gested that Mrf-2 might also play important roles in adipogenesis and obesity, which are also critical aspects for the pathogenesis of atherosclerosis.

Taking together, we therefore hypothesized genetic variations in the Mrf-2 gene may predispose humans to CAD. The aim of the present study was to confirm whether genetic variations of the Mrf-2 gene are associated with susceptibility to CAD.

METHODS

Subject characteristics: This study was approved by the institutional ethics committee of the University of Tokyo, and written informed consent was obtained from all participants.

To examine the associations between Mrf-2 SNPs and susceptibility to CAD, we recruited 475 CAD patients (372 men and 103 women, aged 65.3 ± 8.6 years old) and 310 control subjects (173 men and 137 women, aged 68.1 ± 9.8 years old) as the study population. The CAD patients were consecutively enrolled from the Department of Cardiovascular Medicine of the University of Tokyo Hospital, and the control subjects were recruited consecutively from the Institute for Adult Diseases Asahi Life Foundation, which is located close to the University of Tokyo Hospital. All subjects were of Japanese ancestry and were not first- or second-degree relatives. All female participants were postmenopausal.

The patients with CAD were enrolled from October 1999 to March 2002 and were diagnosed when at least one of the 3 major coronary vessels had more than 50% narrowing on the basis of coronary angiography. The control subjects were recruited in 2000 based on having a normal ECG pattern and having no medical history of coronary artery disease or stroke in the monthly follow-up or health check-up program at the outpatient clinic of the Institute for Adult Diseases Asahi Life Foundation Hospital, Tokyo. The control subjects who had two or more coronary risk factors (DM, hypertension (HT), hypercholesterolemia, smoking) or had a history of chest pain were further evaluated using the Master or Treadmill exercise test, and those who had an ECG abnormality in the additional testing were excluded.

At the point of enrollment, the baseline profiles of both the CAD patients and control subjects were collected, and past medical history and smoking habits were obtained from all study participants. HT was diagnosed according to World Health Organization criteria. Hypercholesterolemia was diagnosed when total plasma cholesterol levels were > 220 mg/dL or when a subject was already being treated with lipid-lowering medication. DM in the CAD group and control group was diagnosed based on HbA1c > 6.5% or FBS ≥ 126 mg/dL. Fasting venous blood samples were drawn for biochemical and genetic analysis.

Genomic DNA extraction and genotyping of SNPs used in the association study: Venous blood samples were collected in tubes containing Na2EDTA and applied to genomic DNA extracting columns (Genomix kit, Talent) according to the manufacturer's protocol.

The SNPs were identified based on the sequences reported in the JSNPs (Japanese Single Nucleotide Polymorphisms) database (http://snp.ims.u-tokyo.ac.jp/index_ja.html) or the NCBI GeneBank database (http://www.ncbi.nih.gov/index.html). No exon SNP of Mrf-2 was reported in the above databases for the Japanese population, therefore, 11 intron SNPs which covered the whole length of the Mrf-2 gene were almost equally selected from the public databases to detect the associations between genetic variations of Mrf-2 and CAD.

Two JSNPs (nomenclatures of SNP8, SNP11 represent JSNPs 025551 and 025550, respectively) as well as exon 3, exon 4 and their exon-intron boundaries were genotyped by direct sequencing as described in detail previously.[15] Briefly, PCR was performed under standard conditions and the sequencing reactions were performed using a BigDye terminator kit (Applied Biosystems, Foster City, CA), and resolved using an ABI 3700 automated DNA sequencer (Applied Biosystems). The results were integrated using a Sequencher (Gene Codes Corporation, Ann Arbor, MI) and individual SNPs were manually genotyped. Ambiguous base callings were eliminated from further analysis. The primer sequences are available from the authors.

Meanwhile, nine SNPs of the Mrf-2 gene from the public GeneBank database (nomenclatures of SNP1, SNP2, SNP3, SNP4, SNP5, SNP6, SNP7, SNP9 and SNP10 represent rs6415872, rs10821929, rs7901348, rs2893880, rs10740055, rs7087507, rs10761600, rs12357548 and rs10761604, respectively) were selected and genotyped using a MassARRAY system. When using the MassARRAY system, primer extension products were analyzed by chip based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) following PCR amplification as described in detail previously.[16]

Statistical analysis: Continuous parameters such as age, body mass index (BMI), total cholesterol (T-chol), high density cholesterol (HDL), and triglycerides (TG) are presented as the mean ± SD and were compared using Student's *t* test, while characteristics such as sex, HT, hyperlipidemia (HL), DM, and smoking are given as proportions of the entire samples and were compared using the chi-square (χ^2) test. The genotype and allele association for each SNP and CAD were tested using the χ^2 test with 2 and 1 degree (s) of freedom. The odds ratios and 95% CI, with adjustment for classical risk factors of CAD, were calculated by multiple logistic regression analysis. The differences among more than 2 average values were statistically tested using one-way ANOVA. The statistical analyses, except for the linkage disequilibrium and haplotype estimation, were performed using

SPSS 13.0 or Statview v4.0 software.

The statistical significance level was corrected for multiple testing, and we took potential overestimation into account when evaluating the most statistically significant value among those tested under different models. A two-tailed value of $P < 0.05$ was considered to be significant unless otherwise indicated.

Haplotype analysis: To examine the pair-wise linkage disequilibrium (LD) structure, linkage disequilibrium coefficients (D') and r^2 between the SNPs and haplotype frequencies were estimated via the method of maximum likelihood from two-locus genotype data using the EM algorithm under the assumption of Hardy-Weinberg equilibrium.[17,18] For the estimation of haplotype frequencies, we selected one of the SNPs as a tagging SNP from every set of SNPs with $r^2 > 0.80$. All haplotypes were jointly tested for association with disease status by performing a 2 x n χ^2 test of independence in a permutation procedure, where "n" indicates the number of haplotypes with a frequency > 0.2.

