differences within the study population, environmental factors, misdiagnosed disease, and genetic mistyping, greatly facilitating molecular-level genetic analysis of naturally selected thrifty genes.

Identification of type 2 diabetes susceptibility genes

Nearly 50% of Mexican Americans aged 35 years or older have diabetes mellitus or family history in first-degree relatives. A genome-wide linkage study with affected sib-pairs for type 2 diabetes genes in a Mexican American population localized a type 2 diabetes susceptibility gene (*NIDDM1*) to a 12-cM 1-lod support interval in the distal long arm of chromosome 2 near *D2S140* [3]. A physical map of the *NIDDM1* region was generated to identify the SNPs within the region by resequencing the expressed sequence tags (EST). SNPs were surveyed in eight patients of families with evidence of linkage at *NIDDM1* and in two patients of families without such evidence. SNPs with minor allele frequency of more than 10% or showing a unique pattern were genotyped in a patient group of 110 Mexican Americans with type 2 diabetes and a control group of 112 randomly sampled subjects to compare allele and haplotype frequency distributions between the groups as described below. Haplotype structure was determined by the expectation maximization (EM) algorithm [4]. We then classified the patients into three subgroups for comparisons of association with the *NIDDM1* allele. The first patient subgroup included all 110 patients, the second subgroup included the 37 patients from families showing evidence of linkage at *NIDDM1*, and the third subgroup included the 20 patients from families showing evidence of linkage at both *NIDDM1* and *CYP19*, a marker located on the second peak of chromosome 15 [5] (Fig. 1).

Of these, no putative single SNP was significantly associated with increased risk of diabetes. However, when three SNPs were combined in a computed LD block, haplotypes composed of the three intron SNPs in the region of calpain-10, namely SNP-43, -19, and -63, showed a significant association with type 2 diabetes. Specifically, the 112/121-haplotype combination (1 = major allele, 2 = minor allele) was associated with increased risk of diabetes in our Mexican Ameri-

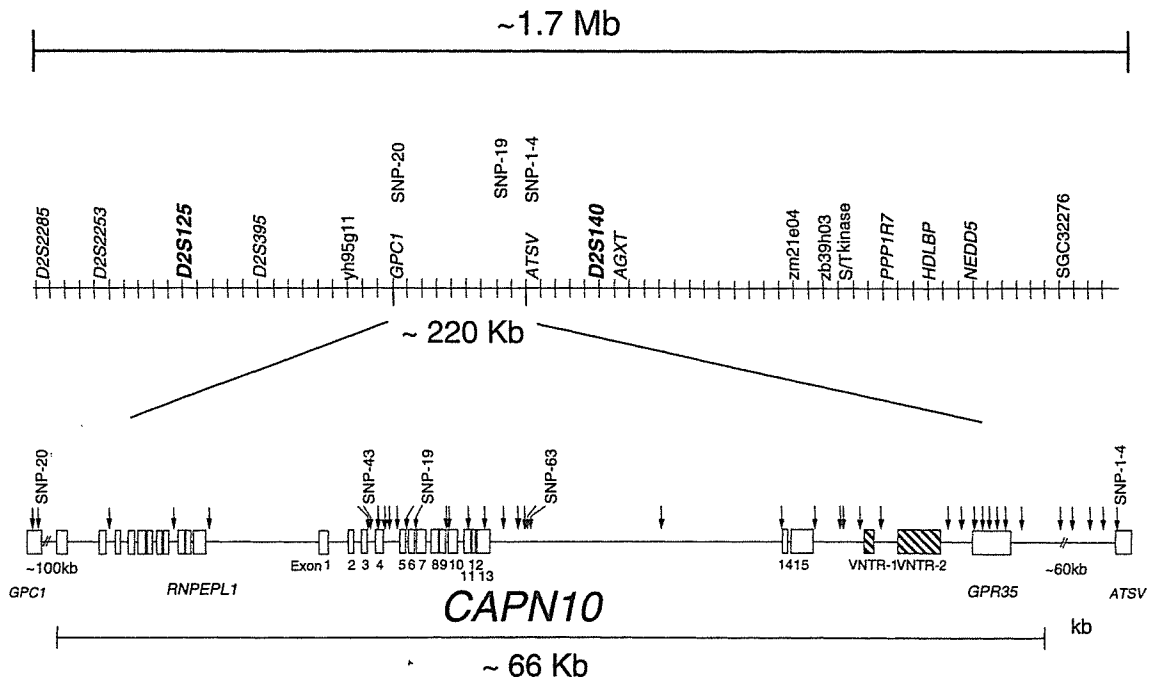

Fig. 1. The guide map of identification of *NIDDM1*
SNPs are numbered in the order in which they were identified. Genes and ESTs were found on screening the GenBank database with the indicated STS. The locations of the SNPs typed in 110 patients and the 112 random samples are shown by arrows.

can group (O.R., 3.02; 95% C.I., 1.37 to 6.64). This combination also was associated significantly with diabetes in Finnish and German populations [5]. The identification of *NIDDM1* demonstrates the following: 1) several susceptibility alleles can be found in one gene; 2) some combinations of SNP increase risk of development of diabetes while other combinations decrease risk; 3) risk that cannot be associated with a single SNP can be identified by a haplotype including the SNP; and 4) SNPs in introns affect regulation of the transcription level of the gene.

Genetic analysis of *NIDDM1* in various populations

An analysis of *NIDDM1* including SNP-44, which is located near SNP-43 and affects transcriptional activity, in British and Irish whites found a significant association of SNP-44 with type 2 diabetes. SNP-44 showed significant LD transmission singly and was in complete LD with the missense mutation T504A. Thus, proteins with mutations or altered transcription expression levels contribute to the development of type 2 diabetes in this population [6]. A recent meta-analysis with additional genotyping in 4213 individuals (2056 type 2 diabetes patients and 2157 healthy individuals) found the O.R. of the development of diabetes to be 1.17 (95% C.I., 1.02 to 1.34) for SNP-44 with 80% statistical power [7]. Another large-scale meta-analysis involving 2288 type 2 diabetes patients and 3041 healthy individuals found O.R. of 1.19 (95% C.I., 1.07 to 1.33) for SNP-43 alone [8]. Racial differences were reported in the association of three SNPs (SNP-43, -19, and -63) with the incidence of diabetes [9–16]. An analysis in Japanese found no significant association between diabetes and these haplotype combinations [17]. In addition, a case-control association analysis in nearly 1000 patients and controls found that the minor allele of SNP-19 and the 121 haplotype was associated with reduced risk in Japanese diabetes patients aged 50 years or over [18]. Unfortunately, most of the original studies included in the these meta-analyses were based on genotyping only SNP-43, SNP-19, and SNP-63, without consideration of LD blocks specific to each ethnic group. Thus, the frequencies of the haplotypes in each population remain to be carefully assessed. For example, the pattern of LD in *CAPN10* was evaluated by calculation of r^2 both in Japanese and in Mexican Americans from the genotype data using 17 SNPs for all possible pairs with 96 control subjects (Fig. 2A & B). The distribution of LD was similar in the two populations, and at least four major SNP subgroups with minor differences were present. Since more SNPs are found in tight linkage with each other in Japanese than in Mexican Americans (Fig. 2B), Japanese may have higher LD in this locus.

