Combination of Multiple Genetic Risk Factors Is Synergistically Associated With Carotid Atherosclerosis in Japanese Subjects With Type 2 Diabetes

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OBJECTIVE — Several genetic risk factors, such as single nucleotide polymorphisms (SNPs), in candidate genes have been reported to be responsible for intima-media thickness (IMT), which is one of the surrogate end points of cardiovascular events. However, the synergistic effects of SNPs have not been evaluated in detail.

RESEARCH DESIGN AND METHODS — We measured the average IMT of the common and internal carotid artery in Japanese type 2 diabetic patients ($n = 690$) (>50 years old) using ultrasonography. We also determined their genotypes regarding 106 SNPs in candidate genes responsible for cardiovascular diseases. Among the 106 SNPs, we selected 40 common (frequency of minor allele ≥10%) SNPs. We compared the average IMT of subjects with and without any pairs of four genotypes selected from the 40 common SNPs.

RESULTS — The combination of methylen-tetrahydrofolate reductase 677 TT genotype and lymphotoxin-α (LTA) 252 GG genotype and that of ACE DD genotype and LTA 252 GG genotype were evaluated as responsible for a statistically significant ($P = 2.7 \times 10^{-9}$ and 3.5×10^{-6}, respectively) increase in average IMT (mean [±SD] 1.54 ± 0.60 and 1.43 ± 0.58 mm, respectively) compared with those of the subjects without these combinations (1.04 ± 0.34 and 1.04 ± 0.34 mm, respectively). No single genotype was shown to be responsible for the statistically significant difference in average IMT after Bonferroni's multiple comparison procedure.

CONCLUSIONS — The present analysis demonstrates an approach to evaluate combinations of multiple genetic risk factors that are synergistically associated with carotid atherosclerosis.

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Abbreviations: IMT, intima-media thickness; LTA, lymphotoxin-α; MTHFR, methylene-tetrahydrofolate reductase; SNP, single nucleotide polymorphism; TNF, tumor necrosis factor.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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In Westernized countries, a large proportion of the patients with diabetes die from cardiovascular diseases, mainly due to markedly advanced atherosclerosis (1). Although the risk of cardiovascular disease increases additively with the number of conventional risk factors, including diabetes, hypertension, and dyslipidemia, these conventional risk factors cannot fully account for the risk of cardiovascular diseases. Because atherosclerosis is a complex multifactorial and polygenic disorder that is thought to result from interactions among individual genetic risk factors and various environmental factors (2,3), to identify disease-susceptibility genes, genome-wide scanning of single nucleotide polymorphisms (SNPs) and candidate gene analyses have been performed. As a result, various single candidate genes and a locus involved in the predisposition to cardiovascular diseases have been identified (4–6). Although it is possible that some of these atherosclerosis-susceptibility gene polymorphisms are synergistically responsible for advanced atherosclerosis, few studies have reported on the involvement of a combination of potentially susceptible genes in cardiovascular diseases (6,7).

The objective of the present study was to identify specific combination(s) of single SNPs associated with carotid atherosclerosis of type 2 diabetic patients, which can be considered as a surrogate end point of cardiovascular events (8–10).

RESEARCH DESIGN AND METHODS

The study enrolled type 2 diabetic subjects ($n = 690$, ≥50 years old) who visited the outpatient clinics of two participating hospitals (Osaka University Hospital and Juntendo University Hospital). The determination of type 2 diabetes was based on World Health Organization criteria. The patients' characteristics are listed in Table 1. Smoking habit was evaluated as follows: value of 0 and 1 were assigned to subjects when the number of

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Table 1—*Patient characteristics*

n	690
Female/male	321/359
Age (years)	62.7 ± 7.1
Duration (years)	12.5 ± 9.4
BMI (kg/m^2)	23.0 ± 3.2
A1C (%)	7.3 ± 1.5
Total cholesterol (mg/dl)	198 ± 36.9
Triglycerides (mg/dl)	147 ± 113
HDL cholesterol (mg/dl)	53.7 ± 17.3
Systolic blood pressure (mmHg)	134 ± 14.9
Diastolic blood pressure (mmHg)	77.2 ± 8.4
Smoking habit (0/1)*	547/143
Average IMT (mm)	1.06 ± 0.35
Previous myocardial infarction (no/yes)	610/80

