Original Article

Single Nucleotide Polymorphisms in the Interleukin-6 Gene Associated with Blood Pressure and Atherosclerosis in a Japanese General Population

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It is known that increased plasma levels of inflammatory markers, such as interleukin-6 (IL-6), are associated with atherosclerosis and myocardial infarction. The aim of this study was to reveal the contribution of the single nucleotide polymorphisms (SNPs) of the *IL-6* gene on the blood pressure regulation and progression of atherosclerosis in a general Japanese population. In order to evaluate the potential implications of genetic variability of the *IL-6* gene, we explored eight SNPs by direct sequencing for the entire coding region and the promoter region in the *IL-6* gene and genotyped two SNPs, −636G>C in the promoter region and 1691C>G in intron 3, for a total of 2,421 Japanese subjects (1,162 men and 1,259 women). As a consequence, −636 G>C was significantly associated with systolic blood pressure (SBP) and carotid intima-media thickness (IMT) in women, and 1691C>G showed a relationship with SBP and carotid IMT in men after adjustment for all confounding factors. Although neither SNP had a significant correlation to the prevalence of hypertension, the haplotype frequency analysis indicated that the number of hypertensive men with a G allele at both −636 and 1691 was significantly greater than the number of nonhypertensive men with this combination. Thus, these two SNPs in the promoter region and intron 3 of the *IL-6* gene might play a role in the blood pressure regulation and progression of atherosclerosis in the Japanese. (*Hypertens Res* 2005; 28: 35–41)

***Key Words*: interleukin-6, single nucleotide polymorphism, blood pressure, atherosclerosis, Japanese general population**

Introduction

Recent epidemiological studies have shown that chronic inflammation plays a key role in cardiovascular disease (CVD) (*1*). Several epidemiological studies show that increased plasma levels of inflammatory markers such as C-reactive protein (CRP) and interleukin-6 (IL-6) are associated with atherosclerosis, myocardial infarction (*2*), endothelial dysfunction (*3*) and high blood pressure (*4*, *5*).

IL-6 is a pleiotropic cytokine involved in not only immunity and inflammation but also bone metabolism and neural development (*6*). IL-6 also stimulates the proliferation of cultured vascular smooth muscle cells (*7*), indicating that this cytokine may play an important role in the development of arteriosclerosis.

Regarding the pathophysiological contribution of the gene polymorphisms of *IL-6* to CVD, the positive correlation between two single nucleotide polymorphisms (SNPs) in the promoter region of *IL-6* and CVD has been previously

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Table 1. Clinical Features of Study Participants

Variables	Men (n=1,162)	Women (n=1,259)	p
Age (years old)	61.0±12.2	58.9±11.7	<0.0001*
Body mass index (kg/m^2)	23.1±2.8	22.4±3.1	<0.0001*
SBP (mmHg)	129.2±19.1	128.5±21.1	0.443*
DBP (mmHg)	81.0±10.8	78.8±10.6	<0.0001*
TC (mg/dl)	204.5±32.0	215.9±32.9	<0.0001*
HDL cholesterol (mg/dl)	54.5±14.3	64.4±15.4	<0.0001*
Hypertension (%)	39.7	35.5	0.0343†
Diabetes mellitus (%)	9.0	3.4	<0.0001†
Hyperlipidemia (%)	35.2	51.4	<0.0001†
Current alcohol consumer (%)	71.5	29.3	<0.0001†
Current smoker (%)	38.2	8.7	<0.0001†
Antihypertensive medication (%)	17.7	17.2	0.7500†
−636G>C (n)			
GG	87	79	0.109†
GC	359	439	
CC	648	681	
1691C>G (n)			
CC	14	12	0.785†
GC	175	198	
GG	938	1,028	