To clarify the ancestral role of these high-risk haplotypes in the development of diabetes, a genotyping survey was undertaken in human individuals from various populations and other primates. The presence of positive natural selection at the calpain-10 locus that cannot be explained by genetic drift has previously been established [19]. Comparison of human individuals from various populations with primates revealed that the 111-haplotype was likely to carry the ancestral allele in all populations, while the 112-haplotype was selectively favored in African populations, and the 121- and 221-haplotypes were selected in populations outside Africa in the process of racial migration toward Europe, Asia, and America. In addition, the survey showed that the four major haplotypes (111-, 112-, 121-, and 221-) occur in Native Americans, an ancestral population of Mexican Americans, and that recent admixture between populations was not a factor [19].

Haplotypes with minor allele SNP-44 have almost no other polymorphisms, suggesting recent and rapid positive selection of these haplotypes. According to the common disease common variant hypothesis, major alleles can be disease susceptibility alleles, represented by PPARγ (Pro12Ala) for type 2 diabetes [20]; minor alleles as susceptibility alleles are represented by ApoE (ε4<ε3) for coronary artery diseases [21] or Alzheimer's disease [22] and the calpain-10 gene (SNP-44 C<T) for type 2 diabetes [6, 7]. For the latter two genes, the major allele may have originally been beneficial by reducing the risk of common metabolic syndromes but become detrimental relatively recently.

Summary statistics in various populations for polymorphisms on the 33465 base pairs in the *NIDDM1* region including calpain-10 and *GPR35* show a higher frequency of mutation at the calpain-10 locus than at the *GPR35* locus. In addition, sliding window analysis reveal a high frequency of polymorphisms in intron 13 of calpain-10 that cannot be fully characterized by the neutral hypothesis. Simulation analysis indicates that this locus cannot be explained with a simple two-allele

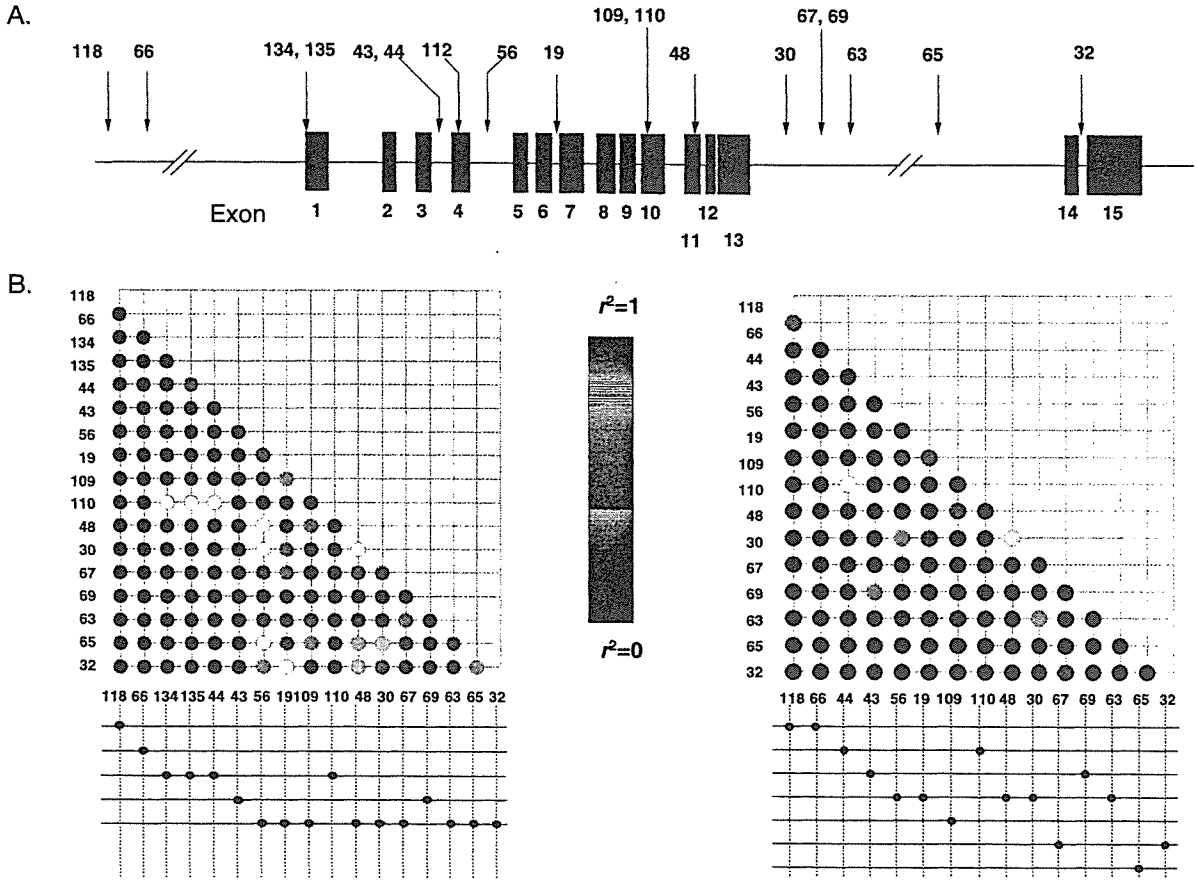

Fig. 2. A. Exon-intron organization of *CAPN10*. The physical distance between SNP-118 and SNP-32 is approximately 40 kb. The locations of the SNPs are shown.
B. Pairwise linkage disequilibrium in *CAPN10* evaluated by r^2. All SNP numbers are denoted in Fig. 2A. Pairwise LD was determined using 136 and 105 marker pairs in Japanese (left panel) and Mexican Americans (right panel), respectively. SNP-134 and -135 were not identified in the previous study with Mexican Americans [5]. The color gradations from red (perfect LD, *i.e.*, $r^2 = 1$) to blue (no LD, *i.e.*, $r^2 = 0$) reflect the degree of the observed LD. Subgroups of SNPs found in tight linkage (r^2>0.5) also are shown. Since there was no suitable polymorphic site upstream of *CAPN10* [5], we used SNP-118 and -66 in the study, despite their low allele frequencies.

selection model but requires a multiallele selection model with sequential turnover of polymorphisms. Thus, a complicated population structure other than natural selection may be involved in this feature, but that alone cannot explain the rapid decline in LD. For loci such as intron 13 of calpain-10 that exhibit a rapid decline in LD, a high recombination rate is generally reported. Although no studies have reported a correlation between the recombination rate and the frequency of polymorphisms, exceptional recombination or mutation in a neutral state is possible [23]. Recently, a close analysis of intron 13 showed that a selection model consisting of five clusters of haplotypes can explain the genetic findings in Mexican Americans, two of the five clusters appearing 2 to 3 million years ago

