Mitochondrial Haplogroup N9a Confers Resistance against Type 2 Diabetes in Asians

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Because mitochondria play pivotal roles in both insulin secretion from the pancreatic β cells and insulin resistance of skeletal muscles, we performed a large-scale association study to identify mitochondrial haplogroups that may confer resistance against or susceptibility to type 2 diabetes mellitus (T2DM). The study population comprised 2,906 unrelated Japanese individuals, including 1,289 patients with T2DM and 1,617 controls, and 1,365 unrelated Korean individuals, including 732 patients with T2DM and 633 controls. The genotypes for 25 polymorphisms in the coding region of the mitochondrial genome were determined, and the haplotypes were classified into 10 major haplogroups (i.e., F, B, A, N9a, M7a, M7b, G, D4a, D4b, and D5). Multivariate logistic-regression analysis with adjustment for age and sex revealed that the mitochondrial haplogroup N9a was significantly associated with resistance against T2DM ($P = .0002$) with an odds ratio of 0.55 (95% confidence interval 0.40–0.75). Even in the modern environment, which is often characterized by satiety and physical inactivity, this haplogroup might confer resistance against T2DM.

Type 2 diabetes mellitus (T2DM [MIM 125853]) is a complex disorder characterized by impaired insulin secretion from pancreatic β cells and reduced insulin action or insulin resistance in the peripheral tissue. There is a growing body of evidence indicating that mitochondrial dysfunction plays a pivotal role in β-cell dysfunction, as well as in insulin resistance. Mitochondrial metabolism, which produces ATP, is essential in insulin secretion through metabolism-secretion coupling.[1] A pancreatic β-cell line lacking mitochondrial function exhibits impaired insulin secretion,[2] and mice with pancreatic β-cell–specific knockout of mitochondrial transcription factor Tfam show a diabetic phenotype with severe mtDNA depletion.[3] Decreased capacity of the mitochondrial oxidative phosphorylation (OXPHOS) is associated with the insulin resistance found in aged people and in offspring of individuals with T2DM.[4,5] Microarray studies have shown that insulin resistance and T2DM are associated with decreased expression of genes related to OXPHOS in the skeletal muscle.[6,7] Therefore, mitochondrial dysfunction can explain not only impaired insulin secretion but also reduced insulin action.

Proteins composing the mitochondrion are encoded by both nuclear DNA and mtDNA. The latter encodes 13 subunits of the OXPHOS machinery and also encodes 2 ribosomal RNA (rRNA) and 22 tRNA genes essential for the translation process in mitochondria.[8] There are many lines of evidence indicating that mtDNA is responsible for the pathogenesis of diabetes. A point mutation at nucleotide position 3243 in mitochondrial tRNA-Leu (UUR) is well known to cause maternally inherited diabetes and deafness, as well as mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike (MELAS) episodes in patients with high mutant loads.[1] However, it remains questionable whether mitochondrial dysfunction originating from common mtDNA polymorphisms is responsible for T2DM. In this regard, it should be noted that many epidemiologic studies have reported a maternal excess in the transmission of T2DM.[9,10] In addition, a control-region polymorphism, such as the 16189T→C substitution in the noncoding region, is known to be associated with insulin resistance, obesity, and diabetes in both Europeans[11] and Asians.[12,13] A meta-analysis of European studies, however, has indicated that genetic variation of the 16184–16193 poly-C tract is unlikely to have a major role in the cause of T2DM.[14]

The geographic region–specific variations of mtDNA haplogroups are now known to have been formed by natural selection, possibly to allow habitation in cold climatic environments.[15,16] Although mtDNA variations might have permitted our ancestors to adapt to more-northern or colder climates, they are also suggested to play a detrimental role in modern human diseases related to bioenergetics or mitochondrial dysfunction.[15–17] Therefore,

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Received October 18, 2006; accepted for publication December 16, 2006; electronically published January 22, 2007.

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Am. J. Hum. Genet. 2007;80:407–415. 0002-9297/2007/8003-0003$15.00
DOI: 10.1086/512202

some mtDNA haplogroup might actually confer susceptibility to T2DM. It has been very recently reported that there is no evidence of association between common mtDNA polymorphisms and T2DM, at least not in Europeans.[18] Since Asians have different mtDNA haplogroups and since T2DM is the result of complex interactions between genes and the environment, the above finding cannot be extended to the Asian populations. In the present study, we performed a large-scale association study on T2DM and 10 major haplogroups in both Japan and Korea, on the basis of comprehensive analysis of polymorphisms in the coding region of the mitochondrial genome.

Material and Methods

Study Population

The study population comprised 2,906 Japanese and 1,365 Korean subjects. Unrelated Japanese individuals (1,938 men and 968 women) aged ⩾40 years were enrolled from the population of individuals who had either visited outpatient clinics of or been admitted to one of the participating hospitals (Gifu Prefectural Gifu, Tajimi, and Gero Hotspring Hospitals) between October 2002 and March 2005. The patients with T2DM had a fasting plasma glucose (FPG) concentration of ⩾7.0 mmol/liter (126 mg/dl) and/or a blood glycosylated hemoglobin (HbA1c) level of ⩾6.5% or were taking antidiabetes medication. T2DM was defined according to the criteria accepted by the World Health Organization. Members of families with diabetes mellitus and sib pairs with this condition were excluded from the study. Although some of the patients with T2DM who were taking antidiabetes medication had a normal HbA1c level or a normal FPG concentration when the blood samples were obtained, they had exhibited abnormally high levels of HbA1c and FPG before starting the antidiabetes medication. We excluded the patients with type 1 diabetes who required insulin within 1 year after the initial diagnosis or episode of diabetic ketoacidosis.

On the basis of these criteria, 1,289 subjects (890 men and 399 women) in the Japanese study population were given diagnoses of T2DM. The control group comprised the remaining 1,617 individuals (1,048 men and 569 women) in the Japanese study population who visited the outpatient clinics of the participating hospitals for an annual health checkup. They had an FPG concentration of <6.1 mmol/liter (110 mg/dl) and a blood HbA1c level of <5.6%, and they had no history of T2DM or of taking antidiabetes medication. The study protocol was approved by the Committee on the Ethics of Human Research of Gifu International Institute of Biotechnology, and written informed consent was obtained from each participant.