Values are mean±SD or percentage. Hypertension: SBP ≥140 mmHg, DBP ≥90 mmHg or antihypertensive medication; diabetes mellitus: fasting plasma glucose ≥126 mg/dl, HbA1c ≥ 6.5% or antidiabetic medication; hyperlipidemia: TC ≥220 mg/dl, TG ≥150 mg/dl or antihyperlipidemia medication. *p was calculated by Student's t-test. †p was calculated by χ^2 test. SBP; systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride.

reported. An association of the −174G>C polymorphism has been found with systolic blood pressure (SBP) and diastolic blood pressure (DBP) in middle-aged healthy men (*8*). Several studies have suggested that the −174G allele was associated with a higher plasma level of IL-6 in patients with abdominal aortic aneurysms (*9*), in patients undergoing coronary artery bypass graft surgery (CABG) (*10*), and in healthy women (*11*). Another polymorphism, −572G>C, which was identical to −634G>C, was associated with the progression of diabetic nephropathy (*12*) and increased plasma levels of IL-6 after CABG (*10*). Functional studies using the reporter gene showed that −174G>C, −572G>C, and the A and T repeat variation, AnTn tract, which lies between the −174G>C and −572G>C, intricately cooperate in regulating *IL-6* gene expression (*13*).

Although several association studies on −174G>C in Caucasians have been reported, this polymorphism is recognized only with low allele frequency in South Chinese, Korean, and Japanese populations (*12*, *14–16*). In the present study, we identified the common SNPs of the *IL-6* gene in the Japanese, and investigated the association between SNPs of the *IL-6* gene and blood pressure and atherosclerosis in the Suita Study, which employed a representative general Japanese population.

Methods

Subjects

The selection criteria and design of the Suita Study have been previously described (*17–20*). The protocol of study was approved by the Ethics Committee of the National Cardiovascular Center. Only the participants who gave their written informed consent for genetic analysis were included in this study. DNA from leukocytes was collected from 2,421 participants who visited the Division of Preventive Cardiology at the National Cardiovascular Center between May 1996 and February 1998.

The characteristics of the subjects analyzed in the present study are summarized in Table 1. Blood pressure was measured in the subjects after at least 10 min of rest in a sitting position. The blood pressure value is the mean of two physician-obtained measurements (recorded >3 min apart). Carotid intima-medial thickness (IMT) was measured by ultrasonography using previously described methods (*21*) as an indicator of atherosclerosis. Hypertension was defined as SBP ≥140 mmHg or DBP ≥90 mmHg, or the current use of antihypertensive medication, diabetes mellitus (DM) was defined as fasting blood glucose ≥126 mg/dl, HbA1c ≥6.5%

Table 2. Identified Polymorphisms in the *IL-6* Gene

SNPs	Amino acid	Region	Allele 1	Hetero	Allele 2	Total	Allele frequency		Flanking sequence	dbSNP ID
Allele 1>Allele 2	change		Homo		Homo		Allele 1	Allele 2		
−636G>C*,†		promoter	7	16	25	48	0.313	0.688	tctacaacagcc[g/c]ctcacagggaga	
1338C>T*		intron 2	8	14	23	45	0.333	0.667	ttttcttagaga[c/t]tttcctggctgt	
1368G>T*		intron 2	8	15	23	46	0.337	0.663	aacaatgaaaag[g/t]ccctctagtggt	rs2066992
1394A>G		intron 2	44	2	0	46	0.978	0.022	tttgttttaggg[a/g]cacttaggtgat	
1691C>G†		intron 3	39	7	0	46	0.924	0.076	tgaggaggccaa[c/g]ttcaagcttttt	rs2069840
2099T>G		intron 3	45	1	0	46	0.989	0.011	tatttaaaatgg[t/g]gctgtccaatgt	
4158T>A	Asp162Glu	exon 5	33	3	0	36	0.958	0.042	aaagaatctaga[t/a]gcaataaccacc	
4415G>A	3′ UTR	exon 5	34	1	0	35	0.986	0.014	ggagaactaaaa[g/a]tatgagcgttag	