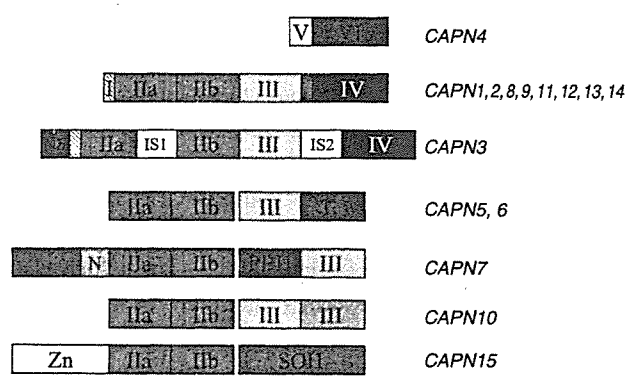

Fig. 3. Domain structures of the human calpain family
Typical calpains are composed of four domains (I–IV), but in the case of atypical calpains, certain domains have been deleted or replaced. The small subunit of calpain is composed of two domains.

in the glacial ages. Comparisons of the partial sequence at intron 13 among 10 species show that four USF1 and one HNF1 binding sites in a segment share common sequence motifs. In addition, an unknown gene is expressed around the segment that might influence calpain-10 expression [24].

Calpains

Calpains are a family of cytoplasmic cysteine proteases activated by Ca^{2+}. At least 15 calpains have been identified as eight typical calpains, six atypical calpains, and one small subunit of calpain [25, 26]. The domain structures of the calpain family are shown in Fig. 3. Known substrates for calpains include cytoskeletal proteins, actin-binding proteins, calmodulin-binding proteins, hormone receptors, cell membrane hormone receptors, glucose-metabolizing enzymes, enzymes regulating signal transmission, and transcription factors. Calpains are known to play a physiological role in reconstruction of cytoskeleton, apoptosis, and reconstruction (proliferation, differentiation, and transformation) of tissue cells. Mutations in genes encoding calpains cause various disorders including diabetes related to calpain-10, gastric cancer related to calpain-9, muscular dystrophy related to calpain-3, neurodegenerative diseases (*e.g.*, Alzheimer's disease), cerebral infarction, spinal injury, myocardial infarction, hepatic ischemia, and renal impairment. Animal tests show death in the fetal period in calpain-4 (small subunit) knockout mice due to impaired development of the cardiovascular system. Calpain-1 knockout mice develop normally because the lost function of calpain-1 is compensated by calpain-2, but the mice often show platelet aggregation disorder.

Calpain-10

The human calpain-10 gene is located in chromosome band 2q37.3 and consists of 15 exons. There are at least eight isoforms (calpain-10a to calpain-10h) of the gene. The longest isoform, calpain-10a, consists of 672 amino acids. Calpain-10 is an atypical calpain that lacks domain IV and instead has a tandem linking domain, domain III. Calpain-10a is expressed most strongly in the heart, but is present in various tissues including those playing an important role in glucose metabolism, including liver, muscle, pancreatic islets, and adipocytes [5, 25]. Although calpain-10c and 10g can be detected in many tissues, calpain-10b, 10d, 10e, and 10f are much less abundant [5]. Because calpain-10 lacks calcium-binding sites in domains II and III, it is not known whether the protein is activated by calcium. Calpain-10 may react with calcium in a separate mechanism, as it can be found in sarcomere, the calcium storage in muscle fibre, and its expression is increased or its distribution is altered following calcium stimulation in the epithelium of crystalline [27].

Effects of calpain-10

Effects of calpain-10 on β cells

1) Calpain and apoptosis

Involvement of calpain-10 in ryanodine-induced apoptosis was reported based on finding that apoptosis was enhanced by ryanodine or palmitin acid in pancreatic β cell-specific calpain-10 transgenic mice by the rat insulin promoter but was not enhanced in calpain-10 knockout mice [28].

2) Calpain and insulin secretion

The relationship between calpain and insulin secretion was assessed with calpain inhibitors. Assessed with a nonspecific calpain inhibitor, glucose-responsive insulin secretion was enhanced in short-term culture with the inhibitor added, while insulin secretion was inhibited by reduced mitochondrial glucose metabolism in 48-hour culture with the inhibitor added [29, 30].

Calpain-1, a typical calpain, is reported to break ICA512, a tyrosine phosphatase-like protein located in insulin granules, dependent on the intracellular Ca^{2+} concentrations. A calpain inhibitor blocked the breakage, thereby impairing insulin secretion [31]. In a recently reported assay system in which calpain-10 was overexpressed stably in INS1 cells, glucose-responsive insulin secretion was enhanced. The breakage was reduced by the addition of E64, a calpain inhibitor, to the cells. When Ca^{2+} was removed from the supernatant, these reactions were not induced. These results indicate that the intracellular Ca^{2+} concentration increased by glucose stimulation can activate calpain-10 and break SNAP25 (a SNARE protein), thereby inducing fusion of insulin granules to the membrane of the β cells [32]. However, considering the

absence of key 54-kD proteins among the reported calpain-10 isoforms, further analysis is required.

Effects of calpain-10 on muscles

In clinical research assessing the relationship between SNP-43G/G and the level of calpain-10 mRNA expression in Pima Indians, individuals with the SNP-43G/G allele were found to have a low level of expression. A significant association was also found between oxidative utilization of glucose and the level of calpain-10 expression [33]. In an animal study using mice expressing calpastatin (a calpain inhibitor) in muscles, muscular glucose uptake and general glucose tolerance remained unchanged despite increased expression of GLUT4 protein and muscular hypertrophy mainly due to loss of insulin action resulting from reduced AKT kinase activity [34]. However, while calpastatin inhibits the activity of calpain-1 and calpain-2, action on calplain-10 is unlikely since calpain 10 does not bind with the calpain small subunit (calpain-4), the target of calpastatin. In an *in-vitro* study with human myoblasts to assess the relationship between calpain-10 and muscle differentiation, 60 kD calpain-10 protein levels were found to increase as differentiation progressed [35]. As differentiation of the myoblasts progressed, calpain-1 levels also increased and calpastatin expression decreased. In L8 cells over-expressing calpastatin, muscle differentiation was inhibited [36–38]. These findings demonstrate the involvement of calpain-10 as well as the calpain-calpastatin system in the process of myoblast differentiation.

