAIL* and plasma sTRAIL levels with UC in Chinese patients (Figure 1A and B). Our present study suggested that the genetic mutations of *TRAIL* (G1525A/G1588A/C1595T) and decreased plasma levels of sTRAIL might engender a lower risk of UC attack.

Patients and Methods

Study Subjects

Patients (n=393) with UC, admitted during May 2007 to December 2012 to the Second and First Affiliated Hospitals of Wenzhou Medical University and The Center Hospital of Wenzhou City, Zhejiang Province of Southeast China were recruited for this study. Diagnosis of UC was made by endoscopic, radiological, and pathological examinations in accordance with Lennard-Jones criteria. The severity of the disease was evaluated using the Truelove & Witt Activity Index. The location of the disease was assessed by colonoscopy at the initial diagnosis and follow up. Extensive colitis was defined as disease lesion location beyond spleen flexure and distal colitis was defined as disease lesion distal to spleen flexure.

During the same period, a total of 1292 age- and sex-matched healthy controls were recruited from Physical Examination Center at The Second Affiliated Hospital of Wenzhou Medical University. The demographic data of patients with UC and healthy controls are presented in Table I. The study protocol was approved by the Ethics Committees of the three hospitals mentioned above. Informed consent was obtained from all the patients and healthy individuals.

Blood Samples

Venous blood (~5 ml) were collected from each subject into EDTA tubes after a 12-hour fast. Within 30 min after sampling, plasma was isolated by centrifugation (400 ↔ g, for 10 min, 4°C) and immediately frozen at –20°C pending