*These SNPs are in linkage disequilibrium ($r^2 > 0.95$). †These SNPs were used for genotyping analysis. The A of the initiator Met codon is denoted nucleotide +1, as recommended by the Nomeclature Working Group (*Hum Mut* 1998; **11**: 1–3). The genome sequence retrieved from GenBank (accession ID: NT_007819.14, GI: 37538470) was used as a reference sequence. IL-6, interleukin-6; SNP, single nucleotide polymorphism.

or the current use of insulin or oral anti-diabetic agents, and hyperlipidemia was defined as total cholesterol ≥ 220 mg/dl or triglyceride ≥ 150 mg/dl, or the current use of antihyperlipidemia medication at the time of the first examination.

Direct Sequencing for the Detection and Genotyping of Polymorphisms in the *IL-6* Gene

We sequenced the entire coding region of the *IL-6* gene and the 5′-flanking region upstream, approximately 1.5 kb from the transcription start site, using DNA samples from 48 volunteers after obtaining their written informed consent. The method of direct sequencing has been previously described (*22*). The identified polymorphisms were numbered from the A of the initiator codon, as recommended by the Nomenclature Working Group (*23*). The genotype of the SNPs having a 5% or greater minor allele frequency was determined by the TaqMan-polymerase chain reaction (PCR) system (*24*). The sequences of PCR primers and probes for the TaqMan-PCR method were as follows. For −636G>C, the primers were 5′-GTAACTGCACGAAATTTGAGGGT-3′ (sense) and 5′-GTTTCCTCTGACTCCATCGCA-3′ (antisense), and the probes were Fam-ACAGCCCCTCACAGG-MGB (for the C allele) and Vic-ACAGCCGCTCACAG-MGB (for the G allele). For 1691C>G, the primers were 5′-TCTGGCCAT ACCTGTCCAAGA-3′ (sense) and 5′-CAGCAACAAAAAG TGGGTAAATGT-3′ (antisense), and the probes were Fam-AAGCTTGAAGTTGGCCT-MGB (for the C allele) and Vic-AAGCTTGAACTTGGCCT-MGB (for the G allele).

Statistical Analysis

The association of genotypes with blood pressure and IMT was investigated by ANCOVA considering potential confounding factors. For multivariate risk predictors, the adjusted odds ratios were given with 95% confidence intervals. The relationship between genotype and hypertensive risk was expressed as an odds ratio adjusted by possible confounding factors. All analyses were performed with SAS statistical software (release 8.2; SAS Institute Inc., Cary, USA). Linkage disequilibrium (LD) was evaluated by obtaining r^2 values between polymorphisms, and haplotype analysis was performed using the program SNPAlyze, ver. 3.1Pro (DYNACOM Co., Ltd., Mobara, Japan).

Results

Polymorphisms in the *IL-6* Gene

Direct sequencing using DNA samples from 48 volunteer subjects identified eight SNPs in the *IL-6* gene, including one SNP in the promoter region, one missense mutation in exon 5, one SNP in the 3′-untranslated region, and five SNPs in introns (Table 2). One SNP, −636G>C, was identical to the previously described −572G>C (−634G>C) (*13*, *16*). Three SNPs, −636G>C, 1338C>T and 1368G>T, were tightly in LD ($r^2 > 0.95$). An AnTn polymorphism in the promoter region was also detected, but it was difficult to detect the precise number of A and T residues by direct sequencing. We could not detect −174G>C in our Japanese population, as reported previously (*12*, *16*). A missense mutation, 4158T>A (Asp162Glu), may be functionally important. However, since the allele frequency of this mutation was low, this SNP was not genotyped in the present study. Finally, −636G>C in the promoter region and 1691C>G in intron 3, both of which showed a minor allele frequency of 0.05 or more, were selected for genotyping.