The above calculations were carried out using SNPAlyze v5.1 standard software (Dynacom, Yokohama, Japan). We considered a $P < 0.05$ to be statistically significant.

RESULTS

Baseline characteristics of the CAD and control populations: The baseline characteristics of the CAD patients and the control subjects are shown in Table I.

Table I. Clinical Characteristics of the Study Subjects

	CAD subjects (n = 475)	Control subjects (n = 310)	P
Males (%)	372 (78.3%)	173 (55.8%)	< 0.0001*
Age	65.3 ± 8.6	68.1 ± 9.8	< 0.0001*
BMI	24.1 ± 3.2	23.5 ± 4.1	< 0.0001*
Hypertension	399 (84.0%)	249 (80.3%)	< 0.016*
Hyperlipidemia	320 (67.3%)	128 (41.3%)	< 0.0001*
T-chol (mg/dL)	191.6 ± 31.0	212.9 ± 47.9	
HDL (mg/dL)	50.6 ± 14.9	54.4 ± 14.7	
TG (mg/dL)	183.6 ± 230.4	136.4 ± 94.0	
Diabetes mellitus	180 (37.9%)	42 (13.5%)	< 0.0001*
HbA1c (%)	5.9 ± 1.2	6.0 ± 0.6	
Cr (mg/dL)	0.93 ± 0.68	0.88 ± 0.19	
Smoking	350 (73.7%)	105 (33.9%)	< 0.0001*

Continuous parameters such as age, BMI, T-chol, HDL, and TG are presented as the mean ± SD and were compared using Student's t test. Characteristics such as sex, HT, HL, DM, and smoking are presented as proportions of the entire sample and were compared using the χ^2 test. A $P < 0.05$ (*) was considered to indicate statistical significance.

Baseline characteristics including age of the CAD patients were calculated at the point of enrollment when patients underwent the coronary angiography. For the control subjects, ages and other clinical characteristics were adopted at the enrollment point in 2000. As indicated, the patients in the CAD group had more conventional risk factors such as being male, smoking, HT, hypercholesterolemia, and DM in comparison to the control subjects. The average age of the control subjects was older than that of the patients with CAD.

Associations between Mrf-2 SNPs and CAD: We identified a total of 17 candidate SNPs composing at least 4 haplotype blocks which covered almost the full length

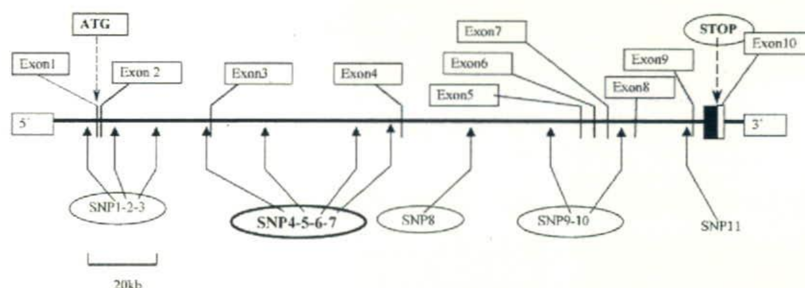

Figure 1. Gene structure and haplotype blocks of Mrf-2. Exons differing from <100 bp to >1000 bp are indicated by lines or closed boxes. Each circle indicates a haplotype block which represents a cluster of SNPs with D' > 0.8. SNP1-11 represents the 11 SNPs in database, as shown below:
SNP1: rs6415872 SNP2: rs10821929 SNP3: rs7901348
SNP4: rs2893880 SNP5: rs10740055 SNP6: rs7087507
SNP7: rs10761600 SNP8: JSNP025551 SNP9: rs12357548
SNP10: rs10761604 SNP11: JSNP025550

Table II. Association Between Allele Frequencies of Mrf-2 SNPs and CAD

		Allele Frequency			*P*
	Allele	Case	Control	Overall	
SNP4	C	640 (67.7%)	389 (62.9%)	1029 (65.8%)	0.055
	G	306 (32.3%)	229 (37.1%)	535 (34.2%)	
SNP5	A	470 (495%)	303 (49.0%)	773 (49.3%)	0.86
	C	480 (50.5%)	315 (51.0%)	795 (50.7%)	
SNP6	A	630 (66.3%)	380 (61.5%)	1010 (64.4%)	0.041*
	G	320 (33.7%)	238 (38.5%)	558 (35.6%)	
SNP7	T	385 (40.5%)	233 (37.8%)	618 (39.5%)	0.29
	A	565 (59.5%)	383 (62.2%)	948 (60.5%)	

A total of 475 CAD subjects and 310 control subjects were observed. Values are given as numbers of alleles (portions of the whole observed subjects x 2). *indicates statistical significance.

of the Mrf-2 gene as shown in Figure 1. All polymorphisms were in accordance with Hardy-Weinberg equilibrium both in the CAD and control groups with each minor allele frequency > 5%.

