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The IL-33-ST2L Pathway Is Associated with Coronary Artery Disease in a Chinese Han Population

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The effects of interleukin-33 (IL-33) on the immune system have been clearly demonstrated; however, in cardiovascular diseases, especially in coronary artery disease (CAD), these effects have not yet been clarified. In this study, we investigate the genetic role of the IL-33-ST2L pathway in CAD. We performed three-stage case-control association analyses on a total of 4,521 individuals with CAD and 4,809 controls via tag SNPs in the genes encoding IL-33 and ST2L-IL-1RL1. One tag SNP in each gene was significantly associated with CAD (rs7025417^{T} in *IL33*, $p_{adj} = 1.19 \times 10^{-28}$, OR = 1.39, 95% CI: 1.31–1.47; rs11685424^{G} in *IL1RL1*, $p_{adj} = 6.93 \times 10^{-30}$, OR = 1.40, 95% CI: 1.32–1.48). Combining significant variants in two genes, the risk for CAD increased nearly 5-fold ($p_{adj} = 8.90 \times 10^{-21}$, OR = 4.98, 95% CI: 3.56–6.97). Traditional risk factors for CAD were adjusted for the association studies by SPSS with logistic regression analysis. With the two variants above, both located within the gene promoter regions, reporter gene analysis indicated that the rs7025417 C>T and rs11685424 A>G changes resulted in altered regulation of *IL33* and *IL1RL1* gene expression, respectively ($p < 0.005$). Further studies revealed that the rs7025417 genotype was significantly associated with plasma IL-33 levels in the detectable subjects ($n = 227$, $R^2 = 0.276$, $p = 1.77 \times 10^{-17}$): the level of IL-33 protein increased with the number of rs7025417 risk (T) alleles. Based on genetic evidence in humans, the IL-33-ST2L pathway appears to have a causal role in the development of CAD, highlighting this pathway as a valuable target for the prevention and treatment of CAD.

Introduction

Coronary artery disease (CAD [MIM 608320, 610947, 611139, 608901, 607339, 608316]), a complex trait that results from multiple genetic and environmental factors and their interactions, is the leading cause of morbidity and mortality worldwide.[1,2] In 2002 and 2007, Zdravkovic et al. estimated that the total heritability of CAD was 0.57 in men and 0.38 in women for death[3] and 0.39 in men and 0.43 in women for angina, based on a longitudinal study of more than 20,000 Swedish twins.[4] Recently, large-scale genome-wide association studies (GWASs) have identified approximately 50 risk loci for CAD.[5–15] However, these risk loci primarily have modest effect sizes (odds ratios = 1.04–1.28) and collectively explain only approximately 10.6% of the total CAD heritability.[15–17] Therefore, a large proportion of the heritability of CAD remains unexplained and is called "missing heritability."[17]

Inflammation has been demonstrated to play a key role in the pathogenesis of atherosclerotic CAD.[18] Considerable evidence from animal studies has indicated that many cytokines are involved in the development of atherosclerosis.[19] Most population-based studies have observed that variants in the genes encoding interleukin-1 receptor antagonist, IL-6, IL-10, IL-16, IL-17A, and IL-18 are also genetically associated with atherosclerosis and CAD.[20–24]

Recently, it was confirmed that the novel cytokine IL-33, a member of the IL-1 family, and its receptor ST2L-IL-1RL1 play important roles in inflammatory diseases. Furthermore, Miller et al. demonstrated that the IL-33-ST2L pathway might inhibit the development of atherosclerosis.[25] Therefore, we speculated that the IL-33-ST2L pathway might be genetically associated with CAD. To confirm this possibility, we performed the following steps: (1) a case-control association study for CAD based on the GeneID Chinese Han population, by using tag SNPs covering *IL33* (MIM 608678) and *IL1RL1* (MIM 601203) with a large discovery sample size; (2) an interaction analysis between the most significant variants of *IL33* and *IL1RL1* in the association with CAD; (3) a reporter gene assay to investigate the function of the reference variants; and (4) a circulation level study of IL-33 in the individuals with CAD.

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http://dx.doi.org/10.1016/j.ajhg.2013.08.009.

