Original Article

Association between Low-density Lipoprotein Receptor-related Protein 5 Polymorphisms and Type 2 Diabetes Mellitus in Han Chinese: a Case-control Study*

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Abstract

Objective To investigate the association between low-density lipoprotein receptor-related protein 5 (*LRP5)* variants (rs12363572 and rs4930588) and type 2 diabetes mellitus (T2DM) in Han Chinese.

Methods A total of 1842 T2DM cases (507 newly diagnosed cases and 1335 previously diagnosed cases) and 7777 controls were included in this case-control study. PCR-RFLP was conducted to detect the genotype of the two single nucleotide polymorphisms (SNPs). Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated to describe the strength of the association by logistic regression.

Results In the study subjects, neither rs12363572 nor rs4930588 was significantly associated with T2DM, even after adjusting for relevant covariates. When stratified by body mass index (BMI), the two SNPs were also not associated with T2DM. Among the 3 common haplotypes, only haplotype TT was associated with reduced risk of T2DM (OR 0.820, 95% CI 0.732-0.919). In addition, rs12363572 was associated with BMI (P<0.001) and rs4930588 was associated with triglyceride levels (P=0.043) in 507 newly diagnosed T2DM cases but not in healthy controls.

Conclusion No *LRP5* variant was found to be associated with T2DM in Han Chinese, but haplotype TT was found to be associated with T2DM.

Key words: Low-density lipoprotein receptor-related protein 5; Gene polymorphism; Type 2 diabetes mellitus; Haplotype; Metabolic characteristics

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic disease, which is characterized by hyperglycemia resulting from impaired insulin secretion and insulin resistance[1]. Long-term chronic hyperglycemia often leads to multiple organ dysfunction and tissue structure abnormalities, which would pose a serious threat to the health of patients[2]. It is commonly believed that T2DM is caused by both genetic factors and environmental factors, but T2DM pathogenesis remains to be further elucidated, genetic susceptibility seems to play a crucial role in the development of this multifactorial disorder[3]. In the past few years, genome-wide association studies (GWAS) have revealed that many single nucleotide polymorphisms (SNPs) were associated with T2DM, including SNPs in the regions near *TCF7L2*, *KCNQ1*, *PGC-1* and so on[3-7]. More recently, many other SNPs have been identified by other studies in addition to GWAS and meta-analyses[8-10].

Low-density lipoprotein receptor-related protein 5 (LRP5) is a member of the low-density lipoprotein receptor family, which is the co-receptor ligand of the WNT signaling pathway[11]. One study found that WNT and insulin signaling pathways exhibit cross-talk at multiple levels and the WNT co-receptor LRP5 has a positive effect on insulin signaling; altered WNT and LRP5 activity can modify insulin action and insulin resistance in the pathop- hysiology of diabetes and metabolic syndrome[12]. Experiments also showed that LRP5 was highly expressed in hepatocytes and pancreatic β cells[13-14].

In the past, many researchers[15-18] have focused on the role of *LRP5* in osteoporosis. But DNA sequence analysis[19] indicated that the *LRP5* locus is on chromosome 11q13, a region related with type 1 diabetes mellitus, so *LRP5* might confer susceptibility to diabetes. Animal models[20] indicated that *LRP5* was required for normal cholesterol and glucose metabolism, and *LRP5*-deficient mice showed increased plasma cholesterol levels when fed with high-fat diet and markedly impaired glucose tolerance when fed with normal diet. In addition, many SNPs have been identified in human *LRP5* and were reported to be associated with metabolic diseases, such as obesity[21], hypercholesterolemia[22] and hypertension[23-24]. However, studies investigating the role of *LRP5* in people's susceptibility to type 2 diabetes mellitus (T2DM) are limited, which were conducted only in Japanese[25], United Kingdom populations[26] and Chinese[27], and the results were opposite.

In this study, the association between polymorphisms of *LRP5* and T2DM in Han Chinese was investigated. Overweight and obesity are strong risk factors for T2DM[28-30] and a previous study[21] had reported that *LRP5* was associated with obesity. To understand the *LRP5* effect on risk of T2DM other than the effect on obesity, we compared the genotypes for patients with diabetes mellitus and controls with different BMI. In addition, *LRP5* was found to be associated with diabetes-related metabolic characteristics[26], so we investigated such association in 507 newly diagnosed T2DM cases.