The allele and genotypic frequencies of each SNP are shown in Table II and Table III. The distributions of G-allele in SNP4 (rs2893880) and G-allele in SNP6 (rs7087507) were more frequent in the control group than in the CAD group, and

Table III. Association Between Genotype Frequencies of Mrf-2 SNPs and CAD

		Genotypic Frequency		*P*	
	Genotype	Case ($n = 475$)	Control ($n = 310$)		
SNP4	CC	209 (44.2%)	136 (43.7%)	[1]CC+CG/GG	0.00020*
	CG	222 (46.9%)	119 (38.5%)	[2]CC/CG/GG	0.00050*
	GG	42 (8.9%)	55 (17.8%)	[3]CC/CG+GG	0.89
SNP5	AA	110 (23.2%)	84 (27.2%)	[1]AA+AC/CC	0.20
	AC	250 (52.6%)	135 (43.7%)	[2]AA/AC/CC	0.050*
	CC	115 (24.2%)	90 (29.1%)	[3]CC+AC/AA	0.13
SNP6	AA	203 (42.7%)	123 (39.8%)	[1]AA+AG/GG	0.0058*
	AG	224 (47.2%)	134 (43.4%)	[2]AA/AG/GG	0.022*
	GG	48 (10.1%)	52 (16.8%)	[3]GG+AG/AA	0.38
SNP7	TT	78 (16.4%)	43 (14.0%)	[1]TT+AT/AA	0.35
	AT	168 (35.4%)	147 (47.7%)	[2]AA/AT/TT	0.55
	AA	229 (48.2%)	118 (38.3%)	[3]AA+AT/TT	0.40

Values are *n* (*n*%). *P* for genotype frequency are calculated as three models and are unadjusted. The superscripts [1], [2] and [3] represent dominant, codominant, and recessive models, respectively. * indicates statistical significance.

Figure 2. Pairwise linkage disequilibrium (LD). The pairwise LD between SNPs in the Mrf-2 gene and D' are indicated by the color of the block. Block structure is indicated at the upper right of the figure as a closed box.

G allele in SNP6 was significantly more prevalent in the control subjects (P = 0.041) (Table II). We also detected statistically significant differences for the genotype distribution of SNP4 (rs2893880) and SNP6 (rs7087507) for both the dominant model (P = 0.0002 and 0.0058, respectively) and the codominant model (P = 0.0005 and 0.022, respectively), and for the codominant model (P = 0.050) of SNP5, in the comparison of the CAD group and the control group (Table III).

Linkage disequilibrium between Mrf-2 SNPs and CAD: Linkage disequilibrium (LD) was estimated between pairs of SNPs by using an absolute value of D' (|D'|). There was almost complete linkage disequilibrium among SNP4 (rs2893880), SNP5 (rs10740055), SNP6 (rs7087507), and SNP7 (rs10761600). The LD of the above four SNPs covered over 60 kb of the Mrf-2 gene and constituted 1 haplotype block (Figure 2).

We then performed haplotype analysis, which is considered to be a more useful method for detecting associations than the assessment of individual SNPs. Seven major combinations (with a frequency > 2% for the entire sample) of the haplotype block consisting of SNP4, SNP5, SNP6, and SNP7 are shown in Table IV. Haplotype case-control analysis revealed a significant association between CAD and the haplotype G-C-G-A (SNP4-5-6-7) with a minor G in SNP4, a major C in SNP5, a minor G in SNP6, and a major A in SNP7 (P = 0.049). This association was consistent with the observations that GG genotype for SNP4 and GG for SNP6 were negatively associated with susceptibility to CAD.

Logistic regression analysis for the associations of Mrf-2 SNPs with CAD: The CAD group showed more conventional confounding risk factors. In order to

Table IV. Haplotype Analysis for Mrf-2 SNPs and CAD

	Frequency of Major Haplotypes			P
SNP4-5-6-7	Overall	CAD	Control	
C-A-A-T	0.3410	0.3432	0.3377	0.82
G-C-G-A	0.3123	0.2937	0.3408	0.049*
C-A-A-A	0.1423	0.1442	0.1397	0.80
C-C-A-A	0.0922	0.1031	0.0754	0.064
C-C-A-T	0.0413	0.0470	0.0322	0.15
C-C-G-A	0.0298	0.0297	0.0300	0.97
G-C-A-A	0.0215	0.0206	0.0230	0.75

Seven major haplotypes were detected for SNP4 to SNP7. The G-C-G-A haplotype is negatively associated with susceptibility to CAD. * indicates statistical significance. This association is relevant to the fact that the GG (SNP4) and GG (SNP6) are negatively associated with susceptibility to and severity of CAD.

eliminate these confounding influences on the presence of CAD, logistic regression analysis was performed for the four SNPs in the risk haplotype block. After adjusting for confounding conventional risk factors such as sex, age, BMI, HT, HL, DM, and smoking, subjects with GG genotype for SNP4 and GG genotype for SNP6 were still at decreased risk for CAD compared with those having C-allele for SNP4 and A-allele for SNP6 (P = 0.0040, OR: 2.20, 95%CI: 1.29-3.77 and P = 0.029, OR: 1.82, 95%CI: 1.07-3.12, respectively) (Table V).

Tendency of associations between Mrf-2 SNPs and severity of CAD: To evaluate whether Mrf-2 SNPs were associated with the severity of CAD, individuals in the CAD group who had previously undergone coronary artery bypass graft surgery

Table V. Logistic Regression Analysis for Mrf-2 SNPs and CAD

		P	Odds ratio	95%CI
SNP4	CC+CG/GG	0.0040*	2.20	1.29-3.77
	CC/CG/GG	0.0010*	1.56	1.20-2.03
SNP6	AA+AG/GG	0.029*	1.82	1.07-3.12

Odds ratios (OR) were calculated using three genetic models. CC+CG/GG and AA+AG/GG are dominant models for SNP4 and SNP6, respectively. OR for each SNP was adjusted simultaneously for sex, age, BMI HT, HL, smoking, and DM. * indicates statistical significance. SNP4 and SNP6 were indicated to be potential independent predictors for susceptibility to CAD after eliminating confounding factors.

