RESEARCH COMMUNICATION

Associations between Serum C-reactive Protein (CRP) Levels and Polymorphisms of CRP, Interleukin 1B, and Tumor Necrosis Factor Genes among Japanese Health Checkup Examinees

Masato Kikuchi[1,2]*, Asahi Hishida[3], Kohei Ishikawa[1], Hiroe Sagawa[1], Koji Suzuki[4], Yoshinori Ito[2], Hiroko Terasaki[1], Nobuyuki Hamajima[2]

Abstract

C-reactive protein (CRP) is a sensitive marker of acute inflammation, which is associated with risk of cardiovascular and other chronic diseases. Some CRP polymorphisms are reported to affect the basal and stimulated CRP levels. Thus we conducted a population-based cross-sectional study to examine the associations of CRP levels with CRP C1444T polymorphism and two cytokine polymorphisms (IL-1B C-31T and TNF-A T-1031C), according to sex, age, smoking, alcohol, and BMI, in a total of 489 Japanese health checkup examinees (156 males and 333 females). Serum CRP levels were measured by high sensitivity latex-enhanced nephelometry. CRP C1444T, IL-1B C-31T and TNF-A T-1031C genotypes were genotyped by PCR-CTPP (polymerase chain reaction with confronting two-pair primers). Males, aged, smokers, and those with high BMI had a higher CRP on average. All genotype frequencies among the 489 subjects were in Hardy-Weinberg equilibrium. No significant associations of serum CRP levels with the genotypes of CRP C1444T and IL-1B C-31T were observed. TNF-A -1031CC polymorphism was significantly associated with high CRP values. For the females, those aged 61-69 years, never smokers, non-drinkers, or those with body mass index 24 or less, the association was remarkable. Since the biological mechanism is not clear, further investigations are required to confirm the association.

Key Words: C-reactive protein - *IL-1B* - *TNF-A* - polymorphism

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Introduction

C-reactive protein (CRP), which is mainly produced in liver, is a calcium-dependent ligand-binding protein with 206 amino acid residues, belonging to pentaxin family of plasma proteins. It is a sensitive systemic marker of inflammation and tissue damage, such as infections, inflammatory diseases, tissue necrosis, trauma, and neoplasia (Hirschfield and Pepys, 2003). After a single stimulus, the serum concentration begins to rise by about 6 hours, and reaches the peak about 48 hours (Kushner et al., 1978). CRP is induced in cell lines by interleukin (IL) 6 predominantly, but IL-1 or tumor necrosis factor α (TNFα) may also induce it (Mackiewicz et al., 1991). CRP gene is expressed with transcription factors C/EBP β/δ, STAT3, and Rel p50 (Agrawal et al., 2003).

Although the serum CRP concentration is elevated responding to inflammation, the concentration in the absence of such pathogenic factors was reportedly influenced by age (Mendall et al., 1996; Danesh et al., 1999; Berger et al., 2002), sex (Berger et al., 2002; Slade et al., 2000), smoking (Mendall et al., 1996; Danesh et al., 1999; Berger et al., 2002; Slade et al., 2000; Harris et al., 1999; Koenig et al., 1999), and body mass index (BMI) (Mendall et al., 1996; Danesh et al., 1999; Harris et al., 1999; Visser et al., 1999; Suzuki et al., 2005). Females, aged, smokers, and those with high BMI had a higher CRP on average. It was observed in many prospective studies that the level of CRP was associated with risk of cardiovascular diseases (Koenig et al., 1999; Kuller et al., 1996; Ridker et al., 1997; Strandberg et al., 2000). The associations were also reported with the risk of the mortality among the aged (Harris et al., 1999), colorectal cancer (Erlinger et al., 2004), and age-related macular degeneration (Seddon et al., 2004).