Data are means ± SD. *Smoking habit was evaluated as follows: value of 0 and 1 were assigned to subjects when the number of cigarettes per day × smoking years was <200 and ≥200, respectively.

cigarettes per day × smoking years was <200 and ≥200, respectively.

The study protocol was approved by the committees on the ethics of human research of Osaka University Graduate School of Medicine and Juntendo University School of Medicine.

Carotid atherosclerosis measurement

A series of ultrasonographic scannings of the carotid artery were performed using an echotomographic system (EUB-450; Hitachi Medico, Tokyo, Japan) with an electrical linear transducer (midfrequency of 7.5 MHz). All the images were photographed. The resolution limit of this system using 7.5 MHz was ~0.1 mm. The conventional B-mode imaging of the extracranial common carotid artery, the carotid bulb, and the internal carotid artery in the neck was performed bilaterally from three different longitudinal projections (i.e., anterior-oblique, lateral, and posterior-oblique) as well as the transverse projection, as reported previously (11,12). The carotid IMT was measured as the distance from the leading edge of the first echogenic line to the leading edge of the second echogenic line (13). Three determinations of IMT were conducted at the site of the greatest thickness and at two points, 1 cm upstream and 1 cm downstream from the site of the greatest thickness. These three values were averaged. The greatest value among the six averaged IMT (three from the left and three from the right) was used as the representative value (average IMT) for each individual. Physicians conducted all scans and different physicians performed the determinations of IMT using the photographs. The physicians were unaware of the clinical characteristics of the subjects. The reproducibility of the IMT measurements was examined by conducting another scan 1 week later on eight subjects. The mean difference in IMT between these two determinations was 0.01 mm and the SD was 0.04 mm, demonstrating good reproducibility of repeated measurements.

Assessment of other parameters

Fasting blood was withdrawn for analyses of serum total cholesterol, serum HDL cholesterol, serum triglyceride, plasma glucose, and HbA_{1c} (A1C) levels by standard laboratory techniques. Blood pressure and BMIs were also measured. Examination for the occurrence of old myocardial infarction (major Q-QS changes) was performed based on the results of the resting 12-lead electrocardiogram and double Master two-step tests and the existence of previous symptoms of myocardial infarction (14).

Selection of SNPs

With the use of PubMed, we selected 106 candidate gene SNPs that are potentially associated with atherosclerosis, diabetes, hypertension, dyslipidemia, or diabetic microangiopathy (Table 2). Of these 106 polymorphisms, we selected 40 common SNPs in which the frequency of the minor allele was ≥10% of sample population.

Genotyping of SNPs

Venous blood was collected from each subject and genomic DNA was isolated with a DNA isolation kit (Qiagen). The genotypes of the SNPs in each subject were determined with a fluorescence- or colorimetry-based allele-specific DNA-primer probe assay system (Toyobo Gene Analysis) as described in detail by Yamada et al. (5).

Statistical analysis

The data are expressed as means ± SD, and all statistical tests were two-sided. The Student's *t* test was applied to compare the average IMT of subjects with and without the SNP genotype or the SNP combination.

Genotypes of 40 common SNPs were classified into two categories by four ways, including the major allele's dominant model, minor allele's dominant model, major allele's recessive model, and minor allele's recessive model. Then, 16 SNP combination models consisting of one combination from each category for the two SNPs and the set of the other combinations were also built. In each model, the continuous data of two of the categories were compared by Student's *t* test. The categorical data of the two categories were compared by Pearson's χ^2 test. The data of age, systolic and diastolic blood pressure, and SNP combination as independent variables were also analyzed by multivariate regression analysis with the data of average IMT as a dependent variable. The association between SNP combination and the history of coronary heart disease was evaluated by the multiple logistic model.

