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The Association Between Innate Immunity Gene (*IRAK1*) and C-Reactive Protein in the Diabetes Heart Study

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Abstract

Evidence suggests that interleukin-1 receptor associated kinase-1 (*IRAK1*), fundamental in the toll-like receptor pathway (TLR), may play a more specific role in atherosclerosis.

Methods—Caucasian women (N=529) and men (N=467) from the Diabetes Heart Study (DHS) were genotyped at four loci within the *IRAK1* gene located on the X chromosome. Generalized estimating equations (GEE1) were used to evaluate association with C-reactive protein (CRP) for both single SNP and haplotype analyses.

Results—For each SNP genotyped, Caucasian women carrying one or two copies of the variant allele had greater CRP concentrations than those carrying the common genotype in both crude and adjusted models. There were 2 major haplotypes, CTTT (82%) and its complement, TCCG (13%). The presence of the TCCG haplotype was associated with greater CRP concentrations in Caucasian women (p=0.0004) and this relationship was maintained after adjustment for age, BMI, smoking, diabetes, and cholesterol-lowering therapy (p=0.003). There was no association between CRP and *IRAK1* SNPs in Caucasian men.

Conclusion—Variation in the *IRAK1* gene is associated with CRP concentration in Caucasian women in DHS. Further studies are needed to reproduce the current finding and to understand the biological relationship between *IRAK1* and inflammation related to atherosclerosis.

Keywords

Interleukin-1 receptor-associated kinase-1; *IRAK1*; C-reactive protein; CRP; innate immunity

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INTRODUCTION

Through a family of Toll-like-receptors (TLRs), the human innate immune system can recognize diverse microbial pathogen-associated molecular patterns (such as lipopolysaccharide, lipoteichoic acid, viral RNAs, etc) as well as various endogenous human substances (such as heat shock proteins, fatty acids, and mildly oxidized low density lipoprotein) (Medzhitov and Janeway, 2000). Ligated TLRs activate a series of downstream signal transduction processes that lead to the expression of various pro- and anti-inflammatory mediators. Through these mechanisms the innate immune system serves as a first line of defense toward microbial infections and helps regulate various aspects of cellular metabolism.

Several lines of evidence suggest that the innate immune system may play a specific role in the pathogenesis of atherosclerosis. For example, elevated expression of TLRs has been observed in human atherosclerotic plaques (Edfeldt et al., 2002) and genetic variants of TLRs have been associated with increased intima-media thickness (Kiechl et al., 2002). In addition, in MyD88-null mice on a high cholesterol diet, the absence of the intracellular TLR downstream adaptor molecule, MyD88, attenuated the development of atherosclerosis (Bjorkbacka et al., 2004).

The interleukin-1 receptor associated kinase 1 (IRAK1) is one of the key downstream signaling proteins that can be recruited to MyD88 and activated upon challenges by TLR ligands. IRAK1 activation leads to transcription factor Stat3 phosphorylation and activation of Stat3 in the presence of IL-6 has been shown to activate CRP in Hep3B cells (Zhang et al., 1996). Gene array analyses have revealed that human coronary arteries express high levels of IRAK1 (Satterthwaite et al., 2005), and IRAK1 has been shown to be constitutively activated in human blood mononuclear cells isolated from atherosclerosis patients (Huang et al., 2004). In addition, recent evidence suggests that IRAK1 may play a novel role in regulating anti-inflammatory cytokine, interleukin-10 (IL-10) (Huang et al., 2004).

These observations suggest that activation of the innate immune system in general, and IRAK1, in particular, may contribute to the increased levels of inflammation proteins associated with coronary heart disease. To further explore this hypothesis, we sought to determine whether genetic variation in the IRAK1 gene is associated with C-reactive protein, a marker of inflammation and predictor of cardiovascular risk (Ridker et al., 2000; Pai et al., 2004). Caucasian individuals from the Diabetes Heart Study (DHS) were genotyped at four loci in high linkage disequilibrium within the *IRAK1* gene and associations were determined between plasma concentrations of CRP and *IRAK1* sequence variants.