Genetic traits also seem to be factors that determine the level of CRP. A twin study estimated that the hereditability was 52% (95% confidence interval, 40-62%) (MacGregor et al., 2004). Recent studies examined the associations between serum level and polymorphisms

*[1]Department of Ophthalmology, [2]Department of Preventive Medicine / Biostatistics and Medical Decision Making, Nagoya University Graduate School of Medicine, Nagoya, [3]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, [4]Department of Public Health, Fujita Health University, Toyoake, Japan. *Corresponding author:TEL: +81-(0)52-744-2275 FAX: +81-(0)52-744-2278 E-mail address: kikuchi@med.nagoya-u.ac.jp*

Table 1. List of Primers and Conditions for Polymerase Chain Reaction with Confronting Two-pair Primers

Polymorphism (GenBank accession number), common band length, polymerase, and annealing temperature Allele-specific band length and primers		
CRP C1444T (AF449713), common band: 280bp, AmpliTaq Gold, and 66˚C		
C allele: 187bp	F1:5' TCT CTG TCT CTG GTA CCT CCC	R1:5' AAA TTC TGA TTC TTT TGG ACC G
T allele: 137bp	F2:5' GCT CGT TAA CTA TGC TGG GAA AT	R2:5' CCT TTC TGG GTG TTA GTT TTC TC
IL-1B C-31T (X04500), common band: 588bp, AmpliTaq Gold, and 66˚C		
C allele: 370bp	F1:5' GAC ATC AAC TGC ACA ACG ATT GT	R1:5' CAG TTT CTC CCT CGC TGT TTT TAT A
T allele: 266bp	F2:5' CTC CTA CTT CTG CTT TTG AAA GCC	R2:5' TGA CAC TAA CCT TTA GGG TGT CAG C
TNF-A T-1031C (AB048818), common band: 444bp, AmpliTaq Gold, and 66˚C		
T allele: 316bp	F1:5' AAG GCT CTG AAA GCC AGC TG	R1:5' CCA GAC CCT GAC TTT TCC TTC A
C allele: 174bp	F2:5' GAA GCA AAG GAG AAG CTG AGA AGA C	R2:5' CTT CCA TAG CCC TGG ACA TTC T

of CRP gene located in chromosome 1. Brull et al reported that those with TT genotypes of CRP C1444T in the 3'UTR had a higher CRP than those with CT or CC (Brull et al., 2003). Russell et al also found that two CRP polymorphisms (CRP2 and CRP4) were associated with serum CRP concentration (Russell et al., 2004). In addition, functional polymorphisms of cytokines could influence the CRP level possibly through the different production of cytokines; IL-1B 3954 2/2 was reportedly a high CRP genotype (Eklund et al., 2003).

This study was conducted to examine the associations with one CRP polymorphism (CRP C1444T) and two cytokine polymorphisms (IL-1B C-31T and TNF-A T-1031C), according to sex, age, smoking, alcohol, and BMI, in a series of Japanese health checkup examinees.

Materials and Methods

Study subjects

In 1982, we launched a health checkup examination with a research purpose for inhabitants aged 39 or over in a rural town of Hokkaido. The examination has been conducted annually during a period of 3 to 5 days, under the support of the local government (Ito et al., 2002). Subjects were those who attended the annual examination on August 2nd to 4th, 2003.

The study subjects comprised 864 subjects (309 males and 555 females). Of these, we selected 489 subjects (156 males and 333 females), aged 39-79 years, with a CRP value less than 1.0 mg/dl, who had no history of stroke, hypertension, coronary heart disease, diabetes mellitus, cancer or liver and kidney disorders. We obtained written informed consent from each participant for providing information and serum for our epidemiological study.

Daily lifestyle habits and health condition were evaluated using a questionnaire by trained public health nurses. Hypertension was defined as either self-reported high blood pressure with treatment or systolic blood pressure over 140 mmHg, or diastolic blood pressure over 90 mmHg. Smoking status was grouped into three categories defined as "current smoker", "former smoker" and "never smoker". Diabetes was defined as fasting glucose>125 mg/dl or self-reported use of insulin or oral diabetes medication. Body height, weight and blood pressure were measured during the health examinations. Body mass index (BMI) was calculated as the weight (kg) divided by height (m) squared.

Biochemical analysis

Serum samples were taken for the health checkup, and sera were separated from blood cells by centrifugation within 1 hour. Within the day when sera were separated, blood samples were conveyed by the nearby hospital and biochemical analysis was performed using an auto-analyzer (JCS-BM1650, Nihon Denshi Co. Ltd.).

