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Association of an Anti-inflammatory Cytokine Gene *IL4* Polymorphism with the Risk of Type 2 Diabetes Mellitus in Korean Populations

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Abstract

Chronic inflammation has been implicated as one of the important etiological factors in insulin resistance and type 2 diabetes mellitus (T2DM). To investigate the role of anti-inflammatory cytokines in the development of T2DM, we conducted a case-control study to assess the association between *IL4*/*IL4R* polymorphisms and disease risk. We firstly identified single nucleotide polymorphisms (SNP) at *IL4* and *IL4RA* loci by sequencing the loci in Korean participants. Case-control studies were conducted by genotyping the SNPs in 474 T2DM cases and 470 non-diabetic controls recruited from community-based cohorts. Replication of the associated signals was performed in 1,216 cases and 1,352 controls. We assessed effect of *IL4-IL4RA* interaction on T2DM using logistic regression method. The functional relevance of the SNP associated with disease risk was determined using a reporter expression assay. We identified a strong association between the *IL4* promoter variant *rs2243250* and T2DM risk (OR=0.77; 95% CI, 0.67～0.88; $p=1.65\times10^{-4}$ in the meta-analysis). The reporter gene expression assay demonstrated that the presence of *rs2243250* might affect the gene expression level with ～1.5-fold allele difference. Our findings contribute to the identification of *IL4* as a T2D susceptibility locus, further supporting the role of anti-inflammatory cytokines in T2DM disease development.

Keywords: type 2 diabetes mellitus, single nucleotide polymorphisms, *IL4*, *IL4R*, anti-inflammatory cytokine, gene-gene interaction

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Introduction

Type 2 diabetes mellitus (T2DM) is one of the diseases for which the etiological factors have been revealed by recent progress in genome-wide association studies (GWAS). More than 20 genetic loci have been identified as being associated with the risk of T2DM, and this finding has been replicated across different ethnic populations. Many of the loci are involved in pancreatic β-cell function, suggesting the technical validity of GWAS and predominant contribution of these loci to T2DM (Prokopenko *et al.*, 2008). However, given the genetic heterogeneity of the disease, hypothesis-based candidate gene approaches have been successfully applied along with GWAS to identify additional loci that are moderately associated with T2DM.

Among the candidates, genes involved in inflammation have been intensively studied, with most studies examining the role of subclinical chronic inflammation in the development of insulin resistance and T2DM (Shoelson *et al.*, 2007). It is known that chronic over-nutrition results in local inflammation in adipose tissue, mediated by macrophage infiltration and increased production of pro-inflammatory mediators, the occurrence of which potentiates insulin resistance (Weisberg *et al.*, 2003). Consistently, genetic variants in pro-inflammatory markers and cytokines have been tested in many studies, supported by clinical observation including elevated levels of tumor necrosis factor-α (TNF-α), C-reactive protein (CRP) and interleukin-6 (IL6) in patients with obesity-related insulin resistance (Genco *et al.*, 2005; Plomgaard *et al.*, 2005; Pradhan *et al.*, 2001).

Based on the identification of homeostatic regulatory mechanisms in the inflammatory system that involve both pro- and anti-inflammatory cytokines, it has been proposed that anti-inflammatory cytokines, such as insulin-like growth factor-1 (IGF-1), IL10, IL13 and IL4, play a role in the pathogenesis of insulin resistance and T2DM. However, the underlying mechanism of their disrupted functions in chronic inflammation in relation to T2DM remains to be defined. Interestingly, there has been a report showing disruption of anti-inflammatory responses in type 2 diabetic model animals, suggesting a role of anti-inflammatory processes in T2DM (O'Connor *et al.*, 2007).