Effects of calpain-10 on adipocytes

Some groups have investigated the function of adipocytes in association with calpains, but no relationship between SNP43 and obesity was noted. In an *in-vitro* study using human adipocytes, adipogenesis in adipocytes was increased in a group with the SNP-43G/G allele irrespective of the level of GLUT4 expression. Lipolysis function from β3 adrenalin receptors was reduced in a group with the SNP19-deficient allele to one-thirtieth that in the normal group [39, 40]. In a clinical study, the level of calpain-10 mRNA expression in adipocytes decreased dependent on the blood triglyceride level in obese patients with the SNP43G/G allele [41]. In calpain-10 antisense-expressing stable cell line 3T3L1 adipocytes, actin reconstruction was inhibited by insulin with an unchanged level of GLUT4 expression, resulting in reduced glucose uptake and inhibited transfer of GLUT4 to the membrane by insulin [42]. As in other reports on the relationship between calpains and adipocytes, the level of calpain-1 expression increased while calpastatin expression decreased as adipocyte differentiation progressed, and when the action of calpain was inhibited by forced expression of calpastatin or the addition of the calpain inhibitor, C/EBPα expression and adipocyte differentiation were inhibited [43].

Clinical assessment of calpain-10

An analysis in nondiabetic British subjects revealed that genetic variation in the *CAPN10* gene influences blood glucose levels and that this is, at least in part, due to the effects of calpain-10 on early insulin secretory response [44]. An analysis in Finns showed that individuals with the1121/1121-haplotype combination for SNP-44, -43, -19, or -63 have approximately two times higher risk of development of diabetes, and that SNP-43 is associated with high fasting insulin levels, high HOMA-R levels, and high fasting fatty acid levels [45]. An analysis in Japanese found no significant association between diabetes and these haplotype combinations, but did find an association with insulin resistance and high fatty acid levels under euglycemic hyperinsulinemic clamp in individuals with the 112/121 haplogenotype [17] (Table 1).

Relationship between calpain-10 and diabetes-related diseases

For polycystic ovary syndrome, no phenotypic differences were noted among non-diabetic European Americans with a single polymorphism or haplotype of SNP-43, -19, or -63. In non-diabetic African American probands, no single polymorphism of SNP-43, -19, or -63 was associated with any phenotype, but individuals with the 112/121-haplotype combination showed a significantly greater area under the insulin-time curve on oral glucose tolerance test. This result was evident after data adjustment for body mass index. In African Americans and European Americans, the 112/121-haplotype combination was associated with approximately two times higher risk of polycystic ova-

Table 1. Association studies of *CAPN10* in various populations

Population	Genotype	Odds Ratio	Phenotype
Mexican American	112/121	3.02 (1.37–6.64)	
Pima Indian	SNP-43 G/G		Glucose utilization ↓
	111/111	2.04 (1.22–3.39)	
British/Irish Whites	2111/2111	2.52 (1.06–5.97)	
	2111/1111	2.36 (1.19–4.66)	A.I.R ↓
	112/121		HOMA-R ↑
Samoans	112/121	1.42 (0.68–2.98)	
Utah-Caucasian	111/221	1.48 (1.06–1.91)	Insulin AUC ↑
	SNP-19-63		HOMA-R ↑
African-American	SNP-43 G/G	1.38 (1.04–1.83)	Insulin AUC ↑
	112/121	2.18 (1.06–4.45)	PCOS Odds Ratio ↑
Spanish	SNP-44 CC, TC	2.57 (1.22–5.44)	PCOS Odds Ratio ↑
Polish	121/121	1.93 (1.03–3.54)	
Finnish	1121/1121	1.93 (1.07–3.47)	Fasting Insulin HOMA-RFFA ↑
Japanese	112/121		FFA ↑
	121/121		Protective against T2DM ↑

The results of studies with various populations are shown in this slide. The major (1) and minor (2) alleles are denoted as in Mexican Americans.
A.I.R, Acute Insulin Response; A.U.C, Area Under Curve; HOMA-R, Homeostasis Model Assessment-Resistance; FFA, Free Fatty Acid

ry syndrome [46]. In Spanish, an association between UCSNP44 and the incidence of polycystic ovary syndrome was reported [47].

Protease inhibitors and diabetes

Protease inhibitors are effective in the treatment of HIV infection. However, long-term use appears to induce pathology of hyperlipidemia or diabetes, including peripheral fatty atrophy and central fatty hypertrophy [48]. The mechanism is thought to be inhibited adipocyte differentiation. Calpain inhibitors, a type of protease inhibitor, are considered in the treatment of several disorders including spinal injury, liver transplantation, and myocardial infarction. Since calpain inhibitors inhibit adipocyte differentiation, attention must be paid to the possibility of diabetes and hyperlipidemia when they are used.

Conclusions

Ethnic comparison of polymorphisms can clarify the association of genetic structure with disease and play an important role in supporting case-control results. The approach is especially useful in screening putative susceptibility genes for common diseases that may have undergone recent natural selection, including those for type 2 diabetes. Sequence determination of a naturally selected segment can both identify a susceptibility gene and the mechanism of its regulation. Because of the recent, remarkable progress in sequencing techniques, identification of all of the polymorphisms on the entire human genome, SNPs in particular, may soon be possible. However, the enormous cost of SNP typing remains a limiting factor for their use in investigations of ethnic variants in common diseases. Association studies based on haplotype analysis can identify susceptibility genes, calculate developmental risks, and predict drug responsiveness. Translational research on function using interactome, proteome, and model mice can be used to apply the results clinically as individualized therapy.

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Brief Genetics Report

Association Studies of Variants in the Genes Involved in Pancreatic β-Cell Function in Type 2 Diabetes in Japanese Subjects

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Because impaired insulin secretion is characteristic of type 2 diabetes in Asians, including Japanese, the genes involved in pancreatic β-cell function are candidate susceptibility genes for type 2 diabetes. We examined the association of variants in genes encoding several transcription factors (*TCF1*, *TCF2*, *HNF4A*, *ISL1*, *IPF1*, *NEUROG3*, *PAX6*, *NKX2–2*, *NKX6–1*, and *NEUROD1*) and genes encoding the ATP-sensitive K^+ channel subunits Kir6.2 (*KCNJ11*) and SUR1 (*ABCC8*) with type 2 diabetes in a Japanese cohort of 2,834 subjects. The exon 16 −3c/t variant rs1799854 in *ABCC8* showed a significant association ($P = 0.0073$), and variants in several genes showed nominally significant associations ($P < 0.05$) with type 2 diabetes. Although the E23K variant rs5219 in *KCNJ11* showed no association with diabetes in Japanese (for the K allele, odds ratio [OR] 1.08 [95% CI 0.97–1.21], $P = 0.15$), 95% CI around the OR overlaps in meta-analysis of European populations, suggesting that our results are not inconsistent with the previous studies. This is the largest association study so far conducted on these genes in Japanese and provides valuable information for comparison with other ethnic groups. *Diabetes* 55:2379–2386, 2006

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HWE, Hardy-Weinberg equilibrium; K_{ATP} channel, ATP-sensitive K^+ channel; LD, linkage disequilibrium; SNP, single nucleotide polymorphism.

