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IL6R gene, plasma CRP, and diabetes risk in women

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ABSTRACT

Objective: Recent genome-wide association studies (GWAS) related common variants in the interleukin 6 receptor (*IL6R*) gene to plasma C-reactive protein (CRP) concentrations. Because *IL6R* variants were previously associated with IL-6 levels, we tested whether the associations with CRP were independent of IL-6 and the interactions between *IL6R* variants and CRP in relation to diabetes risk.

Research Design and Methods: Plasma CRP and IL-6 levels and ten *IL6R* polymorphisms were determined in a nested case-control study of 633 diabetic and 692 healthy Caucasian women.

Results: In both non-diabetic and diabetic women, *IL6R* polymorphisms were associated with plasma CRP levels, independent of IL-6 concentration. After adjustment of IL-6 levels, CRP concentrations in the genotype AA, AC, and CC of the GWAS polymorphism rs8192284 were 0.32, 0.26, and 0.24 pg/mL among non-diabetic women (P for trend=0.003; and FDR=0.01); and 0.63, 0.48, 0.43 pg/mL among diabetic women (P for trend<0.0001; and FDR=0.0001). Haplotypes inferred from polymorphisms within a linkage disequilibrium (LD) block including rs8192284 were also significantly associated with CRP levels (P=0.0002). In an exploratory analysis, rs8192284 showed significant interactions with CRP levels in relation to diabetes risk (P for interaction=0.026). The odds ratios across increasing quartiles of CRP were 2.19 [95% confidence interval (CI), 1.42-3.36], 2.03 (1.27-3.23), and 2.92 (1.77-4.82) in the carriers of allele-C; and were 2.21 (1.18-4.12), 3.77 (1.87-7.57), and 5.02 (2.4-10.5) in the non-carriers.

Conclusions: IL6R variants were significantly associated with plasma CRP, independent of IL-6 levels. *IL6R* variants may interact with CRP in predicting diabetes risk.

Chronic systemic inflammation can induce insulin resistance and is a key mechanism linking obesity and diabetes (1). As a nonspecific marker of systemic inflammation, C-reactive protein (CRP) is an acute phase reactant synthesized in the liver in response to cytokines (2) especially interleukin 6 (IL-6) (3). In epidemiological studies, circulating CRP levels significantly predict the risk of type 2 diabetes (4-6).

In an earlier analysis (7), we found that the common variants in the interleukin 6 receptor (*IL6R*) gene, especially a single nucleotide polymorphism (SNP) rs8192284, were significantly related to high plasma IL-6 concentration. Similar associations between *IL6R* SNPs and IL-6 concentration were also observed in an admixture study (8). In a recent genome-wide association study (GWAS) on plasma CRP levels among 6,345 apparently healthy women, common SNPs in the *IL6R* gene including rs8192284 were associated with CRP concentration at genome-wide significance level ($p < 5 \times 10^{-8}$) (9). Because of the close relationship between IL-6 and CRP, we hypothesized that the *IL6R* gene-CRP associations observed in the GWAS might be secondary to the changes in circulating IL-6 levels.

To test this hypothesis, we examined the associations between *IL6R* gene variants and CRP concentrations in healthy and diabetic women, controlling for IL-6 levels. We also assessed the interactions between *IL6R* SNPs and plasma CRP levels in predicting the risk of type 2 diabetes.

MATERIALS and METHODS

Study population. The Nurses' Health Study (NHS) began in 1976 with the recruitment of 121,700 female registered nurses (30 to 55 years). 32,826 women provided blood between 1989 and 1990. The medical history and lifestyle information were updated every two years using questionnaire (10). Samples for the present study were selected from women who provided a blood sample and were free from diabetes, cardiovascular disease, stroke, or cancer at the time of blood collection. Incident cases of type 2 diabetes were defined as self-reported diabetes confirmed by a validated supplementary questionnaire and diagnosed at least 1 year after blood collection. We used National Diabetes Data Group criteria (11) to define diabetes because our subjects were diagnosed before the release of the American Diabetes Association criteria in 1997. The validity of this method has been confirmed (12). We used the American Diabetes Association diagnostic criteria for diagnosis of diabetes cases after 1998 cycle (13). The incident cases were matched to control subjects who did not report physician-diagnosed diabetes on age, month and year of blood draw, and fasting status (14; 15). In total 1,325 European Caucasian women (633 diabetic patients and 692 controls) with plasma IL-6 and CRP measures were included.