To investigate the implication of anti-inflammatory cytokines in the development of T2DM, we conducted case-control association studies to examine the correlation between disease risk and single nucleotide polymorphisms (SNP) in the relevant genetic loci including *IL4* and the IL4 receptor-coding gene, *IL4RA*, which express an anti-inflammatory cytokine and its receptor that play a key role in directing macrophages to an 'alternatively activated' state that is characterized by low expression patterns of pro-inflammatory cytokines and high levels of anti-inflammatory cytokines (Gordon, 2003). Our findings indicate that a promoter variant of an anti-inflammatory cytokine *IL4* gene is associated with risk of T2DM in the Korean population, which, we believe, lends significant insight into the role of anti-inflammatory cytokines in the T2DM etiology.

Methods

Subjects

Study subjects for SNP discovery and the first and second stages of association analyses were recruited from two community-based cohorts established as part of the Korean Genome and Epidemiology Study (KoGES) conducted by Korea Centers for Disease Control and Prevention (KCDC). The Institutional Review Board of KCDC approved the study, and informed consent was obtained from every subject prior to enrollment. The case subjects with T2DM were selected in accordance with the 1999 World Health Organization criterion. Control subjects were composed of individuals with no history of diabetes or first-degree relatives with diabetes, a fasting plasma glucose level≤126 mg/dL and a plasma glucose level≤140 mg/dL 120 min after glucose ingestion. The parameters measured were height, weight, waist and hip circumference and blood pressure. Body mass index (BMI) was calculated by dividing weight (kg) by height squared (m^2). Waist-to-hip ratio (WHR) was calculated from these measurements. Blood samples were collected at three separate times (0, 60, 120 min) from the cohort participants who were subjected to oral glucose tolerance tests (OGTT) and analyzed for glucose and insulin concentration at the given time. Cholesterol, triglyceride, and high-density lipoprotein (HDL) cholesterol were measured at baseline. Low-density lipoprotein (LDL) cholesterol level was calculated using the Friedewald formula for subjects with a triglyceride level less than 400 mg/dL (LDL=total cholesterol−(triglyceride/5+HDL)). Clinical parameters of the study subjects are summarized in Table 1.

Sequencing and genotyping

To identify SNPs of *IL4* and *IL4RA*, we sequenced entire exons of the genes including the coding and the 5'- and 3'-untranslated regions (up to approximately 2 kb upstream of the start codon and 0.5 kb downstream of the stop codon) as well as exon-intron boundary regions using genomic DNA samples collected from 24 unrelated Korean subjects. The ABI PRISM 3730 DNA analyzer (Applied Biosystems™) was used for sequencing and the primer sequences were designed based on GenBank sequences (NG_023252.1 for *IL4* and NG_

Table 1. Clinical profile of the study subjects

Features	Stage 1		Stage 2	
	Non-diabetic control (n=470)	T2DM (n=474)	Non-diabetic control (n=1,352)	T2DM (n=1,216)
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Age	64.00±2.88	64.62±2.80	64.68±2.37	59.05±6.88
Sex (male/female)	208 (44.3)/262 (55.7)	204 (43.0)/270 (57.0)	570 (42.2)/782 (57.8)	575 (47.3)/641 (52.7)
BMI (kg/m^2)	23.31±3.12	25.12±3.15	23.62±3.05	25.22±3.33
WHR	0.91±0.07	0.93±0.07	0.89±0.06	0.91±0.06
Systolic BP (mmHg)	121.01±17.27	129.23±18.37	129.09±18.29	134.08±18.58
Diastolic BP (mmHg)	75.37±9.86	77.66±10.34	81.31±11.56	84.17±11.39
Triglyceride (mg/dL)	149.18±71.01	200.25±141	144.72±85.85	196.77±147.86
Total cholesterol (mg/dL)	180.48±31.74	195.01±42.50	203.66±37.35	206.78±44.1
LDL cholesterol (mg/dL)	106.61±27.69	115.68±38.63	120.69±32.31	119.19±36.01
Glucose 0 (mg/dL)	74.53±3.54	118.07±35.29	87.43±6.39	120.03±36.57
Glucose 60 (mg/dL)	124.22±36.05	249.45±52.94	146.59±39.35	257.61±61.97
Glucose 120 (mg/dL)	98.69±22.26	247.71±61.95	105.84±21.94	243.67±61.88
Insulin 0 (μU/mL)	6.72±6.57	8.82±6.87	7.41±4.03	9.35±5.36
Insulin 60 (μU/mL)	30.56±29.50	26.25±28.18	41.52±39.28	29.48±26.63
Insulin 120 (μU/mL)	22.55±23.13	33.39±40.69	26.21±23.56	40.82±38.61