Unrelated Korean patients with T2DM were enrolled from the Diabetes Clinic of Seoul National University Hospital (n = 732). Control subjects without diabetes were recruited from the group of individuals who had visited Seoul National University Hospital for a routine annual checkup (n = 633). T2DM was diagnosed according to World Health Organization criteria. Subjects with positive glutamic acid decarboxylase antibodies were excluded. The control subjects without diabetes were selected according to the following criteria: age ⩾60 years, no past history of diabetes, no diabetes in first-degree relatives, an FPG concentration of <6.1 mmol/liter, and an HbA1c value of <5.8%. The Institutional Review Board of the Clinical Research Institute in Seoul National University Hospital approved the study protocol, and written informed consent for genetic analysis was obtained from each subject. All study subjects were examined in the morning after an overnight fast. The clinical characteristics of Japanese and Korean subjects are shown in tables 1 and 2, respectively.

Selection of Mitochondrial Polymorphisms for Haplogroup Classification

In earlier studies, we aimed to identify mitochondrial SNPs (mtSNPs) associated with age-related conditions, such as longevity,[19] Parkinson disease,[20,21] and Alzheimer disease, as well as those related to energy metabolism—such as obesity,[22,23] thinness, and T2DM[22]—or to atherosclerosis. For this purpose, we sequenced the entire mitochondrial genomes of 672 individuals belonging to seven different groups, with 96 individuals in each group—namely, centenarians, patients with Parkinson disease, patients with Alzheimer disease, young obese or nonobese males, and patients with T2DM with or without severe vascular involvement.[24] From our findings, we constructed a human mitochondrial genome polymorphism database (mtSNP). On the basis of these mtSNP data, we have developed a comprehensive mtSNP analysis system that uses fluorescent beads.

By using our mtSNP database and a phylogenetic tree of the Japanese,[24] we selected 149 polymorphic sites that have been useful for classification of mitochondrial haplogroups. We selected a further 25 mtSNPs that define 10 major haplogroups (i.e., F, B, A, N9a, M7a, M7b, G, D4a, D4b, and D5) found in this area (table 3). Then, we examined the relationship between these haplogroups and T2DM in the 4,271 participants.

Genotyping of Polymorphisms

Venous blood (7 ml) was collected from each subject into tubes containing 50 mmol/liter EDTA (disodium salt), and genomic DNA was isolated with the use of a commercial kit (Genomix [Talent]). For amplifying mtDNA fragments, we performed 28-plex PCR. The reaction mixture (25 μl) contained 1 ng of genomic DNA, 5 pmol of each primer, 0.2 mmol/liter of each deoxynucleoside triphosphate, 2 mmol/liter $MgCl_2$, and 1 U of DNA polymerase (FastStart Taq DNA Polymerase [Roche Diagnostics]) in the PCR buffer supplied by the manufacturer. The amplification protocol consisted of an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 7 min. The primers used are shown in table 4. Mitochondrial polymorphisms were determined with sequence-specific oligonucleotide probes (G&G Science) by use of suspension-array technology (Luminex 100 [Luminex]). The methodology used for genotyping was described in detail elsewhere.[25] Probes used for haplotyping are shown in tables 5 and 6. To confirm the accuracy of genotyping by this method, we subjected 91 DNA samples whose entire sequence of the mitochondrial genome had been determined by direct sequencing to the Luminex method. In each instance, the genotype determined by the Luminex sequence-specific oligonucleotide-hybridization assay system was identical to that determined by direct sequencing.

Statistical Analysis

Quantitative clinical data were compared between patients with diabetes and control individuals by use of the unpaired Student's t test. Qualitative data were compared using the χ^2 test. We performed multivariate logistic-regression analysis to adjust for risk

Table 1. Characteristics of Japanese Patients with T2DM and Controls

	All			Women			Men		
Variable	T2DM (n = 1,289)	Controls (n = 1,617)	P	T2DM (n = 399)	Controls (n = 569)	P	T2DM (n = 1,289)	Controls (n = 1,048)	P
Age (years)	63.5 ± 11.6 (25–92)	65.5 ± 11.0 (18–95)	<.0001	65.2 ± 11.9 (26–90)	66.1 ± 11.4 (18–95)	.2290	62.7 ± 11.3 (25–92)	65.2 ± 10.8 (22–94)	<.0001
Sex (% female/% male)	30.9/69.1	35.2/64.8	.0140	…	…	…	…	…	…
BMI (kg/m^2)	23.7 ± 3.5 (13.2–42.6)	23.1 ± 3.2 (13.6–34.2)	<.0001	23.5 ± 3.9 (13.2–39.4)	23.0 ± 3.5 (13.6–34.2)	.0270	23.8 ± 3.3 (14.6–42.6)	23.2 ± 3.0 (14.1–34.1)	<.0001
Blood pressure (mmHg):									
Systolic	146 ± 27 (82–256)	142 ± 26 (70–254)	.0004	150 ± 29 (88–256)	145 ± 26 (89–254)	.0200	145 ± 26 (82–250)	141 ± 25 (70–244)	.0030
Diastolic	77 ± 15 (30–166)	76 ± 15 (31–146)	.0420	77 ± 15 (41–166)	76 ± 15 (38–130)	.1710	77 ± 15 (30–132)	76 ± 14 (31–146)	.1300
Total cholesterol (mmol/liter)	5.21 ± 1.01 (2.26–10.50)	5.24 ± .98 (2.60–9.02)	.6470	5.50 ± 1.15 (2.94–10.50)	5.43 ± 1.02 (2.81–9.02)	.4300	5.10 ± .93 (2.26–8.22)	5.12 ± .95 (2.60–8.87)	.5630
Triglycerides (mmol/liter)	1.80 ± 1.37 (.15–19.62)	1.58 ± 1.04 (.13–16.90)	<.0001	1.60 ± .93 (.44–7.90)	1.42 ± .84 (.29–5.54)	.0170	1.94 ± 1.50 (.15–19.62)	1.66 ± 1.12 (.13–16.90)	.0020
HDL cholesterol (mmol/liter)	1.26 ± .44 (.42–6.01)	1.33 ± .45 (.36–9.31)	.0005	1.40 ± .43 (.62–3.64)	1.45 ± .38 (.65–3.22)	.1440	1.20 ± .42 (.42–6.01)	1.26 ± .46 (.36–9.31)	.0110
FPG (mmol/liter)	9.32 ± 3.98 (3.80–33.72)	5.40 ± .76 (2.81–6.88)	<.0001	9.44 ± 3.94 (3.63–26.40)	5.41 ± .76 (3.25–6.88)	<.0001	9.27 ± 4.00 (3.80–33.72)	5.40 ± .76 (2.81–6.88)	<.0001
HbA1c (%)	7.5 ± 2.2 (4.4–16.4)	5.3 ± .4 (3.8–6.4)	<.0001	7.9 ± 2.3 (4.7–15.0)	5.2 ± .4 (3.8–6.4)	<.0001	7.3 ± 2.2 (4.4–16.4)	5.3 ± .4 (4.1–6.2)	<.0001

NOTE.—Values are given as means ± SDs, with ranges in parentheses.