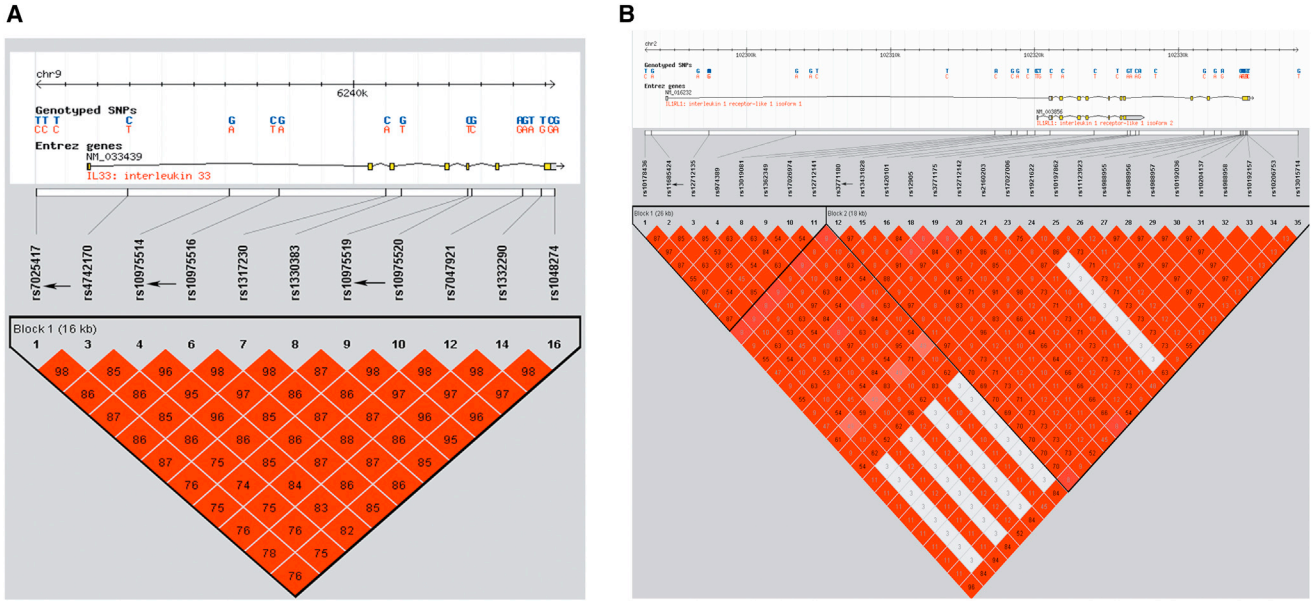

Figure 1. LD Blocks of *IL33* and *IL1RL1* Based on the HapMap CHB and JPT Data Sets
LD blocks of (A) *IL33* and (B) *IL1RL1* are shown. The arrows indicate the selected tag SNPs. Each diamond represents the LD degree between the SNPs. The color indicates the D' (a redder color represents a higher D') and the numbers within the diamonds are the r^2 values ($r^2 > 0.55$ and $D' > 0.83$ in the blocks of both genes).

Material and Methods

Study Population

The GeneID Chinese Han population is an ongoing database that was established for studying the genetic basis of various cardiovascular diseases.[13,26–28] The study subjects were 4,521 CAD cases and 4,809 controls, who were acquired from this database in three stages: first, the discovery population of 860 cases and 707 controls was assembled from eastern China; second, the validation population of 575 cases and 471 controls was assembled from northern China; and third, two replication populations with much larger sample sizes (2,016 cases and 1,647 controls in replication 1 and 1,070 cases and 1,984 controls in replication 2) were assembled from central China.

The criterion for the enrolment as a CAD case was >70% luminal stenosis in at least one main vessel, as identified by coronary angiography, coronary artery bypass graft, percutaneous coronary intervention, and/or myocardial infarction. Individuals were excluded from the study if they had experienced myocardial spasms or had a myocardial bridge, as identified by angiography, or had congenital heart disease, childhood hypertension, or type 1 diabetes mellitus.

The control subjects in the discovery and validation studies were defined as individuals without atherosclerotic lesions, as confirmed by angiography, and without any history of CAD. In the large replication studies, the control subjects were selected from the general population among individuals undergoing physical examinations. Study subjects who had notes of potential CAD or myocardial infarction in their medical records or from electrocardiographic tests were excluded.

The disease status of hypertension and diabetes mellitus were evaluated, and the lipid profile (total cholesterol [Tch], low-density lipoprotein cholesterol [LDL-c], high-density lipoprotein cholesterol [HDL-c], and triglyceride [TG]) was measured according to guidelines and standard methods.[29–31] Other clinical data, such as age, gender, body mass index (BMI), and smoking history, were obtained via direct interviews or a medical records review.

The study complied with the ethical principles set forth by the Declaration of Helsinki, and approval was obtained from the local institution review boards on human subject research. Informed consents were obtained from all of the participants.

Genetic Analysis

The DNA samples were collected from peripheral blood. Tag SNPs were selected according to the following principles: (1) linkage disequilibrium (LD) between SNPs according to Haploview (v.4.2) based on HapMap CHB and JPT data sets (v.3, release 2) with the thresholds of $r^2 > 0.4$ and $D' > 0.7$ to reduce the redundancy;[32,33] (2) potential functional sites predicted by bioinformatics (Promoter and Genevar); and (3) a minor allele frequency (MAF) threshold of greater than 0.05. Data were excluded if the allele call rate was less than 95% or the Hardy-Weinberg equilibrium (HWE) χ^2 p value was less than 0.001. The selected tag SNPs were rs7025417, rs10975514, and rs10975519 in *IL33* and rs11685424 and rs3771180 in *IL1RL1* (Figure 1). rs7025417 and rs11685424 were located in the predicted promoter regions, which might regulate the expression of the genes.