012086 for *IL4RA*). SNPs with a minor allele frequency ≥0.02 and a pair-wise r^2 coefficient≥0.8 were selected for further genotyping. The VeraCode GoldenGate (Illumina, Inc.) or TaqMan® (Life technologies) genotyping assay was employed using genomic DNA samples extracted from blood of study subjects. Genotypes with concordance rates≥99% and call rates≥98% were only included for association analyses.

Statistical analysis

Deviation from Hardy-Weinberg equilibrium (HWE) was tested using the χ^2 test. We examined linkage disequilibrium (LD) between all pairs of biallelic loci using Lewontin's D' (|D'|) and r^2 coefficients, widely used measures for LD analysis. Haplotypes of each individual were inferred using HaploView (Barrett *et al.*, 2005). Logistic regression analyses were conducted to determine the odds ratio (OR) (95% confidence interval (CI)) for the risk of T2DM, controlling for the covariates of age, sex, and BMI. Multivariate linear regression analyses were performed to test for associations among T2DM-related intermediate phenotypes such as BMI, serum glucose and insulin level among the unaffected group, controlling for age and sex. Statistical analyses were performed using SAS (SAS institute). The inverse-variance meta-analysis method was employed for combined analysis of the two-stage association study, assuming fixed effects. The R program (version 2.7.1) was used for the analysis. Heterogeneity was verified using Cochran's Q test. Gene-gene interaction between the *IL4* and *IL4RA* SNPs was analyzed using the logistic regression method. A combination of the homozygous genotype of the major allele of both genes (TT for *rs2243250T>C* and CC for *rs3024610C>T*) was used as a reference of epistasis. Correction for multiple testing was conducted following the false discovery rate (FDR)-controlling methods of Benjamini and Hochberg. The transcription factor binding of the SNP-containing region was analyzed using FASTSNP (http://fastsnp.ibms.sinica.edu.tw/pages/input_CandidateGeneSearch.jsp).

Reporter gene plasmid construction and expression assay

The genomic region (−433 to +353 with respect to the transcription initiation site of NM_000589 as +1) of *IL4* was amplified using standard PCR methods. DNA samples extracted from *rs2243250T* or *rs2243250C* carriers were used as a template for PCR. Sequences of the oligonucleotide primers used for amplification are as follows: 5' to 3' primer, CCTGATCACAACATTGCATTTCAGC; 3' to 5' primer, CAGTGACAATGTGAGGCAATTAGTTTATC. Each PCR product was thoroughly sequenced prior to insertion into the upstream region of the *bla* (*M*) reporter gene, coding for β-lactamase (GeneBLAzer™, Invitrogen), to ensure the SNP sequence and the proper sequence identity of the constructs except for the SNP of interest. Plasmid DNA of the reporter constructs was prepared for transfection using an endotoxin-free DNA preparation kit (Qiagen). For transfection, cells of the human hepatocellular carcinoma cell line SNU398 or the human adipocyte cell line SW872 (3×10^4 per well) were plated in a black-walled 96-well tissue-culture plate with a clear bottom (Costar). After a 24 hr-incubation period, the cells were transfected with 400 ng of purified reporter plasmid DNA using Lipofectamine 2000 transfection reagent (Invitrogen). The reporter β-lactamase activity was quantitatively measured from the transiently transfected cells after 48 hrs of incubation at 37°C, 5% CO_2, following the manufacturer's instructions (Invitrogen, GeneBLAzer™ *in vivo* detection kit). The normalized activity was presented as a relative ratio with respect to that of cells transfected without DNA.