Assessment of plasma levels of CRP and IL-6, and covariates. Blood sample collection (between 1989 and 1990) and processing were previously described (14; 16). The assays were performed in 2003 using the stored blood samples (in the vapor phase of liquid nitrogen freezers; the highest temperature is - 130°C). Frozen plasma aliquots from case and control subjects were selected for simultaneous analysis. Study samples were analyzed in randomly ordered case-control pairs to further reduce systematic bias and interassay variation. CRP levels were measured via a high-sensitivity latex-enhanced immunonephelometric assay on a BN II analyzer (Dade Behring, Newark, DE). Plasma concentrations of IL-6 were measured using a quantitative sandwich enzyme immunoassay technique (Quantikine HS Immunoassay kit). The coefficient of variation

(CV) was 3.8% for CRP and 5.9% for IL-6 (7; 16). Body mass index was calculated as weight in kilograms divided by the square of height in meters. Physical activity was expressed as metabolic equivalent task (MET)–hours based on self-reported types and durations of activities over the previous year.

SNPs selection and genotype determination. DNA was extracted from the buffy coat fraction of centrifuged blood using the QIAmp Blood Kit (Qiagen, Chatsworth, CA). Ten linkage disequilbrium (LD) tagging-SNPs (rs4845618, rs12083537, rs4075015, rs6684439, rs4845622, rs8192284, rs4329505, rs4240872, rs2229238, and rs4845617) for *IL6R* were selected as previously described (7). The tagging-SNPs cover ~94% allele variance. The polymorphisms were genotyped using Taqman SNP allelic discrimination by means of an ABI 7900HT (Applied Biosystems, Foster City, CA). Replicate quality control samples (10%) were included and genotyped with >99% concordance. All genotypes fit Hardy-Weinberg Equilibrium (HWE).

Statistical analyses. A chi-square test was used to assess whether the genotypes were in HWE. General linear models were used to compare geometric mean values of quantitative traits across groups. Plasma CRP and IL-6 were logarithmically transformed to improve the normality. We adjusted for covariates including age (continuous), BMI (<23, 23–24.9, 25–29.9, 30–34.9, or ≥ 35 kg/m^2), physical activity (<1.5, 1.5–5.9, 6.0–11.9, 12–20.9, and ≥21.0 metabolic equivalent hours/week), smoking (never, past, and current), alcohol intake (nondrinker and drinker [0.1–4.9, 5–10, or >10 g/day]), family history of diabetes, menopausal status (pre- or postmenopausal [never, past, or current hormone use]), and IL-6 levels (in quartiles). Because of the missing data in biomarkers and genotyping, some matching pairs in the case-control design were broken. Therefore, we used unconditional logistic regression as the primary analysis in estimating odds ratios (ORs) for diabetes risk to avoid the loss of unpaired samples. Conditional logistic regression yielded similar results (data not shown). The interactions between polymorphisms and biomarkers were examined using likelihood ration test (LRT), with a comparison of the log likelihood of the two models with and without the interaction terms.

To account for multiple statistical testing, we calculated false discovery rate (FDR) for the analyses on the polymorphisms by the method of Benjamini and Hochberg (17) using SAS procedure PROC MULTTEST. FDR estimates the proportion of results declared positive that are actually false (18). The SAS statistical package was used for the analyses (SAS, Version 8.2 for UNIX). Haplotype analysis was conducted based on the Stochastic-EM (SEM) algorithm using THESIAS program (19). All *P*-values are two-sided.

RESULTS

Table 1 shows age and age-adjusted baseline characteristics according to rs8192284 genotypes for women with both CRP and IL-6 measurements available. IL-6 levels were higher in women with AC and CC genotypes compared with those with AA genotype. The genotypes were not associated with any other characteristics.

In non-diabetic women, the three SNPs rs6684439, rs4845622 and rs8192284, which were in strong LD, were significantly associated with low plasma CRP levels after adjusting for IL-6 and other covariates (**Table 2**). SNPs rs12083537 and rs4329505 were associated with high plasma CRP levels. We calculated FDR by the method of Benjamini and Hochberg (17) to adjust for the multiple testing. The FDRs for all these associations were less than 0.05. In most cases similar associations between *IL6R* polymorphisms and CRP levels were observed in the diabetic patients.

We inferred the haplotypes from the polymorphisms within the LD block (including SNPs rs6684439, rs4845622, rs8192284, rs4329505, rs4240872, rs2229238 and rs4845617). Because rs6684439, rs4845622 and rs8192284 are in nearly perfect LD (r^2>0.9), only rs8192284 was kept in haplotype inference, together with the other four SNPs in this LD block (rs4845618, rs4329505, rs4240872, and rs2229238). Five common haplotypes accounted for about 96% allele variance of the LD block. Haplotypes 21122 (P=0.039; '1' represents the common allele and '2' represents the minor allele) and 11211 (P=0.0006), were associated with 0.13 (0.01-0.26) and 0.23 (0.10-0.37) pg/mL higher CRP levels compared with the most common haplotype 12111, adjusting for IL-6 and other covariates (**Figure 1**). The global test for haplotype associations was statistically significant (P=0.0002).

