Association of a polymorphism of *CYP3A4* with type 2 diabetes mellitus

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Abstract. Although several environmental factors, including a high-calorie diet and physical inactivity, influence the development of type 2 diabetes mellitus, genetic factors have been shown to contribute to individual susceptibility to this condition. The purpose of the present study was to identify gene polymorphisms that confer susceptibility or resistance to type 2 diabetes mellitus, and thereby to contribute to assessment of the genetic risk for this condition. The study population comprised 5259 unrelated Japanese individuals (2980 men, 2279 women), including 1640 subjects with type 2 diabetes mellitus (1071 men, 569 women) and 3619 controls (1909 men, 1710 women). The genotypes for 94 polymorphisms of 67 genes were determined with a method that combines the polymerase chain reaction and sequence-specific oligonucleotide probes with suspension array technology. Evaluation of genotype distributions by the chi-square test revealed that the 13989A→G (Ile118Val) polymorphism of the cytochrome P450, subfamily IIIA, polypeptide 4 gene (*CYP3A4*) was significantly (false discovery rate, 0.000009) associated with the prevalence of type 2 diabetes mellitus. Multivariable logistic regression analysis with adjustment for age and sex also revealed that the 13989A→G (Ile118Val) polymorphism of *CYP3A4* was significantly (P=0.00002) associated with the prevalence of type 2 diabetes mellitus, with the *AG* genotype being protective against this condition. Genotyping for *CYP3A4* may thus prove informative for assessment of the genetic risk for type 2 diabetes mellitus.

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Introduction

Type 2 diabetes mellitus is a multifactorial and polygenic disease. Although several environmental factors, including a high-calorie diet and physical inactivity, influence the development of type 2 diabetes mellitus, genetic factors have been shown to contribute to individual susceptibility to this condition. A combination of genes thus likely influences the underlying level of glucose intolerance or insulin resistance and thereby contributes to the overall susceptibility to type 2 diabetes mellitus. Although genetic linkage analyses (1-5) and association studies (6-10) have implicated several loci and candidate genes in predisposition to type 2 diabetes mellitus, the genes that contribute to genetic susceptibility to this condition remain to be identified definitively. In addition, given the ethnic differences in lifestyle and environmental factors as well as in genetic background, it is important to examine polymorphisms related to type 2 diabetes mellitus in each ethnic group.

We have now performed a large-scale association study for 94 polymorphisms of 67 candidate genes and type 2 diabetes mellitus in 5259 Japanese individuals. The purpose of the present study was to identify gene polymorphisms that confer susceptibility or resistance to type 2 diabetes mellitus, and thereby to contribute to the assessment of genetic risk for this condition.

Materials and methods

Study population. The study population comprised 5259 unrelated Japanese individuals (2980 men, 2279 women) who either visited outpatient clinics of or were admitted to one of the participating hospitals (Gifu Prefectural General Medical Center, Gifu Prefectural Tajimi Hospital, and Gifu Prefectural Gero Hotspring Hospital in Gifu Prefecture, Japan; and Hirosaki University Hospital, Reimeikyo Rehabilitation Hospital, and Hirosaki Stroke Center in Aomori Prefecture, Japan) between October 2002 and March 2007 because of various symptoms or for an annual health checkup. The 1640

Table I. Primers, probes, and other PCR conditions for genotyping.

Gene symbol	Polymorphism	Sense primer	Antisense primer	Probe 1	Probe 2	Annealing (˚C)	Cycles
CYP3A4	13989A→G (Ile118Val)	CAACCATggAgACCTCCACAA	TggTgAAggTTggAgACAgCA	TgCCATCTCTATAgCTgAg	TgCCgTCTCTATAgCTgA	60	50
GNB3	1429C→T	CCCCCAgAgCCACTACCTTTg	gCATgAATAAgAAgAgggCCAggA	gACCCTAgTggTgCCAgAg	gACCCTAgTAgTgCCAgAgC	60	50
HNRPUL1	A→T	AgCCACCgCgCCCgCCCTT	ggTCAgTACCTTCTgAAgTCTggTC	TCTgTCTATgTCTTTCTTATTC	TCTgTCTTTTgTCTTTCTTATTC	60	50
TNFRSF4	A→G	CCCCTTCTCCTATTCgggTTgg	CggCCCgTggAggTCCCCg	ggCCCCACTgggCTgggC	ggCCCCgCTgggCTgggC	60	50
APOA5	-3A→G	AAgTCCCCTgCgCCATgT	gCAgCCATgCTTgCCATTA	CTCAgAgCAggTAATggCA	CTCAgAgCAgATAATggCAA	60	50
ABCA1	102555G→A (Val771Met)	CACgCTgTACCTgCCCTACg	gCCACTgAAgAAAggCCAgA	CAggACTACgTgggCTTCA	CAggACTACATgggCTTCAC	60	50
KCNMB1	G→A (Glu65Lys)	AgTgCCACCTgATTgAgACCAACATC	gTCCTCCgTgTggTACAgCACAg	CAgggACCAggAgAAgCTg	ggACCAggAggAgCTg	60	50
CYP3A5	6986A→G (intron 3)	TggAgAgTggCATAggAgATACCC	TgTggTCCAAACAgggAAgAgA	TTgTCTTTCAgTATCTCTT	TTgTCTTTCAATATCTCTTC	60	50