Results

Association of an *IL4* polymorphism with the risk of T2DM

To explore the possibility that *IL4* and *IL4RA* are associated with the risk of T2DM, we first attempted to identify SNPs in the Korean population by sequencing the gene loci including 5'- and 3'-flanking regions. Among the identified SNPs (Supplementary Fig. and Supplementary Tables 1-1 and 1-2), we selected two and eight SNPs from the *IL4* and *IL4RA* loci, respectively, considering LD and allele frequencies for large-scale genotyping. We genotyped 474 unrelated T2DM cases and 470 non-diabetic control subjects to test for association of the polymorphisms with host susceptibility to the disease. The subjects were recruited from Ansan and Ansung community-based cohorts as part of KoGES (Table 1).

In the case-control study using logistic regression analysis with adjustment for age, sex, and BMI, we found no differences in the distribution of genotype frequencies between the control and T2DM cases for any of the SNPs tested, with exception of a promoter variant on the *IL4* locus, *rs2243250T>C*. The analysis revealed that the minor allele C had a protective effect on T2DM risk compared to that of the major allele T (protective allele OR=0.70; 95% CI, 0.55～0.89; p=0.004 (FDR-P < 0.05) in an additive model, Table 2).

To confirm these results, we conducted a second

Table 2. Logistic analysis of the *IL4* and *IL4RA* polymorphisms for association with T2DM

Gene	rs number	Position	Amino acid change	Stage 1		Stage 2		Meta-analysis		
				OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	Heterozygosity P
IL4	*rs2243250*	*Promoter*		**0.70 (0.55～0.89)**	**0.004**[a]	**0.80 (0.67～0.94)**	**0.007**[a]	**0.77 (0.67～0.88)**	**1.65E-04**[a]	**0.39**
IL4R	*rs1805010*	*exon5*	*Ile75Val*	1.06 (0.87～1.28)	0.568	-	-	1.06 (0.87～1.28)	0.568	-
	rs2074571	*intron5*		1.14 (0.86～1.5)	0.367	1.09 (0.91～1.3)	0.367	1.10 (0.94～1.28)	0.220	0.80
	rs3024575	*intron6*		0.87 (0.71～1.06)	0.169	-	-	0.87 (0.71～1.06)	0.169	-
	rs3024610	*intron7*		1.09 (0.9～1.32)	0.391	1 (0.88～1.14)	0.986	1.02 (0.92～1.14)	0.694	0.47
	rs3024658	*intron9*		1.12 (0.81～1.53)	0.502	1.09 (0.87～1.37)	0.443	1.10 (0.91～1.32)	0.322	0.92
	rs1801275	*exon11*	*Gln576Arg*	1.07 (0.82～1.39)	0.616	-	-	1.07 (0.82～1.39)	0.616	-
	rs2074570	*exon11*		0.92 (0.65～1.30)	0.621	-	-	0.92 (0.65～1.30)	0.621	-
	rs8832	*exon11*		0.96 (0.79～1.18)	0.710	-	-	0.96 (0.79～1.18)	0.710	-

[a]FDR $p < 0.05$.