CRP levels were measured by using a latex-enhanced nephelometry (LT Auto Wako CRP, Wako Pure Chemical Industries, Ltd.). The lower detection limit of this assay is 0.003mg/dl. In order to avoid the influence of acute inflammation, we adopted CRP levels below 1.0 mg/dl.

Genotyping

CRP C1444T , IL-1B C-31T, and TNF-A T-1031C were genotyped by PCR-CTPP (polymerase chain reaction with confronting two-pair primers) (Hamajima et al., 2000). The primers are listed in Table 1. The PCR conditions for CRP C1444T were as follows; initial denature at 95˚C for 10 minutes, followed by 30 cycles of denature at 95˚C for 1 minute, annealing at 66˚C for 1 minute, and extension at 72˚C for 1 minute, and a final extension at 72˚C for 5 minutes. A representative gel for CRP C1444T polymorphism is depicted in Figure 1. IL-1B C-31T, and TNF-A T-1031C were genotyped by duplex PCR-CTPP, as described previously (Atsuta et al., 2005).

Statistical Analysis

STATA Version 8 (STATA, College Station, TX, USA) was used for statistical ananlysis. Because of the non-normal distribution of CRP levels, difference in CRP levels was tested using a Kruskal-Wallis test among the

Table 2. Characteristics of the Study Subjects

		Males (156)	Females (333)
Smoking	Never	33 (21.2)#	269 (80.8)
	Former	68 (43.6)	29 (8.7)
	Current	55 (35.3)	34 (10.2)
	Unknown	0 (0.0)	1 (0.3)
Drinking	Not habitual	70 (44.9)	275 (82.6)
	Habitual	86 (55.1)	58 (17.4)
Age* (years)		59.4 ± 10.7	58.6 ± 10.2
Body mass index* (kg/m^2)		23.7 ± 2.8	23.4 ± 3.0
Total cholesterol* (mg/dl)		209.3 ± 30.8	219.0 ± 34.9
Triglyceride* (mg/dl)		109.1 ± 66.0	87.6 ± 45.9
HDL-cholesterol* (mg/dl)		54.1 ± 12.1	62.3 ± 13.1

*Mean ± SD # Percentages

Table 3. CRP Levels According to the Polymorphisms

Genotype	Observed	Arithmetric Mean	Geometric Mean	Min.	Max.	*p-value
CRP C1444T						
CC	392	0.082	0.041	0.006	0.965	0.374
CT	66	0.062	0.043	0.008	0.313	
TT	1	0.044	0.044	0.044	0.044	
IL-1B C-31T						
CC	106	0.077	0.040	0.006	0.965	0.957
CT	229	0.072	0.040	0.006	0.848	
TT	124	0.091	0.043	0.007	0.951	
TNF-A T-1031C						
TT	315	0.076	0.041	0.006	0.965	0.004
TC	136	0.076	0.038	0.007	0.766	
CC	8	0.242	0.159	0.013	0.791	

*with the Kruskal-Wallis test.

genotypes. Command "genhwi" of STATA was used for examining Hardy-Weinberg equilibrium. A $p<0.05$ was considered significant.

Results

The characteristics of study subjects (156 males and 333 females) are shown in Table 2. Males, when compared with females, had a slightly higher mean age, and higher rates of smokers and habitual drinkers. The difference was not seen for the levels of BMI between genders.

Table 3 presents the CRP levels according to the three polymorphisms. Genotyping was successful of all 489 persons. The genotype frequencies for the present study subjects were in Hardy-Weinberg equilibrium; x^2=1.068; p=0.301 for CRP C1444T, x^2=0.000; p=0.989 for IL-1B C-31T, and x^2=2.398; p=0.122 for TNF-A T-1031C. Although the CRP 1444TT genotype had a higher arithmetric mean of CRP than CC and CT genotypes, it was not significant in a Kruskal-Wallis test. While no difference was observed for IL-1B C-31T, a significant difference was observed for TNF-A T-1031C.

