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Association between *TNFSF4* tagSNPs and myocardial infarction in a Chinese Han population

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ABSTRACT. Tumor necrosis factor superfamily member 4 (*TNFSF4*) plays an important role in atherosclerosis development. However, the biological significance of *TNFSF4* variants on myocardial infarction (MI) pathogenesis remains poorly understood. We investigated the influence of 5 *TNFSF4* tagging single nucleotide polymorphisms (rs3861950, rs17346501, rs7518045, rs1234313, and rs3850641) on individual susceptibility to MI in a Chinese population of 285 MI patients and 645 controls. Genotyping was performed using the polymerase chain reaction-ligase detection reaction method. In multivariate logistic regression analysis, only the *TNFSF4* tagging single nucleotide polymorphism rs7518045 exhibited a significant effect on MI risk; A allele

(odds ratio = 0.68, 95% confidence interval = 0.46-1.00, P = 0.048) and AA genotype (odds ratio = 0.64, 95% confidence interval = 0.42-0.97, P = 0.036) were associated with a decreased risk of MI compared with the G allele and the combined AG/GG genotype, respectively. Moreover, the haplotype rs3861950C-rs17346501C-rs7518045A-rs1234313G containing the rs7518045 A allele also exhibited a significant association with a decreased risk for MI (odds ratio = 0.42, 95% confidence interval = 0.21-0.84, P = 0.011). Our study showed that the A allele of the rs7518045 and haplotype rs3861950C-rs17346501C-rs7518045A-rs1234313G in the *TNFSF4* gene were associated with decreased MI risk in a Chinese Han population. Further studies using larger sample sizes and in diverse ethnic populations are needed to confirm the general validity of our findings.

Key words: Myocardial infarction risk; Single nucleotide polymorphism; Tumor necrosis factor superfamily member 4

INTRODUCTION

Myocardial infarction (MI) is the leading cause of death in humans worldwide and its incidence is rapidly increasing (Watkins and Farrall, 2006). Numerous risk factors, including smoking, alcohol intake, diabetes, hypertension, hypercholesterolemia, obesity, physical inactivity, and psychosocial situation, have been reported to contribute to the pathogenesis of MI (Anand et al., 2008; Zhang et al., 2008). However, aside from these modifiable risk factors, accumulating studies have demonstrated that host genetic background plays a critical role on MI development (Zdravkovic et al., 2002; Kangas-Kontio et al., 2010).

Tumor necrosis factor superfamily member 4 (*TNFSF4*), also known as *OX40L*, is located in human chromosome 1 and encodes a type II glycoprotein that is expressed in various cells, including professional antigen-presenting cells, $CD4^+$ T cells, $CD8^+$ T cells, macrophages, dendritic cells, and vascular endothelial cells (Stuber et al., 1995; Imura et al., 1996; Ohshima et al., 1997; Murata et al., 2000). TNFSF4 functions as a ligand for TNFRSF4, and thus plays a crucial role in regulating T lymphocyte proliferation and survival, natural killer T cells and natural killer cell function, and regulatory T cell differentiation and activation (Ito et al., 2006; Duan et al., 2008). Accumulating experimental evidence has confirmed that activated T cells and inflammatory responses play an essential role in atherosclerosis development (Hansson, 2001; Hansson et al., 2002; de Boer et al., 2003). The inflammatory process is also largely responsible for plaque rupture and thrombus formation, which clinically manifests as ischemia, stroke, or MI (van der Wal et al., 1994; Nakajima et al., 2002; Hansson, 2005). In addition, *TNFSF4*-deficient mice had significantly smaller atherosclerotic lesions and higher levels of plasma total cholesterol and high-density lipoprotein cholesterol (HDL-C) than controls, while mice over-expressing TNFSF4 exhibited significantly larger atherosclerotic lesions (Wang et al., 2005). Interruption of the TNFSF4/TNFRSF4 pathway attenuates atherogenesis in low-density lipoprotein (LDL) receptor-deficient mice (van Wanrooij et al., 2007). Thus, the TNFSF4-TNFRSF4 pathway may play a key role in MI pathogenesis by participating in T cell activation and the inflammatory response.