Study Population

The clinical characteristics of the study subjects, 1,162 men and 1,259 women, are shown in Table 1. There were significant differences between men and women in some variables, including age, body mass index, DBP, serum total choles-

Table 3. Multivariate-Adjusted Blood Pressure Levels on Genotype of SNPs in *IL-6* Gene

SNP	Men			Women		
	Genotype group		*p*	Genotype group		*p*
-636G>C	GG	GC+CC		GG	GC+CC	
DBP (mmHg)	80.5±1.1	81.0±0.3	0.656	80.4±1.1	78.8±0.3	0.160
SBP (mmHg)	127.9±1.8	129.3±0.5	0.461	133.3±2.0	128.4±0.5	0.017
	GG+GC	CC		GG+GC	CC	
DBP (mmHg)	81.1±0.5	80.9±0.4	0.656	78.6±0.4	79.1±0.4	0.383
SBP (mmHg)	129.7±0.8	128.8±0.7	0.363	128.7±0.8	128.8±0.7	0.935
1691C>G	CC	CG+GG		CC	CG+GG	
DBP (mmHg)	81.0±0.3	81.7±0.7	0.360	78.9±0.3	78.6±0.7	0.727
SBP (mmHg)	128.9±0.5	131.2±1.2	0.072	128.6±0.6	127.8±1.2	0.571
	CC+CG	GG		CC+CG	GG	
DBP (mmHg)	81.1±0.3	81.1±2.7	0.982	78.8±0.3	80.3±2.8	0.597
SBP (mmHg)	129.3±0.5	126.7±4.4	0.559	128.5±0.5	123.8±5.1	0.356

Values are mean±SD. All adjusted for age, body mass index, hyperlipidemia, diabetes mellitus, smoking, drinking, and antihypertensive medication. Il-6, interleukin-6; SNP, single nucleotide polymorphism; DBP, diastolic blood pressure; SBP, systolic blood pressure.

terol, and high desity lipoprotein cholesterol levels, prevalence of hypertension, DM, and hyperlipidemia, and percentage of current alcohol drinking and smoking.

Association of the Polymorphisms with Blood Pressure

DBP and SBP levels were evaluated in men and women by genotypes after adjustment for the confounding risk variables. After adjustment for age, the −636G>C polymorphism was significantly associated with both SBP and DBP in women, and the 1691C>G polymorphism was significantly associated with SBP in men (data not shown). After adjustment for all confounding factors, only −636G>C was significantly associated with SBP in women ($p=0.017$) (Table 3). Mean SBP levels of women with the GG genotype were approximately 5 mmHg higher than those of women with the C allele. There were no significant positive associations between −636G>C and blood pressure in men. In order to exclude the influence of antihypertensive medication, we performed the same analysis in subjects who did not receive medication. There was no difference in the distribution of the genotypes of the two poly-

Table 4. Multivariate-Adjusted Blood Pressure Levels in Subjects without Antihypertensive Medication

SNP	Genotype group		*p*	Adj
−636G>C Women	GG (*n*=59)	GC+CC (*n*=932)		
DBP (mmHg)	80.2±1.3	77.4±0.3	0.037	age
	79.7±1.3	77.4±0.3	0.073	all
SBP (mmHg)	130.5±2.3	124.2±0.6	0.008	age
	130.2±2.3	124.3±0.6	0.012	all
1691C>G Men	CC (*n*=780)	CG+GG (*n*=145)		
DBP (mmHg)	79.9±0.4	80.4±0.9	0.620	age
	79.9±0.4	80.6±0.8	0.419	all
SBP (mmHg)	125.5±0.6	128.4±1.4	0.045	age
	125.4±0.6	128.6±1.3	0.027	all

Values are mean±SD. All adjusted for age, body mass index, hypertension, diabetes mellitus, smoking, and drinking. SNP, single nucleotide polymorphism; Adj, adjustment; DBP, diastolic blood pressure; SBP, systolic blood pressure.