Table 2. Characteristics of Korean Patients with T2DM and Controls

	All			Women			Men		
Variable	T2DM (n = 732)	Controls (n = 633)	P	T2DM (n = 393)	Controls (n = 351)	P	T2DM (n = 339)	Controls (n = 282)	P
Age (years)	59.5 ± 9.4 (32–83)	64.7 ± 3.6 (60–93)	<.0001	60.0 ± 9.1 (32–81)	64.4 ± 3.4 (60–75)	<.0001	59.0 ± 11.3 (32–83)	64.9 ± 3.8 (60–93)	<.0001
Sex (% female/% male)	53.8/46.3	55.5/44.6	.5295	…	…	…	…	…	…
BMI (kg/m^2)	24.4 ± 2.8 (16.5–35.0)	23.6 ± 3.1 (14.7–32.8)	<.0001	24.8 ± 3.1 (16.5–35.0)	24.1 ± 3.2 (14.7–32.8)	.0026	24.1 ± 2.5 (16.5–32.2)	23.0 ± 2.8 (16.0–32.0)	<.0001
Blood pressure (mmHg):									
Systolic	135 ± 20 (88–200)	128 ± 20 (87–203)	<.0001	135 ± 21 (88–200)	129 ± 20 (88–202)	<.0001	135 ± 19 (90–199)	128 ± 19 (87–203)	<.0001
Diastolic	81 ± 12 (36–120)	80 ± 11 (51–120)	.0834	80 ± 12 (40–120)	79 ± 11 (51–120)	.1017	82 ± 12 (36–113)	81 ± 11 (51–113)	.4883
Total cholesterol (mmol/liter)	5.15 ± .97 (1.87–9.33)	4.98 ± .91 (2.47–8.74)	.0011	5.29 ± .98 (2.68–9.33)	5.10 ± .89 (3.20–8.09)	.0063	5.00 ± .94 (1.87–8.97)	4.84 ± .92 (2.47 –8.74)	.0039
Triglycerides (mmol/liter)	1.88 ± 1.28 (.36–12.23)	1.39 ± .70 (.36–5.83)	<.0001	1.83 ± 1.15 (.42–11.41)	1.42 ± .71 (.36–5.83)	<.0001	1.93 ± 1.43 (.36–12.23)	1.36 ± .68 (.50–4.92)	<.0001
HDL cholesterol (mmol/liter)	1.23 ± .05 (.34–2.60)	1.20 ± .07 (.52–2.52)	.1463	1.28 ± .05 (.60–2.60)	1.19 ± .05 (.60–2.26)	<.0001	1.16 ± .02 (.34–2.31)	1.21 ± .09 (.52–2.52)	.0497
FPG (mmol/liter)	8.54 ± 2.53 (3.74–21.29)	4.96 ± 0.49 (3.69–6.05)	<.0001	8.61 ± 2.55 (3.74–18.37)	4.95 ± .49 (3.85–6.05)	<.0001	8.47 ± 2.51 (3.96–21.29)	4.97 ± .50 (3.69–6.05)	<.0001
HbA1c (%)	8.0 ± 1.6 (4.2–14.4)	5.3 ± .3 (4.1–5.8)	<.0001	8.1 ± 1.6 (4.2–14.4)	5.3 ± .3 (4.1–5.8)	<.0001	7.9 ± 1.6 (4.4–14.3)	5.3 ± 1.3 (4.1–5.8)	<.0001

NOTE.—Values are given as means ± SDs, with ranges in parentheses.

Table 3. Polymorphic Sites Characteristic to 10 Major Haplogroups

Haplogroup	Polymorphism(s)[a]
F	3970C→T (ND1: syn), 13928G→C (ND5: S531T), 10310G→A (ND3: syn)
B	8272 (9-bp deletion in noncoding region)
A	663A→G (12S rRNA), 8794C→T (ATP6: H90Y)
N9a	5231G→A (ND2: syn), 12358A→G (ND5: T8A), 12372G→A (ND5: syn)
M7a	2772C→T (16S rRNA), 4386T→C (tRNA-Gln)
M7b	4071C→T (ND1: syn), 4048G→A (ND1: D248N), 6680T→C (CO1: syn), 12811T→C (ND5: Y159H)
G	709G→A (12S rRNA), 4833A→G (ND2: T122A), 5108T→C (ND2: syn)
D4a	4883C→T (ND2: syn), 5178C→A (ND2: L237M), 3010G→A (16S rRNA), 14979T→C (Cytb: I78T),[b] 8473T→C (ATP8: syn)
D4b	4883C→T (ND2: syn), 5178C→A (ND2: L237M), 3010G→A (16S rRNA), 1382A→C (12Ss rRNA)
D5	4883C→T (ND2: syn), 5178C→A (ND2: L237M), 10397A→G (ND3: syn)

[a] syn = Synonymous mutation.
[b] Ctyb = cytochrome *b*.

factors, with T2DM as a dependent variable and independent variables including age, sex (0 = female and 1 = male), and genotype of each mtSNP. The *P* value, odds ratio (OR), and 95% CI were calculated. Unless indicated otherwise, a *P* value <.05 was considered statistically significant. Because of multiple comparisons of haplogroups, we applied Bonferroni correction. Since we examined 10 haplogroups, we divided .05 by 10 to get .005. Thus, a *P* value <.005 was considered statistically significant.

Results

The characteristics of the 2,906 Japanese subjects are shown in table 1. BMI, systolic and diastolic blood pressure, serum concentration of triglycerides, FPG concentration, and blood HbA1c level were significantly higher in patients with T2DM than in the controls ($P < .05$). Age, female:male ratio, and serum concentration of high-density lipoprotein (HDL) cholesterol were lower in the patients with diabetes than in the controls ($P < .05$).