METHODS

The DHS was conducted in Forsyth County, NC, to study the genetic epidemiology of cardiovascular disease-related phenotypes in families with multiple individuals who have a diagnosis of type 2 diabetes. Type 2 diabetes was clinically defined as diabetes developing after age 34 and treated with insulin and/or oral agents, in the absence of historic evidence of ketoacidosis. Siblings who were concordant for type 2 diabetes were recruited, along with one nondiabetic sibling in each family when possible. Nondiabetic siblings had fasting blood glucose (<126 mg/dL) to confirm nondiabetic status. Diabetic index cases with renal insufficiency, defined by a serum creatinine ≥ 1.5 mg/dl or blood urea nitrogen ≥ 35 mg/dL, were excluded.

The participant examinations included interviews for medical history and health behaviors, anthropometric measures. Use of cholesterol-lowering medication was assessed by reviewing all medications that participants reported to be currently taking and coded by a single reviewer. BMI was calculated from the equation weight (kg)/height (m^2).

CRP was measured using an ELISA (high sensitivity) kit from American Laboratory Products Company (ALPCO, Windham, NH 03087). The intra-assay variability was 6.7% and the inter-assay coefficient of variation was 8%. The sensitivity was 0.002 mg/L. CRP was log transformed and geometric mean CRP concentrations were reported.

Genotyping of *IRAK1*

Although the *IRAK1* gene is located in the pseudoautosomal (PAR2) region of the X chromosome (Xq28), it does not have an active counterpart on chromosome Y. Single nucleotide polymorphisms (SNPs) across the *IRAK1* gene were chosen based on a minor allele frequency of at least 5% at a resolution of 1 SNP per kb across *IRAK1*, including 2.5 kb of the promoter, all the exons, introns and 3' untranslated region (UTR). Initially, 16 SNPs were chosen based on the above criteria, including all the SNPs reported in the coding region. The 16 SNPs were originally genotyped among 96 randomly selected samples in a separate study of Swedish individuals. Twelve SNPs were eliminated, 1 SNP whose reaction did not perform well and 11 SNPs that were monomorphic in the sample, including all the 6 SNPs identified in 2.5 kb promoter region. The four remaining SNPs are listed in Table 1.

PCR and primer extension reactions were performed by using the Mass ARRAY genotyping system (Sequenom Inc, San Diego, CA). Fragment sizes were determined by means of mass spectrometry (Bruker Daltonics, Billerica, MA), and the genotypes were viewed and analyzed with SpectroTyper software (Sequenom).

Statistical Methods

Although the DHS has recruited both Caucasians and African Americans, the African American sample is small (58 African American males from 40 pedigrees and 119 African American females from 62 pedigrees). Given the allele frequency of some of these polymorphisms, the tests associated with the African American sample have low statistical power. Therefore, the current study only reports the Caucasians sample available for genotyping (467 males from 277 pedigrees, 529 females from 294 pedigrees). Focusing the analysis on the Caucasian samples minimizes the potential confounding effects of population admixture and genetic heterogeneity.

To account for the familial correlation, generalized estimating equations (GEE1) assuming exchangeable correlations and using a robust estimator of the variance were calculated (O'Connell 2000a). As noted above, the *IRAK1* gene is 8.4 kbp in length and located within the pseudoautosomal (PAR2) region of the X chromosome (Xq28) but does not have an active counterpart on chromosome Y. Therefore analyses were also stratified by gender given potential gender-specific effects. For each gender, multivariable GEE1 models were computed adjusting for age, BMI, smoking, and cholesterol-lowering medications. To help control type I errors, analyses focused on common alleles and haplotypes (e.g., frequency greater than 5%) emphasizing the dominant or additive genetic models in females and the presence or absence of the allele (haplotype) in males. In females, other models are presented for completeness. To best approximate the distributional assumptions of the tests (e.g., conditional normality and homogeneity of variance), the natural log of serum CRP concentrations plus one was analyzed.