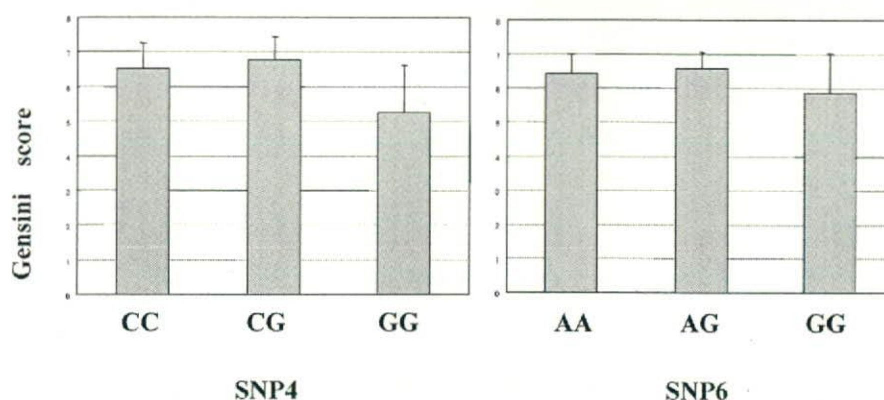

Figure 3. Mrf-2 SNPs and severity of CAD. The carriers of GG genotype in SNP4 and GG genotype in SNP6 had slightly less severe coronary artery diseases, although statistical significance was not observed.

(CABG) or percutaneous coronary intervention (PCI) were excluded, and only the patients who underwent a coronary arteriogram for the first time without revascularization were selected for this analysis.

The associations between genotypes of Mrf-2 and the modified Gensini score,[19,20] which is an index of coronary atherosclerosis severity and is computed by assigning the severity score to each coronary stenosis according to the degree of luminal narrowing and its geographic importance, were investigated. Although the differences among Mrf-2 polymorphisms and severity of CAD did not reach statistical significance, individuals with GG genotype for SNP4 and GG for SNP6 had lower Gensini scores, which was relevant to the correlations that these SNPs were negatively associated with susceptibility to CAD (Figure 3).

No genetic variations were found in exon3 and exon4: Exon 3 is the only exon included in the haplotype block consisting of SNP4-6, and exon 4 is located close to SNP6, therefore, we screened exon3 (226 bp), exon4 (231 bp) and their exon-intron boundaries to investigate whether there was functional SNP in these exon regions by comparing sequences of 100 randomly chosen samples of CAD subjects. No SNP was found in these regions.

DISCUSSION

In the present study, we evaluated whether the polymorphisms of the Mrf-2 gene were associated with susceptibility to CAD. We first demonstrated that genetic variations of Mrf-2 in SNP4 (rs2893880) and SNP6 (rs7087507) themselves and in the haplotype block, consisting of SNP4 (rs2893880), SNP5 (rs10740055), SNP6 (rs7087507), and SNP7 (rs10761600) were significantly associated with susceptibility to CAD. Carrying G for SNP4, C for SNP5, G for SNP6, and A for SNP7 was much more likely to play protective roles against CAD.

It is well accepted that the pathogenesis of CAD is multi-factorial. In addition to the conventional coronary risk factors, other factors such as vasoconstriction,[21] and platelet function[22,23] are also well established. Furthermore, studies have indicated a greater genetic risk in monozygotic twins compared with dizygotic ones, and adoption studies showed evidence that much of the excess risk was genetic rather than environmental for CAD.[24,25] A number of initial studies attempted to address the possibility that polymorphisms at various candidate genes might be associated with the pathogenesis of CAD and some concluded there was a significant association between them. For example, polymorphisms in renin-angiotensin system gene polymorphisms (angiotensin-type receptor genes, angiotensin II converting enzyme genes) are well known to be associated with hypertension,[26] CAD[27-31] and MI,[32-34] though dissenting opinions still exist.

In the case of transcriptional factors, only a few of them were concluded to be associated with the presence of CAD. Within them, controversial data of associations such as the findings between mutations in the myocyte enhancer factor (MEF) gene and the presence of myocardial infarction (MI) have been reported.[35-38] The contribution of genetic polymorphisms to the pathogenesis of CAD still remains to be definitively established. Our findings provided the first evidence for disease relevance of the polymorphisms of a novel transcription factor, Mrf-2.

Some genetic polymorphisms have a direct effect on the occurrence or development of a disease, whereas others have a modifying effect. We previously reported the full length of the Mrf-2 gene consisting of 11 exons and including two nonoverlapped isoforms. The two isoforms differ at the 5' end and share the same sequence from exon5. Exon 1α (specific for Mrf-2α), together with exon5-10 constitute the shorter isoform named Mrf-2α whose open reading frame was 2835 bp, and exon 1-10 constitute the longer one named Mrf-2β whose open reading frame was 3564 bp. SNPs involved in this study were located in the second and third intron regions of the Mrf-2β gene, and no coding SNP was observed in exon3 caught in the 4 disease associated SNPs and exon4, therefore, these findings suggest the intronic polymorphisms in the Mrf-2β gene are much more likely to have a modifying effect by influencing the expression level of Mrf-2 by themselves or by other unknown causative genetic variations located within or nearby the haplotype block. Since the two isoforms conserve the same sequences from exon5 and the Mrf-2 α specific exon located between exon 4 and exon 5, there are 2 possibilities: one could be that these intronic SNPs of Mrf-2β might be related to probable variations in the promoter region of the Mrf-2α specific exon, as yet unidentified; the other is that they might also regulate the upstream promoter of Mrf-2β as these SNPs are located in the second and third intron region, and by which influence the Mrf-2 expression level and regulate its function. In addition, Mrf-2 is a gene of approximately 200 kb, and the haplotype block containing the disease associated SNPs is located in the mid portion of the Mrf-2 gene, which excluded the possibility that other nearby genes were functionally causative concerning the associations between the Mrf-2 variations and susceptibility to CAD in the present study.