METHODS

Study Subjects

The case group included 1842 T2DM patients in Henan province, i.e. 810 patients selected from rural area in Luoyang during 2007-2008 and 1032 patients selected from outpatient departments of three hospitals in Zhengzhou during 2010-2011, and the control group included 7777 people with normal glucose-tolerant level selected from rural area in Luoyang during 2007-2008. The cases and controls were from the same rural areas, but they had no personal relationship. The case group included previously diagnosed T2DM patients and newly diagnosed T2DM patients, T2DM was diagnosed according to the American Diabetes Association (ADA) criteria[31]. The patients with type 1 diabetes mellitus, gestational diabetes and other types of diabetes mellitus were excluded. The controls had no history of T2DM and were not diagnosed with diabetes according to the 2005 ADA criteria. Any people with cancer, malnutrition (BMI<18.5 kg/m^2), mentally disturbed, handicapped, obesity (caused by disease) and those who were taking certain drugs or were pregnant were excluded from the case group and control group. All the subjects belonged to Han ethnic group. In addition, considering that metabolic characteristics of previously diagnosed patients were different from true levels due to the treatment, we analyzed genotypes for only 507 newly diagnosed T2DM patients with different metabolic characteristics. The study was approved by the Ethics Committees of Zhengzhou University, and informed consent was obtained from all the subjects before data collection.

Research Design

This case-control study consisted of questionnaire interview, anthropometric measurement and laboratory test. Data were collected by trained staff. Body weight, body height and waist circumference (WC) were measured by using standard techniques. BMI was calculated as weight in kilograms divided by height in meters squared (kg/m^2). Blood samples were collected with vacuum tubes containing ethylene diamine tetraacetic acid anticoagulant in the morning after an overnight fast. Plasma was separated to detect fasting plasma glucose (FPG) level and the levels of total cholesterol (TC), triglyceride (TG) and high-density lipoprotein-cholesterol (HDL-C); low-density lipoprotein-cholesterol (LDL-C) levels were calculated by using Freidwald formula[32].

DNA Extraction and Genotyping

Genomic DNA was extracted from whole blood by using genomic DNA purification kit (Yaneng BIO, Shenzhen, China). The tagging SNPs were selected for genotyping from the Chinese Han Beijing (CHB) population data of HapMap (HapMap Data Rel#27/phase II+III, Feb09, on NCBI B36 assembly, dbSNP b126) by using Haploview 4.2 (http://www.broad.mit.edu/haploview) according to the inclusion criteria as follows: minor allele frequencies (MAF) >0.01 and linkage disequilibrium by r^2<0.8. Primers were designed by using Primer Premier v5.0 (PREMIER Biosoft International, Palo Alto, CA, USA). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was conducted to detect the genotype. In fact, 12 tagging SNPs of *LRP5* were selected in this study, but only rs12363572 and rs4930588 can be genotyped successfully by PCR-RFLP. So only this two SNPs were used in this study.

The primer sequences for rs12363572 were 5'-AGT GGG AAG ATC CCT TGA GTC C-3' (forward), 5'-TCT ATT CAC GTC CTT TGC CCA T-3' (reverse). All the reactions were performed in a total volume of 20 μL reaction volume containing 50 ng genomic DNA, 5 pmol each primer, 10 μL 2×Taq PCR mix (Laifeng BIO, Shanghai) containing 1 mmol/L $MgCl_2$, 100 μmmol/L dNTPs, and 0.5 U Taq polymerase. The amplification program was initial precycling denaturation at 95 °C for 5 min, denaturation for 30 cycles at 95 °C for 30 s, then annealing at 55.5 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 8 min. The PCR product was digested overnight at 65 °C with 5 U restriction enzyme TaiI (Fermentas, USA), and resolved in 4% agarose gel ethidium bromide (Solarbio, Beijing) with electrophoresis for genotype analysis.