Single nucleotide polymorphisms (SNPs) are the most frequent variants in the human

genome and may contribute to individual susceptibility to some diseases. Increasing evidence suggests that SNPs within candidate genes may influence MI risk (Fujimaki et al., 2010; Ghaderian et al., 2010; Kallel et al., 2010). Wang et al. (2005) found that the *TNFSF4* rs3850641 G allele contributed to the risk of developing MI in a Swedish population. Furthermore, the T allele of a promoter polymorphism (rs45454293), consistent with the linked rs3850641G-allele, was found to be associated with an increased risk of MI in Swedish women (Ria et al., 2011). To date, no study of *TNFSF4* SNPs with MI risk in the Chinese population has been reported. Thus, we conducted a case-control study to elucidate the association between *TNFSF4* polymorphisms and MI risk in a Chinese population. Because many SNPs have shown with correlated genotypes or linkage disequilibrium (LD), only a subset of SNPs (known as tagging SNPs, or tagSNPs) must be genotyped for disease association studies. In this study, we focused on 5 tagSNPs (rs3861950, rs17346501, rs7518045, rs1234313, and rs3850641) in the *TNFSF4* gene.

MATERIAL AND METHODS

Study subjects

A total of 930 unrelated Chinese Han subjects were included in this study. A total of 285 patients with MI were recruited from the First People's Hospital of Foshan (Foshan, China) and the Affiliated Hospital of Guangdong Medical College (Zhanjiang, China) from March 2011 to July 2013. MI was diagnosed based on clinical symptoms and typical electrocardiographic changes, as well as on increases in the serum cardiac markers such as creatinine kinase, aspartate aminotransferase, lactate dehydrogenase, and troponin T. The diagnosis was confirmed by identifying the responsible stenosis in any of the major coronary arteries or in the left main trunk by coronary angiography. A total of 645 control subjects were consecutively recruited from the participating hospitals for regular physical examinations during the same period when MI patients were recruited. The unaffected controls were judged to be free of MI by questionnaires, medical history, clinical examination, and electrocardiography. Individuals with congestive heart failure, peripheral vascular disease, rheumatic heart disease, pulmonary heart disease, chronic kidney disease, hepatic disease, or any malignancy were excluded from the study.

All study subjects were interviewed after written informed consent was obtained, and structured questionnaires were administered by interviewers at enrollment to collect information on demographic data and risk factors related to MI. The study was approved by the Medical Ethics Committee of the First People's Hospital of Foshan and the Affiliated Hospital of Guangdong Medical College.

Analysis of biochemical parameters

An approximately 2 mL venous blood sample was drawn from each subject into tubes containing ethylenediaminetetraacetic acid after an overnight fast. The blood sample was centrifuged at 2000 *g* for 15 min immediately after collection and stored at -80°C until analysis. The levels of plasma total cholesterol, triglyceride, HDL-C, and LDL-C were measured enzymatically using a chemistry analyzer (Olympus, Tokyo, Japan). Glucose was analyzed using the glucose oxidase method with an Abbott V/P Analyzer (Abbott Laboratories, Abbott Park, IL, USA).

DNA extraction

Genomic DNA was extracted from peripheral whole blood using the TIANamp blood DNA extraction kit (TianGen Biotech, Beijing, China) according to manufacturer instructions. All DNA samples were dissolved in water and stored at -20°C until use.

TagSNP selection and genotyping

The Chinese Han population's SNP data of the *TNFSF4* gene were downloaded from the HapMap database (http://www.hapmap.org). We then analyzed these data using the Haploview software version 4.2 (Barrett et al., 2005). A minor allele frequency (MAF) >0.05 and LD measure (r^2) >0.8 were prerequisites for tagSNP selection. We obtained 5 tagSNPs, including rs3861950, rs17346501, rs7518045, rs1234313, and rs3850641. Among the 5 tagSNPs, rs3861950, rs1234313, and rs3850641 had been previously investigated in 2 case-control samples from Sweden (Wang et al., 2005). The other 2 tagSNPs (rs17346501 and rs7518045) captured a broader extension (Table 1). Together, the 5 tagSNPs represented the information of more than 80% of *TNFSF4* SNPs with an MAF > 0.05 (Table 1). Haplotype analysis was performed using the SHEsis platform (Shi and He, 2005).