Table 5. Odds Ratio of −636G>C and 1691C>G for Hypertension

SNP	Genotype group	OR (95% CI)	*p*
−636G>C			
Men	GG	1	
	GC+CC	1.10 (0.63–1.93)	0.745
	GG+GC	1	
	CC	1.30 (0.95–1.77)	0.103
Women	GG	1	
	GC+CC	1.64 (0.90–2.98)	0.105
	GG+GC	1	
	CC	0.92 (0.67–1.26)	0.605
1691C>G			
Men	CC	1	
	CG+GG	0.69 (0.46–1.02)	0.065
	CC+CG	1	
	GG	2.04 (0.43–10.00)	0.371
Women	CC	1	
	CG+GG	1.09 (0.72–1.64)	0.69
	CC+CG	—	
	GG	—	—

*Conditional logistic analysis, adjusted for age, body mass index, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence intervals.

Table 6. Haplotype Frequency of *IL-6* Gene in Hypertensives (HT) and Normotensives (NT)

Haplotype	−636/1691	Men				Women			
		HT (866 alleles)	NT (1,300 alleles)	χ^2	*p*	HT (854 alleles)	NT (1,532 alleles)	χ^2	*p*
1	C/C	73.1	77.2	4.63	0.031	74.1	75.5	0.52	0.47
2	G/C	17.8	16.1	1.08	0.298	17.9	16.4	0.84	0.36
3	G/G	9.1	6.8	4.04	0.044	7.8	8.0	0.03	0.874
4	C/G	0.0	0.0	—	—	0.1	0.1	0.18	0.675

Haplotype frequencies are expressed as percentage.

Table 7. Multivariate-Adjusted Carotid IMT on Genotype of SNPs in *IL-6* Gene

SNP	Men				Women			
	Genotype group			*p*	Genotype group			*p*
−636G>C	GG	GC	CC		GG	GC	CC	
Mean IMT (mm)	0.91±0.01	0.91±0.01	0.90±0.00	0.288	0.89±0.01	0.86±0.00	0.85±0.00	0.005
Max-IMT (mm)	1.70±0.08	1.78±0.04	1.70±0.03	0.357	1.47±0.06	1.36±0.02	1.33±0.02	0.025
1691C>G	CC	CG	GG		CC	CG	GG	
Mean IMT (mm)	0.91±0.00	0.92±0.01	0.95±0.03	0.021	0.86±0.00	0.86±0.01	0.86±0.03	0.538
Max-IMT (mm)	1.71±0.03	1.86±0.06	1.60±0.22	0.108	1.34±0.02	1.38±0.04	1.26±0.15	0.515

Values are mean±SD. Adjusted for age, body mass index, hypertension, hyperlipidemia, diabetes mellitus, smoking, and drinking. IMT, intima-medial thickness; SNP, sigle nucleotide polymorphism; Max-IMT; maximum-IMT.

morphisms among the subjects administered antihypertensive drugs. After adjustment for all confounding factors, -636G>C and 1691C>G were significantly associated with SBP in women and men, respectively (Table 4).

The association between these two genotypes and hypertension was not significant in either men or women (Table 5). Table 6 indicates the results of haplotype frequency analysis for the *IL-6* gene polymorphisms between hypertensives and normotensives. We identified that haplotypes 1 and 3 had significantly lower ($p=0.031$) and higher ($p=0.044$) frequency in hypertensive men than in normotensive men, respectively (Table 6). In other words, the prevalence of men with a G allele at both -636 and 1691 in the *IL-6* gene was significantly higher in the hypertensives. In contrast, there was no difference in the haplotype frequency between hypertensive and normotensive women.

Association of the Polymorphisms with Carotid IMT

We also evaluated the relationship between the *IL-6* gene polymorphisms and mean IMT and maximum-IMT. In men, 1691G was associated with greater mean IMT. In women, on the other hand, mean IMT and maximum-IMT significantly decreased as the copy number of the C allele at −636 decreased (Table 7).