The primer sequences for rs4930588 were 5'-TGG CCT TTA ATT GCC TGC ACC AG-3' (forward), 5'-CAA AGC AGA TGT GGA ACG GAC T-3' (reverse). The genotyping conditions were same as for rs12363572, but the annealing temperature was 58.1 °C, and the PCR product was digested overnight at 37 °C with 5 U restriction enzyme TaiI (Fermentas, USA).

Statistical Analysis

Quantitative variables with non-normal distribution are presented as median [25th-75th percentile (Q1-Q3)], and categorical data are shown as number (%). Mann-Whitney Wilcoxon test was used to assess the differences in quantitative variables and chi-square test was used for categorical data. The two SNPs were tested for Hardy-Weinberg equilibrium with chi-square goodness-of-fit test in the control group. Allele frequency was calculated by using genotype counting, and chi-square test was used to determine the difference in allele frequency between case group and control group. Logistic regression analysis was conducted to calculate ORs and 95% CI for the association between SNPs and T2DM after controlling for covariates (sex, age, BMI, WC, TC, TG, HDL-C, and LDL-C), Bonferoni correction was used for multiple testing due to the increased risk of a type I error ($\alpha'=\alpha/n$). Data were analyzed with software SAS 9.1 for Windows (SAS Inst. Inc., Cary, NC, USA). Sample Size Calculations (Mark Woodward, The George Institute International Health; Lesley Francis, MIS Consultants) was used to calculate the sample size and power. Calculation of pairwise linkage disequilibrium (LD) coefficients and haplotype analysis involved use of SHEsis[33]. All the tests were two tailed and P<0.05 was considered statistically significant.

RESULTS

Characteristics of Study Subjects

The characteristics of the study subjects are shown in Table 1. A total of 1842 T2DM patients and 7777 controls with normal glucose-tolerant level were included. The proportion of males was higher in case group than in control group (50.22% *vs.*

41.33%, *P*<0.001) and the median (Q1-Q3) age of the cases was older than that of controls [54.0 (45.0-61.0) *vs*. 49.0 (40.0-58.0) years, *P*<0.001]. As expected, BMI, WC, FPG, and levels of TC, TG, and LDL-C were higher in case group, but HDL-C level was higher in control group (all *P*<0.001).

Association between LRP5 Polymorphisms and T2DM

Both rs12363572 and rs4930588 were in agreement with Hardy-Weinberg equilibrium in 7777 controls (*P*=0.055 and *P*=0.067, respectively). Neither rs12363572 nor rs4930588 SNP was associated with risk of T2DM in terms of allele, genotype, dominant model or recessive model analysis (Table 2).

It was estimated that with a sample of 1842 T2DM patients and 7777 controls, the prevalence of the risk factor in control was 7.69%, the study would have 99.5% power to detect an increase of 50% in the risk of cases than the controls, and would have 100% power to detect an increase of 60%, at a significance level of 0.05 for a two-sided test. So our sample size was adequate to achieve sufficient power.

Association between LRP5 Polymorphisms and T2DM by BMI

Because of the low minor allele frequency (MAF) for the rs12363572 T allele and rs4930588 G allele in controls (0.077 and 0.139, respectively), a dominant model was used in this study for stratified analysis (Table 3). In the normal-weight subgroup (BMI<25 kg/m^2), rs12363572 was not associated with risk of T2DM. However, in the overweight/obesity subgroup (BMI≥25 kg/m^2), the CT+TT genotype seemed to be associated with reduced risk of T2DM after adjustment for the relevant covariates (CT+TT *vs*. CC, OR=0.809, 95% CI: 0.661-0.990, *P*=0.039), but this association was found to be false-positive after used Bonferoni correction (*P*>0.025). In addition, rs4930588 was not associated with the risk of T2DM in any subgroups (all *P*>0.05).