Genomic DNA was genotyped by polymerase chain reaction (PCR)-ligase detection reaction (LDR) method (Shanghai Biowing Applied Biotechnology Company, Shanghai, China). Primer and probe sequences are summarized in **Table S1**. PCR was carried out on the ABI 9600 (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 µL, including 50 ng genomic DNA, 1X PCR buffer, 3 mM $MgCl_2$, 2 mM of each dNTP, 0.5 mM/mL primer mix, and 1 U hot-start *Taq* DNA polymerase (Qiagen, Hilden, Germany). Cycling parameters were as follows: 95°C for 2 min; 40 cycles at 94°C for 30 s, 56°C for 1 min and 30 s, 65°C for 30 s; and a final extension step at 65°C for 10 min. The ligation reaction for each PCR product was carried out in a final volume of 10 µL containing 1 µL 1X ligation buffer, 4 µL PCR product, 2 pmol of each discriminating probe, 2 U *Taq* DNA ligase (New England Biolabs, Ipswich, MA, USA). The LDR parameters were as follows: 95°C for 2 min, 40 cycles at 94°C for 15 s, and 50°C for 25 s. Following the LDR, 1 µL LDR product was mixed with 1 µL ROX and 1 µL loading buffer. The mixture was then analyzed using an ABI Prism 377 DNA Sequencer (Applied Biosystems). Approximately 10% of the samples were randomly selected to perform repeated assays and the results were 100% concordant.

Table 1. Alleles captured by the 5 *TNFSF4* tagSNPs.

tagSNPs	Alleles captured
rs3861950	rs7525284, rs7514229, rs4081545, rs10912564, rs3861950
rs17346501	rs10912561, rs13343108, rs10489268, rs11808060, rs10798264, rs17300347, rs17346501, rs17372309, rs10912560
rs7518045	rs6661173, rs16845543, rs7518045, rs16845585, rs10489267
rs1234313	rs1234313
rs3850641	rs3850641

Statistical analysis

The statistical power analysis was performed using Power and Sample size calculations, version 3.0.43 (Dupont and Plummer Jr., 1990). Both *TNFSF4* tagSNPs were tested for conformity to Hardy-Weinberg equilibrium using a goodness-of-fit χ^2 test among the control subjects. Quantitative variables are reported as means ± standard deviation, and qualitative variables are reported as percentages. The differences in the demographic characteristics between the cases and controls were estimated using the χ^2 test (for categorical variables) and the Student *t*-test (for continuous variables). For individual tagSNP association analyses, genotype frequencies were assessed using multivariate methods based on logistic regression analysis. We estimated odds ratios (ORs) and 95% confidence intervals (CIs) for the effect of *TNFSF4* tagSNPs on MI risk adjusted by age, gender, smoking, drinking, hypertension, diabetes, and hyperlipidemia. Statistical analyses were performed using the SPSS software version 21 (SPSS, Inc., Chicago, IL, USA). Haplotype analysis on the polymorphisms was conducted using the SHEsis software (http://analysis.bio-x.cn/myAnalysis.php) (Shi and He, 2005). A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Characteristics of the study population

Characteristics of patients with MI (N = 285) and controls (N = 645) are summarized in Table 2. There was no statistically significant difference between cases and controls in terms of age. Compared with the control group, more patients in the MI group were males, smokers, alcohol consumers, and individuals with hypertension, diabetes, or hyperlipidemia. The patient group also had significantly higher serum triglycerides, total cholesterol, and LDL-C, whereas serum HDL-C levels were significantly higher among controls. The average levels of systolic blood pressure, diastolic blood pressure, and fasting plasma glucose in the patients were significantly higher than those in controls. These data demonstrated that male gender, smoking, alcohol intake, hypertension, hyperlipidemia, and diabetes mellitus were important risk factors in the development of MI in our Chinese population.

Table 2. General characteristics in the MI cases and controls.

