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Original Article

Genetic polymorphisms and haplotypes of TRAIL gene correlate with NSCLC susceptibility in a group of Chinese patients

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Abstract: The association between genetic polymorphisms and haplotypes of TNF-related apoptosis-inducing ligand (TRAIL) and the NSCLC development was investigated in 592 Chinese patients and the prevalence of G1525A, G1588A, and C1595T gene polymorphisms compared between the NSCLC patients and control group in this study. It was found that the frequencies of variant allele A and genotype GA+AA of G1525A were significantly lower and those of variant alleles A and T of G1588A and C1595T significantly higher in the NSCLC patients compared with those in control. The frequencies of variant allele T and genotype CT+TT of C1595T were significantly higher in stage III and IV than in stage I and II of the patients. Moreover, the frequencies of variant allele A and genotype GA+AA of G1525A were significantly higher in stage III and IV than in stage I and II of the patients. In addition, TRAIL gene variants G1525A/G1588A/C1595T were found to be in complete linkage disequilibrium in all patients. Compared with the healthy people, the frequency of AAT haplotype was significantly lower whereas that of GAT haplotype significantly higher in NSCLC patients. The results indicated that the genetic polymorphisms and haplotypes of TRAIL gene correlated significantly with the NSCLC susceptibility in the group of Chinese patients.

Keywords: Non-small cell lung cancer, TNF-related apoptosis-inducing ligand, correlation, genetic polymorphism, haplotype

Introduction

Lung cancer is known as the most frequent and mortal human cancer. Non-small cell lung cancer (NSCLC), with an overall 5-yr survival rate of only 15% [1], accounts for about 80%-85% of lung cancer [2] and leads to 1.2 million death annually worldwide in both sexes [1]. Although the exact etiology of NSCLC remains unclear, environment factor including smoking is recognized as an important pathogenic cause and even four lung cancer susceptibility loci are found to be associated and interacted with smoking in a large sample (7436 cases) of Chinese patients [3]. In recent studies, significant progresses have been made on the genetic pathogenesis and the correlation between specific genes to the susceptibility of lung cancer [1, 4-6]. It has been further confirmed that genetic mechanisms, including gene mutation, gene deletion and gene polymorphism, determine the individual susceptibility to lung cancer of different people [7, 8].

Programmed cell death (apoptosis) is a complicated biological process by which unwanted cells including malignant cells are eliminated from multicellular organisms in a non-harmful way. Importantly, cancer cells proliferate out of control but tend to exhibit more apoptosis than normal cells [9-11]. Dysregulation of apoptosis is crucial for carcinogenesis. Endogenous TNF-related apoptosis-inducing ligand (TRAIL), a new member of the TNF cytokine family [12, 13], is expressed in natural killer (NK) cells, macrophages, T cells, and dendritic cells and is thought to involve in self-defense mechanism by killing virus-infected and cells [14] or inducing apoptosis of the malignant cells without affecting normal cells. TRAIL has demonstrated an association with several autoimmune diseases, such as ulcerative colitis [15], thyroiditis