The characteristics of the 1,365 Korean subjects are shown in table 2. The subjects with diabetes were significantly younger than the controls ($P < .05$). BMI, systolic blood pressure, serum concentrations of total cholesterol and triglycerides, FPG concentration, and blood HbA1c level were significantly higher in the subjects with T2DM than in the controls ($P < .05$).

Ten common mtDNA haplogroups accounted for 72.4% and 68.2% of haplogroups in Japanese and Korean subjects, respectively (table 7). When we combined Japanese and Korean subjects, multivariate logistic-regression analysis with adjustment for age and sex (table 8) showed that the subjects in the mitochondrial haplogroup N9a had a

Table 4. Primers Used for 28-Plex PCR

	Primer				
	Forward		Reverse		Product
Fragment	Position	Sequence (5′→3′)	Position	Sequence (5′→3′)	(bp)
1	631	ACATCACCCCATAAACAAATAggTT	931	gCTTCTATTgACTTgggTTAATCg	301
2	1272	AgCAAACCCTgATgAAggCTAC	1781	TATATCTATTgCgCCAggTTTCAAT	510
3	2698	AgAggCgggCATgACACAgCA	3066	gATCACgTAggACTTTAATCgTTgA	369
4	3215	CCAAgAACAgggTTTgTTAAgATg	3569	ggggTTCATAgTAgAAgAgCgAT	355
5	3611	TCCTATTTATTCTAgCCACCTCTAg	3862	ATCATATTATggCCAAgggTCATg	252
6	3916	gAgTCCgAACTAgTCTCAggCT	4255	gAgggggAATgCTggAgATTgTA	340
7	4344	TCgAACCCATCCCTgAgAATCC	4577	gTTTATTTCTAggCCTACTCAggTAA	234
8	4623	TCCACAgAAgCTgCCATCAAgTA	4940	gAgAgTgAggAgAAggCTTACgT	318
9	4989	CAgCTACgCAAAATCTTAgCATAC	5257	TTgggCAAAAAgCCggTTAgCg	269
10	5921	ACTATTCTCTACAAACCACAAAgAC	6284	TgTTCAACCTgTTCCTgCTCCg	364
11	6535	CAgACCgCAACCTCAACACCAC	6807	gTgTgTCTACgTCTATTCCTACTg	273
12	7567	CTAAATCCTATATATCTTAATggCAC	7895	ATTggTggCCAATTgATTTgATggT	329
13	8153	ggggTATACTACggTCAATgCTC	8530	TCATTTTggTTCTCAgggTTTgTTAT	378
14	8628	CAAATATCTCATCAACAACCgACTA	8994	CAgggCTATTggTTgAATgAgTAg	367
15	9044	TAATTggAAgCgCCACCCTAgC	9414	ggCCTTggTATgTgCTTTCTCgT	371
16	9673	gAAACCAAATAATTCAAgCACTgCT	9987	ACCCTCATCAATAgATggAgACAT	315
17	10277	ACCCCTACCATgAgCCCTACAA	10515	gTgAgATggTAAATgCTAgTATAATAT	239
18	10983	TCACAATCATggCAAgCCAACgC	11280	AgTgAgCCTAgggTgTTgTgAg	298
19	11667	TCCAAACCCCCTgAAgCTTCAC	12137	AAgAggAAAACCCggTAATgATgT	471
20	12274	AggATAACAgCTATCCATTggTCT	12545	gTggCTCAgTgTCAgTTCgAgAT	272
21	12582	AgACTACTTCTCCATAATATTCATCC	12858	gTATAggATTgCTTgAATggCTgC	277
22	13077	CCACTCAAgCACTATAgTTgTAgC	13591	TCAgggAggTAgCgATgAgAgTA	515
23	13711	gCCggAAgCCTATTCgCAggAT	13980	CAggTTTTggCTCgTAAgAAggC	270
24	14217	CTAATCAACgCCCATAATCATACAA	14562	gTCgggTgTgTTATTATTCTgAATTT	346
25	14829	TCCgCATgATgAAACTTCggCT	15175	ggCCCCTCAgAATgATATTTggC	347
26	15257	gACAgTCCCACCCTCACACgAT	15600	gggACggATCggAgAATTgTgT	344
27	15696	TTCgCCCACTAAgCCAATCACTT	16037	TCCCCATgAAAgAACAgAgAATAgT	342
28	16421	ATATCCCgCACAAgAgTgCTACT	45	TggAgAgCTCCCgTgAgTggTT	194