Variable	Controls (N = 645)	MI (N = 285)	P value[a]
Age (years)	61.89 ± 11.95	62.07 ± 11.99	0.493
Gender (male)	380 (58.0%)	221 (77.5%)	<0.001
Smoking	169 (25.8%)	171 (60.0%)	<0.001
Drinking	95 (14.5%)	77 (27.0%)	<0.001
Hypertension	233 (35.6%)	179 (62.8%)	<0.001
Diabetes	106 (16.2%)	136 (47.7%)	<0.001
Hyperlipidemia	247 (37.7%)	201 (70.5%)	<0.001
Systolic blood pressure (mmHg)	132.55 ± 18.94	140.02 ± 19.16	<0.001
Diastolic blood pressure (mmHg)	72.84 ± 10.46	75.66 ± 11.56	<0.001
FPG (mM)	5.82 ± 1.92	6.64 ± 1.72	<0.001
TG (mM)	1.49 ± 0.82	2.06 ± 0.97	<0.001
TC (mM)	4.63 ± 1.16	4.71 ± 1.21	0.297
HDL-C (mM)	1.37 ± 0.67	1.18 ± 0.36	<0.001
LDL-C (mM)	2.64 ± 0.92	3.03 ± 0.97	<0.001

[a]Two-sided chi-square test or independent-sample Student *t*-test. FPG, fasting blood glucose; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Multivariate association between *TNFSF4* tagSNPs and MI risk

Five *TNFSF4* tagSNPs (rs3861950, rs17346501, rs7518045, rs1234313, and rs3850641) were genotyped in 285 MI patients and 645 control subjects. The primary information for the tagSNPs is shown in Table 3. The MAF of these tagSNPs in our controls was similar to the MAF in the Chinese in HapMap database (Table 3). The genotype frequency distributions of the tagSNPs in our control subjects were in Hardy-Weinberg equilibrium (all P values ≥0.05; Table 3).

Table 3. Primary information for the 5 *TNFSF4* tagSNPs.

Genotyped tagSNPs	Chr Pos (Genome Build 106)	Pos in *TNFSF4* gene	MAF for Chinese (CHB) in HapMap	MAF in our controls (N = 645)	P value for HWE test in our controls
rs3861950	173187153	Intron 2	0.128	0.138	0.058
rs17346501	173188402	Intron 2	0.057	0.040	0.333
rs7518045	173194373	Intron 1	0.067	0.091	0.198
rs1234313	173197108	Intron 1	0.326	0.376	0.328
rs3850641	173206693	Intron 1	0.107	0.215	0.769

[a]MAF: minor allele frequency. [b]HWE: Hardy-Weinberg equilibrium.

Associations between the 5 tagSNPs and MI were analyzed using multivariate logistic regression analysis after adjusting for age, gender, smoking, drinking, hypertension, diabetes, and hyperlipidemia as covariates. The allele and genotype distributions of the tagSNPs in the cases and controls are listed in Table 4. Only rs7518045 showed significant differences in allele frequency and genotype distribution between MI patients and control subjects. The frequency of the rs7518045 A allele in cases was significantly lower than that in control subjects, indicating that the A allele may be associated with a lower risk of MI (OR = 0.68, 95%CI = 0.46-1.00, P = 0.048; Table 4). Analysis using multivariate logistic regression revealed a similar trend for the decreased risk of MI in the dominant model in which the AG and GG genotypes were combined (OR = 0.64, 95%CI = 0.42-0.97, P = 0.036; Table 4). We further evaluated the genotypes and MI susceptibility after stratifying the subjects by gender, age, and smoking or drinking status. However, no additional evident association between rs7518045 and MI risk was observed among subgroups by age, smoking status, etc. (data not shown).

Association between haplotypes of *TNFSF4* tagSNPs and MI risk

LD analysis for the 5 tagSNPs was performed using the Haploview platform, and showed that 4 tagSNPs (rs3861950, rs17346501, rs7518045, and rs1234313) were located in 1 haplotypic block and could be combined to construct haplotypes (Figure 1). Thus, we further compared the haplotype frequency between MI and controls. Four common haplotypes (frequency >3%) derived from the 4 tagSNPs accounted for nearly 100% of the haplotype variations. Among the 4 common haplotypes, only the haplotype rs3861950C-rs17346501C-rs7518045A-rs1234313G was found to be associated with a decreased risk of MI (OR = 0.42, 95%CI = 0.21-0.84, P = 0.011; Table 5).