Discussion

In the present study, we searched for polymorphisms in the coding and promoter regions of the *IL-6* gene by direct sequencing and identified eight SNPs. Among them, two polymorphisms, −636G>C in the promoter and 1691C>G in intron 3, had positive associations with blood pressure and carotid atherosclerosis in a large-scale Japanese general population. Specifically, −636G>C in women and 1691C>G in men were significantly associated with SBP, mean IMT and maximum-IMT.

Although women with GG of −636G>C showed significantly higher SBP than the C carriers, there was no significant association with the prevalence of hypertension. Nakajima *et al*. previously reported that only −636G>C of *IL-6* showed a trend of association with hypertension in about 300 Japanese women (*16*). Our present study supports their results: no relationship was observed between the haplotypes of *IL-6* and the morbidity of hypertension in the haplotype frequency analysis in women.

This is the first report to show a positive relationship between 1691C>G in intron 3 of *IL-6* and clinical features. Among non-medicated subjects, men with the G allele of 1691C>G showed a significantly higher SBP than did subjects with the CC genotype. Although this SNP did not seem to involve the prevalence of hypertension by itself, the frequency of the haplotype that contains the G allele at both −636 and 1691 was significantly higher in hypertensive than in normotensive men. This suggested that these SNPs should have a cooperative influence on the prevalence of hypertension in men, although each SNP has only a weak effect on blood pressure.

Inflammation is closely related to atherosclerosis (*1*). Recent studies have suggested that the −174G>C polymorphism in the *IL-6* promoter was associated with myocardial infarction in subjects from Northern Ireland and France (*25*), with coronary heart disease in British subjects under pravastatin treatment (*26*), and with ischemic stroke in Italians (*27*). However, this SNP, −174G>C, could not be identified in Japanese. Instead, we found that −636G>C and 1691C>G in the *IL-6* gene were associated with carotid IMT in women and men, respectively. Since a significant association was observed by multivariate analysis with adjustment for confounding risk factors, including hypertension, these two SNPs appear to be independent risk factors of atherosclerosis. Regarding the significant association between *IL-6* gene polymorphisms and blood pressure in men and women, it is possible that atherosclerosis may intervene in the positive relationships between −636G>C and SBP in women and between 1691C>G and SBP in men.

It remains unclear why the influence of SNPs on clinical features differed by gender. It is also unclear whether these SNPs are "functional" or just risk markers. Several studies have reported that the combination of polymorphisms in the promoter region of *IL-6* affects its gene expression (*13*, *28*, *29*). It has been shown that East Asians and African-Americans have different genotype patterns in *IL-6* compared with Caucasians (*12*, *14–16*, *30*). The −174G>C polymorphism in the promoter region of *IL-6* is considered to regulate *IL-6* production (*9*, *26*, *31*) and is associated with juvenile chronic arthritis (*28*) and myocardial infarction in Caucasians (*25*). In the present study, −174G>C was not identified, although this SNP is commonly detected with about a 40% prevalence in Caucasians (*13*, *25*, *28*, *31*, *32*). In contrast, −636G>C, which we clarified to have a significant association with blood pressure and carotid IMT in Japanese women, has been recognized with only about a 5% prevalence in Caucasians (*13*, *25*, *32*). In the African-American population, −636G>C is common (9.5%) and −174G>C is rare (4%), findings which are very different from those for Caucasians (*30*); however, circulating *IL-6* levels in African-Americans are similar to those in Caucasians (*31*). Thus, −174G>C is important in the regulation of *IL-6* production in Caucasians, but other gene polymorphisms, such as −636G>C, may play important roles in *IL-6* production in East Asian and African-American populations. It is necessary to investigate the correlation between the plasma levels of *IL-6* and two SNPs, −636G>C and 1691C>G, in the *IL-6* gene. We also need to clarify whether these SNPs contribute to the morbidity of atherosclerotic CVD by prospective studies in Japanese.

In conclusion, two SNPs, one in the promoter region and the other in intron 3 of the *IL-6* gene, may be involved in blood pressure regulation through their contribution to atherosclerosis in Japanese.

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