To test for an association between the four SNP haplotypes and CRP a parallel GEE1 analysis was computed except that the quasi-likelihood was weighted by the probability for each possible haplo-genotype for an individual. Specifically, we computed the expectation-maximization (EM) algorithm estimates for haplotype frequencies from the family data using the software ZAPLO (O'Connell, 2000a). To reduce the complexity of the problem, ZAPLO assumes zero recombination between markers and generates all possible haplo-genotypes (i.e., haplotype combinations) for each individual. These haplo-genotypes are used as input into

PROFILER (O'Connell, 2000b) to compute the joint probability distribution for the haplotypes of individuals within the pedigree. This joint probability distribution is then used to estimate the probability of each haplotype pair combination (haplo-genotype) possible for each individual, conditional on the family data. Each individual enters into the GEE1 analysis once for each haplo-genotype possibility, weighted by the haplo-genotype probability. Thus, the weight for each individual sums to one. The weighted GEE1 analyses were completed as above using the same transformations and sandwich estimator of the variance to account for the within cluster correlation for each of the haplotypes observed with greater than 5% frequency.

RESULTS

The DHS Caucasian cohort, with an average age of 62 years at the baseline exam, were obese, predominately diabetic, and had a high prevalence of hypertension (87%) (Table 2). Compared to men, women had greater CRP and HDL concentrations and were less likely to be on cholesterol-lowering medications.

The *IRAK1* gene, 8.4 kbp in length, is located in the pseudoautosomal (PAR2) region of the X chromosome (Xq28). The linkage disequilibrium (LD) coefficients, D' and r^2 , were 1 and >0.7 , respectively for each pairwise combination of SNPs. There were no significant departures from Hardy-Weinberg proportions expectations using a χ^2 goodness-of-fit test for each SNP.

Tests for association within the Caucasian females between CRP and *IRAK1* SNPs using a 2-degree of freedom test, and dominant, additive, and recessive models are shown in Table 3. For each SNP genotyped, individuals carrying one or two copies of the variant allele had higher CRP concentrations than those with the common genotype. Using a dominant model, which fit the data well, differences in CRP concentrations by genotype for *IRAK1* SNP, rs1059702, are shown in Figure 1. There was no evidence of an association between CRP and *IRAK1* SNPs in Caucasian men. Removing individuals with CRP >10 mg/L did not change the direction of the associations nor the strength of the associations.

Inferred haplotype frequencies were similar among men and women of DHS (Table 4). Two haplotypes with an estimated frequency $>5\%$ were detected, CTTT and its homologous pair TCCG. Compared to haplotype 1 (CTTT), the presence of haplotype 2 (TCCG) was associated with 69% higher CRP concentrations in women in both crude models or after adjusting for age, diabetes, BMI, and cholesterol-lowering medications. The presence of the rarer haplotype 3(CTTG, $\sim 4\%$) was associated with the lowest CRP concentrations. No differences in CRP by haplotype were demonstrated in Caucasian men.

DISCUSSION

In the current study, variation within the *IRAK1* gene was associated with CRP serum concentration in Caucasian women. There were large effect size differences in CRP by genotype for each SNP tested (1.5 mg/L). Intriguingly, this association was gender specific and was not manifested in Caucasian men.

The exact biochemical and functional implications for the *IRAK1* gene are not clearly understood. IRAK1 is constitutively activated in atherosclerosis patients (Huang et al., 2004). Elevated IRAK1 activity has been shown to contribute to Stat3 activation, which together with other transcriptional regulators, may contribute to CRP gene transcription (Zhang et al., 1996; Heeschen et al., 2003). Besides CRP, there are many other pro and/or anti-inflammatory mediators that are altered during the pathogenesis of atherosclerosis. IL-10, which has been shown to be regulated in part by IRAK1 (Huang et al., 2004), may be an important cytokine involved in coronary heart disease (Heeschen et al., 2003). Thus, future biochemical studies are warranted to determine the contribution of *IRAK1* gene variants to IRAK1 protein

expression and function, and its contribution to inflammatory processes. It may also be useful to examine the effect of *IRAK1* gene variation on the expression of other inflammatory genes as well.

It is not clear why an association is present between *IRAK1* and CRP in DHS women but not men. The fact that *IRAK1* gene is located on the X chromosome, implies a set of unique properties. Secondly, the phenotype for the current study, CRP, is known to vary by gender (Khera et al., 2005; Lakoski et al., 2006). Thus, it is possible that an association between genetic variation in *IRAK1* and CRP is actually due to unmeasured confounding variables in women, which are genotype-specific. In general, sex differences in genetic association studies are present in the literature and require further understanding and investigation in multiple cohorts to attain reproducible results (Newton-Chen and O'Donnell, 2004).