subjects (1071 men, 569 women) with type 2 diabetes mellitus had a fasting plasma glucose concentration of ≥6.93 mmol/l (126 mg/dl) or a blood glycosylated hemoglobin (HbA_{1c}) content of ≥6.5% (or both) or were taking antidiabetes medication. Type 2 diabetes mellitus was defined according to the criteria accepted by the World Health Organization and described previously (11,12). Individuals with type 1 diabetes mellitus, maturity-onset diabetes of the young, other metabolic or endocrinologic diseases, or severe liver or renal dysfunction were excluded from the study. Individuals taking drugs that cause secondary diabetes mellitus were also excluded. The control subjects comprised 3619 individuals (1909 men, 1710 women) who had a fasting plasma glucose concentration of <6.05 mmol/l (110 mg/dl) and a blood HbA_{1c} content of <5.6% and who had no history of diabetes mellitus or of taking antidiabetes medication. The study protocol complied with the Declaration of Helsinki and was approved by the Committees on the Ethics of Human Research of Mie University School of Medicine, Hirosaki University School of Medicine, Gifu International Institute of Biotechnology, and participating hospitals, and written informed consent was obtained from each participant.

Genotyping of polymorphisms. Venous blood (7 ml) was collected into tubes containing 50 mmol/l EDTA (disodium salt), and genomic DNA was isolated with a kit (Genomix; Talent, Trieste, Italy). Genotypes of 94 polymorphisms of 67 candidate genes (data not shown) were determined at G&G Science (Fukushima, Japan) by a method that combines the polymerase chain reaction and sequence-specific oligonucleotide probes with suspension array technology (Luminex, Austin, TX, USA) (13-15). Primers, probes, and other PCR conditions for genotyping polymorphisms found to be related ($P<0.05$) to type 2 diabetes mellitus by the chi-square test are shown in Table I. A detailed genotyping methodology was described previously (13).

Statistical analysis. Clinical data were compared between subjects with type 2 diabetes mellitus and controls by the unpaired Student's t-test. Qualitative data were compared by the chi-square test. Allele frequencies were estimated by the gene counting method, and the chi-square test was used to identify departure from Hardy-Weinberg equilibrium. In the initial screen, genotype distributions for each polymorphism were compared between subjects with type 2 diabetes mellitus and controls with the chi-square test. Given the multiple comparisons of genotypes with type 2 diabetes mellitus, we calculated the false discovery rate (FDR) (16) from the distribution of P values for the 94 polymorphisms. Polymorphisms with an FDR of <0.05 were further examined in a more rigorous evaluation of association by multivariable logistic regression analysis with adjustment for covariates, with type 2 diabetes mellitus as a dependent variable and independent variables including age, sex (0, woman; 1, man), and genotype of each polymorphism. Polymorphism genotypes were assessed according to dominant, recessive, and two additive (additive 1 and 2) genetic models, and the P value, odds ratio, and 95% confidence interval were calculated. Each genetic model comprised two groups: the combined group of variant homozygotes and heterozygotes versus wild-type

Table II. Characteristics of the 5259 study subjects.