Table 4. Genotypic and allelic frequencies of *TNFSF4* tagSNPs and MI risk.

Genotyped tagSNPs	Genotype or allele	Controls (N = 645)	Cases (N = 285)	OR (95%CI)	P value[a]
		N (%)	N (%)		
rs3861950	T	1112 (86.2)	495 (86.8)	1.00	-
	C	178 (13.8)	75 (13.2)	0.89 (0.64-1.23)	0.470
	TT	485 (75.2)	214 (75.1)	1.00	-
	CT+CC	160 (24.8)	71 (24.9)	1.25 (0.85-1.83)	0.255
rs17346501	T	1238 (96.0)	560 (98.2)	1.00	-
	C	52 (4.0)	10 (1.8)	0.52 (0.24-1.12)	0.093
	TT	595 (92.2)	275 (96.5)	1.00	-
	CT+CC	50 (7.8)	10 (3.5)	0.52 (0.24-1.14)	0.101
rs7518045	G	117 (9.1)	61 (10.7)	1.00	-
	A	1173 (90.9)	509 (89.3)	0.68 (0.46-1.00)	0.048
	AG+GG	109 (16.9)	58 (20.4)	1.00	-
	AA	536 (83.1)	227 (79.6)	0.64 (0.42-0.97)	0.036
rs1234313	A	805 (62.4)	356 (62.5)	1.00	-
	G	485 (37.6)	214 (37.5)	0.98 (0.77-1.25)	0.878
	AA	257 (39.8)	112 (39.3)	1.00	-
	AG+GG	388 (60.2)	173 (60.7)	1.02 (0.73-1.43)	0.914
rs3850641	G	277 (21.5)	126 (22.1)	1.00	-
	A	1013 (78.5)	444 (77.9)	0.82 (0.63-1.08)	0.164
	GG	31 (4.8)	19 (6.7)	1.00	-
	AG+AA	614 (95.2)	266 (93.3)	0.55 (0.28-1.08)	0.083

[a]Adjusted for age, gender, smoking, drinking, hypertension, diabetes, hyperlipidemia. OR, odds ratio; 95%CI, 95% confidence interval.

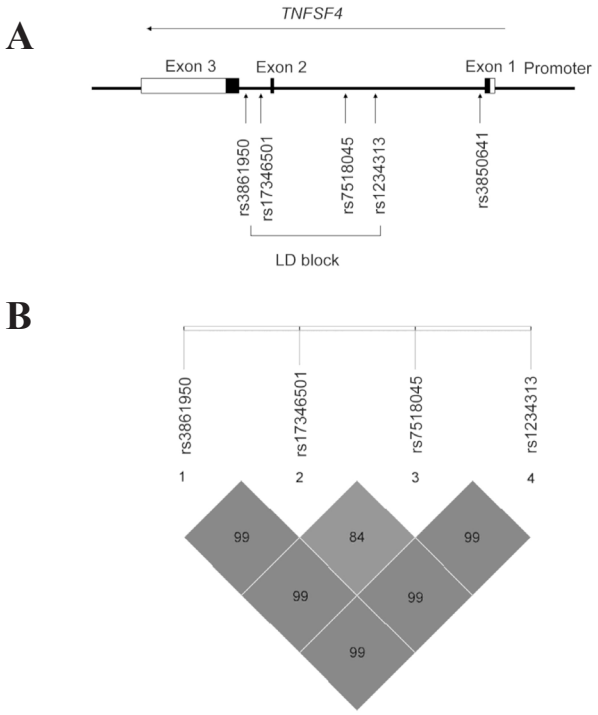

Figure 1. A. Positions of the *TNFSF4* tagSNPs genotyped in the present study. **B.** Linkage disequilibrium analysis among the 4 *TNFSF4* tagSNPs located in one haplotypic block. Numbers within squares indicate the D' value reported as a percentile.