Because the analyses were performed on the four models of 40 SNPs and 16 models of 780 SNP combinations, correction for multiple testing must be required. Bonferroni's multiple comparison procedure was utilized for the correction and gave the corrected level of significance, 0.00031 [$= 0.05/(40 \times 4)$] in the single SNP analyses and 4.01×10^{-6} [$= 0.05/({}_{40}C_2 \times 16)$] in the SNP combination analyses.

These statistical analyses were performed using the SAS statistical package (SAS/STAT 9.1; SAS Institute, Cary, NC).

Simulation for multiple comparisons

The significance level for multiple comparisons was evaluated also by simulation. In a simulation, the virtual average IMT values following the normal distribution with the mean of 1.05 and the SD of 0.35 from the whole enrolled members' population were randomly assigned to all members. Next, the population of the enrolled members was randomly divided between the group with the genotypes' combination (18) and the group without it (646 or 644) by ${}_{40}C_2 \times 16$ patterns. The simulations in which the *P* value by Student's *t* test under the threshold (3.5×10^{-6} or 2.7×10^{-9}) was observed at least once were regarded as false positive. The ratio of the false positive to the whole simulations was regarded as the *P* value for the whole analyses.

RESULTS — In the single SNP analyses, no SNPs were found to be significantly associated with average IMT (mean [±SD] 1.06 ± 0.35 mm) by Bonferroni's multiple comparison procedure in any model, whereas the *P* values of the Student's *t* test were less than the uncor-

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Table 2—*Polymorphisms of candidate genes analyzed* (n = *106*)

Gene	Polymorphism
Adiponectin	T94G*
	C120T (arg112Cys in exon3)
	G276T*
α estrogen receptor	T938C*
α fibrinogen	A4266G (Thr312Ala)*
AMP deaminase	C34T
Angiotensin II type 2 receptor	A1166C
ACE	I/D type*
Angiotensinogen	T704C (Met235Thr)*
Apolipoprotein E	T3932C (Cys112Arg)
	T4070C (Arg158Cys)*
ATP binding cassette A1	G1051A (Arg219Lys)*
ATP binding cassette C6	C3421T
β2 adrenergic receptor	A46G (Arg16Gly)*
	C79G
	C491T
β3 adrenergic receptor	T190C (Trp64Arg)*
β fibrinogen	C148T
Bradykinin B2 receptor	C-58T*
C-C chemokine receptor 2	G190A*
CD14	T-159C*
CD18	C1323T*†
Cholesteryl ester transfer protein	G338A (Arg451Glu)
Connexin 37	C1019T (Pro319Ser)*
C-reactive protein	G1059C
Dopamine-D2 receptor	C3413G (Ser311Cys)
Early growth response factor-1	C-151T
Ecto-nucleotide pyrophosphatase/phosphodiesterase 1	C97A (Lys121Gln)
Endothelial nitric oxide synthase	T-786C
	G894T (Glu298Asp)
Endothelin-1	G5665T*
Epoxide hydrolase	CGT insertion after 1206 (Arg402–403ins)
	G860A
E-selection	G98T
	A561C (Ser128Arg
Factor V	G1691A
Factor XII	C46T*
Fractalkine receptor	G84635A (Val249Ile)
Ghrelin	C247A
GLUT enhancer-2	A45474G
GLUT 1	G283T*
Glutamate-cysteine ligase	C588T*
Glycogen synthase	A260G (Met416Val)
Glycoprotein Ia	A1648G
	C807T*
Glycoprotein IIbIIIa	C1565T
Gylcoprotein VI	C13254C (Ser219Pro)
Hepatic lipase	C-480T*
Hemochromatosis (HFE)	G4762C (His63Asp)
Human atrial natriuretic peptide	T2238C
	C708T
Human paraoxonase	A172T (Met 55Leu)
	A584A (Gln192Arg)*
Human platelet alloantigen-2	C1018T (Thr145Met)
Insulin receptor substrate-1	G3494A (Gly971Arg)
Intercellular adhesion molecule-1	G1548A (Glu469Lys)*
Interleukin-1α	C-889T