Characteristic	Diabetes mellitus	Controls	P
No. of subjects	1640	3619	
Age (years)	63.7±11.6	63.9±11.8	0.6055
Sex (male/female, %)	65.3/34.7	52.8/47.2	<0.0001
Body mass index (kg/m^2)	23.8±3.5	23.4±3.3	0.0001
Current or former smoker (%)	23.5	18.8	0.0001
Systolic blood pressure (mmHg)	145±27	141±26	<0.0001
Diastolic blood pressure (mmHg)	76±15	76±14	0.7915
Serum total cholesterol (mmol/l)	5.23±1.64	5.28±4.60	0.8118
Serum triglycerides (mmol/l)	1.77±1.82	1.47±0.97	<0.0001
Serum HDL-cholesterol (mmol/l)	1.27±0.47	1.40±0.44	<0.0001
Fasting plasma glucose (mmol/l)	9.30±4.02	5.23±0.83	<0.0001
Blood HbA_{1c} (%)	7.8±6.5	5.3±0.4	<0.0001

Quantitative data are means ± SD. Smoker, smoking of ≥10 cigarettes daily.

homozygotes for the dominant model; variant homozygotes versus the combined group of wild-type homozygotes and heterozygotes for the recessive model; heterozygotes versus wild-type homozygotes for the additive 1 model; and variant homozygotes versus wild-type homozygotes for the additive 2 model. For statistical analyses other than the initial screen of polymorphisms, a P value of <0.05 was considered significant. Statistical significance was examined by two-sided tests, and statistical analyses were performed with JMP version 5.1 software (SAS Institute, Cary, NC, USA).

Results

The characteristics of the 5259 study subjects are shown in Table II. The frequency of male subjects, body mass index, prevalence of smoking, systolic blood pressure, serum concentration of triglycerides, fasting plasma glucose level, and blood HbA_{1c} content were greater, whereas the serum concentration of high density lipoprotein (HDL)-cholesterol was lower, in subjects with type 2 diabetes mellitus than in controls. Comparisons of genotype distributions with the chi-square test revealed that the 13989A→G (Ile118Val) polymorphism of the cytochrome P450, subfamily IIIA, polypeptide 4 gene (*CYP3A4*) was significantly (FDR<0.05) associated with the prevalence of type 2 diabetes mellitus (Table III). There were no individuals with the *GG* genotype of this polymorphism. Seven additional polymorphisms were found to be related to type 2 diabetes mellitus on the basis of a P value of <0.05 (Table III). The genotype distributions of the 13989A→G (Ile118Val) polymorphism of *CYP3A4* were in Hardy-Weinberg equilibrium both in subjects with type 2 diabetes mellitus and in controls. Multivariable logistic regression analysis with adjustment for age and sex revealed that the 13989A→G (Ile118Val) polymorphism of *CYP3A4* was significantly associated with the prevalence of type 2 diabetes mellitus, with the *AG* genotype being protective against this condition (dominant and additive 1 models; odds ratio, 0.27; 95% confidence interval, 0.14 to 0.47; P=0.00002). For the total study population, the fasting plasma glucose level was lower in individuals with the *AG* genotype (5.83±2.23 mmol/l) than in those with the *AA* genotype (6.77±3.25 mmol/l, P=0.0394). We also examined the relation of the 13989A→G (Ile118Val) polymorphism of *CYP3A4* to type 2 diabetes mellitus for men and women separately. This polymorphism was significantly associated with type 2 diabetes mellitus both for men (P=0.0001, chi-square test) and for women (P=0.0013).

Discussion

We have examined the possible relations of 94 polymorphisms of 67 genes to type 2 diabetes mellitus in 5259 Japanese individuals. Our large-scale association study revealed that the 13989A→G (Ile118Val) polymorphism of *CYP3A4* was significantly associated with the prevalence of type 2 diabetes mellitus. The chromosomal region containing *CYP3A4* (7q22.1) has not previously been linked to type 2 diabetes mellitus in the Japanese population (17-21), and *CYP3A4* itself has not been identified as a gene that confers predisposition or resistance to this condition.

CYP3A4 is expressed in the prostate, breast, gut, colon, and small intestine, but its expression is most abundant in the liver, where it accounts for 30% of the total CYP protein content (22-25). It exhibits a broad substrate specificity and is responsible for oxidation of many therapeutic drugs and a variety of structurally unrelated compounds, including steroids, fatty acids, and xenobiotics. In liver microsomes, it contributes to a nicotinamide adenine dinucleotide phosphate (NADP)-dependent electron transport pathway. CYP3A4 plays an important role in the oxidation of both testosterone (2ß-, 6ß-, or 15ß-hydroxylation) and estrogen (4α- and 16α-hydroxylation) (26-28). Its expression is induced by various compounds including drugs, pesticides, and carcinogens, resulting in high CYP3A4 levels in liver and other tissues, including the mammary gland. *CYP3A4* is highly polymorphic, with at least 78 genetic variations having been identified (29). Such variation in *CYP3A4* may lead to a reduced potential for oxidation of testosterone, increasing the amount of this hormone available for metabolism to the biologically active form of dihydroxytestosterone, the principal androgenic hormone responsible for regulation of prostate growth. The 13989A→G (Ile118Val) polymorphism of *CYP3A4* has been associated with the activity of CYP3A4, with the activity in heterozygotes (*AG* genotype) being lower than that in wild-type homozygotes (*AA* genotype) (30).