[16], multiple sclerosis [17], systemic lupus erythematosus [18] and pathological liver disease [19, 20]. Kikuchi et al. have shown that the TRAIL C1595T polymorphism is associated with the susceptibility to multiple sclerosis of Japanese population [17]. Therefore, the induced apoptosis of TRAIL may explain the self-defensiveness of human body and contribute to the clinical effectiveness of some promising targeted agents including recombinant human TRAIL (rhTRAIL) [21]. TRAIL gene is located on chromosome 3q26 with five exons encoding an approximately 1.77 kb mRNA [22]. Previous studies have shown that the 3' untranslated region (3'UTR) of TRAIL has important role in TRAIL gene regulation. Five single-nucleotide polymorphisms (SNPs) have been identified in TRAIL exons. Three of them are in the 3'UTR at loci 1525, 1588 and 1595, while the other two are in exon 1 at site of 1192 and 5912, which do not alter the encoded amino acid sequence [22, 23]. According to increasing evidence, the function of TRAIL relays on the genetic polymorphisms of TRAIL, and death receptors (DRs) of TRAIL including DR4, DR5, DR2 and DR3 [24]. The significant association was found between the DR4 haplotype 626 C-683 C and increased risk of the breast cancer onset in a German population (OR=3.52, 95% CI=1.45-8.52, P=0.003). However, neither Thr209Arg (626 C>G) nor Glu228Ala (683 A>C) (both as the DR4 variants) alone was significantly associated with breast cancer risk [OR=0.84, 95% CI=0.65-1.08, P=0.18 and OR=0.89, 95% CI=0.72-1.12, P=0.30] in the same patient group [25], and DR5 expression is inducible by cancer therapeutic agents, including platinum agents and taxanes [24], indicating the genetic complexity of DRs in the pathogenesis of cancer. And a single nucleotide polymorphisms (SNPs) and haplotypes of TRAIL have been studied in a group of Japanese patients with gastric cancer [26]. Some SNPs have been reported to be associated with the clinical performance of NSCLC in a group of mostly non-Hispanic Whites [24]. And a recent immunohistochemical study proved a significantly less staining of TRAIL protein in cancer tissue than in normal tissue (45.0% vs. 60.0%, χ^2-test, P<0.01) in 60 Chinese patients of NSCLC, and more intensive staining in highly differentiated tissue than in moderately and primarily differentiated tissue (60.0% vs. 18.0%, χ^2-test, P<0.01) of these patients, and that the TRAIL protein expression was positively correlated to the apoptosis index of NSCLC tissues (r=0.663, P=0.000) [27], but these results are based on an immunohistochemical study, but the research needs to be verified by molecular biological support. And, the relation between some single nucleotide polymorphisms in the non-coding region of TRAIL genes of Chinese patients with NSCLC has not been studied by means of genotype and haplotype analysis. The present study is designed to analyze the relationship between three polymorphic sites G1525A/G1588A/C1595T in the 3'UTR of *TRAIL* gene and the disease onset and prognosis in 592 Chinese patients with NSCLC.

Materials and methods

Subjects

Totally 592 patients (484 females and 108 males Chinese) with NSCLC from Zhongnan Hospital and Renming Hospital of Wuhan University and the Cancer Hospital of Hubei Province in China were included in this study. They are all offspring of the Han Chinese, without consanguinity, and smoking history unclear. They are diagnosed as NSCLC and their clinical stages assessed according to criteria of other researchers [28], and 308 patients were at stage I and II, and 284 patients at stage III and IV. A total of 636 age- and sex-matched healthy controls (132 females and 504 males) were recruited from the Health Examination Center of Zhongnan Hospital. The study protocol was approved by the Ethics Committees of the three hospitals mentioned above. Informed consent was obtained from the patients and healthy individuals.

DNA extraction

Peripheral blood of 5 mL was collected each from the NSCLC patients and from the healthy persons. EDTA was used as an anticoagulant for blood samples. Genomic DNA was extracted from peripheral blood lymphocytes using DNeasy Blood and Tissue Kit (QiaGen, Valencia, CA) following the manufacturer's instruction and stored at 4°C for subsequent identification of genetic mutations by PCR.

Genotyping and haplotype analysis

The PCR product was amplified using 5'-GTAGTAGCCTCCAGGTTTCC-3' and 5'-AACTCAACCCAGAACAAAGG-3' as sense and antisense

Table 1. Allelic and genotypic distribution of TRAIL in the NSCLC patients and the control

TRAIL Gene	Control group (n=636)	NSCLC group (n=592)	*P*	OR (95% CI)
G1525A				
GG	139 (21.86)	290 (48.99)	<0.001	3.433 (2.680-4.398)
GA	298 (46.86)	83 (14.02)		
AA	199 (31.28)	219 (36.99)		
GA+AA	497 (78.14)	302 (51.01)		
G allele	576 (45.28)	663 (56.00)	<0.001	1.538 (1.311-1.803)
A allele	696 (54.72)	521 (44.00)		
G1588A				
GG	160 (25.16)	208 (35.14)	<0.001	1.611 (1.260-2.061)
GA	323 (50.79)	118 (19.93)		
AA	153 (24.05)	266 (44.93)		
GA+AA	476 (74.84)	384 (64.86)		
G allele	643 (50.55)	534 (45.10)	0.007	1.121 (1.031-1.218)
A allele	629 (49.45)	650 (54.90)		
C1595T				
CC	180 (28.30)	189 (31.93)	0.166	0.836 (0.659-1.074)
CT	316 (49.69)	177 (29.90)		
TT	134 (22.01)	226 (38.18)		
CT+TT	456 (71.70)	343 (68.07)		
C allele	676 (53.14)	555 (46.88)	0.002	1.285 (1.097-1.506)
T allele	596 (46.86)	629 (53.13)		

primers respectively. The PCR reactions contain approximately 34 ng of genomic DNA, 0.4 mM of 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 in a final volume 25 μL. The PCR conditions were set as the followings: initial denaturation at 95°C for 2 min, followed by 45 cycles at 95°C for 15 s, 55°C for 30 s, and 70°C for 34 s, with a final extension at 70°C for 7 min. The genetic polymorphisms of TRAIL G1525A/G1588A/C1595T were examined by direct Sanger sequencing. The haplotype analysis was performed using PHASE2.3 software.