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Table 2—*Continued*

Gene	Polymorphism
Interleukin-1β	C3953T
Interleukin-4 receptor α	A398G (Ile50Val)
Interleukin-6	C-634G
	G-174C*
Interleukin-10	G-1082A*
	C-819T
Interleukin-13	G4166A (Arg10Gln)
Interleukin-18	C-607A*
	G-137C
LDL receptor–related protein	C766T
Lipoprotein lipase	C3150G (Ser447 STOP)
Lymphotoxin α	A252G*†
Manganese superoxide dismutase	C1183T (Val16Ala)
Matrilysin promoter	A-181G
Matrix Gla protein	G-7A*
Matrix metalloproteinase-7	C-153T
Matrix metalloproteinase-9	C-1562T*
Matrix metalloproteinase-12	A-82G
Methionine synthase	A2756G (Asp919Gly)*
Methylenetetrahydrofolate reductase	C677T*†
Microsomal triglyceride transfer protein	G-493T
Monocyte chemoattractant protein-1	A-2518G
Myeloperoxidase	G-463A*
Neuropeptide Y	T1128C (Leu7Pro)
p22phox	C242T (His72Tyr)
Peroxisome proliferators–activated receptor α	C696G (Leu162Val)
	C892G (Pro12Ala)
Peroxisome proliferators–activated receptor α coactivator-1	G1302A (Thr394Thr)*
	G1564A (Gly482Ser)*
Plasminogen activator inhibitor-1	4G-668/5G*
Pronatriodilatin	C2238T
Prothrombin	G20210A
P-selectin	A37674C (Thr715Pro)
Receptor for advanced glycation end products	T-429C
	A7221G (Gly82Ser)
Regulated upon activation, normal T-cell expressed and secreted (RANTUS)	C-28G
Scavenger receptor class B type I (CLA-1)	G4A (Gly2Ser)
	G403A (Val135Ile)
Serotonin 2A receptor	T102C*
Thrombomodulin	G33A
Thrombopoietin	A5713G*
Thrombospondin-1	A2210G (Asn700Ser)
Thrombospondin-4	G1186C (Ala387Pro)
Toll-like receptor 2	C2029T
Transforming growth factor β	T29C (Leu10Pro)*
TNF-α	G-238A
	G-308A
Vascular endothelial growth factor	C-634G*†
Von Willebrand factor	G-1051A*

*Common polymorphism in subjects with type 2 diabetes (minor allele frequency ≥10%); †polymorphism associated ($P < 0.05$) with an increase in IMT in the carotid artery.

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rected level of significance (0.05) in seven SNPs, including the ACE DD genotype (1.13 ± 0.43 vs. 1.04 ± 0.34 mm, $P = 0.03284$), CD18 1323 CC and CT genotypes (1.07 ± 0.36 vs. 0.93 ± 0.24 mm, $P = 0.01768$), factor XII 46CC genotype (1.13 ± 0.40 vs. 1.05 ± 0.34 mm, $P = 0.04173$), glycoprotein Ia 807 TT and CT genotypes (1.08 ± 0.39 vs. 1.01 ± 0.29 mm, $P = 0.01179$), LTA 252 GG genotype (1.15 ± 0.42 vs. 1.04 ± 0.33 mm, $P = 0.00109$), methylene-tetrahydrofolate reductase (MTHFR) 677 TT genotype (1.15 ± 0.45 vs. 1.04 ± 0.33 mm, $P = 0.00220$), and vascular endothelial growth factor −634 GG genotype (1.10 ± 0.40 vs. 1.03 ± 0.33 mm, $P = 0.01829$).