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Impaired insulin secretion and insulin resistance both contribute to the pathogenesis of type 2 diabetes. The former is a characteristic feature of type 2 diabetes, especially in Asians including Japanese (1), and genes encoding proteins critical in pancreatic β-cell function are therefore particularly good candidate susceptibility genes for type 2 diabetes for this population. Studies of maturity-onset diabetes of the young in humans (2) and knockout mice (3) have shown that mutations of transcription factors required for development, differentiation, and maintenance of the pancreatic β-cells can cause diabetes. Pancreatic β-cell ATP-sensitive K^+ channels (K_{ATP} channels) are crucial in the regulation of insulin secretion by coupling cell metabolism to membrane electrical activity. The pancreatic β-cell K_{ATP} channel comprises two subunits, the inwardly rectifying potassium channel Kir6.2 (*KCNJ11*) and the sulfonylurea receptor SUR1 (*ABCC8*) (4). Mutations in the genes (*ABCC8* and *KCNJ11*) can cause familial persistent hyperinsulinemic hypoglycemia of infancy (5) and permanent neonatal diabetes (6). Several polymorphisms in these genes also have been reported to be associated with type 2 diabetes in populations with distinct ethnic backgrounds (7–20). However, a large-scale association study of these genes has not been performed in type 2 diabetes in the Japanese population. Here, we report on the association of variants in genes encoding various transcription factors and pancreatic β-cell K_{ATP} channel subunits with type 2 diabetes in a large Japanese cohort.

A case-control association study using 1,590 Japanese diabetic subjects and 1,244 nondiabetic control subjects was performed. All subjects were genotyped for 33 variants of 12 genes including transcription factors (*TCF1*, *TCF2*, *HNF4A*, *ISL1*, *IPF1*, *NEUROG3*, *PAX6*, *NKX2–2*, *NKX6–1*, and *NEUROD1*) and β-cell K_{ATP} channel subunits (*KCNJ11* and *ABCC8*) (Table 1).

Results of Hardy-Weinberg equilibrium (HWE) tests are shown in Table 1 of the online appendix (available at http://diabetes.diabetesjournals.org). All genotypes were in HWE, except for departures in cases at *TCF2*_SNP (single nucleotide polymorphism) 5 rs2689, *TCF2*_SNP6

TABLE 1
Summary of association studies of 33 variants for 12 genes with type 2 diabetes

Number	Locus	HapMap data	Subject	Allele data (frequency) 1	Allele data (frequency) 3	Genotype data (frequency) 1	Genotype data (frequency) 2	Genotype data (frequency) 3	*P* value Allele 2*2	*P* value Genotype 2*3	OR (95% CI)
1	TCF1_SNP1			A	C	A/A	A/C	C/C			
				1,590	1,590						
	rs1169288	JPT	Case	(0.50)	(0.50)	385 (0.24)	820 (0.52)	385 (0.24)	0.4508	0.2388	1.04 (0.94–1.16)
				1,270	1,218						
	I27L		Control	(0.51)	(0.49)	332 (0.27)	606 (0.49)	306 (0.25)			
2	TCF1_SNP2			G	A	G/G	G/A	A/A			
				1,702	1,478						
	rs1169294	none	Case	(0.54)	(0.46)	443 (0.28)	816 (0.51)	331 (0.21)	0.3247	0.1566	1.06 (0.95–1.17)
				1,298	1,190						
	IVS1 −42		Control	(0.52)	(0.48)	348 (0.28)	602 (0.48)	294 (0.24)			
3	TCF1_SNP3			A	T	A/A	A/T	T/T			
					2,698			1,146			
	rs2071190	JPT	Case	482 (0.15)	(0.85)	38 (0.02)	406 (0.26)	(0.72)	0.8925	0.8617	1.01 (0.87–1.17)
					2,115						
	IVS2 −51		Control	373 (0.15)	(0.85)	26 (0.02)	321 (0.26)	897 (0.72)			
4	TCF2_SNP1			G	A	G/G	G/A	A/A			
				2,079	1,101						
	rs757210	JPT	Case	(0.65)	(0.35)	697 (0.44)	685 (0.43)	208 (0.13)	0.4565	0.2695	1.04 (0.93–1.17)
				1,651							
	IVS2 + 2916		Control	(0.66)	837 (0.34)	546 (0.44)	559 (0.45)	139 (0.11)			
5	TCF2_SNP2			A	G	A/A	A/G	G/G			
				1,460	1,720						
	rs757211	none	Case	(0.46)	(0.54)	342 (0.22)	776 (0.49)	472 (0.30)	0.6994	0.5473	1.02 (0.92–1.14)
				1,156	1,332						
	IVS2 + 2953		Control	(0.46)	(0.54)	262 (0.21)	632 (0.51)	350 (0.28)			
6	TCF2_SNP3			G	A	G/G	G/A	A/A			
				2,288							
	rs718960	JPT	Case	(0.72)	892 (0.28)	824 (0.52)	640 (0.40)	126 (0.08)	0.6121	0.8597	1.03 (0.92–1.16)
				1,774							
	IVS4 + 14307		Control	(0.71)	714 (0.29)	632 (0.51)	510 (0.41)	102 (0.08)			
7	TCF2_SNP4			T	A	T/T	T/A	A/A			
				2,823		1,260					
	rs1016991	JPT	Case	(0.89)	357 (0.11)	(0.79)	303 (0.19)	27 (0.02)	0.0105*	0.0399*	1.23 (1.05–1.45)
				2,152							
	IVS8 + 929		Control	(0.87)	336 (0.14)	938 (0.75)	276 (0.22)	30 (0.02)			
8	TCF2_SNP5			A	T	A/A	A/T	T/T			
				1,722	1,458						
	rs2689	JPT	Case	(0.54)	(0.46)	488 (0.31)	746 (0.47)	356 (0.22)	0.5195	0.3582	1.04 (0.93–1.15)
				1,325	1,163						
	+274 TGA		Control	(0.53)	(0.47)	355 (0.29)	615 (0.49)	274 (0.22)			
9	TCF2_SNP6			A	C	A/A	A/C	C/C			
				1,840	1,340						
	rs2688	JPT	Case	(0.58)	(0.42)	552 (0.35)	736 (0.46)	302 (0.19)	0.0563	0.0291*	1.11 (1.00–1.24)