Table 5. Association between haplotypes of rs3861950, rs17346501, rs7518045, and rs1234313 and MI risk.

Haplotype	Controls (N = 645)	Cases (N = 285)	OR (95%CI)	P value
	N (%)	N (%)		
rs3861950C-rs17346501C-rs7518045A-rs1234313G	52.00 (4.0)	9.99 (1.8)	0.42 (0.21-0.84)	0.011
rs3861950C-rs17346501T-rs7518045G-rs1234313G	115.99 (9.0)	61.00 (10.7)	1.21 (0.87-1.68)	0.251
rs3861950T-rs17346501T-rs7518045A-rs1234313A	804.99 (62.4)	355.99 (62.5)	1.00 (0.81-1.23)	0.986
rs3861950T-rs17346501T-rs7518045A-rs1234313G	306.00 (23.7)	139.01 (24.4)	1.04 (0.82-1.30)	0.769

OR = odds ratio; 95%CI, 95% confidence interval.

DISCUSSION

The principal pathogenesis of MI is coronary atherosclerotic plaque formation and rupture. Recent studies have provided evidence that the TNFSF4-TNFRSF4 pathway plays a key role in MI pathogenesis by participating in T cell activation and the inflammatory response. However, the association between polymorphisms in the *TNFSF4* gene and MI risk remains unknown. In the present study, we performed a genetic association analysis on 5 *TNFSF4* tagSNPs (rs3861950, rs17346501, rs7518045, rs1234313, and rs3850641) in 285 MI patients and 645 controls. We found that only rs7518045 had a significant effect on MI risk. In addition, the haplotype rs3861950C-rs17346501C-rs7518045A-rs1234313G containing the rs7518045 A allele also exhibited a significant association with a decreased risk of MI.

Despite the association between rs7518045 and decreased MI risk, the underlying mechanism of the rs7518045 polymorphism influencing disease susceptibility remains unclear. Because the rs7518045 SNP is located in intron 1, it is not expected to change the functional properties of the TNFSF4 protein. However, rs7518045 is a tagSNP, and the haplotype block in which it lies also covers exon 2 of the gene. Thus, this SNP is in LD with a causal SNP that may either result in a change in amino acid or affect a possible splice variant or the stability of the mRNA. Future fine mapping and re-sequencing of the *TNFSF4* gene may detect such functional variants. Further studies involving large samples and more variations are warranted to clarify the impact of the gene on MI risk.

With respect to the tagSNPs rs3861950, rs17346501, rs1234313, and rs3850641 of the *TNFSF4* gene, we observed no association between MI risk in the Chinese Han population. This result agrees with two previous reports that showed no evidence of an association between *TNFSF4* gene variation (5 SNPs, including rs3861950 and rs1234313) and the risk for coronary heart disease or MI (Koch et al., 2008; Cheng et al., 2011). However, the minor allele of rs3850641 was more significantly frequent in individuals with MI than in the controls in 2 independent populations from Sweden (Wang et al., 2005), while no significant difference was observed in a study by Cheng et al. (2011) and our studies in the Chinese Han population, as well as in a study by Koch et al. (2008) in a German population. The main reason for this discrepancy may be because of varying genetic backgrounds, differences between the studies in geographical ethnic groups, sample size, and study design.

There were several limitations to our study. First, the patients and controls were enrolled from hospitals and may not represent the general population. Nonetheless, the genotype distribution of the controls was in Hardy-Weinberg equilibrium. Second, the moderate sample size limited the statistical power of our study, particularly in the case subjects. Third, based on functional considerations, the tagSNPs investigated were non-coding, and we therefore assume these variants to be linked with 1 or more functional variants within the *TNFSF4* gene

or its regulatory regions. Finally, further studies in different population may establish the true significance of the association between these SNPs and MI risk.

In summary, the tagSNP rs7518045 and the haplotype rs3861950C-rs17346501C-rs7518045A-rs1234313G were associated with a decreased risk of MI, suggesting that these genetic variants can be used as biomarkers for assessing the risk of developing MI. Further studies with larger sample sizes and in diverse ethnic populations are necessary to confirm the general validity of our findings.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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