Table 3—*Patient characteristics of subjects with or without the two combinations of SNPs*

	ACE DD and LTA 252 GG genotype			MTHFR 677 TT and LTA 252 GG genotype		
	Without (646)	With (18)	*P*	Without (644)	With (18)	*P*
n	646	18		644	18	
Female/male	315/331	8/10	NS	312/332	8/10	NS
Age (years)	62.6 ± 7.2	64.6 ± 15.2	NS	62.6 ± 7.1	66.3 ± 8.0	0.0286
Duration (years)	12.5 ± 9.4	12.3 ± 9.3	NS	12.5 ± 9.4	14.0 ± 9.4	NS
BMI (kg/m^2)	23.0 ± 3.2	22.7 ± 3.6	NS	23.0 ± 3.2	22.7 ± 3.2	NS
A1C (%)	7.3 ± 1.5	7.3 ± 1.0	NS	7.3 ± 1.5	7.1 ± 1.1	NS
Total cholesterol (mg/dl)	198 ± 36.5	193 ± 33.4	NS	198 ± 37.1	197 ± 40.2	NS
Triglycerides (mg/dl)	148 ± 115	143 ± 108	NS	148 ± 115	156 ± 99.0	NS
HDL cholesterol (mg/dl)	53.5 ± 16.4	54.5 ± 18.4	NS	53.9 ± 17.5	50.0 ± 12.8	NS
Systolic blood pressure (mmHg)	134 ± 14.9	143 ± 15.2	0.01082	134 ± 14.9	137 ± 16.1	NS
Diastolic blood pressure (mmHg)	77.2 ± 8.4	82.9 ± 7.4	0.00458	77.1 ± 8.3	82.1 ± 8.0	0.01114
Smoking habit (0/1)*	514/132	15/3	NS	513/131	NS	NS
Average IMT (mm)	1.04 ± 0.34	1.43 ± 0.58	3.5×10^{-6}	1.04 ± 0.34	1.54 ± 0.60	2.7×10^{-9}
Previous myocardial infarction (no/yes)	573/73	12/6	0.01318	569/75	13/5	0.0883

Data are means ± SD. Student's *t* test was used to compare continuous data between groups, and Pearson's χ^2 test to compare categorical data, sex, smoking habit, and previous myocardial infarction. *Smoking habit was evaluated as follows: value of 0 and 1 were assigned to subjects when the number of cigarettes per day × smoking years was <200 and ≥200, respectively. NS, not significant ($P > 0.05$).

Next, to investigate the association between average IMT and the combinations of potential susceptible genes, we compared the average IMT data of two categories classified by the combinations of SNP genotypes with Student's *t* test. In 780 combinations of SNPs, only two genotype combinations including LTA 252 GG–MTFHR 677 TT ($P = 2.7 \times 10^{-9}$) and ACE DD–LTA 252 GG ($P = 3.5 \times 10^{-6}$) were found to be associated with a significant ($P = 2.7 \times 10^{-9}$ and 3.5×10^{-6}, respectively) increase in average IMT (1.54 ± 0.60 and 1.43 ± 0.58 mm, respectively) compared with those of the subjects without these combinations (1.04 ± 0.34 and 1.04 ± 0.34 mm, respectively).

The characteristics of the patients with and without these two genotype combinations are shown in Table 3. The diabetic subjects with the combination of ACE DD and LTA 252 GG showed significantly higher systolic and diastolic blood pressure than those without this combination. However, multivariate regression analysis demonstrated that the combination of ACE DD and LTA 252 GG is still the independent determinant of average IMT (partial regression coefficient = 0.344 mm, $F = 18.53$, $P = 0.00002$) after adjustment of systolic and diastolic blood pressure. A multiple logistic model demonstrated that this genotype combination is significantly responsible for a high frequency of history of coronary heart disease (odds ratio 3.11 [95% CI 1.16–8.38], $P = 0.0247$).