	+444 TGA		Control	1,503 (0.60)	985 (0.40)	448 (0.36)	607 (0.49)	189 (0.15)			
10	HNF4A_SNP1			T	C	T/T	T/C	C/C			
	rs717247	JPT	Case	2,277 (0.72)	903 (0.28)	811 (0.51)	655 (0.41)	124 (0.08)	0.2071	0.4223	1.08 (0.96–1.22)
	−4229		Control	1,820 (0.73)	668 (0.27)	661 (0.53)	498 (0.40)	85 (0.07)			
11	HNF4A_SNP2			A	G	A/A	A/G	G/G			
	rs736820	none	Case	1,207 (0.38)	1,973 (0.62)	230 (0.14)	747 (0.47)	613 (0.39)	0.4482	0.6472	1.04 (0.94–1.16)
	IVS1 + 3889		Control	919 (0.37)	1,569 (0.63)	176 (0.14)	567 (0.46)	501 (0.40)			
12	HNF4A_SNP3			T	C	T/T	T/C	C/C			
	rs745975	JPT	Case	643 (0.20)	2,537 (0.80)	53 (0.03)	537 (0.34)	1,000 (0.63)	0.0470*	0.0769	1.15 (1.00–1.31)
	IVS1 −5		Control	450 (0.18)	2,038 (0.82)	39 (0.03)	372 (0.30)	833 (0.67)			
13	ISL1_SNP10			G	A	G/G	G/A	A/A			
	rs2303750	none	Case	2,842 (0.89)	338 (0.11)	1,271 (0.80)	300 (0.19)	19 (0.01)	0.5101	0.4453	1.06 (0.90–1.26)
	IVS3 −4		Control	2,209 (0.89)	279 (0.11)	987 (0.79)	235 (0.19)	22 (0.02)			
14	ISL1_SNP11			A	G	A/A	A/G	G/G			
	rs2303751	none	Case	2,466 (0.78)	714 (0.22)	958 (0.60)	550 (0.35)	82 (0.05)	0.0539	0.1224	1.14 (1.00–1.29)
	P165P		Control	1,983 (0.80)	505 (0.20)	787 (0.63)	409 (0.33)	48 (0.04)			
15	IPF1_SNP3			G	T	G/G	G/T	T/T			
	rs4430606	none	Case	1,688 (0.53)	1,492 (0.47)	451 (0.28)	786 (0.49)	353 (0.22)	0.1807	0.3405	1.06 (0.90–1.26)
	IVS1 + 512		Control	1,366 (0.55)	1,122 (0.45)	371 (0.30)	624 (0.50)	249 (0.20)			
16	IPF1_SNP4			A	C	A/A	A/C	C/C			
	rs1124607	JPT	Case	2,527 (0.79)	653 (0.21)	1,007 (0.63)	513 (0.32)	70 (0.04)	0.7609	0.6259	1.02 (0.90–1.16)
	IVS1 + 539		Control	1,968 (0.79)	520 (0.21)	788 (0.63)	392 (0.32)	64 (0.05)			
17	IPF1_SNP7			G	A	G/G	G/A	A/A			
	none	none	Case	2,836 (0.89)	344 (0.11)	1,260 (0.79)	316 (0.20)	14 (0.01)	0.1309	0.2318	1.14 (0.97–1.34)
	IVS1 + 1787		Control	2,186 (0.88)	302 (0.12)	953 (0.77)	280 (0.23)	11 (0.01)			

Continued on following page

TABLE 1
Continued

Number	Locus	HapMap data	Subject	Allele data (frequency) 1	Allele data (frequency) 3	Genotype data (frequency) 1	Genotype data (frequency) 2	Genotype data (frequency) 3	*P* value Allele 2*2	*P* value Genotype 2*3	OR (95% CI)
18	NEUROG3_SNP1			A	G	A/A	A/G	G/G			
	rs3812704	JPT	Case	1,472 (0.46)	1,708 (0.54)	337 (0.21)	798 (0.50)	455 (0.29)	0.2674	0.4687	1.06 (0.96–1.18)
	−1822		Control	1,114 (0.45)	1,374 (0.55)	252 (0.20)	610 (0.49)	382 (0.31)			
19	NEUROG3_SNP2			T	C	T/T	T/C	C/C			1.06 (0.95–1.19)
	rs4536103	JPT	Case	2,268 (0.71)	912 (0.29)	798 (0.50)	672 (0.42)	120 (0.08)	0.3129	0.2040	
	F199S		Control	1,743 (0.70)	745 (0.30)	616 (0.50)	511 (0.41)	117 (0.09)			
20	PAX6_SNP1			A	T	A/A	A/T	T/T			1.08 (0.97–1.20)
	rs2239789	none	Case	1,725 (0.54)	1,455 (0.46)	483 (0.30)	759 (0.48)	348 (0.22)	0.1697	0.2391	
	IVS6 + 282		Control	1,396 (0.56)	1,092 (0.44)	392 (0.32)	612 (0.49)	240 (0.19)			
21	PAX6_SNP2			C	T	C/C	C/T	T/T			1.01 (0.86–1.18)
	rs667773	none	Case	2,791 (0.88)	389 (0.12)	1,228 (0.77)	335 (0.21)	27 (0.02)	0.9358	0.8923	
	IVS7 + 218		Control	2,181 (0.88)	307 (0.12)	961 (0.77)	259 (0.21)	24 (0.02)			
22	NKX2-2_SNP1			T	C	T/T	T/C	C/C			1.08 (0.74–1.56)
	none	none	Case	3,117 (0.98)	63 (0.02)	1,530 (0.96)	57 (0.04)	3 (0.002)	0.7650	0.8727	
	+856 TGA		Control	2,435 (0.98)	53 (0.02)	1,193 (0.96)	49 (0.04)	2 (0.002)			
23	NKX2-2_SNP2			C	T	C/C	C/T	T/T			1.13 (1.02–1.25)
	rs3746741	none	Case	1,666 (0.52)	1,514 (0.48)	452 (0.28)	762 (0.48)	376 (0.24)	0.0251*	0.0563	
	+1163 TGA		Control	1,228 (0.49)	1,260 (0.51)	305 (0.25)	618 (0.50)	321 (0.26)			
24	NKX6-1_SNP1			A	C	A/A	A/C	C/C			1.03 (0.92–1.16)
	rs1017560	JPT	Case	2,182 (0.69)	998 (0.31)	747 (0.47)	688 (0.43)	155 (0.10)	0.6052	0.0144*	
	−15606		Control	1,724 (0.69)	764 (0.31)	625 (0.50)	474 (0.38)	145 (0.12)			
25	NKX6-1_SNP2			T	G	T/T	T/G	G/G			1.01 (0.83–1.23)
	none	none	Case	2,939 (0.92)	241 (0.08)	1,359 (0.85)	221 (0.14)	10 (0.01)	0.9750	0.9966	
	−8823		Control	2,298 (0.92)	190 (0.08)	1,062 (0.85)	174 (0.14)	8 (0.01)			