Genotyping was performed with a Roto-gene 6000 High-Resolution Melt (HRM) system (Corbett Life Science),[26–28] with a total of 25 μl PCR volume containing 1 μl of LC Green dye, 5 pmol of each primer, 25 ng of genomic DNA, 2.5 μl of 10× PCR buffer with 1.5 mmol/l $MgCl_2$, 5 mmol deoxynucleotide triphosphates, and 1 unit of Taq polymerase. Two positive controls for each genotype were included in each run, and the data with call rates of less than 95% were excluded. For each SNP, a total of 48 cases and controls were randomly selected for the verification of genotyping results by direct DNA sequencing analysis. DNA sequence analysis was performed with forward and/or reverse primers with the BigDye Terminator v3.1 Cycle Sequencing Kits on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Reporter Gene Assay

Because rs7025417 and rs11685424 were both located in the promoter region, which could influence gene expression, we conducted a reporter gene assay.[34] We first subcloned a 1,556 bp 5′ UTR sequence of *IL33* containing the rs7025417 C allele into a pGL3 basic vector (Promega Corporation). After confirmation by restriction mapping and direct sequencing, the pGL3 vector containing the "C" allele of rs7025417 was used as a template to generate the insert containing the T allele by using a site-directed mutagenesis kit (Promega). Direct sequencing was used to verify the two plasmids (*IL33*-C and *IL33*-T). The human lung adenocarcinoma cell line A549 and the human embryonic kidney cell line 293 (HEK293) (American Type Culture Collection [ATCC]) were transfected with 500 ng of each plasmid simultaneously with 50 ng of a pRL-TK vector (Promega) using Lipofectamine 2000 (Invitrogen). After 48 hr, firefly luciferase activity was measured with a Dual Luciferase Reporter Assay Kit (Promega). Three independent experiments were conducted, and each was performed in triplicate. The empty pGL3-basic vector was used as the negative control.

For *IL1RL1*, the constructed plasmids contained the rs11685424 A or G allele and the distal promoter sequence, totaling 1,218 bp continuous with exon 1a of *IL1RL1*. The two plasmids carrying *IL1RL1* (*IL1RL1*-A and *IL1RL1*-G) were transfected into the human mast cell line CHMAS (ATCC) by nucleofection (Amaxa Cell Line Nucleofector Kit R).

Circulation Level Test for IL-33

Blood samples for the determination of IL-33 were collected in tubes containing potassium EDTA at the time that the subjects enrolled in the GeneID Chinese Han Population database. After centrifugation, plasma samples were frozen at −80°C for less than 2 months. The concentrations of IL-33 were measured in 440 individuals with CAD by enzyme-linked immunosorbent assay (ELISA) with commercially available LEGEND MAX kits (BioLegend). The detection sensitivity was 4.14 pg/ml.

Statistical Analyses

Hardy-Weinberg disequilibrium tests were performed among controls for each population by PLINK. The allelic and genotypic associations for each SNP were analyzed by Pearson's 2 × 2 and 2 × 3 contingency table chi-square tests, and the odds ratio (OR) and 95% confidence interval (CI) were also calculated. Traditional risk factors for CAD, including age, gender, BMI, hypertension, diabetes mellitus, smoking history, Tch, HDL-c, LDL-c, and TG, were analyzed as covariates by multiple logistic regression models. Testing for interactions used logistic regression analysis under the genotypic model as suggested.[35] Statistical power analysis and sample size estimation for validation and replication populations were performed with a free power and sample size calculation program (PS v.3.0.12) for single-variant association analysis and a free software program, QANTO, for interaction analysis (QANTO v.1.2.4). The luciferase activity difference was tested with Student's t test (independent samples test, four degrees of freedom), and statistical significance was defined as a p value of less than 0.05. Analysis of association between the rs7025417 genotypes and IL-33 plasma concentrations was performed with a linear regression model, assuming an additive effect of the allele. The Mann-Whitney U test was used to test for differences in the IL-33 levels in the subjects with different genotypes of rs7025417.

Results

Population Characteristics

Table 1 illustrates the detailed clinical features of the three cohorts. The age, gender, and BMI did not differ between the case subjects and control subjects; the LDL-c, smoking, hypertension, and diabetes mellitus were significantly different. The case individuals had higher levels of LDL-c and a higher incidence of smoking, hypertension, and diabetes mellitus than those of the controls in all four populations.

Under the population parameter settings of the effect size (OR = 1.2 for CAD),[28] the allele frequency of 0.46 (HapMap CHB and JPT data sets, the minimum minor allele frequency for rs11685424 between the two significant variants in the discovery study), our sample size provides a statistical power of 60% in the validation population, 97.3% in the replication 1 population, and 92.4% in the replication 2 population.

The prior statistical power used to detect the interaction between rs7025417 in *IL33* and rs11685424 in *IL1RL1* under the genotypic model for their association with CAD was greater than 80% in the combined population.