Table 3. Gene-gene interaction analysis between *rs2243250T>C* of *IL4* and *rs3024610C>T* of *IL4RA*

Polymorphism	Stage1				Stage 2			
	Case	Control	OR (95% CI)	p	Case	Control	OR (95% CI)	p
IL4 T/T×IL4R C/C	105	88	-	-	230	261	-	-
IL4 T/T×IL4R C/T	166	148	0.92 (0.63～1.35)	0.670	379	370	1.07 (0.82～1.40)	0.638
IL4 T/T×IL4R T/T	64	50	1.05 (0.64～1.72)	0.859	135	144	1.14 (0.81～1.61)	0.455
IL4 T/C×IL4R C/C	23	54	**0.40 (0.22～0.73)**	**0.003**	142	155	0.92 (0.66～1.29)	0.632
IL4 T/C×IL4R C/T	67	69	0.93 (0.58～1.49)	0.760	181	250	**0.72 (0.533～0.98)**	**0.034**
IL4 T/C×IL4R T/T	29	32	0.61 (0.33～1.12)	0.111	74	82	0.75 (0.49～1.16)	0.193
IL4 C/C×IL4R C/C	3	6	0.27 (0.06～1.35)	0.112	18	23	0.93 (0.43～1.980	0.843
IL4 C/C×IL4R C/T	7	15	0.39 (0.15～1.05)	0.063	22	24	0.64 (0.61～1.34)	0.236
IL4 C/C×IL4R T/T	5	2	2.19 (0.37～13.02)	0.388	12	7	1.33 90.43～4.14)	0.621

stage case-control association analysis by genotyping samples from another community-based cohort, Health 2 of KoGES. The genotyping of 1,216 cases and 1,352 controls generated additional evidence supporting the association of *rs2243250T>C* with T2DM (OR=0.80; 95% CI, 0.67～0.94; p=0.007 (FDR-P<0.05)). A meta-analysis of the data of the two cohorts produced stronger combined evidence of association (OR=0.77; 95% CI, 0.67～0.88; $p=1.65\times10^{-4}$ (FDR-P<0.05); Table 2).

Effect of *IL4-IL4RA* gene-gene interaction on T2DM risk

Although our analyses of individual SNPs or haplotypes revealed no evidence of *IL4RA* association with T2DM, the linear regression analyses for T2DM-related intermediate phenotypes such as BMI, WHR, plasma glucose and insulin level in the controls showed modest association signals of *IL4RA* polymorphisms with BMI and insulin level in either or both populations (Supplementary Table 2). This result led us to examine the effect of *IL4-IL4RA* interaction on disease susceptibility. Logistic analysis for epistasis between *rs2243250T>C* of *IL4* and the tagging SNPs of *IL4RA* (Table 2) showed existence of an interaction between the *IL4* SNPs and an intronic SNP, *rs3024610C>T* (Table 3). The heterozygous genotype for *rs2243250T>C* (TC) and the homozygous genotype for *rs3024610C>T* (CC) showed significant association with a protective effect in the first stage, compared to the TT×CC as a reference (OR=0.40; 95% CI, 0.22～0.73; p=0.003). In the second stage analysis, a combination of the heterozygous genotypes for both SNPs (TC×CT) displayed a moderate association in the same direction (OR=0.72; 95% CI, 0.5333～0.98; p=0.034). Although the same combination of genotypes was not replicated, these results suggest that interaction of *IL4* and *IL4RA* has a cumulative effect on T2DM risk, compared to the effects from the individual SNPs.

Functional association of an *IL4* promoter variant with the gene expression

The strong association of *rs2243250T>C* with T2DM

and its close proximity to the *IL4* transcription initiation site (−219 bp with respect to +1) led us to investigate the effect of this polymorphism on *IL4* promoter activity. Results from searches for transcription factor binding sites (TFBS) suggested that *rs2243250* C allele to T allele alteration would generate a SOX5 (SRY (sex determining region Y) box 5) binding site in the proximal promoter region of the gene. To this end, we prepared reporter gene constructs carrying β-lactamase-coding sequences whose expression was controlled by the *IL4* promoter region. These reporter constructs were then used for transfection of cells to measure reporter gene expression. As shown in Fig. 1, the cells transiently expressing the T allele-carrying reporter gene showed greater reporter activity than those with the C allele-carrying construct, which was consistent with the TFBS prediction results. The allelic differential expression was consistently observed in two different cell lines, SW872, a human adipocyte cell line and SNU398, a human hepatocellular carcinoma cell line, which excluded the possibility of cell line-specific indirect effects. This result suggests that the *IL4* promoter variant *rs2243250T>C* might affect the gene expression level by modulating promoter activity in the SNP-containing region.