26	NKX6-1_SNP3			G	A	G/G	G/A	A/A			1.05 (0.95–1.17)
	rs1545330	JPT	Case	1,719 (0.54)	1,461 (0.46)	452 (0.28)	815 (0.51)	323 (0.20)	0.3348	0.5938	
	−8797		Control	1,312 (0.53)	1,176 (0.47)	338 (0.27)	636 (0.51)	270 (0.22)			
27	NKX6-1_SNP4			A	C	A/A	A/C	C/C			1.01 (0.90–1.13)
	rs2278671	JPT	Case	2,094 (0.66)	1,086 (0.34)	681 (0.43)	732 (0.46)	177 (0.11)	0.8884	0.5354	
	IVS2 + 28		Control	1,633 (0.66)	855 (0.34)	541 (0.43)	551 (0.44)	152 (0.12)			
28	NEUROD1_SNP1			C	G	C/C	C/G	G/G			1.03 (0.91–1.16)
	rs3916026	JPT	Case	893 (0.28)	2,287 (0.72)	132 (0.08)	629 (0.40)	829 (0.52)	0.6685	0.3769	
	−5425		Control	685 (0.28)	1,803 (0.72)	87 (0.07)	511 (0.41)	646 (0.52)			
29	NEUROD1_SNP2			G	T	G/G	G/T	T/T			1.03 (0.90–1.18)
	rs7420169	JPT	Case	2,538 (0.80)	642 (0.20)	1,022 (0.64)	494 (0.31)	74 (0.05)	0.6684	0.6459	
	−5084		Control	1,998 (0.80)	490 (0.20)	803 (0.65)	392 (0.32)	49 (0.04)			
30	ABCC8_SNP1			C	T	C/C	C/T	T/T			1.08 (0.97–1.20)
	rs1799854	JPT	Case	1,507 (0.47)	1,673 (0.53)	371 (0.23)	765 (0.48)	454 (0.29)	0.1664	0.0073†	
	IVS15 −3		Control	1,226 (0.49)	1,262 (0.51)	280 (0.23)	666 (0.54)	298 (0.24)			
31	ABCC8_SNP2			G	A	G/G	G/A	A/A			1.07 (0.91–1.25)
	rs4148643	JPT	Case	2,795 (0.88)	385 (0.12)	1,232 (0.77)	331 (0.21)	27 (0.02)	0.4419	0.7059	
	R1273R		Control	2,169 (0.87)	319 (0.13)	950 (0.76)	269 (0.22)	25 (0.02)			
32	ABCC8_SNP3			T	G	T/T	T/G	G/G			1.07 (0.96–1.19)
	rs757110	JPT	Case	1,884 (0.59)	1,296 (0.41)	570 (0.36)	744 (0.47)	276 (0.17)	0.2432	0.4293	
	S1369A		Control	1,513 (0.61)	975 (0.39)	463 (0.37)	587 (0.47)	194 (0.16)			
33	KCNJ11_SNP1			G	A	G/G	G/A	A/A			1.08 (0.97–1.21)
	rs5219	none	Case	1,954 (0.61)	1,226 (0.39)	610 (0.38)	734 (0.46)	246 (0.15)	0.1513	0.3343	
	E23K		Control	1,576 (0.63)	912 (0.37)	503 (0.40)	570 (0.46)	171 (0.14)			

Locus: experimental name of the SNP, followed by rs number and position. HapMap data: JPT, genotyped on Japanese in Tokyo. Nominal *P* values are listed for allele or genotype frequencies (**P* <0.05; †*P*<0.01). IVS, intron variant sequence.

TABLE 2
Magnitude of LD (D′ and r^2) between *ABCC8* and *KCNJ11* variants

D′/r^2	ABCC8_SNP1	ABCC8_SNP2	ABCC8_SNP3	KCNJ11_SNP1
ABCC8_SNP1 rs1799854	—	0.0012	0.0177	0.0151
ABCC8_SNP2 rs4148643	0.0867	—	0.0919	0.0808
ABCC8_SNP3 rs757110	0.1708	0.9867	—	0.8703
KCNJ11_SNP1 rs5219	0.1653	0.9711	0.9794	—

rs2688, and *NKX2-2*_SNP1 (+856 TGA) and in controls at *NKX6-1*_SNP1 rs1017560 and *ABCC8*_SNP1 rs1799854 (online appendix Table 1). Although none of these are significant with correction for multiple comparisons, we reanalyzed several of the variants, including *NKX2-2*_SNP1 (+856 TGA) and *NKX6-1*_SNP1 rs1017560, and confirmed that there was no typing error for these variants. We also tested whether the observed departures were consistent with the genotype frequencies expected for a genetic disease model (21). The genotype distributions for *TCF2*_SNP5 rs2689, *TCF2*_SNP6 rs2688, *NKX2-2*_SNP1 (+856 TGA), and *ABCC8*_SNP1 rs1799854 are consistent with genetic models that best fit these data. In contrast, the departure from HWE observed in the control samples for *NKX6-1*_SNP1 rs1017560 is not consistent with any genetic model for disease. Thus, the observed departure from HWE in controls at *NKX6-1*_SNP1 rs1017560 is likely to be a chance observation. The remaining departures are unlikely to be attributable to genotyping errors and are consistent with the possibility that the selection of case and control samples from a population in HWE at a susceptibility locus (at the test marker or a polymorphism in strong linkage disequilibrium [LD]) has generated genotype distributions with the observed departures from HWE.

Among the 33 variants of 12 genes, 6 variants (*TCF2*_SNP4 rs1016991, *TCF2*_SNP6 rs2688, *HNF4A*_SNP3 rs745975, *NKX2-2*_SNP2 rs3746741, *NKX6-1*_SNP1 rs1017560, and *ABCC8*_SNP1 rs1799854) showed at least nominally significant associations ($P < 0.05$) with type 2 diabetes (Table 1 and online appendix Table 2). *ABCC8*_SNP1 rs1799854 showed the strongest association ($P = 0.0073$) with diabetes among the SNPs examined in this study. By further analysis of the variant, the T/T genotype was found in 454 (28.6%) and 298 (24.0%) subjects in the diabetic and control groups, respectively, a significant difference in the frequency of individuals with the T/T genotype between the two groups (C/C + C/T vs. T/T, $P = 0.0068$) (online appendix Table 2). The odds ratio (OR) for the T/T genotype was 1.27 (95% CI 1.07–1.50; C/C + C/T vs. T/T), indicating that the T/T genotype in *ABCC8*_SNP1 rs1799854 is associated with type 2 diabetes in Japanese subjects.

There was no association of other variants in *ABCC8* and *KCNJ11* with diabetes (Table 1 and online appendix Table 2). These include the E23K variant in *KCNJ11* (*KCNJ11*_SNP1 rs5219: for the K allele, OR 1.08 [95% CI 0.97–1.21], $P = 0.15$). To determine the extent of LD between the four variants in *ABCC8* and *KCNJ11*, we calculated D′ and r^2 (Table 2). There was modest LD between *ABCC8*_SNP2 rs4148643 and *ABCC8*_SNP3 rs757110. Strong LD was found between *ABCC8*_SNP3 rs757110 and *KCNJ11*_SNP1 rs5219. In the latter, we tested two-locus haplotypes having a frequency of >5% for association with diabetes and found no association of any of the haplotypes with diabetes (data not shown).