Association Analysis of Single Variants with CAD in the GeneID Chinese Han Population

There was no deviation from the Hardy-Weinberg equilibrium for all of the SNPs in the control subjects. In the GeneID-discovery population, both *IL33* and *IL1RL1* possessed a promoter variant that was significantly associated with CAD (rs7025417^{T} in *IL33*, $p_{adj} = 1.38 \times 10^{-4}$, OR = 1.32, 95% CI: 1.14–1.52; rs11685424^{G} in *IL1RL1*, $p_{adj} = 2.12 \times 10^{-3}$, OR = 1.25, 95% CI: 1.08–1.44). In the GeneID-validation population, the SNPs rs7025417 and rs11685424 were also significantly associated with CAD (rs7025417^{T} in *IL33*, $p_{adj} = 7.69 \times 10^{-6}$, OR = 1.49, 95% CI: 1.25–1.77; rs11685424^{G} in *IL1RL1*, $p_{adj} = 1.25 \times 10^{-4}$, OR = 1.40, 95% CI: 1.18–1.67). In the large GeneID-replication 1 population, the associations between the two variants and CAD were still significant (rs7025417^{T} in *IL33*, $p_{adj} = 2.32 \times 10^{-7}$, OR = 1.28, 95% CI: 1.16–1.40; rs11685424^{G} in *IL1RL1*, $p_{adj} = 1.15 \times 10^{-12}$, OR = 1.40, 95% CI: 1.28–1.53). In the GeneID-replication 2 population, the associations between the two variants and CAD was confirmed (rs7025417^{T} in *IL33*, $p_{adj} = 1.06 \times 10^{-12}$, OR = 1.47, 95% CI: 1.33–1.66; rs11685424^{G} in *IL1RL1*, $p_{adj} = 2.17 \times 10^{-13}$, OR = 1.49, 95% CI: 1.34–1.65) (Table 2). In addition, genotypic association analysis also demonstrated that rs7025417 in *IL33* and rs11685424 in *IL1RL1* were significantly associated with CAD in all three population stages, as well as in the combined population (Table S1 available online).

rs10975514 and rs10975519 in *IL33* and rs3771180 in *IL1RL1*, which failed to show significant associations with CAD in the GeneID-discovery population (the lowest adjusted p value of the three SNPs is 0.20 for rs10975514) (Table S2), were excluded from the next two stages of the study.

Table 1. Clinical Characteristics of the Studied GeneID Chinese Han Population

Characteristics	GeneID Discovery			GeneID Validation			GeneID Replication 1			GeneID Replication 2			GeneID Combined		
	CAD	Control	p	CAD	Control	p	CAD	Control	p	CAD	Control	p	CAD	Control	p
Number of subjects	860	707	–	575	471	–	2,016	1,647	–	1,070	1,984	–	4,521	4,809	–
Age (years)[a]	59.4 ± 11.2	58.2 ± 13.7	0.12	60.8 ± 11.0	59.9 ± 11.5	0.24	60.5 ± 9.08	59.9 ± 8.49	0.14	60.2 ± 10.2	59.6 ± 9.12	0.10	60.2 ± 9.85	59.7 ± 9.25	0.10
Percent of subjects male	56.8	59.1	0.36	69.7	65.7	0.17	61.7	63.9	0.16	61.5	59.6	0.21	61.9	61.6	0.80
Percent of subjects smoking	44	19	0.00	21.7	6.4	0.00	24.1	0.4	0.00	23.8	7.1	0.00	27.5	5.8	0.00
BMI (kg/m^2)	24.7 ± 3.49	23.8 ± 2.92	0.34	24.5 ± 2.80	23.7 ± 9.41	0.39	24.8 ± 2.93	23.5 ± 3.09	0.46	26.3 ± 2.10	25.7 ± 8.24	0.11	25.1 ± 3.31	24.5 ± 5.16	0.10
Hypertension (%)	61	29.6	0.00	48.6	16	0.00	69.6	1.2	0.00	49.3	11	0.00	60.5	10.7	0.00
DM	16.8	9.9	0.00	17.5	6	0.00	23.1	0.4	0.00	16.8	3	0.00	19.7	3.4	0.00
Tch (mmol/l)	4.49 ± 1.07	4.35 ± 0.89	0.00	4.59 ± 1.11	4.39 ± 0.93	0.00	4.57 ± 1.17	4.53 ± 0.85	0.13	4.69 ± 1.21	4.55 ± 0.88	0.00	4.58 ± 1.10	4.50 ± 0.73	0.00
TG (mmol/l)	1.84 ± 1.36	1.52 ± 0.88	0.00	1.92 ± 1.48	1.65 ± 0.90	0.00	1.86 ± 1.39	1.57 ± 0.89	0.00	1.74 ± 1.10	1.46 ± 0.70	0.00	1.84 ± 1.16	1.53 ± 0.65	0.00
HDL-c (mmol/l)	1.08 ± 0.24	1.11 ± 0.24	0.08	1.10 ± 0.21	1.16 ± 0.22	0.00	1.11 ± 0.23	1.14 ± 0.25	0.00	1.14 ± 0.27	1.23 ± 0.20	0.00	1.12 ± 0.32	1.18 ± 0.21	0.00
LDL-c (mmol/l)	2.66 ± 0.77	2.41 ± 0.83	0.01	2.57 ± 0.98	2.40 ± 0.82	0.00	2.60 ± 0.84	2.37 ± 0.84	0.00	2.67 ± 0.91	2.52 ± 0.65	0.00	2.60 ± 0.84	2.44 ± 0.60	0.00

The data are presented as the means ± standard deviation or percent.
[a]Age for the case subject is at diagnosis; age for the control subject is at enrollment.