Statistical analysis

Hardy-Weinberg equilibrium was analyzed with chi square test for the distribution of genotype frequencies in the healthy persons. All statistical analyses were conducted using SPSS 13.0 software. Association of *TRAIL* genotypes with NSCLC was each evaluated with χ^2-test or fisher exact test. For all the analyses, a two-tailed *p* value less than 0.05 was considered as statistically significant.

Results

Allele and genotype frequencies of TRAIL G1525A/G1588A/C1595T in the NSCLC patients and the healthy persons

The distributions of *TRAIL* G1525A, G1588A and C1595T genotypes were coincident with Hardy-Weinberg equilibrium in the healthy controls (*P*>0.05). The frequencies of genotype GA+AA and variant A of G1525A were each significantly lower in NSCLC group compared with the control group [51.01% vs. 78.14%, OR=3.433, 95% CI: 2.680-4.398, *P*<0.01; 44% vs. 54.72%, OR=1.538, 95% CI: 1.311-1.803, *P*<0.01, respectively]. In the NSCLC group, the frequencies of mutation genotype GA+AA of G1588A was significantly lower than that of the control (64.86% vs.74.84%, OR=1.611, 95% CI: 1.260-2.061, *P*<0.01); but the mutation genotype CT+TT of C1595T in NSCLC group was insignificantly lower than that in control group, (68.07% vs. 71.70%, OR=0.836, 95% CI: 0.659-1.074, *P*=0.166). However, the frequencies of these two mutation alleles A and T of G1588A and C1595T respectively were each significantly higher in NSCLC patients than in control group (54.90% vs. 49.45%, OR=1.121, 95% CI: 1.031-1.218, *P*<0.01; 53.13% vs. 46.86%, OR=1.285, 95% CI: 1.097-1.506, *P*<0.01), all shown in **Table 1**.

Haplotype analysis of TRAIL gene polymorphisms G1525A/G1588A/C1595T

The *TRAIL* G1525A, G1588A and C1595T polymorphic sites (**Figure 1**) were in complete linkage disequilibrium (G1525A/G1588A, *D'*=0.978, r^2=0.818; G1525A/C1595T, *D'*=0.917, r^2=0.827; G1588A/C1595T, *D'*=0.976, r^2=0.967) respectively. In eight haplotypes, frequencies that were simultaneously less than 3% in the NSCLC patients as well as in the healthy controls were not taken into account. The highest frequency of haplotypes in the