The Mrf-2 SNPs exhibited a tendency to be associated with the severity of CAD with GG genotype for SNP4 and GG genotype for SNP6 had slightly lower Gensini scores. However, these findings did not reach statistical significance. One probable reason might be a reduced statistical power due to the small available sample size because only the CAD patients who underwent coronary arteriogram for the first time without revascularization were selected for this analysis. Individuals who had previously undergone CABG or PCI were excluded, which also indicated another possibility that the reduced statistical power was due to the

fact that the more serious patients were excluded from this analysis.

Within the population studied, the definition of CAD was based on coronary angiography, while the control subjects were defined on the basis of having a normal ECG pattern and having no medical history of coronary artery disease or stroke. It remained a possibility that the control group might not be completely free from significant coronary atherosclerosis. However, if it were not for the ethics reason, we would have obtained much clearer results by performing coronary angiography in the control group to exclude the subjects with more than 50% narrowing. Therefore, the results on the associations of Mrf-2 SNPs with CAD in the present study were considered to be reliable.

The positive disease associations were adjusted by performing logistic regression analysis to eliminate conventional confounding risk factors. The significant differences that remained indicated genetic variations of the Mrf-2 may contribute risk to CAD independent from traditional coronary risk factors. Within the studied population, assessments on Mrf-2 variations and each of the traditional coronary risk factors revealed weak associations of Mrf-2 SNPs with DM and BMI. However, statistical significance was not attained after multiple analysis, which might be a result of 1) reduced statistical power due to the small available investigating sample size; and 2) the definition of DM for the CAD and its control groups was based on only one time FBS level or HbA1c value, which was inadequate for obtaining more reliable results. Thus, the possibility that they are associated cannot be excluded, and this should be confirmed in future studies.

At present, although we could not provide definite evidence to elucidate the mechanisms of how the Mrf-2 gene contributes to the pathogenesis of CAD, there are a number of possible explanations. First, the phenotypic change (ie, differentiation and dedifferentiation) of SMCs has recently been considered critical in the pathogenesis of atherosclerotic lesions. Since Mrf-2 was cloned as a key regulator for smooth muscle cell differentiation,[4)] we hypothesized that Mrf-2 might be involved in the pathogenesis of atherosclerosis via its regulation of SMC differentiation. This question would be resolved by constructing a targeting mouse model of Mrf-2 and examining the changes of SMCs. Second, it was reported that mice lacking the Mrf-2 gene exhibited severe growth retardation, reduced weight gain, and reduced lipid accumulation,[8,13,14)] which indicate the possibility that the association between Mrf-2 and CAD might be mediated partially through metabolic syndrome and DM. Third, Mrf-2 is a transcriptional factor which preferentially binds to the sequence AATAC/T and is expressed in the cardiovascular system. Thus, it is conceivable that this gene product might regulate other important vasoactive and pathogenetic molecules, which should be investigated in future studies. Fourth, Mrf-2 was shown to be an early marker for nephrogenic tissues, and broad expression of Mrf-2α was observed in lung, heart, brown adi-

pose tissue, brain and kidney, which suggested widespread biological functions. Gene targeting of Mrf-2 indicated that the homozygous mutant showed reduced viability, displayed female and male reproductive organ abnormalities, and adrenal gland abnormalities,[8,13] suggesting that Mrf-2 might presumably play important roles in the endocrine system through which it has an effect on the pathogenesis of CAD.

Overall, the present results demonstrate for the first time disease associations of the Mrf-2 SNPs with the presence of CAD. One limitation of the present study is the number of subjects studied. To validate the present findings, more individuals need to be recruited. In addition, much more basic research should be conducted to elucidate the mechanisms of the Mrf-2 gene. Furthermore, a prospective study would be a more effective means and should be performed to obtain reliable information on the clinical utility of this possible genetic risk factor of CAD.

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ORIGINAL ARTICLE

Association of *TCF7L2* polymorphisms with susceptibility to type 2 diabetes in 4,087 Japanese subjects

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Abstract Transcription factor 7-like 2 (*TCF7L2*) has been shown to be associated with type 2 diabetes mellitus in multiple ethnic groups. Regarding the Asian population, Horikoshi et al. (Diabetologia 50:747–751, 2007) and Hayashi et al. (Diabetologia 50:980–984, 2007) reported that single nucleotide polymorphisms (SNPs) in *TCF7L2* were associated with type 2 diabetes in the Japanese population, while contradictory results were reported for Han Chinese populations. The aim of this study was to investigate the associations of the *TCF7L2* gene with type 2 diabetes using a relatively large sample size: 2,214 Japanese individuals with type 2 diabetes and 1,873 normal controls. The minor alleles of rs7903146, rs11196205, and rs12255372 showed significant associations with type 2

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Table 1 Clinical characteristics of each sample set. Data are means ± SD. *BMI* Body mass index