The diabetic patients with the combination of LTA 252 GG and MTHFR 677 TT showed a significantly higher age and diastolic blood pressure than those without this combination (Table 3). However, multivariate regression analysis demonstrated that the combination of LTA 252 GG and MTHFR 677 TT is still the independent determinant of average IMT after adjustment of age and diastolic blood pressure (partial regression coefficient = 0.438 mm, $F = 29.91$, $P = 6.43 \times 10^{-8}$). A multiple logistic model demonstrated that this genotype combination was not significantly responsible for a high frequency of history of coronary heart disease.

Additionally, the *P* value for the each combination of genotypes was empirically evaluated by the simulation for multiple comparisons. The simulation indicated that *P* value of 3.5×10^{-6} for ACE DD and LTA 252 GG combination corresponded to the *P* value of 2.8×10^{-2} for the whole analysis, and *P* value of 2.7×10^{-9} for MTHFR 677 TT and LTA 252 GG combination was stricter than *P* value of 1.0×10^{-5} for the whole analysis.

CONCLUSIONS — In this study, we selected 106 candidate gene polymorphisms that may contribute to coronary heart disease, diabetes, and diabetes vascular complications. After genotyping of these candidate gene polymorphisms in all subjects, we found that 40 SNPs were relatively common (the frequency of the minor allele was ≥10%) in Japanese subjects with type 2 diabetes. Because multiple comparisons must be performed on these 40 SNPs in order to evaluate their association with carotid atherosclerosis, Bonferroni's multiple comparison procedure was applied for statistical tests to control the family-wise type 1 error to <0.05.

In the single SNP analyses, we found that no single SNP genotypes were statistically significantly associated with carotid IMT after Bonferroni's multiple comparison procedure. Although this procedure may be too strict to find out candidate SNP genotypes related to disease susceptibility, these results suggest the fact that any single candidate genotype is not critical in determining carotid IMT and that it is difficult to predict the risk of carotid atherosclerosis by simply diagnosing single SNP genotypes independently.

In the SNP combination analyses, two SNP combinations (MTHFR 677 TT genotype plus LTA 252 GG genotype and ACE DD genotype plus LTA 252 GG genotype) were found to be statistically ($P < 4.01 \times 10^{-6}$) significantly associated with carotid atherosclerosis in Japanese type 2 diabetic subjects after Bonferroni's multiple comparison procedure.

LTA, formerly named tumor necrosis factor (TNF)-β, is structurally similar to TNF-α and plays a crucial role in the inflammatory response by inducing monocyte migration and lymphocyte activation (4). Furthermore, reduction of atherosclerotic lesions was observed in LTA

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knockout mice but not in TNF-α knockout mice, suggesting that LTA is more important in the progression of atherosclerosis (15). It is known that the A252G polymorphism in the LTA gene is to an amino acid–coding polymorphism, leading to an increase of C-reactive protein, vascular cell adhesion molecule (VCAM)-1, and selectin E (4), all of which are closely associated with the inflammatory process and the progression of atherosclerosis. However, the association between the LTA polymorphism and the progression of atherosclerosis has yet to be reported. Hyperhomocysteinemia is well known to be an independent risk factor for atherosclerosis, and MTHFR is an enzyme that is involved in the remethylation of homocysteine to methionine (16,17). The MTHFR 677 TT genotype results in a reduction of MTHFR activity and an increase in serum homocysteine level, and thus is possibly involved in the progression of atherosclerosis (18,19). In this study, we could not find that the LTA 252 GG genotype or the MTHFR 677 TT genotype was significantly associated with average IMT. These results indicate that a polymorphism of each genotype alone is not enough to lead to a substantial progression of atherosclerosis and that it is very important to examine both polymorphisms when we estimate whether each subject is predisposed to atherosclerosis.