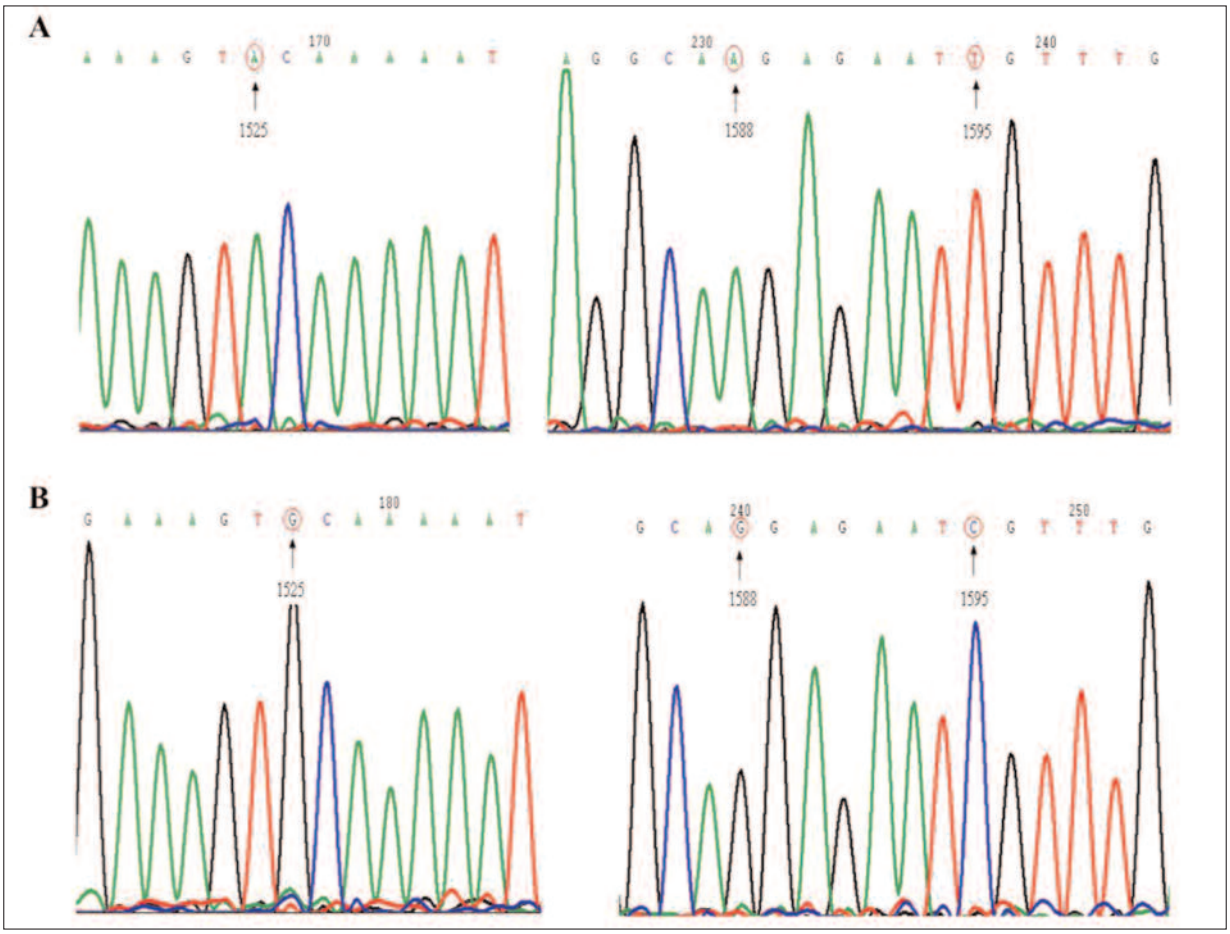

Figure 1. The genotypes of TRAIL presented in panel ***A,*** are 1525AA, 1588AA, and 1595TT (*showed by black arrows*). The genotypes shown in the panel ***B,*** are 1525GG, 1588GG and 1595CC (*showed by black arrows*).

Table I. Demographic characteristics of patients with Ulcerative Colitis (UC) and Healthy Controls (HC).

Characteristics	UC	HC	*p*
Total number	393	1292	
Sex (female/male)	167/226	572/720	n.s.
Age, years [mean (SD)]	42 (14)	43 (13)	n.s.
Age of onset, years [mean (SD)]	33 (17)		
Duration of the disease, years [mean (SD)]	15.7 (6.4)		
Location of UC, n (%)			
Distal colitis	253 (64.37)		
Extensive colitis	140 (35.63)		
Severity of UC, n (%)			
Mild	174 (44.27)		
Intermediate	119 (30.28)		
Severe	100 (25.45)		
Treatment, n (%)			
SASP/5-ASA	314 (79.89)		
Prednisone	157 (39.94)		
Antibiotics	137 (34.86)		
Immunosuppressive	20 (5.09)		
Colectomy, n (%)	11 (2.79)		

SD: standard deviation; SASP: sulfasalazine; 5-ASA: 5-aminosalicylic acid; n.s.: not statistically significant.

analyses. Genomic DNA was extracted from peripheral blood lymphocytes using the DNeasy Blood & Tissue kit (Qiagen GmbH, Hilden, Germany) and then stored at 4°C for subsequent identification of genetic mutations.

Genotyping and Haplotype Analysis of the TRAIL (G1525A/G1588A/C1595T)

The PCR product was amplified using 5'-GTAGTAGCCTCCAGGTTTCC-3' and 5'-AACTCAACCCAGAACAAAGG-3' as sense and antisense primer, respectively. The PCR reactions were carried out in a final volume of 25 L, containing approximately 40 ng genomic DNA, 0.4 mM each primer, 200 mM dNTP, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.4) and 0.2 U KOD-Plus polymerase. The PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 45 cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 40 s, with a final extension at 68°C for 7 min. Then, the genetic polymorphisms of *TRAIL* (G1525A/G1588A/C1595T) were examined by direct sequencing, and the haplotype analysis was also performed in all the subjects using PHASE2.1 software.