	Kobe		Gunma		Consortium	
	Diabetes	Control	Diabetes	Control	Diabetes	Control
n	465	323	576	576	1,173	974
Male participants (%)	59.6	45.8	56.1	40.4	56.6	43.1
Age at study (years)	60.5 ± 10.7	75.6 ± 8.1	60.2 ± 11.5	67.3 ± 6.5	62.5 ± 8.8	69.2 ± 7.0
BMI	24.3 ± 3.9	21.4 ± 3.5	23.9 ± 4.2	23.0 ± 2.9	23.1 ± 2.9	22.6 ± 3.0
HbA_{1c} (%)	8.1 ± 2.0	5.0 ± 0.4	7.8 ± 3.5	5.0 ± 0.4	7.5 ± 1.5	4.9 ± 0.4

diabetes (OR = 1.48, $P = 2.7 \times 10^{-4}$; OR = 1.39, $P = 4.6 \times 10^{-4}$; OR = 1.70, $P = 9.8 \times 10^{-5}$, respectively) in the combined sample sets. However, neither rs11196218 nor rs290487 showed a significant association. These results indicate that *TCF7L2* is an important susceptibility gene for type 2 diabetes in the Japanese population.

Keywords Type 2 diabetes · Polymorphism · β-cell function · Transcription factor 7-like 2 (*TCF7L2*) · Association study

Introduction

The transcription factor 7-like 2 gene (*TCF7L2*) is one of the most convincing susceptibility genes for type 2 diabetes. Following the initial report (Grant et al. 2006), there have been a number of association studies in various ethnic groups (Florez et al. 2006; Zhang et al. 2006; Saxena et al. 2006). Regarding the Asian population, Horikoshi et al. (2007) reported that a single nucleotide polymorphism (SNP), rs7903146, in *TCF7L2* is associated with type 2 diabetes in the Japanese population but that other SNPs (rs7895340, rs11196205, rs12255372) are not. The minor allele frequencies of these SNPs in Japanese were also found to be much lower than those of Caucasians. Hayashi et al. (2007) replicated the association of *TCF7L2* with type 2 diabetes in Japanese. Contradictory results were reported for Han Chinese populations (Ng et al. 2007; Chang et al. 2007), but these two reports found that other common SNPs (rs11196218 and rs290487, respectively) were associated with type 2 diabetes. This apparent difference between Asian populations could be due to the relatively small sample sizes involved. Recently, variants in the *TCF7L2* gene also were reported to be associated with β-cell function (Schäfer et al. 2007; Lyssenko et al. 2007) and response to sulfonylureas in Caucasians (Pearson et al. 2007). To clarify the association of the *TCF7L2* gene with type 2 diabetes and β-cell function in an Asian population, we have performed association studies using a relatively large Japanese sample set: 2,214 Japanese individuals with type 2 diabetes and 1,873 normal controls.

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Subjects and methods

Subjects

Three sample sets were involved. The Kobe set and the Gunma set samples were recruited from hospitals in Hyogo and Gunma prefecture, respectively. The Consortium set samples were recruited from seven districts in Japan by the Study Group of the Millennium Genome Project for Diabetes Mellitus. The Kobe, Gunma, and Consortium sets were independent of one another. The inclusion criteria for normal, control subjects of the Consortium set were as follows: (1) >60 years of age; (2) HbA_{1c} values <5.8%; and (3) no family history of type 2 diabetes in first- or second-degree relatives. In the Kobe and Gunma control samples, the inclusion criteria were (1) no past history of diabetes and (2) HbA_{1c} values < 5.8%. The control subjects were hospital patients for annual medical checkup or unrelated disorders. Type 2 diabetes was diagnosed in accordance with WHO criteria. Other forms of diabetes were excluded based on the clinical data. The clinical and laboratory characteristics of the study subjects are shown in Table 1. Written, informed consent was obtained from all participants. The study was approved by the ethics committee of each participating institute.

Genotyping

Five SNPs (rs7903146, rs11196205, rs12255372, rs11196218, rs290487) were genotyped using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City,

CA) or SSP-FCS (sequence specific primer-fluorescence correlation spectroscopy) Assays (Bannai et al. 2004). Of the original five SNPs (rs7903146, rs11196205, rs12255372, rs7901695, rs7895340) in the first report (Grant et al. 2006), we selected three SNPs (rs7903146, rs11196205, rs12255372) for the following reasons: the original five SNPs are located in one linkage disequilibrium (LD) block surrounding exon 4 in the Japanese population (Supplementary Figure 1), which is similar to the case in Caucasians (Grant et al. 2006); rs7901695 and rs7895340 are in almost complete LD with rs7903146 ($r^2 = 1$) and rs11196205 ($r^2 = 0.90$), respectively, in the Japanese population (Horikoshi et al. 2007); there is no common (minor allele frequency > 10%) SNP in this LD block (HapMap JPT data). We also genotyped rs11196218 and rs290487, which were associated with type 2 diabetes in Han Chinese, to replicate this association in Japanese. To evaluate our genotyping, 180 samples in the Consortium set were genotyped by both TaqMan SNP Genotyping Assays and SSP-FCS Assays. The concordance rate between these two assays was 100%: genotypes determined by TaqMan or SSP-FCS methods were identical to those determined by direct sequencing for 48 samples.

The genotyping success rates in the three sample sets were all >93%. All five SNPs were in Hardy–Weinberg equilibrium (HWE; $P > 0.05$ in the Exact test) in both case and control groups of all sample sets.

Clinical assessment

The clinical profile of each subject was directly determined at the time of entry. HOMA-IR and HOMA-β were calculated as follows: HOMA-IR = (fasting insulin [pmol/l]) × glucose [mmol/l])/22.5 × 6 and HOMA-β = (fasting insulin [pmol/l]) × 2)/(glucose [mmol/l] − 3.5) × 6. Diabetic subjects treated with insulin were excluded from analysis of HOMA-IR and HOMA-β. Assessments were performed with the combined three sample sets. Data are expressed as means ± SD.