Association Analysis of rs7025417 in *IL33* and rs11685424 in *IL1RL1* with Subgroups of CAD in the GeneID-Combined Population

We separated the individuals with CAD into two subgroups: GeneID-anatomical-CAD, n = 1,598, individuals with anatomical disease (severe coronary stenosis); and GeneID-clinical-CAD, n = 2,923, individuals with clinical disease (myocardial infarction or revascularization). In GeneID-anatomical-CAD, the associations between the two SNPs and CAD were significant, with OR values of more than 1.3 for both (rs7025417^T in *IL33*, $p_{adj} = 3.79 \times 10^{-11}$, OR = 1.32, 95% CI: 1.22–1.44; rs11685424^G in *IL1RL1*, $p_{adj} = 2.73 \times 10^{-13}$, OR = 1.36, 95% CI: 1.25–1.48). In GeneID-clinical-CAD, the associations of the two SNPs with myocardial infarction or revascularization were also significant, with OR values of approximately 1.4 for both variants (rs7025417^T in *IL33*, $p_{adj} = 8.57 \times 10^{-22}$, OR = 1.39, 95% CI: 1.30–1.49; rs11685424^G in *IL1RL1*, $p_{adj} = 9.48 \times 10^{-22}$, OR = 1.39, 95% CI: 1.30–1.49) (Table 2). Genotypic association analysis also demonstrated that the two SNPs were significantly associated with CAD in both subgroups (Table S1).

Interaction Analysis between rs7025417 in *IL33* and rs11685424 in *IL1RL1* under the Genotypic Model in the GeneID-Combined Population

Because the sample size in the GeneID-combined population was sufficiently large to provide valuable statistical power, we performed an interaction analysis between rs7025417 in *IL33* and rs11685424 in *IL1RL1* under the genotypic model in the combined population. The interaction of the two SNPs (rs7025417 in *IL33* and rs11685424 in *IL1RL1*) in the association with CAD was highly significant, with a p value of 2.81×10^{-32} under the genotypic model. Additionally, the combination with the largest effect under the genotypic model was "TT/GG," which provided a nearly 5-fold increase in the risk for CAD ($p_{adj} = 8.90 \times 10^{-21}$, OR = 4.98, 95% CI: 3.56–6.97) (Table 3, Figure 2A). Figure 2B directly illustrates that the effect of the combination TT/GG was much stronger than that of a single variant in the association with CAD.

Reporter Gene Analysis

Given that rs7025417 and rs11685424 were both located within the gene promoter region and could therefore interfere with the regulation of gene expression, we constructed plasmids carrying *IL33*-C/T and *IL1RL1*-A/G. The assay was performed in triplicate, and a representative result of the three independent experiments is illustrated as the relative luciferase activity in Figure 3. The luciferase activity in the cells transfected with *IL33*-T plasmid was twice that in the cells transfected with *IL33*-C plasmid, and the luciferase activity in the cells transfected with the *IL1RL1*-G plasmid was four times that in the cells transfected with the *IL1RL1*-A plasmid ($p < 0.005$, Figure 3). These results indicate that the rs7025417 C>T change may increase the expression of *IL33* and that the rs11685424 A>G change

Table 2. Allelic Association Analysis of rs7025417 in *IL33* and rs11685424 in *IL1RL1* with CAD in the GeneID Chinese Han Population

Population	n		Gene, SNP (Reference Allele)	Frequency		p_{hwe}	p_{obs}	p_{adj}	OR (95%CI)
	Cases	Controls		Case	Control				
GeneID Discovery	860	707	*IL33*, rs7025417^{T}	0.585	0.516	0.259	1.02×10^{-4}	1.38×10^{-4}	1.32 (1.14–1.52)
			IL1RL1, rs11685424^{G}	0.567	0.515	0.293	3.63×10^{-3}	2.12×10^{-3}	1.25 (1.08–1.44)
GeneID Validation	575	471	*IL33*, rs7025417^{T}	0.584	0.486	0.4	7.42×10^{-6}	7.69×10^{-6}	1.49 (1.25–1.77)
			IL1RL1, rs11685424^{G}	0.57	0.487	0.453	1.48×10^{-4}	1.25×10^{-4}	1.40 (1.18–1.67)
GeneID Replication 1	2,016	1,647	*IL33*, rs7025417^{T}	0.589	0.528	0.008	2.28×10^{-7}	2.32×10^{-7}	1.28 (1.16–1.40)
			IL1RL1, rs11685424^{G}	0.566	0.483	0.776	1.08×10^{-12}	1.15×10^{-12}	1.40 (1.28–1.53)
GeneID Replication 2	1,070	1,984	*IL33*, rs7025417^{T}	0.594	0.497	0.367	4.39×10^{-13}	1.06×10^{-12}	1.47 (1.33–1.66)
			IL1RL1, rs11685424^{G}	0.579	0.481	0.118	1.65×10^{-13}	2.17×10^{-13}	1.49 (1.34–1.65)
GeneID Combined	4,521	4,809	*IL33*, rs7025417^{T}	0.591	0.509	0.001	1.41×10^{-27}	1.19×10^{-28}	1.39 (1.31–1.47)
			IL1RL1, rs11685424^{G}	0.573	0.487	0.027	6.25×10^{-30}	6.93×10^{-30}	1.40 (1.32–1.48)
GeneID Anatomical CAD	1,598	4,809	*IL33*, rs7025417^{T}	0.579	0.509	0.001	6.06×10^{-12}	3.79×10^{-11}	1.32 (1.22–1.44)
			IL1RL1, rs11685424^{G}	0.565	0.487	0.027	2.37×10^{-14}	2.73×10^{-13}	1.36 (1.25–1.48)
GeneID Clinical CAD	2,923	4,809	*IL33*, rs7025417^{T}	0.594	0.509	0.001	2.19×10^{-24}	8.57×10^{-22}	1.39 (1.30–1.49)
			IL1RL1, rs11685424^{G}	0.573	0.487	0.027	4.73×10^{-25}	9.48×10^{-22}	1.39 (1.30–1.49)