Assays for Plasma Levels of sTRAIL

From the above two groups, 134 UC patients and 254 age- and sex-matched controls were randomly selected for determination of plasma sTRAIL concentrations using enzyme-linked immunosorbent assay (Bender Medsystems, Vienna, Austria). The UC patients consisted of 59 patients with extensive colitis and 75 patients with distal colitis, and were divided into 47 cases with mild colitis, 44 cases with intermediate colitis and 43 severe colitis cases, in accordance with activity of the disease.

Statistical Analysis

The results are presented as means ± SD (standard deviation) for continuous variables(age, sTRAIL) and as percentages for categorical variables(allele, genotype and haplotype). Hardy-Weinberg equilibrium for each of the polymorphisms was investigated by the chi-square test in healthy controls. Either the chi-square test or Fisher's exact test was used to compare categorical variables, and the Student's *t* test was used for continuous variables. Furthermore, the associations of genetic and clinical characteristics with sTRAIL in patients were further evaluated by the multiple linear regression analysis. All statistical analyses were conducted using SPSS 13.0 software (SPSS version 13.0, Chicago, IL, USA). A two-tailed p value < 0.05 was considered significant.

Results

Allele and Genotype Frequencies of TRAIL (G1525A/G1588A/C1595T) in UC Patients and the Healthy Controls

As shown in Table II, in healthy controls, the distributions of *TRAIL* G1525A, G1588A and C1595T genotypes were in the Hardy-Weinberg equilibrium (all $p > 0.05$). In UC patients, the frequencies of mutant genotype in *TRAIL* G1525A, G1588A and C1595T genes were significantly lower than in the healthy controls (45.80% vs. 78.57%, OR = 0.231, 95% CI: 0.182-0.293, $p < 0.001$; 62.34% vs. 79.18%, OR = 0.435, 95% CI: 0.341-0.556, $p < 0.001$; 65.40% vs. 79.72%, OR = 0.481, 95% CI: 0.375-0.616, $p < 0.001$, respectively). The mutant allele frequencies of *TRAIL* G1525A and G1588A were also significantly decreased in UC patients (40.08% vs. 54.96%, OR = 0.548, 95% CI: 0.466~0.645, $p < 0.001$; 49.49% *vs*. 55.53%, OR = 0.784, 95% CI: 0.668-0.921, $p = 0.003$, respectively). However, the frequency of mutant allele in *TRAIL* C1595T gene was not significantly lower in UC patients (55.85% vs. 52.80%; OR = 0.884, 95% CI: 0.754-1.038, $p = 0.133$).

The Haplotype Analysis of TRAIL (G1525A/G1588A/C1595T) Gene Polymorphisms

The haplotype analysis was carried out in all study subjects using PHASE2.1 software. The *TRAIL* G1525A, G1588A and C1595T polymorphic sites (Figure 1) were shown to be in a complete linkage disequilibrium (G1525A/G1588A, $D' = 0.985$, $r^2 = 0.816$; G1525A/C1595T, $D' = 0.979$, $r^2 = 0.810$; G1588A/C1595T, $D' = 0.979$, $r^2 = 0.955$, respectively). In eight haplotypes, frequencies that were simultaneously less than 3% in UC patients as well as in the healthy controls, were not taken into account. Thus, the frequency of GAT haplotype was significantly higher in UC patients than in the healthy controls (10.15% vs. 0.18%, OR = 58.448, 95% CI: 23.590-144.815, $p < 0.001$). The frequency of AAT haplotype, nev-

Table II. Genotype and allele frequencies of the TRAIL (G1525A/G1588A/C1595T) gene polymorphisms in patients with Ulcerative Colitis (UC) and Healthy Controls (HC).