Table 5. Probe Set A for Haplotyping

Position	Purpose[a]	Sequence (5′→3′)
681	*a*	TgTAATCTTACTgAgAgCTAAT
681	*b*	TgTAATCTTACTAAgAgCTAA
752	*a*	CgTgCTTgATgCTTATTCCTTTTgA
856	*a*	AAAgTTTAACTAggCTATACTA
1310	*a*	CgTCTTTACgTggATACTTgC
1382	*a*	ggCTATCgTAgTTgTCTggg
1442	*a*	AACTAAgCACTCTATTCTCAgT
1647	*p*	AggAgATTTCAACTTAACTTgA
2766	*a*	gACCTgTgggTTTATTAggTA
3010	*a*	ATCAggACATCCCAATggTg
3010	*b*	ATCAggACATCCCgATggT
3027	*a*	TgCAgCCgCTATCAAAgg
3458	*p*	gCCATAAAACTCTTCACCAA
3496	*a*	CCCTAAAACCCTCCACATc
3497	*a*	CCTAAAACCCgTCACATC
3644	*a*	ggATTgAgTAAgCggCT
3667	*p*	TAgTTTgAgTTTgATgCTCA
4048	*a*	CTAggAACAACATATAACgCACTC
4071	*a*	gACAAAATATgTTgTATAgAgTTC
4071	*b*	ACAAAATATgTTgTgTAgAgTTC
4086	*a*	AAgTAgggTCTTggTAACAAAATA
4386	*a*	ggTgTggTAggTggCAC
4386	*b*	gggTgTgATAggTggC
4491	*a*	CTggCCCAACCCATCATCTA
4505	*a*	gTCATCTACTCTACTATCTTTg
4541	*a*	CAgCgCTAAgCTCACACTgA
4833	*a*	AggTTACCCAAggCgCCCCT
4895	*a*	CCATCTCAATCATgTACCAA
4895	*b*	ATCTCAATCATATACCAAATC
5108	*a*	TTATCCTAACTACCACCgCA
5147	*a*	CTCCAgCACCACAACC
5178	*a*	TgAAACAAgATAACATgAC
5178	*b*	CTgAAACAAgCTAACATgA
5231	*a*	TCCCTAggAggCCTACCCCC
5964	*a*	ATgCgCCgAATAgTAggTAT
6023	*a*	TggCTggCCCAgTTCggCT
6086	*a*	CgTCACAgCCCACgCATTTg
6086	*b*	AgATTATTACAAATgCATgggCT
6253	*a*	CTCgCATCTgCTACAgTggA
6689	*a*	TggTTCTTTTTTTCCAgAgTAgT
6752	*a*	CAATTggCTTCCTggggTT
6752	*b*	CAATTggCTTCCTAgggTTT
8272	*a*	CTCTAgAgggggTAgAggTggTgCT
8272	*b*	TgggCTCTAgAggTggTgCTAT
8392	*a*	gTAATTATggTgggTCATACg
8684	*a*	ATCATTTgTTTTgAgATTAgTTT
8701	*b*	AgTgTTgTgTATggTTATCAT
8731	*p*	TgATTAAggATACTAgTATAAgAg
8784	*a*	TAACCTCCTCgggCTCCTgC
8793	*a*	CggACTCCTgCCCCACTCA
8794	*a*	TggTgTAAATgAgTAAggCAgg
8829	*a*	CAACTATCTATAAATCTAgCC
9123	*a*	gATTTCTAggATAgTTAgTAgAAT
8794	*a*	TggTgTAAATgAgTAAggCAgg
8829	*a*	CAACTATCTATAAATCTAgCC
9123	*a*	gATTTCTAggATAgTTAgTAgAAT
9219	*p*	ATCACATgCCTATCATATAgTA
9296	*a*	CTAATgACCTCCggTCTAgCC
9755	*a*	TCAgAgTACTTCgAATCTCCC
9774	*p*	ATgCCgTCggAAATggTgA
9950	*a*	ATTTTgTAgATgTggTCTgACTA
10310	*a*	ACTATTAgTggTAggTTAgTT
10397	*a*	TACCAATTCAgCCCAgTCTAAT
10400	*m*	ACTATATACCAATTCAgCTCAgT
10400	*n*	ACTATATACCAATTCggTTCAgT
11084	*a*	gTggCTgTgAATgCTATAATTA
11215	*a*	TACTTCCTATTCTATACCCTAg
11215	*b*	TACTTCCTATTCTACACCC
11963	*a*	AggACTCAACATACTAATCACA
12063	*p*	ACCCTCATgTTCATACACCT
12501	*a*	CAACAATATTCATATgCCTAg
12501	*b*	ACAACAATATTCATgTgCCT
12705	*a*	CTACTCATTTTCCTAATTACCA
12775	*p*	TAggAATTATATCCTTCTTgC
12811	*a*	TCATCAgTTgATgACACgCCC
13105	*a*	ATgAgTAAgAAgACTCCTgC
13143	*a*	TggATTAgTgggCTgTTTTC
13156	*p*	CTAAgCATAgTgTTAgAgTTTg
13263	*a*	TATTATgAgTCCTAgCTgACTTg
13563	*a*	gCCTgAgCCCTgTCTAT
13928	*a*	ggATTCTACCCTACCATCA
13928	*b*	gATTCTACCCTAgCATCA
14343	*p*	gTgggTgAAAgAgTATgATg
14476	*a*	CTgTAgTATATCCAAAAACAACC
14893	*a*	TgCATggCTAggAACAgTCCT
14893	*b*	gCATggCTAggAATAgTCC
14927	*a*	ATgAAAAggCggCTgAgg
14944	*a*	gAgTgATgTgggCAATTgAT
14979	*a*	AAggTAgCggATggTTCAgC
15067	*a*	TATATTACggATCATTCCTCTAC
15346	*a*	CACCTCCTATTCTTACACgAAA
15440	*a*	ACgCCCTCggCCTACTTCT
15487	*a*	TATTCTCACCTgACCTCCT
15497	*a*	CCAgACCTCCTAAgCgAC
15524	*a*	TTATACCCTAgCCgACC
15535	*a*	CCAACCCCTTAAATACCCCTC
15535	*b*	AACCCCTTAAACACCCCTCC
15826	*p*	gTTggTATTAggATTAggATTgTT
15860	*a*	gAgTATTTTgTTTTCAACTAgggA
15874	*a*	AggCCCATTTgAgCATTTTgTT
15924	*a*	gTTTTCATCTCCggCTTACAAg
16519	*a*	TTCCTACTTCAgggCCATAAAg
16519	*b*	TCCTACTTCAgggTCATAAAgC

(*continued*)

Table 5. (continued)

NOTE.—Probes used for the first set of hybridization.

[a] Purposes for probes are as follows: *a,* for detecting polymorphism; *b,* for detecting wild type; *p,* for verifying PCR product; *m,* for detecting macrohaplogroup M; and *n,* for detecting macrohaplogroup N.

significantly reduced risk of T2DM (OR 0.55 [95% CI 0.40–0.75], $P = .0002$), whereas those in haplogroup F or D5 tended to have an increased risk of T2DM. We performed multiple-regression analysis of haplogroup N9a associated with T2DM, with adjustment not only for age and sex but also for BMI, systolic and diastolic blood pressure, total cholesterol, triglycerides, and HDL cholesterol (table 9). Even after adjustment for these parameters, logistic-regression analysis demonstrated that haplogroup N9a is an independent protective factor against T2DM for all Korean subjects ($P = .017$, OR 0.47 [95% CI 0.24–0.86]), Korean men ($P = .023$, OR 0.36 [95% CI 0.14–0.83]), all Japanese subjects ($P = .048$, OR 0.57 [95% CI 0.32–0.98]), and Japanese women ($P = .030$, OR 0.19 [95% CI 0.03–0.69]).