Association between LRP5 Haplotypes and T2DM

Haplotype analysis revealed moderate LD between the *LRP5* variants analyzed (D'/r^2= 0.495/0.003) (Figure 1). The 2 SNPs defined 3 common

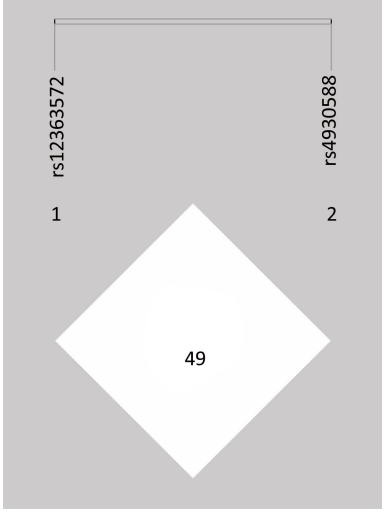

Figure 1. LD plot for rs12363572 and rs4930588 in Han Chinese.

Table 1. Demographic Characteristics of Cases and Controls

Characteristics	Cases (*n*=1842)	Controls (*n*=7777)	*P* Value
Male	925 (50.22)	3,214 (41.33)	<0.001[#]
Age (y)	54.0 (45.0-61.0)	49.0 (40.0-58.0)	<0.001[*]
BMI (kg/m^2)	27.38 (24.53-30.73)	23.83 (21.64-26.24)	<0.001[*]
WC (cm)	90.50 (83.50-101.34)	81.35 (74.90-88.50)	<0.001[*]
FPG (mmol/L)	7.62 (6.13-9.91)	5.19 (4.88-5.51)	<0.001[*]
TC (mmol/L)	4.87 (4.23-5.59)	4.28 (3.74-4.89)	<0.001[*]
TG (mmol/L)	1.73 (1.18-2.60)	1.32 (0.95-1.91)	<0.001[*]
HDL-C (mmol/L)	1.10 (0.96-1.27)	1.14 (0.98-1.31)	<0.001[*]
LDL-C (mmol/L)	2.94 (2.4-3.49)	2.40 (2.20-2.90)	<0.001[*]

Note. Continuous data are presented as median (Q1-Q3) with non-normal distribution; categorical data are shown as *n* (%). BMI, body mass index; WC, waist circumference; FPG, fasting plasma glucose; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. [*] Mann-Whitney Wilcoxon test. [#] Pearson chi-square test.

haplotypes with frequencies >0.03 (Table 4). After analyzing all the estimated haplotypes, only haplotype TT was associated with the reduced risk of T2DM (OR=0.820, 95% CI: 0.732-0.919, *P*<0.001).

Association between LRP5 Polymorphisms and Diabetes-related Metabolic Characteristics

Among 507 newly diagnosed T2DM patients, BMI was lower in the patients with CT+TT genotype of rs12363572 than in the patients with CC genotype [25.45 (22.82-27.96) *vs*. 27.08 (24.41-30.15) kg/m^2, *P*<0.001] (Table 5). The TG level was lower for patients with TG+GG genotype of rs4930588 than in the patients with TT genotype [1.66 (1.15-2.48) *vs.* 1.89 (1.34-2.85) mmol/L, *P*=0.043]. In controls, no metabolic characteristics were associated with rs12363572 or rs4930588 SNPs (data not shown).