Genotypes, n (%)	UC n = 393	HC n = 1292	OR	95% CI	p
TRAIL G1525A					
GG	213 (54.20)	277 (21.43)			
GA	45 (11.45)	610 (47.21)			
AA	135 (34.35)	405 (31.36)			
GA+AA	180 (45.80)	1015 (78.57)	0.231	0.182-0.293	< 0.001
G allele	471 (59.92)	1164 (45.04)			
A allele	315 (40.08)	1420 (54.96)	0.548	0.466-0.645	< 0.001
TRAIL G1588A					
GG	148 (37.66)	269 (20.82)			
GA	101 (25.69)	611 (47.29)			
AA	144 (36.65)	412 (31.89)			
GA+AA	245 (62.34)	1023 (79.18)	0.435	0.341-0.556	< 0.001
G allele	397 (50.51)	1149 (44.47)			
A allele	389 (49.49)	1435 (55.53)	0.784	0.668-0.921	0.003
TRAIL C1595T					
CC	136 (34.60)	262 (20.28)			
CT	99 (25.19)	617 (47.75)			
TT	158 (40.21)	413 (31.97)			
CT+TT	257 (65.40)	1030 (79.72)	0.481	0.375-0.616	< 0.001
C allele	371 (47.20)	1141 (44.15)			
T allele	415 (52.80)	1443 (55.85)	0.884	0.754-1.038	0.133

OR: odds ratio; CI: confidence interval.

ertheless, was significantly lower in UC patients (43.09% vs. 58.41%, OR = 0.537, 95% CI: 0.457-0.632, $p < 0.001$) (Table III).

Associations of Clinical Characteristics of UC patients with the Polymorphisms in TRAIL (G1525A/G1588A/C1595T) Gene

A further investigation was carried out to assess whether the genetic polymorphisms of *TRAIL* (G1525A/G1588A/C1595T) were associated with the clinical characteristics of UC patients. The frequencies of variant allele (T) and genotype (CT+TT) in *TRAIL* C1595T gene were significantly higher in the patients with severe UC than in other patients (63.50% vs. 49.15%, OR = 1.80, 95% CI: 1.294-2.505, $p < 0.001$; 77.00% vs. 61.43%, OR = 2.102, 95% CI: 1.247-3.541, $p = 0.005$, respectively). However, the genetic polymorphisms of *TRAIL* G1525A and G1588A were found not to be significantly related to activity and location of the disease in UC patients ($p > 0.05$) (Table IV).

Plasma Levels of Soluble TRAIL in UC Patients and the Healthy Controls

The average plasma level of sTRAIL in UC patients was significantly higher than in the healthy controls [(1.05 ± 0.48) vs. (0.96 ± 0.90) ng/L, $p < 0.01$], and was significantly related with the genetic polymorphisms of *TRAIL* (G1525A/G1588A/C1595T) (all $p < 0.01$) (Table V). However, no significant associations were found between the sTRAIL levels and the clinical characteristics in UC patients (all $p > 0.05$) (Table V).

Table III. Haplotype frequencies of the *TRAIL* (G1525A/G1588A /C1595T) in patients with Ulcerative Colitis (UC) and Healthy Controls (HC).

Haplotype	n	AAT[a]	AGC	GAC	GAT[b]	GGC[c]	GGT	AAC	AGT
TUC	786	0.431	0.005	0.005	0.102	0.445	0.012	0.000	0.000
HC	2584	0.584	0.001	0.003	0.002	0.407	0.000	0.002	0.001

[a]Odds ratio (OR) = 0.537, 95% confidence interval (CI): 0.457-0.632, $p < 0.001$; [b]OR = 58.448, 95% CI: 23.590-144.815, $p < 0.001$; [c]OR = 1.165, 95% CI: 0.992-1.369, $p = 0.063$.

Table IV. Associations of the TRAIL (G1525A/G1588A/C1595T) gene polymorphisms with the clinical characteristics in UC patients.