In conclusion, we have identified a novel correlation between variation within the *IRAK1* gene and CRP concentration in Caucasian women in DHS. This result provides impetus to further explore the role of *IRAK1* in inflammation and atherosclerosis and provides a plausible mechanism by which alteration in the innate immunity pathway may influence risk for cardiovascular disease.

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Figure 1.

The Association Between *IRAK1* SNP rs1059702 and CRP in Caucasian Women

† adjusted for age, BMI, smoking, diabetes, and cholesterol-lowering medications

Table 1

Nomenclature of SNPs Genotyped in DHS Cohort

NCBI Reference	Relative Position*	Role	Amino Acid
rs3027898	IRAK1_9373G/T	3' UTR	
rs1059703	IRAK1_6434C/T	Exon,non-synonymous	Ser532Leu
rs7061789	IRAK1_4788C/T	Intron	
rs1059702	IRAK1_1071C/T	Exon,non-synonymous	Phe196Ser

*
The SNP number is the relative position of the SNP, based on the sequence available at www.innateimmunity.net

Table 2

Baseline Characteristics of DHS Women and Men

	Women (N=529)	Men (N= 467)	P-Value
Age (mean $\pm$ SD)	61 $\pm$ 10	62 $\pm$ 9	0.8682
Diabetes (%)	80	86	0.0315
Hypertension (%)	87	83	0.0358
Cholesterol Medications (%)	38	47	0.0126
Smoking (%)	16	18	0.0001
BMI (mean $\pm$ SD)	33 $\pm$ 8	31 $\pm$ 6	0.0001
LDL (mg/dL) (mean $\pm$ SD)	109 (32)	102 (32)	0.0003
HDL (mg/dL) (mean $\pm$ SD)	48 (13)	38 (10)	0.0001
CRP (mg/L) (median;IQR)	4.2 (1.8–9.6)	2.4 (1.1–4.6)	0.0001

Table 3
The Association Between *IRAK1* SNPs and CRP in Caucasian Women

rs	AA change	Genotype	no.(%)	CRP ($\pm$ SE) [*]	P-value 2DF [†]	D	A	R
1059702	Phe196Ser	CC	381(70)	3.8 (3.5,4.1)	0.004	0.001	0.001	0.2
		CT	119(22)	5.7(5.0,6.4)				
		TT	9(2)	6.5 (4.3,9.7)				
7061789		TT	331(61)	3.8 (3.4,4.1)	0.02	0.01	0.06	0.7
		CT	163(30)	5.1 (4.6,5.7)				
		CC	16(3)	4.1 (3.0,5.5)				
1059703	Ser532Leu	TT	361(66)	3.7 (3.4,4.0)	0.03	0.009	0.02	0.74
		CT	136(25)	5.3 (4.6,5.7)				
		CC	11(2)	4.5 (3.0,6.7)				
3027898		TT	326(60)	3.7 (3.3,4.1)	0.04	0.03	0.09	0.95
		GT	175(32)	5.0 (4.0,5.5)				
		GG	17(3)	3.7 (2.7,5.0)				

^{*} geometric mean C-reactive protein (mg/L) ($\pm$ standard error)
[†] adjusted for age, bmi, diabetes, smoking, cholesterol-lowering medication

Table 4
The Association Between *IRAK1* Haplotypes and CRP in DHS Women and Men

	rs1059702	rs7061789	rs1059703	rs3027898	Freq	Coefficient	SE	P-value [†]	Homozygote+	Mean by Haplo-Genotype Heterozygote	Homozygote-
Caucasian Women											
Reference haplotype 1	C	T	T	T	0.82				.60	.62	.89
haplotype 2	T	C	C	G	0.13	0.24	0.08	0.0035	.64	.68	.61
haplotype 3	C	C	T	G	0.03	-0.16	0.10	0.1098	-	.47	.66
Caucasian Men											
Reference haplotype 1	C	T	T	T	0.78				.48	-	.52
haplotype 2	T	C	C	G	0.14	-0.04	0.21	0.8514	.54	-	.49
haplotype 3	C	C	T	G	0.04	<0.01	0.36	0.9896	.99	-	.47

[†] adjusted for age, BMI, smoking, diabetes, cholesterol-lowering medications, and other