It is known that the insertion/deletion (I/D) polymorphism of the ACE gene is associated with the level of circulating ACE and that the highest plasma ACE levels are found in subjects with the DD genotype (20). Although the ACE I/D polymorphism possibly influences blood pressure regulation (21) and is involved in the progression of atherosclerosis, it still remains controversial as to whether this SNP actually contributes to the progression of atherosclerosis (20–25). In this study, this SNP was found not to be responsible for an increase in average IMT, and this SNP also did not affect systolic and diastolic blood pressures (data not shown). However, the combination of the ACE DD genotype and the LTA 252 GG genotype contributed to a statistically significant increase in average IMT, and the subjects with this combination showed significantly higher systolic and diastolic blood pressure. Under age-, systolic blood pressure–, and diastolic blood pressure–matched conditions, this combination was still associated with an increase in average IMT. In addition, this combination was significantly related with a high frequency of history of old myocardial infarction in these subjects. Although it remains unknown as to how such a combination of these gene polymorphisms exerts synergistic effects on carotid atherosclerosis and blood pressure, these results indicate that these two genotypes synergistically contribute to the progression of carotid atherosclerosis and predisposition of coronary heart disease partially by elevated blood pressure and that it is very important to examine these polymorphisms when we estimate the predisposition of each subject to atherosclerosis and coronary heart disease.

This cross-sectional study has shown that two synergistic combinations of SNPs predispose subjects with type 2 diabetes to carotid atherosclerosis and that one of these combinations is associated with coronary heart disease. A long-term follow-up study will be required to further establish these combinations of SNPs as responsible for an increased risk of carotid and coronary atherosclerosis.

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References

1. Chait A, Bierman EL: Pathogenesis of macrovascular disease in diabetes. In *Joslin's Diabetes Mellitus*. Kahn CR, Weir GC, Eds. Philadelphia, Lea & Febiger, 1994, p. 648–664
2. Nabel EG: Cardiovascular disease. *N Engl J Med* 349:60–72, 2003
3. Lusis AJ, Fogelman AM, Fonarow GC: Genetic basis of atherosclerosis. II. Clinical implications. *Circulation* 110:2066–2071, 2004
4. Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, Tsunoda T, Sato H, Sato H, Hori M, Nakamura Y, Tanaka T: Functional SNPs in the lymphotoxin-α gene that are associated with susceptibility to myocardial infarction. *Nat Genet* 32:650–654, 2002
5. Yamada Y, Izawa H, Ichihara S, Takatsu F, Ishihara H, Hirayama H, Sone T, Tanaka M, Yokota M: Prediction of the risk of myocardial infarction from polymorphisms in candidate genes. *N Engl J Med* 347:1916–1923, 2002
6. Small KM, Wagoner LE, Levin AM, Kardia SL, Liggett SB: Synergistic polymorphisms of α_1- and β_{2c}-adrenergic receptors and the risk of congestive heart failure. *N Engl J Med* 347:1135–1142, 2002
7. Nakata Y, Katsuya T, Rakugi H, Takami S, Sato N, Kamide K, Ohishi M, Miki T, HIgaki J, Ogihara T: Polymorphism of angiotensin converting enzyme, angiotensinogen, and apolipoprotein E genes in a Japanese population with cerebrovascular disease. *Am J Hypertens* 10:1391–1395, 1997
8. Chambless LE, Heiss G, Folsom AR, Rosamond W, Szklo M, Sharrett AR, Clegg LX: Association of coronary heart disease incidence with carotid arterial wall thickness and major risk factors: the Atherosclerosis Risk in Communities (ARIC) Study, 1987–1993. *Am J Epidemiol* 146: 483–494, 1997
9. Hodis HN, Mack WJ, LaBree L, Selzer RH, Liu CR, Liu CH, Azen SP: The role of carotid arterial intima-media thickness in predicting clinical coronary events. *Ann Intern Med* 128:262–269, 1998
10. O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK Jr, the Cardiovascular Health Study Collaborative Research Group: Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. *N Engl J Med* 340:14–22, 1999
11. Kawamori R, Yamasaki Y, Matsushima H, Nishizawa H, Nao K, Hougaku H, Maeda H, Handa N, Matsumoto M, Kamada T: Prevalence of carotid atherosclerosis in diabetic patients: ultrasound high-resolution B-mode imaging on carotid arteries. *Diabetes Care* 15:1290–1294, 1992
12. Yamasaki Y, Kawamori R, Matsushima H, Nishizawa H, Kodama M, Kajimoto Y, Morishima T, Kamada T: Atherosclerosis in carotid artery of young IDDM patients monitored by ultrasound high-resolution B-mode imaging. *Diabetes* 43:634–639, 1994
13. Pignoli P, Tremoli E, Poli A, Oreste P, Paoletti R: Intimal plus medial thickness of the arterial wall: a direct measurement with ultrasound imaging. *Circulation* 74: 1399–1406, 1986
14. Simonson E, Keys A: The electrocardiographic exercise test: changes in scalar ECG and in mean spatial QRS and T vectors in two types of exercise: effect of absolute and relative body weight and comment on normal standards. *Am Heart J* 52:83–105, 1956
15. Schreyer SA, Vick GM, LeBoeuf RC: Loss of lymphotoxin-alpha but not tumor necrosis factor-alpha reduces atherosclerosis in mice. *J Biol Chem* 277:12364–12368, 2002
16. Robinson K, Mayer EL, Miller DP, Green R, van Lente F, Gupta A, Kottke-Marchant K, Savon SR, Selhub J, Nissen SE, Kutner M, Topol EJ, Jacobsen DW: Hyperhomocysteinemia and low pyridoxal phosphate: common and independent reversible risk