Because SNP rs8192284 was a missense variant, related to CRP in the GWAS, and showed the strongest association with IL-6 levels in our previous study (7), we further tested the interactions between this SNP and CRP levels in relation to diabetes risk and observed significant multiplicative interaction (P for interaction=0.026; **Figure 2**). We then examined the stratified associations between CRP levels and diabetes risk by rs8192284 genotypes. To improve power, we grouped the subjects into carriers and non-carriers of the minor allele-C. After adjusting for IL-6 levels and other covariates, the ORs across increasing quartiles of CRP were 2.21 [95% confidence interval (CI), 1.18-4.12], 3.77 (1.87-7.57), and 5.02 (2.4-10.5) in the non-carriers; while in the carriers, the ORs were 2.19 (1.42-3.36), 2.03 (1.27-3.23), and 2.92 (1.77-4.82) respectively.

DISCUSSION

We found significant associations between *IL6R* variants and plasma CRP levels. Our results were consistent with the findings from recent GWAS (9; 20). We demonstrated that the associations between *IL6R* variants and CRP levels were independent of IL-6 concentration. In an exploratory analysis, we found *IL6R* variant rs8192284 significantly interacted with CRP levels in relation to diabetes risk. The associations between CRP levels and increased diabetes risk were more evident in women carrying the wild-type genotype than in those with the minor allele-C.

IL-6 is a pleiotropic cytokine that performs as the chief stimulator of the production of CRP from the liver (21). In epidemiological studies, circulating levels of IL-6 and CRP are significantly correlated (22). In our earlier analyses, we found *IL6R* variants were significantly related to IL-6 levels (7). The same association was also observed in the Health ABC study (8). Our data from the present study suggest that the association between *IL6R* variants and CRP levels is unlikely due to the changes in IL-6 levels, because the *IL6R* gene-CRP association was independent of IL-6 levels.

As both CRP and IL-6 are not the direct products of the *IL6R* gene, the associations between *IL6R* variants and these biomarkers are likely mediated by other metabolic changes. SNP rs8192284 in the *IL6R* gene has been associated with soluble IL6-receptor levels (8). These data suggest that *IL6R* variant may primarily affect IL6-receptor levels, and the changes in CRP and IL-6 are likely secondary.

The precise mechanisms underlying the opposite associations of *IL6R* variant (rs8192284) with IL-6 and CRP are not clear. The differing associations are particularly puzzling considering that IL-6 may stimulate the product of CRP in the liver. However, the data from our study are highly consistent with the GWAS and previous studies (8; 9; 20). We suspect that the genotype-related changes in CRP and IL-6 levels may be parallel changes, rather than sequential events, both induced by the alterations in IL6-receptor products.

Subclinical systemic inflammation is now considered as an important mechanism leading to insulin resistance and type 2 diabetes (23). Epidemiological studies have documented that circulating inflammatory markers including CRP significantly predicted diabetes risk (4-6). Previous studies indicate that polymorphisms affecting CRP levels may also influence the risk of type 2 diabetes (24). Although *IL6R* SNPs were not significantly associated with the incidence of diabetes in our study sample (7), the exploratory analysis indicated that the genetic variant might modify the association between CRP levels and diabetes risk. The associations between CRP and increased risk of type 2 diabetes were more evident in women carrying genotype AA of rs8192284 compared with those carrying the minor allele-C. This observation reflects a synergic effect of *IL6R* genotype and CRP levels on the development of diabetes. The observed interaction needs to be confirmed in future studies.

Several limitations need to be considered. SNP rs4129267 reported by the GWAS (9) was not typed in the present study. However, this SNP is in near perfect LD with rs8192284 (D'=1 and r^2=0.96; HapMap, CEU). Population stratification arising from ethnic admixture may cause spurious associations. However, the present study was less likely to be influenced by population stratification because the study populations were highly homogeneous including only European Caucasians. In addition, our analyses were restricted to women and therefore may not be generalized to men.

In summary, we demonstrated that the *IL6R* variants were significantly associated with plasma CRP levels, independent of IL-6 levels. In addition, *IL6R* variant interacted with CRP in relation to diabetes risk. Further research is warranted to elucidate the potential mechanisms underlying the associations between *IL6R* variants and the opposite changes in CRP and IL-6 levels.