Table 2. Association between SNPs in *LRP5* and T2DM in Han Chinese

SNPs	Cases n (%)	Controls n (%)	Crude OR (95% CI)	*P* Value	Adjusted OR (95% CI)*	*P* Value*
rs12363572						
Allele						
C	3,435 (93.24)	14,358 (92.31)	Reference			
T	249 (6.76)	1,196 (7.69)	0.870 (0.755-1.003)	0.054		
Genotype						
CC	1,605 (87.13)	6,639 (85.37)	Reference		Reference	
CT	225 (12.21)	1,080 (13.89)	0.862 (0.739-1.005)	0.058	0.924 (0.772-1.107)	0.392
TT	12 (0.65)	58 (0.75)	0.856 (0.459-1.597)	0.625	0.881 (0.405-1.916)	0.750
Dominant model						
CC	1,605 (87.13)	6,639 (85.37)	Reference		Reference	
CT+TT	237 (12.87)	1138 (14.63)	0.861 (0.741-1.001)	0.052	0.922 (0.773-1.100)	0.370
Recessive model						
CC+CT	1,830 (99.35)	7,719 (99.25)	Reference		Reference	
TT	12 (0.65)	58 (0.75)	0.873 (0.468-1.628)	0.669	0.890 (0.410-1.935)	0.769
rs4930588						
Allele						
T	3,150 (85.50)	13,389 (86.08)	Reference			
G	534 (14.50)	2,165 (13.92)	1.049 (0.947-1.162)	0.359		
Genotype						
TT	1,356 (73.62)	5,782 (74.35)	Reference		Reference	
TG	438 (23.78)	1,825 (23.47)	1.204 (0.869-1.668)	0.264	0.974 (0.654-1.450)	0.895
GG	48 (2.61)	170 (2.19)	1.023 (0.908-1.154)	0.706	1.066 (0.926-1.228)	0.372
Dominant model						
TT	1,356 (73.62)	5,782 (74.35)	Reference		Reference	
TG+GG	486 (26.38)	1,995 (25.65)	1.039 (0.926-1.166)	0.516	1.058 (0.923-1.212)	0.420
Recessive model						
TT+TG	1,794 (97.39)	7,607 (97.81)	Reference		Reference	
GG	48 (2.61)	170 (2.19)	1.197 (0.866-1.656)	0.277	0.959 (0.645-1.425)	0.834

Note. -Adjusted for sex, age, BMI, WC, TC, TG, HDL-C, and LDL-C.

Table 3. Association between Genotype and T2DM by BMI

Subgroups	Genotype	Cases n (%)	Controls n (%)	Crude OR (95% CI)	*P* Value	Adjusted OR (95% CI)*	*P* Value*
rs12363572							
BMI<25 kg/m^2	CC	449 (84.72)	4,204 (85.22)	Reference		Reference	
	CT+TT	81 (15.28)	729 (14.78)	1.041 (0.811-1.335)	0.753	1.054 (0.819-1.357)	0.683
BMI≥25 kg/m^2	CC	1,156 (88.11)	2,435 (85.62)	Reference		Reference	
	CT+TT	156 (11.89)	409 (14.38)	0.803 (0.660-0.979)	0.030	0.809 (0.661-0.990)	0.039
rs4930588							
BMI<25 kg/m^2	TT	395 (74.53)	3,660 (74.19)	Reference		Reference	
	TG+GG	135 (25.47)	1,273 (25.81)	0.983 (0.800-1.207)	0.868	0.893 (0.711-1.122)	0.332
BMI≥25 kg/m^2	TT	961 (73.25)	2,122 (74.61)	Reference		Reference	
	TG+GG	351 (26.75)	722 (25.39)	1.073 (0.925-1.245)	0.350	1.141 (0.963-1.353)	0.128

Note. *Adjusted for sex, age, WC, TC, TG, HDL-C, and LDL-C.

Table 4. Association between common Haplotypes of 2 SNPs in *LRP5* and T2DM Risk*

Haplotype	rs12363572	rs4930588	Cases n (%)	Controls n (%)	OR (95% CI)	*P* Value
1	C	G	1010 (13.71)	2089 (13.43)	1.027 (0.947-1.114)	0.517
2	C	T	5860 (79.53)	12,269 (78.88)	1.058 (0.987-1.134)	0.113
3	T	T	440 (5.97)	1120 (7.20)	0.820 (0.732-0.919)	<0.001

Note. *Haplotypes with frequency <0.03 not considered in this analysis.

Table 5. Two SNP Genotypes in Newly Diagnosed T2DM Subjects with Different Metabolic Characteristics (*n*=507)