Table 4 shows the geometric means of serum CRP for CRP C1444T (Table 4-A), IL-1B C-31T (Table 4-B) or TNF-A T-1031C (Table 4-C) according to demographic and lifestyle factors. CRP levels were higher in males than in females, and increased with age. Although the smokers showed higher CRP levels compared with the non-smokers, there was no difference between drinkers and non-drinkers. As for BMI, the group with higher BMI showed higher CRP values. No differences of CRP levels among CRP C1444T or IL-1B C-31T genotypes were observed for any subgroups. For the TNF-A T-1031C genotypes, statistically significant differences of CRP values among the genotypes were observed in females, those with 61-69 years of age, never smokers, and non-habitual drinkers, all of which were in the same trend as the analysis of the whole subjects. For BMI, statistically significant differences were observed both in those with BMI no more than 24 kg/m² and more than 24 kg/m².

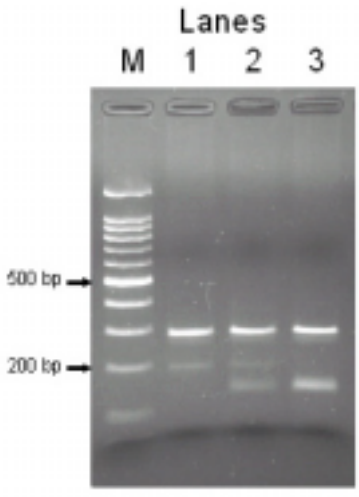

Figure. 1. Polymorphism of the CRP C1444T. Lane M is for a 100-bp DNA ladder. Lane 1 for CC genotype (187- and 280-bp bands), lane 2 for CT genotype (137-, 187- and 280-bp bands), and lane 3 for TT genotype (137- and 280-bp bands).

Discussion

CRP has been recently established both as a marker of cardiovascular risk and as a powerful predictor of outcome after myocardial infarction. CRP reportedly co-operate with low-density lipoprotein, which stimulates the formation of foam cells, leading to the fatty streaks in the endothelium and atherosclerotic plaques (Pepys et al., 2003; Suk et al., 2005). CRP has been reported to play important roles not only in the genesis of cardiovascular diseases but also in the occurrence of other diseases as colorectal cancer or age-related macular degeneration (Erlinger et al., 2004; Seddon et al., 2004).

In the present study, we have examined the associations with a CRP polymorphism and two cytokine polymorphisms according to demographic and lifestyle factors in Japanese health checkup examinees. According to Brull et al., a survey comprised of male army recruit and coronary artery bypass graft (CABG) patients revealed that subjects with CRP 1444TT genotype had higher CRP values both at the baseline and after exercise. Thus we chose this SNP as a candidate for analysis of CRP polymorphisms.

This study could not detect the previously reported association of the subjects with CRP 1444TT genotypes and higher CRP values (Brull et al., 2003). We speculated that this observation would be explained by the small number of the subjects with CRP 1444TT genotype in our study population (1/489 (0.2%)) compared with that

Table 4. Geometric Means of Serum CRP for CRP Genotypes according to Demographic and Lifestyle Factors