Abbreviations are as follows: p_{hwe}, p value from Hardy-Weinberg equilibrium tests; p_{obs}, observed p value; p_{adj}, p value adjusted by covariates; OR, odds ratio after adjustment.

may increase the expression of *IL1RL1*. The results exhibited for *IL33* were tested in A549, and those for *IL1RL1* were tested in CHMAS. The results found in HEK293 cells for *IL33* were similar to those found in A549 cells (data not shown).

Association Analysis between the IL-33 Circulation Level and rs7025417 Genotype

The detection rate of the plasma IL-33 concentration was 51.6% in the 440 individuals with CAD (median, 233.67 pg/ml; range, 12–3,807 pg/ml). As shown in Figure 4A, rs7025417 genotype was significantly associated with plasma IL-33 levels in subjects with a detectable IL-33 level ($n = 227$, $R^2 = 0.276$, $p = 1.77 \times 10^{-17}$). The IL-33 protein levels increased with the number of risk (T) alleles. The comparison between each pair of the three genotypes by the Mann-Whitney U test confirmed this trend, with p values that were less than 0.001 (Figure 4B). The results suggested that rs7025417 could affect the circulation level of IL-33.

Table 3. Interaction Analysis between rs7025417 in *IL33* and rs11685424 in *IL1RL1* under the Genotypic Model in the GeneID Combined Population

Types	Case (%)	Control (%)	p_{adj}	OR (95% CI)
TT/GG	675 (14.9)	416 (8.7)	8.90×10^{-21}	4.98 (3.56-6.97)
TT/GA	594 (13.1)	479 (10.0)	5.20×10^{-9}	2.39 (1.78-3.20)
TT/AA	210 (4.6)	288 (6.0)	3.53×10^{-2}	1.43 (1.02-1.98)
TC/GG	577 (12.8)	490 (10.2)	4.18×10^{-9}	2.4 (1.79–3.22)
TC/GA	1,345 (29.8)	1,518 (31.6)	5.00×10^{-6}	1.92 (1.45–2.54)
TC/AA	442 (9.8)	524 (10.9)	1.35×10^{-3}	1.64 (1.21–2.21)
CC/GG	190 (4.2)	188 (3.9)	2.23×10^{-3}	1.71 (1.21–2.40)
CC/GA	332 (7.3)	499 (10.4)	6.50×10^{-2}	1.34 (0.98–1.83)
CC/AA	156 (3.5)	407 (8.5)	–	1
ADD/ADD	–	–	2.81×10^{-32}	–

The population CAD combined consisted of 4,521 cases and 4,809 controls. Abbreviations are as follows: p_{adj}, p value after adjustment for covariates, such as age, gender, smoking, BMI, hypertension, DM, Tch, TG, HDL-c, and LDL-c; ADD, additive model, rs7025417_TT/TC/CC, rs11685424_GG/GA/AA.

Discussion

In the present study, we demonstrated that rs7025417 in *IL33* and rs11685424 in *IL1RL1* are significantly associated with CAD in three stages of a case-control association study based on the GeneID Chinese Han population. The interaction between the two variants exhibited a much greater effect than that of a single variant in the association with CAD, which indicated that these variants could interact with each other in a biological manner. Given that the two variants were located within the gene promoter regions, we assessed whether these variants could influence gene expression by using reporter gene analysis and found that they did. In addition, rs7025417 genotype affected the level of circulating IL-33, which indicates that rs7025417 might influence the development of CAD by regulating the expression of *IL33*.