We examined the relationships of the three mtSNPs that were used for determination of the haplotype N9a to the prevalence of T2DM in all populations, by multivariable

Table 6. Probe Set B for Haplotyping

Position	Purpose[a]	Sequence (5′→3′)
663	*a*	gCTAATAgAAAggCCAggA
709	*a*	gAACTCACTggAATggggAT
827	*a*	AACAgCAgTgATTAgCCTTTA
1391	*a*	TTTCATAAgggCTgTCgTAgT
1438	*a*	AgCACTCTACTCTTAgTTTACT
1664	*a*	AACTTAACTTgACCACTCTgA
2772	*a*	gTTTAggACCTgTAggTTTg
2835	*a*	TgCTCggAggTTgAgTTCTg
3243	*a*	gATTACCgggCCCTgCCAT
3254	*a*	TTTTAAgTTTTATgCAATTACCg
3421	*a*	gCAAAggCCCCAACATTgTAg
3537	*a*	CCCCgACCTTggCTCTC
3546	*a*	CTTAgCTCTCACTATCgC
3696	*a*	gCTCgCAgTgCgCCAATCAg
3714	*a*	gAgATTgTTTgggCCACTgCT
3714	*b*	gAgATTgTTTgggCTACTgCT
3759	*a*	gCCATCATTCTACTgTCAACA
3970	*a*	ggCTATgAAgAATAAggCgA
4538	*a*	AAAAATCAgTgCgAACTTAgCg
4655	*a*	gCggTTgCTTgTgTgAggA
4688	*a*	TTgTTgAAgAggATggCTATT
4715	*a*	TTATggTTCATTgCCCggAg
4820	*a*	TTCTgAgTCCCAgAAgTTACCC
4850	*a*	CTgACATCCggTCTgCTT
4883	*a*	AACTAgCCCCTATCTCAAT
5127	*a*	ATTCCTACTACTCgACTTAA
6005	*a*	ggCTCgAATAAggAgACTTAgA
6018	*a*	gCCTCCTTATTCgAACCgAgC
6146	*a*	gCTTTggCAACTggCTAgTTC
6146	*b*	gCTTTggCAACTgACTAgTTC
6179	*a*	gTgCCCCCgATATAgCgTTT
6680	*a*	CTCCCATATTgTAACCTACTACT
7600	*a*	TAgACCTACTTgTgCTgC
7698	*p*	CTgCTTCCTAgTCCTgTATgC
7861	*a*	ACgAggTCAACgACCCCT
8188	*a*	AAACTgTggTTTgCCCCACAgA
8200	*a*	ACgATgggCATgAAgCTgTg
8251	*a*	AgggTAAATACgggTCCTATT
8383	*a*	TgggCCATACggTggTATTTAg
8450	*a*	CACCCAACTAAAAATACTAAACAC
8453	*a*	CCCAACTAAAAATATTAgACACA
8473	*a*	AACTACCACCTACCCCCCTC
8701	*a*	AgTgTTgTgTATggCTATCATT
8762	*a*	CCTTAATCATTTTTACTgCCACAA
8856	*a*	ATCCCCTTATgAgCAggCACA
8955	*a*	CCCCATACTAgTTATCATCgAA
9090	*a*	AgATgATAAgTgTggAgggA
9115	*a*	ggATAgTCAgTAgAACTAgAATT
9242	*a*	CTATCATATAgTAAAgCCCAgC
9833	*a*	gACTTCACgTCATCATTggCT
9932	*a*	CCgCCTgATACTgACATTT
10310	*b*	TATTAgTggCAggTTAgTTgTT
10373	*a*	TCCTTTTTgTAgTCATTCATA
10400	*m*	TACCAATTCAgCTCAgTCT
10410	*a*	TTTTgTTTAAACTATgTACCAAT
10454	*a*	gACTCATTAAATTATgACAATCATA
11016	*a*	gATAgTggTTCATTggATA
11017	*a*	TgATAgTggTTCgCTgg
11696	*a*	ATTATgAgAATgATTgCgC
11722	*a*	TAATgAggATgTgAgTCCgT
12026	*a*	CACTCACCCACCACgTTAACA
12085	*a*	TCATACACCTATCTCCCATT
12092	*a*	CTATCCCCCATTATCCTCC
12092	*b*	TATCCCCCATTCTCCTCCT
12358	*a*	gCACACTACTATAgCCACCCT
12372	*a*	AgCCACCCTAACCCTAACTTCC
12406	*a*	ATCCTTACCACCCTCATTAACC
12753	*a*	ACAACCTATTCCAgCTgTTC
12753	*b*	ACAACCTATTCCAACTgTTC
13437	*a*	TCAAAACCATACCCCTCAC
13512	*a*	ggTTTCTACTCCAAggACCAC
13759	*a*	TgTTTggAAgggggATgTgggg
13879	*a*	CAAACTTAAAATAAAACCCCCA
13942	*a*	ATCACACACCgCgCAAT
14287	*a*	ATAATTTATgAAggggAggggT
14308	*a*	TAATAgTgTAgggAgCTgAAT
14364	*a*	AggTAggATTggTgTTgTgg
14914	*a*	TgAggCgTCTggCgAgT
14996	*a*	gAggCgCCATTggTgTgAAg
15047	*a*	ACACATCggACgAAgCCTATA
15314	*a*	gCTgCTAgggCTgTAATAATg
15422	*a*	ACCCTTACTACACAgTCAAAgA
15508	*a*	AggCgACCCAgATAATTAT
15508	*b*	gCgACCCAgACAATTATAC
15850	*a*	TCAATTAgggAgACggTTggTA
15883	*a*	ACAAAATACTCAAATgAgCCTgT

(*continued*)

NOTE.—Probes used for the second set of hybridization.

[a] Purposes for probes are as follows: *a,* for detecting polymorphism; *b,* for detecting wild type; *p,* for verifying PCR product; and *m,* for detecting macrohaplogroup M.

logistic-regression analysis with adjustment for age and sex. All three mtSNPs were significantly associated with resistance against T2DM (5231G→A: $P = .0001$, OR 0.54 [95% CI 0.40–0.74]; 12358A→G: $P = .0026$, OR 0.62 [95% CI 0.46–0.84]; and 12372G→A: $P = .0005$, OR 0.59 [95% CI 0.44–0.79]). The slight differences in the *P* values and ORs among these mtSNPs are due to the occurrence of the same replacement in different haplogroups (homoplasy or parallel mutations). The first mtSNP (5231G→A) was detected not only in haplogroup N9a but also in subhaplogroup D4k3. The second mtSNP (12358A→G) was not detected in some of the subjects with haplogroup N9a, probably because of a revertant substitution. In addition, the second mtSNP was also detected in subhaplogroup D4b2b2 (tentative nomenclature). The third mtSNP (12372G→A) was detected not only in haplogroup N9a but also in subhaplogroup D4h. Thus, the combined analysis of these three mtSNPs is essential for accurate identification of haplogroup N9a.