Metabolic Characteristics	rs12363572			rs4930588		
	CC (*n*=440)	CT+TT* (*n*=67)	*P* Value#	TT (*n*=388)	TG+GG* (*n*=119)	*P* Value#
BMI (kg/m^2)	27.08 (24.41-30.15)	25.45 (22.82–27.96)	<0.001	26.67 (24.05-30.00)	27.34 (24.76-29.83)	0.424
WC (cm)	90.80 (83.75-100.00)	87.50 (82.70-95.25)	0.058	90.25 (83.75-99.50)	91.00 (83.00-99.50)	0.937
FPG (mmol/L)	7.61 (6.54-9.52)	7.48 (6.40-9.40)	0.997	7.67 (6.60-9.73)	7.41 (6.33-9.23)	0.268
TC (mmol/L)	4.97 (4.24-5.69)	4.98 (4.33-5.48)	0.951	4.98 (4.24-5.69)	4.97 (4.28-5.58)	0.604
TG (mmol/L)	1.83 (1.28-2.88)	1.82 (1.33-2.27)	0.551	1.89 (1.34-2.85)	1.66 (1.15-2.48)	0.043
HDL-C (mmol/L)	1.13 (0.98-1.30)	1.08 (0.96-1.29)	0.339	1.13 (0.98-1.29)	1.10 (0.97-1.30)	0.900
LDL-C (mmol/L)	2.90 (2.30-3.50)	2.91 (2.30-3.43)	0.896	2.86 (2.30-3.43)	2.93 (2.30-3.58)	0.517

Note. Data are median (Q1-Q3) for non-normal distributions. *Because of the low proportion of subjects homozygous for the rare allele of rs12363572 and rs4930588 in newly diagnosed diabetes cases (0 and 0.014, respectively), we merged subjects homozygous and heterozygous for the rare allele in this analysis. #Mann-Whitney Wilcoxon test.

DISCUSSION

In this study, *LRP5* variants were not associated with T2DM in a sample of Chinese, the two SNPs were also not associated with T2DM when stratified by BMI, but haplotype TT of rs12363572 and rs4930588 showed an association with T2DM. rs12363572 was associated with BMI and rs4930588 was associated with TG in 507 newly diagnosed T2DM cases but not in healthy controls.

Our findings were similar to that previously reported in the samples of Japanese[25] and Chinese[27], but a United Kingdom cohort study[26]

found a significant association between *LRP5* polymorphisms and increased T2DM incidence. Some factors may explain the discrepant results in different populations. First, different SNPs were selected to represent the *LRP5* gene in different regions due to the geographical and ethnic differences, the Japan study[25] used rs312016, rs4988300, rs7944040, rs638051, rs4930573, rs314750, rs556442, rs3736228, and rs3781579 as the target loci, the China study[27] used rs3736228 and the United Kingdom study[26] used rs491347, rs71166004, and rs3781600. Second, the ethnicity is an important factor in genetic association studies[34-35]; different ethnic groups have different susceptibility to diabetes[36-37]. At last, the effect might be modified by environmental factors or functional polymorphisms in other genes, which would vary across populations. Stratified analysis showed that there was no interaction between *LRP5* variants and overweight or obesity. Further investigations of more regions are needed to elucidate the association between diabetes, obesity and gene variants. In addition, A strong association between haplotype TT and risk of T2DM was found in this study (P<0.001), but the Japan study[25] found no such association.

Animal experiments[20] and population studies[26] indicted that *LRP5* was associated with diabetes-related metabolic characteristics, such as BMI, glucose metabolic parameters and lipid metabolic parameters. In this study, it was found that *LRP5* was associated with BMI and TG level in newly diagnosed T2DM patients but not in healthy controls. However, the Japan study[25] did not find such association in T2DM patients or healthy controls. The Japan study surveyed previously diagnosed T2DM patients and their metabolic indicators could not reflect the true level due to the treatment and long disease duration. Some studies in healthy people also reported no association between *LRP5* and BMI or TG level among Chinese (Shanghai)[38], Japanese women[39] or Caucasians[21]. However, a cohort study[26] found that *LRP5* was strongly associated with TG level in middle-aged United Kingdom men who were healthy at entry and were followed for >15 years. The different results could be explained by the differences in population, gene, dietary habit, lifestyle, and the characteristics of study subjects.

This study is the first one to report the association between *LRP5* SNPs (rs12363572 and rs4930588) and T2DM risk in Han Chinese. In addition, a relatively large sample (9619 subjects) was used, which reduced the possibility of false-positive and false-negative results. The limitation is that the 2 common polymorphisms do not represent the whole *LRP5* gene, so further study (using other tagging SNPs) is needed.

In summary, no association between the 2 SNPs in *LRP5* and T2DM risk was in Han Chinese in this study, but haplotype TT of rs12363572 and rs4930588 was found to be associated with T2DM.

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