A) C1444T		Overall	CC	CT	TT	p-value*
Sex	Males	0.051 (n=156)	0.051 (n=125)	0.060 (n=16)	- (n=0)	0.358
	Females	0.037 (n=333)	0.037 (n=267)	0.038 (n=50)	0.044 (n=1)	0.391
Age	≤ 60 years	0.034 (n=273)	0.033 (n=226)	0.038 (n=34)	0.044 (n=1)	0.282
	61-69 years	0.053 (n=134)	0.054 (n=102)	0.048 (n=21)	- (n=0)	0.780
	≥ 70 years	0.052 (n=82)	0.051 (n=64)	0.049 (n=11)	- (n=0)	0.846
Smoking	Never	0.040 (n=302)	0.038 (n=245)	0.042 (n=40)	0.044 (n=1)	0.354
	Former	0.044 (n=97)	0.046 (n=77)	0.031 (n=13)	- (n=0)	0.442
	Current	0.045 (n=89)	0.043 (n=70)	0.064 (n=12)	- (n=0)	0.145
Drinking	Not habitual	0.042 (n=345)	0.041 (n=282)	0.041 (n=41)	0.044 (n=1)	0.824
	Habitual	0.040 (n=144)	0.038 (n=110)	0.046 (n=25)	- (n=0)	0.096
BMI	≤ 24 kg/m^2	0.032 (n=287)	0.031 (n=227)	0.038 (n=39)	0.044 (n=1)	0.263
	> 24 kg/m^2	0.059 (n=202)	0.059 (n=165)	0.051 (n=27)	- (n=0)	0.949
B) IL-1B C-31T		Overall	TT	TC	CC	p-value*
Sex	Males	0.051 (n=156)	0.056 (n=39)	0.049 (n=70)	0.052 (n=32)	0.857
	Females	0.037 (n=333)	0.039 (n=85)	0.037 (n=85)	0.035 (n=74)	0.904
Age	≤ 60 years	0.034 (n=273)	0.040 (n=77)	0.031 (n=130)	0.035 (n=53)	0.750
	61-69 years	0.053 (n=134)	0.060 (n=22)	0.055 (n=22)	0.047 (n=22)	0.671
	≥ 70 years	0.052 (n=82)	0.044 (n=25)	0.060 (n=25)	0.041 (n=25)	0.373
Smoking	Never	0.040 (n=25)	0.042 (n=75)	0.038 (n=143)	0.037 (n=68)	0.996
	Former	0.044 (n=68)	0.044 (n=24)	0.040 (n=48)	0.052 (n=18)	0.898
	Current	0.045 (n=89)	0.047 (n=89)	0.048 (n=89)	0.039 (n=20)	0.205
Drinking	Not habitual	0.042 (n=345)	0.042 (n=84)	0.040 (n=166)	0.043 (n=74)	0.919
	Habitual	0.040 (n=144)	0.047 (n=40)	0.039 (n=63)	0.033 (n=32)	0.256
BMI	≤ 24 kg/m^2	0.032 (n=287)	0.031 (n=66)	0.032 (n=137)	0.032 (n=65)	0.821
	> 24 kg/m^2	0.059 (n=202)	0.064 (n=58)	0.056 (n=92)	0.055 (n=41)	0.894
C) TNF-A T-1031C		Overall	TT	TC	CC	p-value*
Sex	Males	0.051 (n=156)	0.050 (n=109)	0.055 (n=31)	0.196 (n=1)	0.387
	Females	0.037 (n=333)	0.037 (n=206)	0.033 (n=105)	0.155 (n=7)	0.005
Age	≤ 60 years	0.034 (n=273)	0.036 (n=168)	0.030 (n=90)	0.046 (n=2)	0.171
	61-69 years	0.053 (n=134)	0.045 (n=86)	0.065 (n=31)	0.240 (n=6)	0.001
	≥ 70 years	0.052 (n=82)	0.051 (n=61)	0.044 (n=15)	- (n=0)	0.974
Smoking	Never	0.040 (n=302)	0.038 (n=191)	0.037 (n=89)	0.143 (n=6)	0.034
	Former	0.044 (n=97)	0.047 (n=59)	0.035 (n=30)	0.251 (n=1)	0.213
	Current	0.045 (n=89)	0.044 (n=64)	0.048 (n=17)	0.196 (n=1)	0.361
Drinking	Not habitual	0.042 (n=345)	0.041 (n=211)	0.038 (n=105)	0.159 (n=8)	0.004
	Habitual	0.040 (n=144)	0.041 (n=104)	0.035 (n=31)	- (n=0)	0.768
BMI	≤ 24 kg/m^2	0.032 (n=287)	0.031 (n=186)	0.030 (n=76)	0.121 (n=76)	0.026
	> 24 kg/m^2	0.059 (n=202)	0.060 (n=129)	0.051 (n=60)	0.362 (n=2)	0.048

*Kruskal-Wallis test

of Brull et al. (13/219 (5.9%)). It is also suggested that the frequency of CRP 1444TT genotype in our ethnicity, Japanese, would be fewer than in other ethnic groups. According to Yamada et al., basal CRP values in Japanese are known to be lower than in Caucasians (Yamada et al., 2001). This difference might possibly be explained by the difference in the genotype frequencies of CRP 1444TT polymorphism between the ethnicities, although functional role of CRP 1444TT polymorphism is not clarified in in vitro studies (Brull et al., 2003). Additional studies are required to verify this hypothesis.

As for IL-1B C-31T polymorphism, any significant associations were not observed. To date, the associations between two common IL-1 polymorphisms (IL-1B +3954 and IL-1A +4845) and CRP levels were reported (Berger et al., 2002). This is the first report that investigated the association between IL-1B C-31T polymorphism and CRP value, which suggests that the influence of this polymorphism in the IL-1 gene on the serum CRP levels is limited at least among Japanese.