Japanese subjects in haplogroup F had a significantly increased risk of T2DM (OR 1.53 [95% CI 1.16–2.04], $P = .0032$), whereas those in haplogroup N9a tended to have a reduced risk for the disease. In particular, Japanese women in haplogroup N9a had a significantly reduced risk of T2DM (OR 0.27 [95% CI 0.10–0.62], $P = .0042$), whereas those in haplogroup F or A tended to have an increased risk of T2DM.

Korean subjects in haplogroup N9a had a significantly reduced risk of T2DM (OR 0.43 [95% CI 0.24–0.77], $P = .0048$), whereas those in haplogroup D5 or subhaplogroup

Table 7. Haplogroup Distribution in Controls and Patients with T2DM

	No. of Controls (%)			No. of Patients with T2DM (%)		
Haplogroup	Japanese	Korean	Total	Japanese	Korean	Total
F	96 (5.9)	61 (9.6)	157 (7.0)	112 (8.7)	71 (9.7)	183 (9.1)
B	196 (12.1)	98 (15.5)	294 (13.1)	152 (11.8)	113 (15.4)	265 (13.1)
A	102 (6.3)	46 (7.3)	148 (6.6)	100 (7.8)	63 (8.6)	163 (8.1)
N9a	79 (4.9)	40 (6.3)	119 (5.3)	41 (3.2)	19 (2.6)	60 (3.0)
M7a	115 (7.1)	9 (1.4)	124 (5.5)	92 (7.1)	17 (2.3)	109 (5.4)
M7b	68 (4.2)	18 (2.8)	86 (3.8)	45 (3.5)	21 (2.9)	66 (3.3)
G	188 (11.6)	43 (6.8)	231 (10.3)	141 (10.9)	65 (8.9)	206 (10.2)
D4a	152 (9.4)	38 (6.0)	190 (8.4)	111 (8.6)	32 (4.4)	143 (7.1)
D4b	109 (6.7)	29 (4.6)	138 (6.1)	83 (6.4)	54 (7.4)	137 (6.8)
D5	62 (3.8)	34 (5.4)	96 (4.3)	59 (4.6)	58 (7.9)	117 (5.8)
Others[a]	450 (27.8)	217 (34.3)	667 (29.6)	353 (27.4)	219 (29.9)	573 (28.3)
Total	1,617 (100)	633 (100)	2,250 (100)	1,289 (100)	732 (100)	2,021 (100)

[a] Seventeen other haplogroups with low frequencies, including haplogroups N9b, Y, M10–M12, M7c, M8a, Z, C, and D4d–D4n (except for D4f and D4i).

D4b tended to have an increased risk of the disease. Korean men in haplogroup N9a had a significantly reduced risk of T2DM (OR 0.28 [95% CI 0.11–0.62], $P = .0031$), whereas those in haplogroup D4b had a significantly increased risk of T2DM (OR 3.55 [95% CI 1.65–8.34], $P = .0019$).

We then examined whether the risk of T2DM with haplogroup N9a was related to age; systolic blood pressure; diastolic blood pressure; serum concentration of total cholesterol, triglycerides and/or HDL cholesterol; FPG concentration; or HbA1c level. None of the parameters, other than FPG and HbA1c, showed significant differences between the subjects with haplogroup N9a and those without it. The FPG (mean ± SD) concentration was significantly lower in the individuals with haplogroup N9a than in those with other haplogroups (6.5 ± 3.0 mmol/liter vs. 7.1 ± 3.1 mmol/liter, $P = .021$). The HbA1c level was significantly lower in individuals with haplogroup N9a than in those with other haplogroups (6.1% ± 1.5% vs. 6.8% ± 1.9%, $P = .002$).

Discussion

We examined the relationships between T2DM and each of 10 major mitochondrial haplogroups in a large-scale association study in the Japanese and Korean populations. Haplogroup N9a was significantly associated with reduced susceptibility to T2DM.

Mitochondrial haplogroup N9a has a great diversity in the whole of China and Korea. In Japan, this haplogroup was not detected in aboriginal Ainu and Ryukyuans but only in mainland Honshu Japanese. This distribution suggests that this haplogroup was derived from the new immigrant, or Yayoi, people. These so-called mammoth hunters who had adapted to extremely cold climates in Siberia migrated back to the northern part of China ~6,000 years ago. A part of this continental population immigrated into Japan through the Korean peninsula ~2,900 years ago, and this immigration started the Yayoi period. Haplogroup N9a was not detected in tooth DNA from the remains of an individual from the Japanese Neolithic period, known as the "Jomon" period, whereas N9a was recently detected in the Yayoi remains at the Kuma-Nishioda site in the northern part of Kyushu Island (K. Shinoda [National Science Museum, Tokyo], personal communication). Thus, haplogroup N9a might be one of the mitochondrial haplogroups that had been selected for adaptation to cold climates. This historical character of haplogroup N9a might be relevant to resistance against T2DM by individuals who carry this haplogroup. These hypotheses, however, must be examined further by functional analysis of this haplogroup.

Most of the mtSNPs characteristic to haplogroup N9a are synonymous substitutions, including 5231G→A and 12372G→A, which were used for the present genotyping.

Table 8. Multivariate Logistic-Regression Analysis of Haplogroups Associated with T2DM with Adjustment for Age and Sex in Japanese and Korean Populations

Population and Haplogroup	*P*	OR (95% CI)
Japanese and Korean subjects:		
N9a	**.0002**	.55 (.40–.75)
F	.0114	1.34 (1.07–1.67)
D5	.0475	1.33 (1.00–1.76)
Japanese and Korean women:		
N9a	**.0035**	.43 (.24–.74)
Japanese subjects:		
F	**.0032**	1.54 (1.16–2.04)
N9a	.0206	.63 (.43–.93)
Japanese women:		
N9a	**.0042**	.27 (.10–.62)
F	.0163	1.79 (1.11–2.89)
A	.0407	1.67 (1.02–2.72)
Korean subjects:		
N9a	**.0048**	.43 (.24–.77)
D5	.0483	1.60 (1.01–2.57)
D4b	.0365	1.66 (1.04–2.81)
Korean men:		
N9a	**.0031**	.28 (.11–.62)
D4b	**.0019**	3.55 (1.65–8.34)

NOTE.—Bold font indicates haplogroups with $P < .005$.