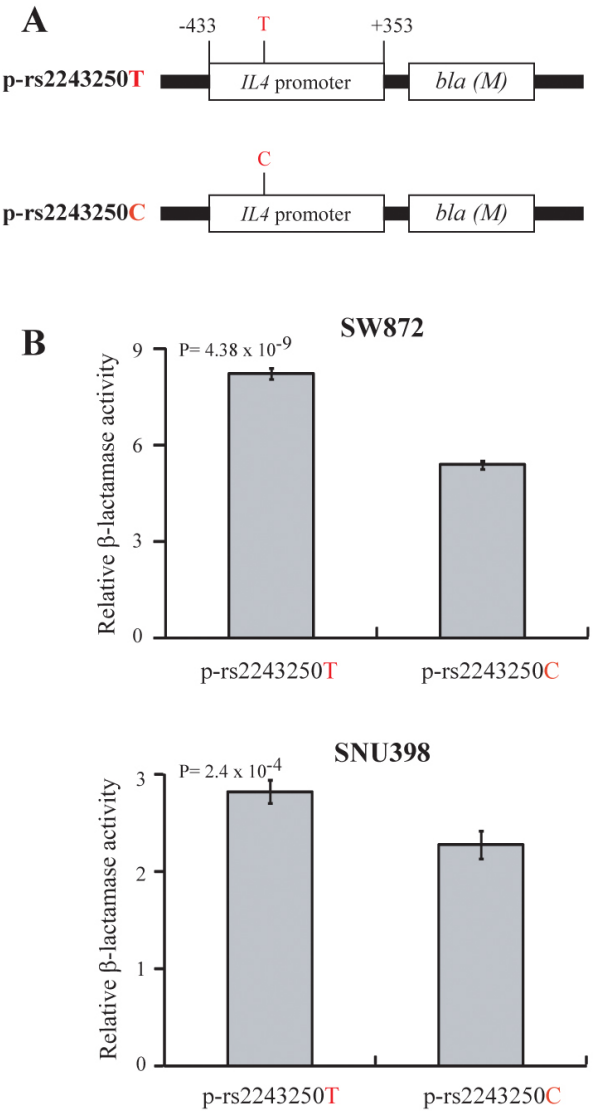

Fig. 1. Functional analysis of the regulatory effect of *rs2243250T>C* on gene expression. (A) Schematic representation of construction of the β-lactamase (*bla(M)*)-plasmid carrying the allele T- or C-containing promoter region. (B) Relative β-lactamase reporter expression is shown with respect to basal level expression from cells transfected without DNA.

Discussion

There is accumulating evidence indicating a critical role for sub-acute chronic inflammation in the pathogenesis of obesity-related insulin resistance and T2DM (Corvera *et al.*, 2006; Dandona *et al.*, 2004; Dandona *et al.*, 2005; Finegood, 2003; Genco *et al.*, 2005; Mangge *et al.*, 2004; Meerarani *et al.*, 2007; WangCai, 2006; Weisberg *et al.*, 2003). In obesity, adipose tissues were shown to be infiltrated by macrophages, which may be a major source of locally-produced pro-inflammatory cytokines (Weisberg *et al.*, 2003). Increased levels of TNFα and IL6 were shown to correlate with reduced insulin- stimulated glucose uptake in obese and T2DM patients (Bastard *et al.*, 2006; Katsuki *et al.*, 1998; Kern *et al.*, 2001; Mishima *et al.*, 2001).