Statistical analysis

The differences for SNPs or estimated haplotypes between type 2 diabetic and non-diabetic subjects were compared using Chi-square test under an allelic model. We also performed multiple logistic regression analysis adjusted for age, sex, and BMI under a dominant model. Statistical analysis was performed with the Stat-View program (version 5.0-J; SAS Institute, Cary, NC). The relation of the variants in *TCF7L2* with BMI and Homeostasis model assessment (HOMA-IR and HOMA-β) by t test under the dominant model for each SNP was then assessed. The HOMA-IR and HOMA-β data were log-transformed for normality. LD and haplotype analyses were performed with SNPAlyze version 5.1 pro software (Dynacom, Mobara, Japan). We considered statistical significance at P values of < 0.01 and < 0.017 in the association study for SNPs and for clinical parameters, respectively, after Bonferroni correction. The prevalence of type 2 diabetes in the Japanese population was assumed to be 0.07. Population attributable risk (PAR) was calculated as PAR = p(RR-1)/ [p(RR-1) + 1], where p and RR are the risk allele frequency in the general population and the relative risk, respectively, estimated by the prevalence. When the frequency of risk allele, OR, and type I error probability are assumed to be 0.03 (Horikoshi et al. 2007), 1.46 (Cauchi et al. 2007), and 0.05, respectively, based upon the previous study, the power of our combined samples (2,214 cases and 1,873 controls) to detect association between SNP rs7903146 and type 2 diabetes is 0.92. In the case of OR assumed to be 1.69 (Horikoshi et al. 2007), the power of our study is 0.99.

Results

We performed association analyses using three independent sample sets. Regarding three SNPs (rs7903146, rs11196205, and rs12255372), which originally showed association with type 2 diabetes, the minor alleles showed a trend toward association with type 2 diabetes in the Kobe set. These SNPs also showed a marginally significant association in the Gunma set and in the Consortium set when multiple testing was considered. In the combined three sample sets (Combined set), the minor alleles of rs7903146, rs11196205, and rs12255372 showed a significant association with susceptibility to the disease (OR = 1.48, $P = 2.7 \times 10^{-4}$; OR = 1.39, $P = 4.6 \times 10^{-4}$; OR = 1.70, $P = 9.8 \times 10^{-5}$, respectively). These associations remained significant after adjustment for age, sex, and BMI (Table 2). As in a previous report (Horikoshi et al. 2007), the MAF and PAR in our study were much lower (MAF: 0.022−0.072, PAR: ~0.02 in the Combined set) than those in Caucasians. Neither rs11196218 nor rs290487 showed a significant association in any sample set (Table 2).

LD among the five SNPs in 974 control subjects in the Consortium set was then analyzed. The D' and r^2 values are shown in Table 3. As reported previously for Japanese, three SNPs (rs7903146, rs11196205, and rs12255372) were found to be in modest to strong LD ($D' = 0.56$–1.0). Haplotypes then were constructed with these SNPs in the Combined set and assessed for association with type 2 diabetes. A haplotype comprising the risk allele of each

Table 2 Association analyses for five single nucleotide polymorphisms (SNPs) in the *TCF7L2* gene. *P* values and OR were calculated with allele data by the Chi-square test. Adjusted *P* values were calculated by multiple logistic regression (dominant model) with adjustment for age, sex and BMI. *MAF* minor allele frequency, *OR* odds ratio, *CI* confidence interval

dbSNP ID	Position on Chr10		Kobe							Gunma						
			n		MAF		OR	*P*	Adjusted *P*	*n*		MAF		OR	*P*	Adjusted *P*
			Case	Control	Case	Control	(95% CI)			Case	Control	Case	Control	(95% CI)		
rs7903146	114748339	CC	426	305	0.043	0.028	1.56	0.12	0.046	475	512	0.060	0.038	1.63	0.015	0.012
		CT	38	18			(0.89–2.76)			63	42			(1.09–2.42)		
		TT	1	0						1	0					
rs11196205	114797037	GG	408	292	0.063	0.047	1.39	0.16	0.093	455	485	0.084	0.055	1.58	0.007	0.023
		GC	55	30			(0.83–2.18)			77	58			(1.13–2.21)		
		CC	2	0						7	1					
rs12255372	114798892	GG	436	312	0.032	0.017	1.92	0.062	0.018	509	538	0.047	0.024	1.99	0.004	0.005
		GT	28	11			(0.96–3.87)			48	27			(1.24–3.20)		
		TT	1	0						2	0					
rs11196218	114830484	GG	271	194	0.23	0.23	1.01	0.92	0.23	317	334	0.22	0.22	1.04	0.72	0.84
		GA	170	106			(0.80–1.29)			184	185			(0.85–1.27)		
		AA	23	21						25	25					
rs290487	114899721	TT	181	124	0.37	0.38	0.94	0.57	0.90	209	236	0.37	0.34	1.18	0.072	0.13
		TC	226	141			(0.76–1.16)			228	235			(0.99–1.41)		
		CC	57	49						78	61					