Table 9. Multivariate Logistic-Regression Analysis of Haplogroup N9a Associated with T2DM

Population and Variable	*P*	OR (95% CI)
Japanese subjects:		
Age	.0003	.22 (.10–.49)
Sex	.0088	1.38 (1.08–1.75)
BMI	<.0001	.13 (.05–.35)
Triglycerides	.0051	.07 (.009–.41)
HDL cholesterol	.0255	14.5 (1.52–167)
Haplogroup N9a	.0478	.57 (.32–.98)
Korean subjects:		
Age	<.0001	1,066 (321–3765)
BMI	.0458	.42 (.18–.98)
Systolic blood pressure	<.0001	.10 (.05–.21)
Triglycerides	<.0001	.01 (.0009–.04)
Haplogroup N9a	.0166	.47 (.24–.86)
Japanese men:		
BMI	.0019	.16 (.05–.51)
Triglycerides	.0114	.08 (.01–.51)
Korean men:		
Age	<.0001	997 (184–6169)
BMI	.0075	.19 (.05–.63)
Systolic blood pressure	<.0001	.06 (.02–.21)
Triglycerides	.0012	.007 (.0003–.12)
Haplogroup N9a	.0233	.36 (.14–.83)
Japanese women:		
Age	.0028	.12 (.03–.47)
BMI	.0158	.19 (.05–.72)
Haplogroup N9a	.0298	.19 (.03–.69)
Korean women:		
Age	<.0001	273 (68.8–1186)
Systolic blood pressure	<.0001	.14 (.05–.37)
Triglycerides	<.0001	.001 (.0001–.02)
HDL cholesterol	<.0001	.03 (.006–.14)

NOTE.—The analysis was adjusted for age, sex, BMI, systolic and diastolic blood pressure, triglycerides, HDL and cholesterol.

Possible candidates for functional polymorphisms in the noncoding region of this haplogroup are 150C→T and 338C→T. The 150C→T substitution was originally reported to occur in Italian centenarians.[26] Also, we reported this substitution to be associated with healthy longevity in both Finland and Japan.[27] Thus, 150C→T might confer resistance against T2DM. Among haplogroup N9a–specific mtSNPs in the coding region, the mtSNP 12358A→G causing the T8A replacement in nicotinamide adenine dinucleotide dehydrogenase subunit 5 (MTND5 [MIM 516005]) may be considered a potentially functional polymorphism. It seems possible that this T8A replacement might influence the function of the ND5 and complex I. The actual effect of the 12358A→G (ND5: T8A) on mitochondrial function remains to be examined. The metabolic characteristics of individuals with haplogroup N9a with both 150C→T and 12358A→G should be examined for better understanding of the mechanisms underlying their resistance against T2DM.

We detected a significant association between haplogroup N9a and a reduced risk of T2DM in all subjects (OR 0.55), and especially low ORs in Japanese women (0.27) and Korean men (0.28) were obtained. Although we cannot exclude the possibility that these associations resulted from the reduced statistical power due to the decreased sample size of subgroups, these sex- and region-specific associations suggest that cultural factors, including nutritional and social customs, modify the protective effect of haplogroup N9a against T2DM. According to the Wallace theory, adaptation to a cold climate might involve uncoupling of electron transfer with ATP production, to increase heat production.[15,16] Thus, increased mitochondrial respiration and energy expenditure is essential to meet the ATP requirement. Such an uncoupling phenotype would be protective against the development of obesity and, consequently, T2DM. However, at present, we do not have evidence that N9a is associated with lean body status. Alternatively, the uncoupling phenotype might be related to decreased mitochondrial oxidative stress, which might in turn exert a protective effect against T2DM. Further functional analysis of cybrids carrying haplogroup N9a will be necessary to verify these hypotheses.

The mitochondrial genome variation is so large that a given haplogroup may consist of various subhaplogroups carrying unique and presumably functional mtSNPs. The frequency of each subhaplogroup is sometimes only a few percent. Therefore, large-scale association studies are necessary for elucidating the impact of each subhaplogroup on the susceptibility to various common diseases.

Although haplogroup F was significantly associated with a risk of T2DM in Japanese subjects (OR 1.53 [95% CI 1.16–2.04], $P = .0032$), this association was not confirmed in Korean individuals. To explain this discrepancy, we hypothesize certain interactions between mitochondrial haplogroups and nuclear polymorphisms and/or environmental factors. Alternatively, the difference in the results between the Japanese and Korean subjects could be ascribable to the difference in the subhaplogroup frequencies between the two countries and to the functional differences among certain subhaplogroups. Our success in detecting a significant association of haplogroup N9a with resistance against T2DM in both Japanese and Korean individuals could be ascribable to the homogeneity of haplogroup N9a (coalescence age of 14,000 ± 5,000 years ago) compared with the heterogeneity of haplogroup F (coalescence age of 47,000 ± 9,000 years ago). Further biomedical and functional studies on mitochondrial polymorphisms should be conducted in conjunction with human phylogenetic studies.

Acknowledgments

In addition to the authors, the following individuals participated in the study: Y. Matsuno and M. Tomita (Gifu Prefectural Gifu Hospital, Gifu); M. Oguri, T. Hibino, and T. Kameyama (Gifu Prefectural Tajimi Hospital, Tajimi); S. Tanihata (Gifu Prefectural Gero Hotspring Hospital, Gero); and the nursing and laboratory staff of the participating hospitals. This work was supported in part by grant-in-aid A-15200051 (to M.T.) and grant 15209021 (to Y.Y.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; by a grant (to M.T.) from the Third-Term Comprehensive 10-Year Strategy for Cancer Control; by

grant 17A-10 (to M.T.) from the program Research Grants for Nervous and Mental Disorders of the Ministry of Health, Labour, and Welfare of Japan; by a grant (to Y.Y.) from the Gifu Prefectural Science and Technology Promotion Center; and by grant 00-PJ3-PG6-GN07-001 (to K.S.P.) from the Korea Health 21 R&D Project of the Ministry of Health and Welfare. Additional support came from the Ministry of Information and Communication and the Institute for Information Technology Assessment through the Information Technology Leading R&D Support Project (to H.K.L).

Web Resources

Accession numbers and URLs for data presented herein are as follows:

mtSNP, http://www.giib.or.jp/mtsnp/index_e.shtml
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for T2DM and MTND5)

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