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factors for coronary artery disease. *Circulation* 92:2825–2830, 1995

17. Boushey CJ, Beresford SA, Omenn GS, Motulsky AG: A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: probable benefits of increasing folic acid intakes. *JAMA* 274:1049–1057, 1995
18. Christensen B, Frosst P, Lussier-Cacan S, Selhub J, Goyette P, Rosenblatt DS, Genest J Jr, Rozen R: Correlation of a common mutation in the methylenetetrahydrofolate reductase gene with plasma homocysteine in patients with premature coronary artery disease. *Arterioscler Thromb Vasc Biol* 17:569–573, 1997
19. Arai K, Yamasaki Y, Kajimoto Y, Watada H, Umayahara Y, Kodama M, Sakamoto K, Hori M: Association of methylenetetrahydrofolate reductase gene polymorphism with carotid arterial wall thickening and myocardial infarction risk in NIDDM. *Diabetes* 46:2102–2104, 1997
20. Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F: An insertion/deletion polymorphism in angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest* 86:1343–1346, 1990
21. Frost D, Pfohl M, Clemens P, Haring HU, Beischer W: Evaluation of the insertion/deletion ACE gene polymorphism as a risk factor for carotid artery intima-media thickening and hypertension in young type 1 diabetic patients. *Diabetes Care* 21: 836–840, 1998
22. Mannami T, Katsuya T, Baba S, Inamoto N, Ishikawa K, Higaki J, Ogihara T, Ogata J: Low potentiality of angiotensin-converting enzyme gene insertion/deletion polymorphism as a useful predictive marker for carotid atherogenesis in a large general population of a Japanese city: the Suita Study. *Stroke* 32:1250–1256, 2001
23. Kogawa K, Nishizawa Y, Hosoi M, Kawagishi T, Maekawa K, Shoji T, Okuno Y, Horii H: Effect of polymorphism of apolipoprotein E and angiotensin-converting enzyme genes on arterial wall thickness. *Diabetes* 46:682–687, 1997
24. Rasmussen LM, Ledet T: Aortic atherosclerosis in diabetes mellitus is associated with an insertion/deletion polymorphism in the angiotensin I-converting enzyme gene: no relation between the polymorphism and aortic collagen content. *Diabetologia* 39:696–700, 1996
25. Yamasaki Y, Tsujino T, Kodama M, Kajimoto Y: DD genotype of the ACE gene as an atherosclerosis-independent risk factor for myocardial infarction. *Circulation* 96:3782, 1997

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