Consortium							Combined						
n		MAF		OR	*P*	Adjusted *P*	*n*		MAF		OR	*P*	Adjusted *P*
Case	Control	Case	Control	(95% CI)			Case	Control	Case	Control	(95% CI)		
1,020	879	0.058	0.041	1.43	0.014	0.06	1,921	1,696	0.055	0.038	1.48	2.7×10^{-4}	0.0011
127	77			(1.07–1.90)			228	137			(1.20–1.84)		
3	1						5	1					
1,011	863	0.071	0.054	1.32	0.031	0.12	1,874	1,640	0.072	0.053	1.39	4.6×10^{-4}	0.0053
153	99			(1.02–1.70)			285	187			(1.16–1.67)		
6	3						15	4					
1,068	906	0.035	0.023	1.52	0.026	0.12	2,013	1,756	0.037	0.022	1.70	9.8×10^{-5}	7.0×10^{-4}
76	42			(1.05–2.21)			152	80			(1.30–2.22)		
2	1						5	1					
728	584	0.20	0.22	0.87	0.076	0.11	1,331	1,115	0.21	0.22	0.94	0.26	0.56
370	321			(0.75–1.01)			730	617			(0.85–1.05)		

Table 2 continued

Consortium							Combined						
n		MAF		OR	*P*	Adjusted *P*	*n*		MAF		OR	*P*	Adjusted *P*
Case	Control	Case	Control	(95% CI)			Case	Control	Case	Control	(95% CI)		
45	54						93	100					
476	381	0.37	0.37	0.99	0.91	0.50	873	744	0.37	0.36	1.04	0.45	0.46
507	448			(0.88–1.13)			977	824			(0.95–1.14)		
169	129						306	239					

SNP, T-C-T, was significantly associated with type 2 diabetes ($P = 5.3 \times 10^{-5}$) (Table 4).

The relation of rs7903146, rs11196205, and rs12255372 to BMI, HOMA-IR, and HOMA-β in the combined cases and controls were then compared. There was no association with BMI in cases or controls. The risk allele of rs7903146 was associated with lower HOMA-β (CC ($n = 789$) versus CT/TT ($n = 83$); 52.0 ± 87.6 versus 35.7 ± 35.9, $P = 0.009$) and lower HOMA-IR (CC vs. CT/TT; 3.2 ± 4.5 vs. 2.2 ± 1.6, $P = 0.01$) in the combined diabetic subjects. However, these associations disappeared after adjustment for age, sex, and BMI. No association was found for HOMA-β or HOMA-IR in the combined control subjects.

Discussion

We have found that three SNPs (rs7903146, rs11196205, rs12255372) of *TCF7L2* are associated with susceptibility to type 2 diabetes in the Japanese population. Our results are consistent with previous reports for Japanese populations (Horikoshi et al. 2007; Hayashi et al. 2007), but not with other reports for Han Chinese populations (Ng et al. 2007; Chang et al. 2007). The apparent difference in the association of these SNPs in Asians could be due to the low frequencies of the SNPs and the relatively small sample sizes used in the previous studies. Since we did not detect any association of rs11196218 or rs290487 in the present study, the associations of the two SNPs in the previous reports for Chinese might be specific to that population. In this study, rs7903146, rs11196205, and rs12255372 were in modest to strong LD. Based on HapMap data (JPT), the LD block surrounding exon 4 of *TCF7L2* in Asians does not exceed the gene (Supplementary Figure 1), which is consistent with findings in Caucasians (Grant et al. 2006). Previous reports (Ng et al. 2007; Chang et al. 2007) also found that the three SNPs were in a single LD block while the other two (rs11196218 and rs290487) were not. According to meta-analysis by Cauchi et al. (2007), *TCF7L2* is the most reproducible susceptibility gene for type 2 diabetes in various ethnic groups. *TCF7L2* also was one of the most significantly associated genes in recent genome-wide association studies (Sladek et al. 2007; WTCCC 2007). While the risk alleles of this gene are not common in East Asians, including Japanese, and the population attributable risk is much lower, *TCF7L2* is nevertheless a risk gene for type 2 diabetes in East Asians as well as in other populations. On the other hand, in a very recent online report, polymorphisms in the *TCF7L2* gene were found not to be associated with type 2 diabetes in a relatively large study of Pima Indians (Guo et al. 2007). Further investigation is required to

Table 3 Pairwise linkage disequilibrium (LD) for five SNPs in the *TCF7L2* gene. Values of D' (left lower) and of r^2 (upper right) for pairwise LD analysis in 974 control subjects of the Consortium set

	rs7903146	rs11196205	rs12255372	rs1196218	rs290487
rs7903146		0.24	0.49	0.002	0.0036
rs11196205	0.56		0.44	0.012	0.0037
rs12255372	0.93	1.00		0.007	0.0036
rs1196218	0.45	0.87	1.00		0.0002
rs290487	0.22	0.19	0.30	0.02	

Table 4 Association analysis for haplotypes with three SNPs (rs7903146, rs11196205, rs12255372). *P* values were calculated by the chi-square test with estimated haplotype data from the Combined set

Haplotype	Case	Control	*P*
C-G-G	0.91	0.93	1.5×10^{-4}
C-C-G	0.032	0.031	0.72
T-C-T	0.032	0.018	5.3×10^{-5}
T-G-G	0.020	0.017	0.30

elucidate the differences in the contribution of the *TCF7L2* gene to type 2 diabetes among various populations.

TCF7L2 regulates expression of the proglucagon gene (*GCG*), which encodes the precursor of glucagon, glucagon-like peptide 1 (GLP-1) (Yi et al. 2005). Several reports have found that polymorphisms of *TCF7L2* are associated with β-cell function (Florez et al. 2006; Saxena et al. 2006; Schäfer et al. 2007; Lyssenko et al. 2007). In this study, the association between the *TCF7L2* gene and HOMA-β was found to disappear after adjustment for the various factors. Although the relationship of this gene to β-cell function is not clear in this study, our results suggest that *TCF7L2* is an important susceptibility gene for type 2 diabetes in Japanese. The pathophysiological mechanism of this gene in susceptibility to type 2 diabetes remains to be elucidated.

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