As a homeostatic mechanism in the immune-inflammatory system, anti-inflammatory agents including IGF-1, IL10, IL13 and IL4 reduce secretion of pro-inflammatory cytokines by macrophages and stimulate the production of various anti-inflammatory molecules (Orino *et al.*, 1992; Sone *et al.*, 1992; Vannier *et al.*, 1992). Several reports have demonstrated that suppression of inflammation by the anti-inflammatory cytokines is impaired in T2DM patients (O'Connor *et al.*, 2007). Insulin resistance due to obesity-related chronic inflammation has been suggested as an underlying mechanism linking anti-inflammatory cytokine dysfunction to T2DM (Shoelson *et al.*, 2007).

IL4, an anti-inflammatory cytokine, is known to direct macrophages toward an 'alternatively activated' state that is characterized by low expression patterns of pro-inflammatory cytokines and high levels of anti-inflammatory cytokines (Gordon, 2003). Although its detailed mechanism is not as well defined as that of pro-inflammatory cytokines, IL4 has received much attention for its potential relationship to insulin resistance and

T2DM, as supported by an observation showing that there is an IL4-dependent insulin signaling pathway in macrophages, and the positive regulatory functions for other anti-inflammatory cytokine production mechanisms were impaired in *db/db* diabetic model mice (Hartman *et al*., 2004; O'Connor *et al*., 2007). Moreover, a recent study clearly demonstrated that knock-out mice defective in the IL4-stimulated alternative activation of macrophage were more susceptible to obesity and insulin resistance than were control mice (Odegaard *et al*., 2007).

These previous results prompted us to investigate whether genetic polymorphisms of the IL4 and IL4 receptor-coding genes would modulate host susceptibility to T2DM. Although there have been several reports controversially linking *IL4* and *IL4R* polymorphisms to risk for autoimmune T1DM in various ethnic backgrounds, there have been no reports such an association with T2DM (Bugawan *et al*., 2003; Maier *et al*., 2005; Nunez *et al*., 2008; Reimsnider *et al*., 2000). In this report, we present results from two-stage case-control association and functional analyses showing that a SNP located in the *IL4* promoter region, *rs2243250T>C*, is associated with the risk of T2DM. Although we found no association of any of the common *IL4RA* SNP with disease susceptibility in our study population, we observed association between intronic variants of *IL4RA* and obesity-related BMI and insulin level. Moreover, our gene-gene-interaction analyses implied that this functional *IL4* promoter SNP could modify disease susceptibility in combination with the intronic SNP in the *IL4RA* gene.

Our reporter gene expression assay indicates that *rs2243250T>C* may affect the overall *IL4* promoter activity modulating the gene expression level. The minor C allele, which is associated with a decreased risk of T2DM, was shown to lower the expression level. This functional consequence of the protective allele reducing *IL4* expression was unexpected, given the previous reports showing links between impaired IL4 function in diabetic model animals and obesity and insulin resistance (Hartman *et al*., 2004; O'Connor *et al*., 2007). A possible explanation for our result is the presence of other interacting genetic and/or environmental effects involved in *IL4* function, other than the expression level itself. It could include additional mechanisms by which increased expression levels of IL4 receptor coding gene and/or other anti-inflammatory cytokine genes would compensate for the decreased *IL4* expression level. Additional in-depth functional and physiological studies will be needed to establish the mechanism of this association.

On the basis of our findings, as well as the functional data of other groups, we propose that the genetic polymorphisms of an anti-inflammatory cytokine coding *IL4* and *IL4RA* may modify an individual's susceptibility to T2DM. Despite the recent success of GWAS in identifying T2DM susceptibility loci, our findings have shown that a hypothesis-based candidate gene approach which complements GWAS is still a powerful way to identify unknown disease-associated loci. We believe our findings contribute to a better understanding of the disease etiology and may help to identify unknown genes in the relevant pathways or mechanism.

Supplementary materials

Supplementary data including a figure and two tables can be found with this article online at http://www.genominfo.org/html/UploadFile/article3_2011306_SP.pdf.

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