Characteristics	Location of disease		Severity of disease	
	Distal colitis	Extensive colitis	Mild + Intermediate	Severe
G1525A genotype				
GG	142 (56.13)	71 (50.71)	162 (55.29)	51 (51.00)
GA+AA	111 (43.87)	69 (49.29)	131 (44.71)	49 (49.00)
G	312 (61.66)	156 (55.71)	359 (61.26)	112 (56.00)
A	194 (38.34)	124 (44.29)	227 (38.74)	88 (44.00)
G1588A genotype				
GG	99 (39.13)	49 (35.00)	112 (38.23)	36 (36.00)
GA+AA	154 (60.87)	91 (65.00)	181 (61.77)	64 (64.00)
G	277 (55.07)	148 (52.85)	300 (51.19)	97 (48.50)
A	229 (44.93)	132 (47.15)	286 (48.81)	103 (51.50)
C1595T genotype				
CC	89 (35.18)	47 (33.57)	113 (37.78)	23 (23.25)
CT+TT	164 (64.82)	93 (66.43)	180 (62.22)	77 (76.75)a
C	257 (50.79)	134 (47.85)	298 (50.85)	73 (36.50)
T	249 (49.21)	146 (52.15)	288 (49.15)	127 (63.5)b

[a]Odds ratio (OR) = 2.102, 95% confidence interval (CI): 1.247-3.541, $p = 0.005$; [b]OR = 1.80, 95% CI: 1.294-2.505, $p < 0.001$.

Discussion

Apoptosis signaling-related genes are known to be involved in IBD. Previous studies have shown that Fas/CD95 was implicated in the intestinal inflammation, particularly in UC[32,33]. The expression of FasL was found to be upregulated on intestinal lymphocytes in UC as well[33]. In addition, although the *Fas*-670 polymorphism was associated with several autoim-

Table V. Associations of the sTRAIL levels with TRAIL polymorphisms and clinical characteristics in patients with Ulcerative Colitis (UC).

TRAIL genotypes/clinical characteristics	sTRAIL levels ($\bar{x} \pm s$) (pg/ml)	95% CI	p
Distal colitis	0.976 ± 0.475	-0.053/0.437	0.122
Extensive colitis	1.168 ± 0.484		
Severity of UC			
Mild	0.957 ± 0.499	-0.204/-0.188	0.127
Intermediate	1.153 ± 0.437	-0.063/-0.036	0.818
Severe	1.007 ± 0.612	0.132/0.159	0.503
G1525A genotype			
GG	1.214 ± 0.482	-0.335/-0.332	0.008
GA	0.886 ± 0.412	-0.588/-0.561	0.010
AA	0.639 ± 0.334	-0.261/-0.233	0.270
G1588A genotype			
GG	1.291 ± 0.538	0.435/0.454	0.005
GA	0.923 ± 0.410	0.359/0.375	0.005
AA	0.845 ± 0.316	-0.087/-0.068	0.613
C1595T genotype			
CC	1.30 ± 0.547	0.366/0.381	0.004
CT	0.927 ± 0.403	0.446-0.465	0.005
TT	0.845 ± 0.316	0.072-0.091	0.591

CI: confidence interval.

mune disorders, such as systemic lupus erythematosus, rheumatoid arthritis[34,35] and multiple sclerosis[36,37], it appears not to be related with IBD[38].

TRAIL has received considerable attention in recent years mainly because, in comparison with TNF-α and FasL, TRAIL selectively induces apoptosis in tumor cells but not in most normal cells[9]. Currently increasing evidence has suggested that TRAIL acts as a fine-tuning regulator of the immune system[39]. The role of TRAIL as an effector molecule in the immune system and its apoptotic potential is reflected in the regulation of the *TRAIL* gene[40]. This study implicates that *TRAIL* gene polymorphisms might be one of the key factors, which influence biological and immunological functions of TRAIL. Clinical observations have demonstrated a role for TRAIL in autoimmune diseases as patients with systemic lupus erythmatosus or multiple sclerosis have elevated serum levels of soluble TRAIL[41,42]. In addition to tumors and autoimmune diseases, TRAIL has also been shown to participate in cardiovascular problems such as atherosclerosis[43,44] and diabetes mellitus[45]. Recent studies revealed that TRAIL may also participate in a variety of pathological liver diseases. For example, Han et al[46] found that plasma sTRAIL concentrations in patients with cirrhosis and liver cancer were significantly higher than those of healthy subjects and hepatitis B virus carriers, but lower than those of the hepatitis B patients. Yan et al[25] suggested that TRAIL should be considered as a novel mediator of fatty liver disease. However, as far as we know, there are few studies carried out on the association of genetic polymorphisms and plasma phenotype of TRAIL with UC.