Several epidemiologic studies have examined the relations between *CYP3A4* genotype and breast or prostate cancer. A US study showed that the *CYP3A4*1B* genotype (392A→G polymorphism in the promoter region) is related to early menarche, a risk factor for breast cancer (31). However, an Australian case-control study failed to detect a relation between *CYP3A4*1B* and breast cancer (32). Two Scottish prospective studies showed *CYP3A4*1B* to be a risk factor for prostate cancer among men with benign prostate hyperplasia (33,34). Three other studies undertaken in the United States [two case-only studies (35,36) and a case-sibling control study (37)] revealed that, although the results for African-Americans

Table III. Genotype distributions of polymorphisms related to type 2 diabetes mellitus as determined by the chi-square test.

Gene symbol	Polymorphism	Diabetes mellitus	Controls	P	FDR
CYP3A4	13989A→G (Ile118Val)			0.0000001	0.000009
	AA	1628 (99.3)	3517 (97.2)		
	AG	12 (0.7)	102 (2.8)		
	GG	0 (0)	0 (0)		
GNB3	1429C→T			0.0016	0.0752
	CC	1068 (65.1)	2480 (68.5)		
	CT	508 (31.0)	959 (26.5)		
	TT	64 (3.9)	181 (5.0)		
HNRPUL1	A→T			0.0078	0.2444
	AA	1072 (65.5)	2527 (69.8)		
	AT	507 (31.0)	978 (27.0)		
	TT	59 (3.6)	117 (3.2)		
TNFRSF4	A→G			0.0096	0.2256
	AA	35 (2.1)	72 (2.0)		
	AG	424 (25.9)	800 (22.1)		
	GG	1181 (72.0)	2753 (75.9)		
APOA5	-3A→G			0.0145	0.2726
	AA	675 (41.2)	1610 (44.5)		
	AG	744 (45.4)	1609 (44.5)		
	GG	221 (13.5)	401 (11.1)		
ABCA1	102555G→A (Val771Met)			0.0298	0.4669
	GG	1465 (89.3)	3147 (86.9)		
	GA	170 (10.4)	452 (12.5)		
	AA	5 (0.3)	21 (0.6)		
KCNMB1	G→A (Glu65Lys)			0.0391	0.5251
	GG	1337 (81.6)	2846 (78.6)		
	GA	282 (17.2)	729 (20.1)		
	AA	20 (1.2)	48 (1.3)		
CYP3A5	6986A→G (intron 3)			0.0446	0.5241
	AA	85 (5.2)	246 (6.8)		
	AG	604 (36.8)	1363 (37.7)		
	GG	951 (58.0)	2011 (55.6)		

Numbers in parentheses are percentages.

were inconsistent, *CYP3A4*1B* was associated with markers of advanced prostate cancer in Caucasians.

We have now shown that the 13989A→G (Ile118Val) polymorphism of *CYP3A4* was significantly associated with type 2 diabetes mellitus, with the *G* allele protecting against this condition. As mentioned above, CYP3A4 plays important roles in the metabolism of testosterone and estrogen (38), and the 13989A→G (Ile118Val) polymorphism is related to the activity of CYP3A4 (30). We previously showed that the association of gene polymorphisms with type 2 diabetes mellitus is sex-specific in Japanese individuals, which may be attributable to differences in the plasma levels of sex hormones (15). The association of the 13989A→G (Ile118Val) polymorphism of *CYP3A4* with type 2 diabetes mellitus might thus be attributable to the effect of this polymorphism on the metabolism of sex hormones. This is the first demonstration of an association of this polymorphism of *CYP3A4* with type 2 diabetes mellitus, although the underlying molecular mechanism remains to be elucidated.

It is possible that the 13989A→G (Ile118Val) polymorphism of *CYP3A4* is in linkage disequilibrium with other polymorphisms of this gene or with those of nearby genes that are actually responsible for the development of type 2 diabetes mellitus. Our present results, however, suggest that *CYP3A4* is a susceptibility locus for type 2 diabetes mellitus in the Japanese population. Determination of the genotype for this polymorphism may prove informative for assessment of the genetic risk for type 2 diabetes mellitus.

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