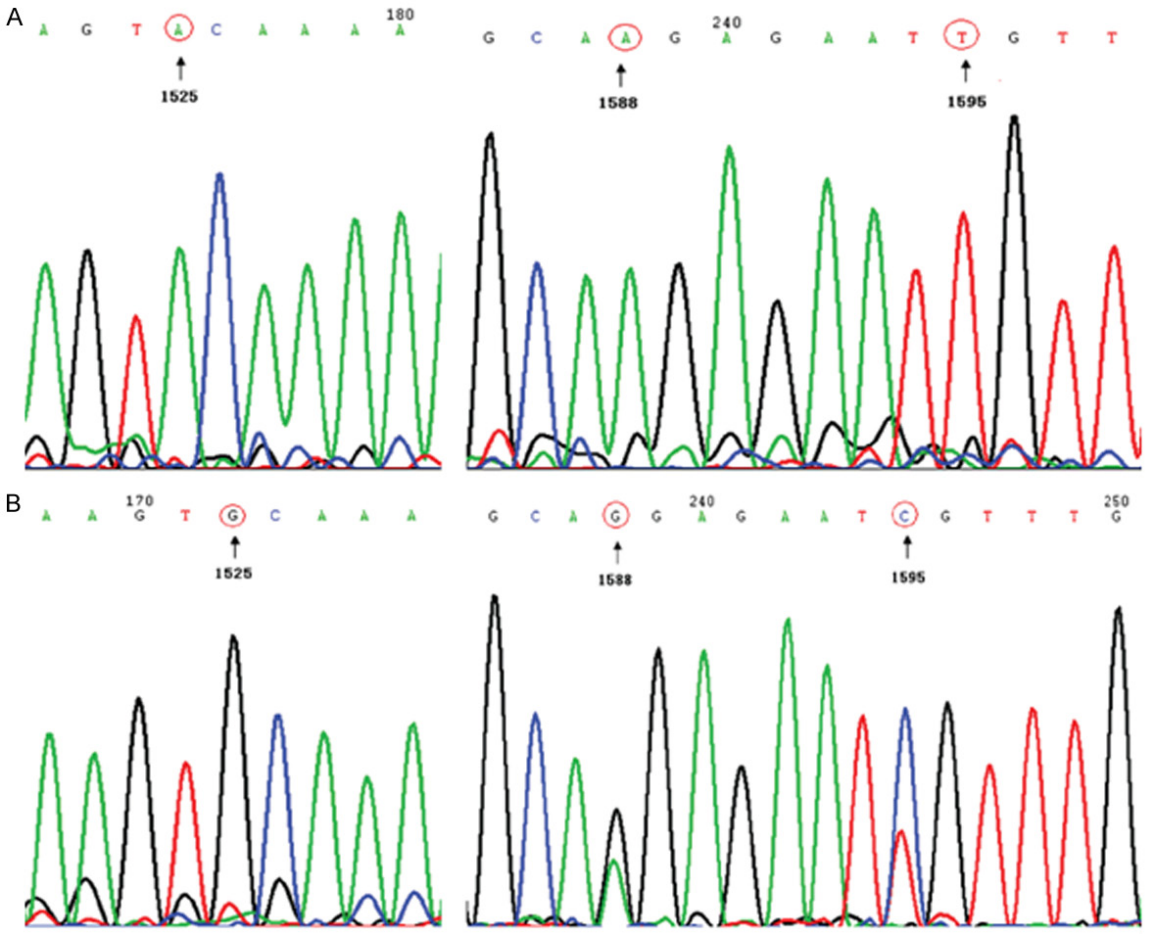

Figure 1. The genotypes of *TRAIL* presented in (A) are 1525AA, 1588AA, and 1595TT. The genotypes shown in (B) are 1525GG, 1588GG and 1595CC (indicated by black arrows).

NSCLC and the control groups are GGC (45.02%) and AAT (58.33%). Even though the frequency of haplotype GGC in the NSCLC groups was higher than that in the control, the difference was statistically indifferent between two groups (45.02% vs. 35.12%, OR=0.864, 95% CI: 0.724-1.031, $P>0.05$). The frequency of AAT haplotype, however, was significantly lower in NSCLC group than in control (36.45% vs. 58.23%, OR=1.977, 95% CI: 1.525-2.284, $P<0.01$). The frequency of GAT haplotype was significantly higher in the NSCLC group than in healthy control (9.98% vs. 0.21%, OR=0.0316, 95% CI: 0.015-0.059, $P<0.01$).

Associations of clinical characteristics of NSCLC patients with the polymorphisms G1525A/G1588A/C1595T

The frequencies of variant allele T and genotype CT+TT of C1595T were significantly higher in stage III and IV NSCLC patients than in stage I and II patients (67.08% vs 34.26%, OR=3.023, 95% CI: 2.383-3.835; 78.52% vs. 58.44%, OR=2.600, 95% CI: 1.809-3.736, $P<0.05$ respectively). Moreover, the frequency of variant allele A and genotype GA+AA of G1525A were significantly higher in stage III and IV than in stage I and II NSCLC patients (58.98% vs. 30.19%, OR=3.322, 95% CI: 1.699-2.618; 63.03% vs. 39.94%, OR=2.560, 95% CI: 1.836-3.571, $P<0.05$, **Table 2**).

However, the genetic polymorphisms of G1588A were not statistically related to the activity and location of the disease in NSCLC patients ($P>0.05$, **Table 3**).