The analysis about the association between TNF-A T-1031C polymorphism and CRP values revealed statistically significant p-values, suggesting that individual differences in the TNF-α activities might influence serum CRP concentrations. There are 5 polymorphisms in the promoter region of the TNF-A gene reported to date, G-238A, G-308A, C-857T, C-863A and T-1031C, which are supposed to play important roles in the transcriptional regulation of the TNF-A gene (Asgher et al., 2004). These polymorphisms have been reportedly associated with increased transcriptional activity and production of TNF-α in several studies (Wilson et al., 1997; Higuchi et al., 1998; Ahmad et al., 2003). As for the associations between the polymorphisms in the TNF-A and CRP values, TNF G-308A promoter polymorphism, which is shown to have higher circulating levels, is reported to modulate serum concentrations of CRP. TNF-α reportedly stimulates IL-6 production by smooth muscle cells in the human atheromatous plaque, which is the main hepatic stimulus for C-reactive protein synthesis (Lagrand et al., 1999).

Ours was the first report that TNF-A T-1031C polymorphism was significantly associated with CRP values. The present study added the novel information that TNF-A T-1031C polymorphism, as well as TNF G-308A promoter polymorphism, might modulate serum CRP values probably through the regulation of serum TNF-α concentrations.

The analyses of serum CRP concentrations for each genotype according to demographic and lifestyle factors demonstrated that CRP levels were higher in males than in females, increased with age, and also revealed higher CRP levels in smokers or in the group with higher BMI values, all of which were in accordance with the previous reports (Mendall et al., 1996; Danesh et al., 1999; Berger et al., 2002; Slade et al., 2000; Harris et al., 1999; Koenig et al., 1999; Visser et al., 1999). No differences in CRP levels among the genotypes of CRP C1444T or IL-1B C-31T were observed in any subgroups. For the TNF-A T-1031C genotypes, significant differences in CRP were observed for females, those with 61- 69 years of age, never smokers, and non-habitual drinkers; all were the subgroups with a larger sample size. Since subjects with -1031CC was limited in number, the insignificant results for the subgroups with a fewer sample size may simply reflect their small statistical power.

Considering the genotype frequencies of these three polymorphisms examined in this study, the C allele frequency of CRP C1444T polymorphism in our Japanese subjects (92.6%) is significantly higher than that in Caucasians (61.8%) (Brull et al., 2003) ($p<0.001$, Fisher's exact test), the T allele frequency of IL-1B C-31T polymorphism in our subjects (52.0%) is found to be significantly higher than that in Caucasians (29.8%) ($p<0.001$) (El-Omar et al., 2000), and the T allele frequency of TNF-A T-1031C polymorphism in our subjects (83.4%) is similar to that of the previous reports in Japanese (81.4% and 84.0%) (Asgher et al., 2004; Negoro et al., 1999; Hamajima et al., 2003), Spanish (84.0%) (Escobar-Morreale et al., 2001) or Swedish (80.0%) (Skoog et al., 1999).

This study had some limitations. First, the number of the subjects with CRP C1444T TT genotype is too few to detect the influence of this polymorphism on CRP values. Second, although statistically significant association between the TNF-A T-1031C polymorphism and serum CRP concentrations was indicated, serum TNF-α concentrations of the participants were not measured, resulting in no direct evidence that this polymorphism modulates serum CRP levels. TNF G-308A polymorphism has been demonstrated to be associated with higher baseline levels of TNF-α or stimulated transcription activity in various cell lines (Araujo et al., 2004), but no report exists as for TNF-A T-1031C polymorphism. Further elucidation of the biological importance of this polymorphism is required.

In conclusion, our study could not detect the previously reported association between CRP 1444TT genotype and higher CRP values. Our analysis between TNF-A T-1031C and CRP values revealed statistically significant association, suggesting that individual differences in the TNF-α activity might influence serum CRP concentrations. Also, our investigation for Japanese proved that CRP levels were influenced by lifestyle. Further investigations with sufficient population and/or in other ethnic groups are required to confirm the present findings, which could be useful information for the prevention of inflammation related disorders like coronary artery diseases.

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