Our present work primarily presented that G/G allele frequencies at position 1525/1588 in UC patients were significantly higher than those of healthy controls. UC patients also showed significantly lower (GA+AA)/(GA+AA)/(CT+TT) genotype frequencies at 1525/1588/1595 sites than healthy subjects, which indicated that Chinese individuals with mutant genotypes of *TRAIL* in the 3' UTR at position 1525/1588/1595 might have a lower risk of UC attack. We also investigated whether or not mutant alleles and genotypes of *TRAIL* in the 3' UTR had an influence on the clinical characteristics in UC patients. Results showed that both the mutant allele (T) and genotype (CT+TT) of *TRAIL* at 1595 site were significantly increased in patients with severe UC compared to other patients, which implied that the polymorphism of *TRAIL* C1595T might exert an impact on severity of the disease. A previous study in Japanese population revealed that the C1595T site of *TRAIL* gene might be a functional variant influencing the susceptibility to multiple sclerosis[31]. Presumably 1595 site of *TRAIL* may alter the binding sequence of microRNA, which could affect the expression of *TRAIL* gene[31].

We also noticed in this study that plasma sTRAIL levels were significantly enhanced in UC patients in contrast with the controls. Furthermore, to explore the causes of the higher levels of sTRAIL in UC patients, we inferred that gene polymorphisms of *TRAIL* in the 3' UTR might affect its expression levels. After adjustment for age and gender, we found that average sTRAIL levels in plasma were significantly related to the three SNPs in the 3' UTR of *TRAIL* in UC patients. UC patients with homozygous variant genotype AA/AA/TT of *TRAIL* at 1525/1588/1595 sites showed lower sTRAIL concentrations in plasma compared to those with other genotypes. Nonetheless, no significant correlation between plasma sTRAIL levels and clinical features in the patients with UC was observed.

Moreover, a further haplotype analysis was also carried out in our study. We found that 1525/1588/1595 sites are in perfect linkage disequilibrium in Chinese population. This finding is in accordance with the study by Yan et al[30], in which 1525/1595 sites are also found to be in complete linkage disequilibrium in Chinese individuals. We also found in all eight haplotypes, that the GAT haplotype was significantly increased, while the AAT haplotype was significantly decreased in UC patients compared to the controls. It implies that Chinese individuals with AAT haplotype of *TRAIL* in 3' UTR at position 1525/1588/1595 have a lower susceptibility to UC.

Conclusions

The current study shows that *TRAIL* (G1525A/G1588A/C1595T) gene polymorphisms and elevated plasma sTRAIL levels are closely linked to UC in Chinese patients. It is worth mentioning that 1595 site of *TRAIL* might be a functional variant, since we found that the *TRAIL* C1595T polymorphism not only have an impact on severity of UC, but also on plasma

sTRAIL concentrations in UC patients. However, whether 1525/1588 sites of *TRAIL* are also functional variants, more function analysis should be further performed in the future. Besides, although the levels of sTRAIL were significantly higher in UC patients than in healthy controls, they had no influence on the clinical characteristics of UC patients in this study. We conjectured that TRAIL might possess another biological function in addition to the induction of apoptosis in Chinese UC patients.

Acknowledgements

The project was supported by grants from Zhejiang Provincial Health Bureau (Grant number: 2012KYA132) and Wenzhou Municipal Science and Technology Bureau (Grant number: Y20080110 and Y20110027). We also appreciate The First Affiliated Hospitals of Wenzhou Medical University and The Central Hospital of Wenzhou City for their supplying of blood samples and clinical data of patients with UC.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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