Discussion

The specific mechanism of NSCLC formation remains unknown and no single mechanism

Table 2. Association of TRAIL genotypes with the clinical features of the NSCLC patients

Characteristics	G1525A		G1588A		C1595T	
	GG	GA+AA[a]	GG	GA+AA	CC	CT+TT[b]
Stage I+II	185 (60.06)	123 (39.94)	111 (36.04)	197 (63.96)	128 (35.56)	180 (58.44)
Stage III+IV	105 (36.97)	179 (63.03)	97(34.15)	187 (65.85)	61 (21.48)	223 (78.52)

[a]*P*<0.001, OR = 2.560, 95% CI: 1.836-3.571; [b]*P*<0.001, OR = 2.600, 95% CI: 1.809-3.736.

Table 3. Associations of TRAIL alleles with the clinical features of NSCLC patients

Characteristics	G1525A		G1588A		C1595T	
	G	A[a]	G	A	C	T[b]
stage I+II	430 (69.81)	186 (30.19)	285 (46.27)	331 (53.73)	368 (59.74)	248 (34.26)
stage III+IV	233 (35.02)	335 (58.98)	249 (43.84)	319 (56.16)	187 (32.92)	381 (67.08)

[a]*P*<0.001, OR = 3.322, 95% CI: 1.699-2.618; [b]*P*<0.001, OR = 3.023, 95% CI: 2.383-3.835.

alone can clearly explain all aspects of NSCLC. On one hand, up to 25% of lung cancer worldwide including about 20% of NSCLC is not attributable to smoking even though there has been no conclusive cause identified for most no-smoking cases. The tumor formation of such cases relates in particular to TP53, KRAS and EGFR genes, which demonstrate strikingly different mutation patterns and frequencies between never-smokers and smokers of lung cancer [1]. On the other hand, the complex pathogenesis of NSCLC in smoking patients, however, covers at least four relatively clear mechanisms, i.e., the environmental and genetic factors, the abnormalities in growth-simulator signaling pathways and in tumor suppressor gene pathways, the evasion of apoptosis, and the epigenetic modifications that alter gene expression without changing the DNA sequence [1]. The environmental and genetic cause of lung cancer mainly refers to tobacco consumption and about 85% of clinically overt lung cancers are attributed to smoking partially because the nicotine-derived nitrosoaminoketone leads to genetic mutations through DNA adduct formation and this formation is resulted from the metabolic activation of some carcinogens. The tumor suppressor gene pathways include p53 gene pathway, $p16^{INK4}$/cyclin D1/Rb pathway and serine/threonine kinase 11 gene, whereas the evasion of apoptosis consists of mitochondrial apoptosis (Bax/Bcl-2), death receptor deregulation and cell immortalisation and telomerase activation [1]. The downstream p53 pathway contains target genes of p53 transcription required for the mitochondrial apoptotic pathway and the death receptor pathway via the following four genes: Bcl-2 (anti-apoptotic), Bax (pro-apoptotic), Fas and TRAIL-death receptor 5 (DR5). Bcl-2 and Bax are up- and downregulated by p53 respectively while Fas and DR5 belong to TRAIL receptor family. TRAI-DR5 and Bcl-2 can play opposite roles during the induction of apoptosis of the cancer cells. The four genes are prominently deregulated, thus leading to strong resistance to mitochondrial and DR-induced apoptosis [1].

A recent immunohistochemical study from NSCLC tissues of 60 Chinese cases proved that the positive staining rate of TRAIL protein was lower in cancer tissue than in adjacent normal tissue (45.0% vs. 60.0%, χ^2-test, P<0.01), and higher in highly differentiated tissue than in moderately and primarily differentiated tissue (60.0% vs. 18.0%, χ^2-test, P<0.01), and TUNEL method for nuclei staining together with the immunohistochemical staining proved that the TRAIL protein expression was positively but the Bcl-2 protein expression negatively correlated to the apoptosis index of NSCLC tissues (r=0.663, P=0.000; r=-0.736, P=0.000), and the TRAIL expression and Bcl-2 expression in NSCLC tissue are negatively correlated to each other (X2=8.929, P=0.000, r=-0.866) [27], thus supporting the opposite roles of TRAIL protein and Bcl-2 protein for the apoptosis-induction. And in the present study in a Han Chinese population, the frequencies of variant A (G1525A) of TRAIL gene were significantly lower and those of the two variants A and T (G1588A and C1595T) are significantly higher in NSCLC patients compared with the control groups, implying that the variant A (G1525A) instead of