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Table 1. Clinical characteristics of non-diabetic women according to the genotypes of SNP rs8192284

	rs8192284			P
	AA	AC	CC	
n of participants	234	342	101	
Age, year	55 (9)	57 (7)	56 (7)	0.56
Body mass index, kg/m^2	26.2 (6.6)	26.3 (5.6)	27.6 (6.6)	0.15
Physical activity, MET hours/week	12.9 (15.4)	16.3 (19.4)	14.8 (13.2)	0.07
Alcohol consumption, g/day	6.34 (11.0)	5.54 (9.62)	5.31 (7.87)	0.54
Family history of diabetes, %	20.9	21	22.8	0.92
Current smoker, %	14.7	10.8	8	0.37
Postmenopausal status, %	76.1	81.3	78.2	0.31
IL-6, ng/mL	1.84 (1.97)	1.99 (2.07)	2.14 (1.82)	0.003

The continuous variables are presented as means (SD)

Table 2. Plasma CRP levels (pg/mL) by IL6R genotypes in the non-diabetic and diabetic women

	Alleles	Non-diabetic			P*	FDR	Diabetic			P*	FDR
	major/minor	11	12	22			11	12	22		
rs4845617	G/A	0.27(0.02)	0.26(0.02)	0.30(0.03)	0.34	0.38	0.52(0.03)	0.51(0.03)	0.57(0.05)	0.94	0.94
rs12083537	T/C	0.27(0.01)	0.28(0.02)	0.29(0.05)	0.009	0.02	0.54(0.03)	0.49(0.04)	0.51(0.10)	0.07	0.11
rs4075015	T/A	0.28(0.02)	0.27(0.02)	0.31(0.03)	0.2	0.27	0.49(0.03)	0.53(0.03)	0.51(0.05)	0.34	0.38
rs6684439	C/T	0.30(0.02)	0.27(0.02)	0.24(0.03)	0.006	0.015	0.60(0.03)	0.49(0.03)	0.41(0.05)	<0.0001	0.0001
rs4845618	A/C	0.27(0.02)	0.28(0.02)	0.28(0.03)	0.55	0.58	0.49(0.04)	0.52(0.03)	0.59(0.05)	0.01	0.018
rs4845622	T/G	0.31(0.02)	0.27(0.02)	0.24(0.03)	0.0002	0.0008	0.61(0.03)	0.49(0.03)	0.41(0.05)	<0.0001	0.0001
rs8192284	A/C	0.32(0.02)	0.26(0.02)	0.24(0.03)	0.003	0.01	0.63(0.03)	0.48(0.03)	0.43(0.05)	<0.0001	0.0001
rs4329505	T/C	0.27(0.01)	0.32(0.02)	0.27(0.07)	0.004	0.011	0.49(0.02)	0.57(0.04)	0.81(0.16)	<0.0001	0.0001
rs4240872	A/G	0.27(0.01)	0.29(0.02)	0.27(0.06)	0.31	0.38	0.51(0.03)	0.53(0.03)	0.61(0.09)	0.07	0.11
rs2229238	G/A	0.27(0.01)	0.30(0.02)	0.32(0.08)	0.13	0.19	0.50(0.02)	0.56(0.04)	0.54(0.11)	0.01	0.018

For each polymorphism, 11 represents the major allele homozygotes, 12 represents the heterozygotes, and 22 represents the minor allele homozygotes; missing genotyping is not included;

*: comparisons between carriers and non-carriers; adjusted for age, BMI, alcohol consumption, smoking, physical activity, family history of diabetes, menopausal status, and interleukin 6

Figure 1. Haplotype (inferred from SNPs rs4845618, rs8192284, rs4329505, rs4240872, and rs2229238) associations with plasma CRP concentration. Haplotype coding, '1' represents the common allele and '2' represents the minor allele; analyses were adjusted for age, BMI, smoking, alcohol consumption, physical activity, family history of diabetes, menopausal status, and interleukin 6.

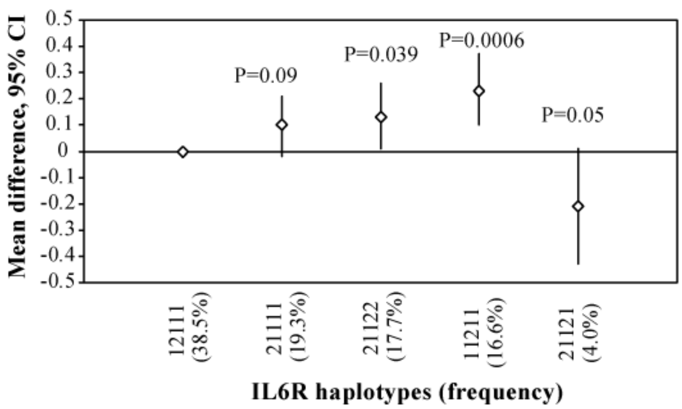

Figure 2. The odds ratios of diabetes risk associated with the plasma CRP levels (in quartiles) by the strata of the genotypes of *IL6R* variant rs8192284 (AA and AC+CC). Error bars represent 95% confidence intervals.