IL-33, a cytokine that belongs to the IL-1 family, has been shown to be expressed in the nucleus of human vascular endothelial cells of most healthy tissues and tumor tissues, as well as epithelial cells exposed to the

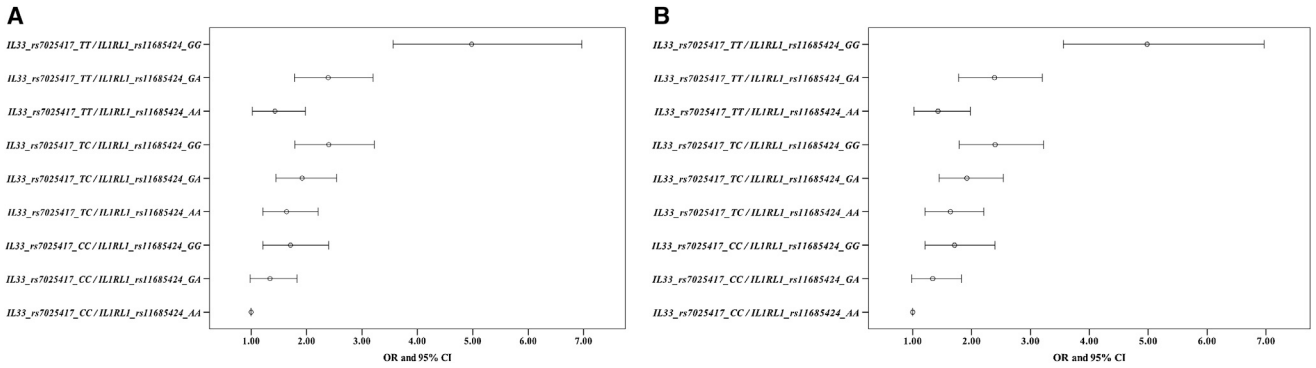

Figure 2. Comparison between OR Values
(A) OR values with a 95% confidence interval (95% CI) of the combination types under the genotypic model.
(B) The comparison of the OR values with a 95% confidence interval (95% CI) between the combination type "TT/GG" and the genotypic models of single variants.
ADD, additive model, rs7025417_TT/TC/CC, rs11685424_GG/GA/AA; DOM, dominant model, rs7025417_TT+TC/CC, rs11685424_GG+GA/AA; REC, recessive model, rs7025417_TT/TC+CC, rs11685424_GG/GA+AA.

external environment and fibroblasts of lymphoid tissues;[36] however, of all the blood cell types, only activated dendritic cells and activated macrophages express IL-33 at low levels.[37] IL-1RL1, previously known as the only receptor for the cytokine of IL-33, has two main protein forms: membrane-bound ST2L and soluble ST2 (sST2). It has been demonstrated in vitro that IL-33 can activate many immune cells by binding to ST2L-IL-1RL1 on the surface of many cells, including Th2 cells, mast cells, basophils, eosinophils, and natural killer cells.[36,38–42] In vivo, the administration of IL-33 can influence multiple inflammatory-immune diseases, such as arthritis, asthma, and inflammatory bowel disease.[43–45] This important evidence, demonstrating the involvement of the IL-33-ST2L pathway in the immune-inflammatory system, indicated that this pathway could also have important effects in the development of atherosclerosis and CAD.

Studies that have addressed the role of the IL-33-ST2L pathway in atherosclerosis and CAD have yielded conflicting results. Animal studies have indicated that the IL-33-ST2L pathway reduces macrophage foam cell formation[46] and inhibits the development of atherosclerosis in apolipoprotein E-deficient ($APOE^{-/-}$) mice.[25] However, other studies have reported that the IL-33-ST2L pathway promotes angiogenesis and vascular leakage and induces adhesion molecule and proinflammatory cytokine expression in human endothelial cells,[47–49] suggesting that the IL-33-ST2L pathway enhances the development of CAD by inducing the activation and injury of the coronary artery endothelium. To our knowledge, there have been few genetic or epidemiological studies on the association between the IL-33-ST2L pathway and CAD. Though Tsapaki et al. investigated the association between the distal promoter variants in *IL1RL1* and CAD with small sample size, their significant results indicated that the IL-33-ST2L pathway might genetically associate with CAD.[50] In the present study, with a large discovery sample size with sufficient statistical power, we detected that two tag SNPs in the two genes analyzed (rs7025417 in *IL33* and rs11685424 in *IL1RL1*) were significantly associated with CAD not only in the subgroup of anatomical disease (severe coronary stenosis) but also in the subgroup of clinical disease (myocardial infarction or revascularization). Furthermore, additional functional studies using reporter gene and circulation level analysis suggested that the rs7025417 genotype affects the level of circulating IL-33, which indicates that rs7025417 might influence the development of CAD by regulating the expression of *IL33*. These results are in agreement with the above in vitro studies with human endothelial cells, suggesting that the IL-33-ST2L pathway enhances the development of CAD.

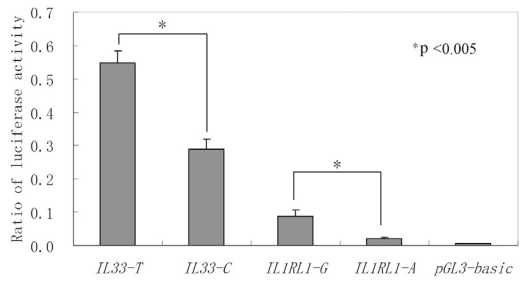

Figure 3. The Reporter Gene Analysis
The luciferase activity was tested by means of cellular extracts. p values less than 0.005 indicate that the difference was statistically significant between the plasmids. Mean ± SD of the relative luciferase activity is shown.