the variants A and T (G1588A and C1595T) may involve in the induction of apoptosis of tumor cells of NSCLC and that the variant TRAIL gene A (G1525A) may be used as the targeted gene of molecular therapy for NSCLC to Chinese patients. On the opposite, we speculate that G1588A and C1595T could facilitate NSCLC susceptibility. And it was concluded after a study in 2331 Chinese patients of lung cancer that the genetic variants in 3q28, 5p15.33, 13q12.12 and 22q12.2 may contribute to the susceptibility of lung cancer in Han Chinese [4], indicating that a series of gene and/or variants influence the risk of NSCLS. And the frequencies of variant allele A and genotype GA+AA of G1525A were significantly higher in stage III and IV than in stage I and II of the patients, indicating that allele A and genotype GA+AA of this TRAIN variant gene are linked to the differentiation degree and prognosis of NSCLC in Chinese population. With haplotype analysis it was found that the three loci in the 3'UTR of TRAIL gene are completely linked, especially GGC and AAT have the highest haplotype frequencies in both the NSCLC group and the control group (45.02% and 58.33% respectively), and that the frequency of GAT haplotype is significantly higher but the frequency of AAT haplotype is significantly lower in NSCLC group. These suggest that the patients who carry GAT haplotype may have higher risk of NSCLC. In contrast, AAT haplotype is likely to reduce the risk of NSCLC. Further clinical analysis of the internal relationship between TRAIL gene polymorphisms (G1525A/G1588A/C1595T) and NSCLC in the patients showed that the frequencies of G1525A and C1595T are significantly higher in the stage III and IV NSCLC patients. And the frequency of C1595T genotype CTTT in the stage III and IV NSCLC patients is also significantly higher. These results suggest that at least C1595T variant may link to the lesion severity and malignant prognosis of NSCLC. In the studies of the polymorphisms of TRAIL, it has been proved in a clinic-based study composed of mostly non-Hispanic Whites that four of the top 15 single nucleotide polymorphisms (SNPs), rs11785599 (T-allele), rs1047275, rs4460370 and rs883369, are located in the TNF-receptor super family member 10b (TNFRSF10B) gene and associated respectively with 35%, 35%, 29% and 24% of the increased death risk of NSCLC patients, and that a most common risk haplotype TCTT was associated with a 78% increased death risk compared with the low risk haplotype CGCC [24]. A recent study in Turkish patients of NSCLC proved an increased risk in those carrying CT genotypes in stage IIB-IIIB patients [29]. And it was newly evidenced in a Turkish population that a significant association exists between TRAIL C1595T and bladder cancer development while the frequency of TRAIL 1595 C allele was significantly lower in patients with bladder cancer compared to controls (χ^2=16.585, P<0.001, OR=1.256, 95% CI=1.138-1.386) [2]. These imply that the genes and their variants affecting the risk of lung cancer vary with regions in the world. And it needs to compare the genetic characteristics and signaling pathway of lung cancer of different races with more systematic and more general methods.

TRAIL binds to the death receptors DR4 and DR5 and induces cell apoptosis in virus-infected hepatocytes through activation of the caspase cascade reaction. Its receptors also include DcR1, DcR2 and three types of decoy receptor osteoprotegerin. Due to the lack of functional death structure, they cannot generate apoptotic signal when bind to TRAIL, which protects cells from TRAIL-induced apoptosis [30]. Some researchers have reported obvious correlation between DR4 variants and cancers, including head and neck cancer, breast cancer, colon cancer, lung cancer, T cell lymphoma, chronic lymphocytic leukemia, bladder cancer, prostate cancer [31-36]. It has been shown that two variants on DR4 gene exon 3 [rs6557634 (A/G)] and exon 4 [rs4871857 (C/G)] are associated with the lung cancer susceptibility [36]. However, due to the complex mechanism and the downstream factors in TRAIL-induced apoptosis, the effect of other SNPs of *TRAIL* gene on NSCLC susceptibility remains to be studied. Identification of NSCLC susceptibility genes will help us to further understand the pathogenesis of NSCLC and prevent the disease.

In summary, two SNPs (G1588A, C1595T) of TRAIL gene are associated with the susceptibility of NSCLC while G1525A is putatively advantageous to the self-defense of NSCLC in a group of Chinese patients. G1525A and C1595T genotype CTTT play similar roles in the differentiation degree and clinical stages of NSCLC.

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Disclosure of conflict of interest

None.

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