The conflicting results about the role of the IL-33-ST2L pathway in the development of atherosclerosis and CAD can be explained as follows. First, evidence provided by Demyanets et al.,[47] Choi et al.,[48] and Aoki et al.[49] suggested that the IL-33-ST2L pathway could contribute to the early events of atherosclerosis development via activation and injury of the coronary artery endothelium. Miller et al.,[25] in contrast, obtained results based on a model in which atherosclerosis had already developed. Second, given that the contradictory findings were obtained from

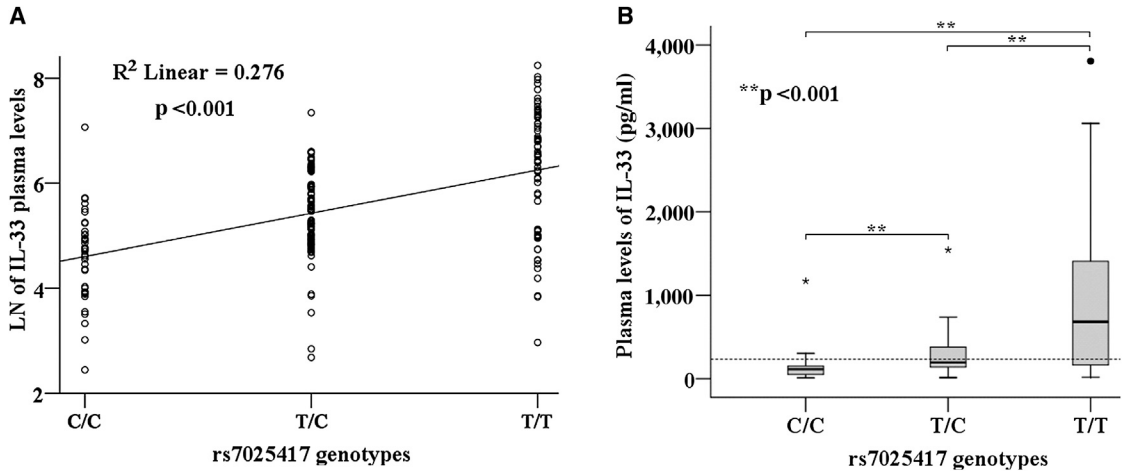

Figure 4. Circulation Level Analysis of IL-33
(A) Association analysis between the circulation levels of IL-33 and rs7025417 genotype in a linear regression model.
(B) The comparison between every pair of the three genotypes, via the Mann-Whitney U test. Special symbols (asterisk or circle) mark the outliers of the values in different genotypes. A broken line indicates the median value of 233.67 pg/ml for IL-33 in the detectable CAD individuals.

experiments that used different species, IL-33 could function differently in human and in mouse tissues. The present study was performed with human cells, producing results that were consistent with those of the human studies described above. The influence of genetic variants on human diseases commences before the occurrence of the diseases and can begin as early as birth.

In 2006, Kabesch et al. reported that individual SNPs might interact with each other in a biological way if their interaction resulted in a more than multiplicative effect.[51] In 2011, Demyanets et al. observed that endothelial cells express both IL-33 and ST2L in the nucleus and that the mRNA expression of these molecules is significantly correlated in the carotid atherosclerotic tissue,[47] suggesting that the crosstalk of IL-33 and ST2L-IL-1RL1 significantly enhances the risk of atherosclerosis. In the present study, we determined that the OR values for the combination types of the two SNPs were much higher than the values for a single one in the associations with CAD (Table 3 and Figure 2). Therefore, we speculated that the two SNPs might interact each other in a biological way, which might influence the development of CAD by regulating the expression of their genes.

There are some limitations to our study. First, although we performed a three-stage population study, a larger sample size in different populations is required to replicate the association results. Second, although the reporter gene analysis and the circulation study suggested that the two promoter variants might regulate the gene expression, a thorough analysis of the loci represented by the two variants is necessary to detect the causal variants that have much larger effect sizes.

In conclusion, this study demonstrated that the two promoter SNPs rs7025417 in *IL33* and rs11685424 in *IL1RL1* are significantly associated with CAD. This finding implies that the IL-33-ST2L pathway may strongly influence the development of CAD, highlighting the IL-33-ST2L pathway as a valuable target for the prevention and treatment of CAD.

Supplemental Data

Supplemental Data include two tables and can be found with this article online at http://www.cell.com/AJHG/.

Acknowledgments

This work was supported by grants from the National Basic Research Program of China (973 Program: 2013CB531103 and 2012CB517800), the National Natural Science Foundation of China (No. 81222002, 81170303, 81070106, and 81270163), the Program for New Century Excellent Talents at the University of China (NCET-09-0380 and NCET-11-0181), and a Hubei Province Natural Science Key Program (2008CDA047).

Received: June 27, 2013
Revised: August 2, 2013
Accepted: August 13, 2013
Published: September 26, 2013

Web Resources

The URLs for data presented herein are as follows:

dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
Genevar (Gene Expression Variation), http://www.sanger.ac.uk/resources/software/genevar/
International HapMap Project, http://hapmap.ncbi.nlm.nih.gov/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/
Promoter 2.0 Prediction Server, http://www.cbs.dtu.dk/services/Promoter/
PS program, http://biostat.mc.vanderbilt.edu/wiki
QANTO, http://hydra.usc.edu/gxe

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