We examined the genes involved in pancreatic β-cell function (transcription factors and K_{ATP} channel subunits) in relation to type 2 diabetes in a large cohort of Japanese subjects. The study included 2,834 subjects, the largest case-control study so far conducted on these variants in a Japanese population. For disease susceptibility allele frequencies in the range of 0.3–0.5, our sample had >99% power to detect a susceptibility gene with a genotype relative risk in the range of 1.5–1.85 (for any genetic model of inheritance). For allele frequencies in this range, we had >80% power to detect susceptibility genes with genotype relative risk in the range of 1.25–1.55. Power was similarly good for dominant models with lower susceptibility allele frequencies (0.1–0.3) or recessive models with higher susceptibility allele frequencies (0.5–0.9). The sample was reasonably powered (>90%) to detect recessive susceptibility alleles at low frequencies (0.1–0.3) for higher genotype relative risks (2.1–4.0) but was not sufficiently powered to detect very common (>0.7) dominant susceptibility genes (genotype relative risk >100).

*ABCC8*_SNP1 rs1799854 (exon 16 −3c/t variant) was significantly associated with type 2 diabetes, primarily due to increased frequency of T/T homozygotes among patients. Since this variant is located in the 3′ splice site, it might impair normal splicing. Alternatively, the variant could be in strong LD with an unidentified functional variant in the unscreened region harboring the *ABCC8* gene. There have been two case-control association studies (22,23) conducted for the variant in Japanese populations, both of which found no association of this variant with type 2 diabetes. However, because these studies were based on a relatively small number of subjects (167 subjects in 22; 456 subjects in 23), their power to detect associations is limited. In Caucasians, several studies (7,8,11–13,17) have reported association of the variant with type 2 diabetes, although other studies found no association of the variant with type 2 diabetes (14–16). On the other hand, several studies have reported an association of the E23K variant in *KCNJ11* (*KCNJ11*_SNP1 rs5219 in this study) with type 2 diabetes in Caucasians (9,10,16,18). Recent meta-analyses (19,20) of the variant support this association. Although our present study finds no association of the E23K variant with diabetes in Japanese subjects (for the K allele, OR 1.08 [95% CI 0.97–1.21], $P = 0.15$), 95% CI around the OR overlaps in meta-analysis of European populations, suggesting that our results are not inconsistent with the previous studies on the E23K variant in *KCNJ11*.

The International HapMap Project aims to determine the common patterns of DNA sequence variation in the human genome (24). In the initial phase of the project, genetic data are being gathered from four populations with African, Chinese, Japanese, and European ancestry. Twenty of 33 SNPs used in this study were genotyped on Japanese subjects in the HapMap project, providing important information for determining whether the genes of interest are associated with type 2 diabetes in a Japanese cohort. To

clarify the relationships between our SNPs and those of the HapMap, the patterns of LD between SNPs for each gene are shown in online appendix Fig. 1. Among nine genes (*TCF1*, *TCF2*, *HNF4A*, *ISL1*, *PAX6*, *NKX6-1*, *NEUROD1*, *ABCC8*, and *KCNJ11*) that have a relatively large number of genotyped SNPs in the HapMap, four genes (*ISL1*, *NKX6-1*, *NEUROD1*, and *KCNJ11*) show a relatively strong LD, while five genes (*TCF1*, *TCF2*, *HNF4A*, *PAX6*, and *ABCC8*) show a weak LD across each gene. For the former genes, our data provide considerable information on the association of genes of interest with type 2 diabetes. However, for the latter genes, we could provide only partial information on their association with type 2 diabetes. In contrast, there are none or few genotyped SNPs in the HapMap for three genes (*IPF1*, *NEUROG3*, and *NKX2-2*). For these genes, our data provide valuable information on both the SNPs and their association with type 2 diabetes.

Associations of *HNF4A* variants in the upstream promoter region with type 2 diabetes have recently been reported in several populations (25–27). Using Japanese samples in the HapMap data, we calculated LD between our SNPs (rs717247 and rs745975) and those (rs1884614, rs2144908, and rs4810424) showing association with type 2 diabetes. However, LD was not found (online appendix Fig. 1), indicating that the association of our SNPs with type 2 diabetes is different from that of other studies. In this study, modest associations ($P < 0.05$) with type 2 diabetes were also detected for several of the candidate genes examined (*TCF2*_SNP4 rs1016991, *TCF2*_SNP6 rs2688, *NKX2-2*_SNP2 rs3746741, and *NKX6-1*_SNP1 rs1017560). As there has been no large association study for these variants, these associations need to be confirmed by further replication studies. Nevertheless, this is the largest association study so far conducted on these genes in Japanese subjects, providing valuable information not only for this population, but also for comparison with other ethnic groups.

RESEARCH DESIGN AND METHODS

We examined 1,590 unrelated Japanese type 2 diabetic subjects recruited from nine university hospitals and affiliated hospitals located in seven prefectures in Japan. Type 2 diabetes was diagnosed using World Health Organization criteria. The clinical data on these type 2 diabetic subjects are as follows (continuous data are given as median [interquartile range]): male 54.2%, age at diagnosis 49 years (40–57), and BMI 22.9 kg/m^2 (21.1–25.2). We also examined 1,244 nondiabetic control subjects matched for geographic region under the following criteria: aged ≥60 years, no past history of diagnosis of diabetes, HbA_{1c} <5.6%, and no diabetes within third-degree relatives. Analyses were performed on the whole population of subjects. Genetic analysis of human subjects was approved by the ethics committee at each university. Appropriate informed consent was obtained from all of the subjects examined.

Selection of SNPs and genotyping. We resequenced several target genes using DNA samples of Japanese subjects and selected SNPs for an association study mainly based on a minor allele frequency >0.10 and the possibilities of haplotype construction with the SNPs used in this study. We also selected several SNPs from previous publications (9,10,22).

Genomic DNA was extracted from peripheral blood samples by standard procedures. Genotyping of SNPs was performed by MassARRAY system (Sequenom, San Diego, CA), chip-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of primer extension products following the PCR amplification. Extension primers, extended across the SNP site, were designed using SpectroDESIGNER software (Sequenom, San Diego, CA). The extension reaction is controlled by a mixture of dideoxy-terminated nucleotides, such that one single-base extension product is created and one double-base extension product is created corresponding to an SNP allele. This scheme creates two peaks in the mass spectrometer that are separated by ~300 Da. The primer extension reaction products were loaded onto SpectroCHIPs preloaded with matrix. SpectroCHIPs were analyzed in fully automated mode by MassARRAY mass spectrometer (Bruker-Sequenom). Quality values are attached to each genotyping result, and samples with low quality value were reanalyzed.

Statistical analyses. Differences in distribution of allele or genotype frequencies between type 2 diabetic and control subjects were assessed using χ^2 tests. The extent of LD and haplotype frequencies were estimated using the Hitagene software (Hitachi Europe, Dublin, Ireland) and PowerMarker software (Kejun Liu and Spencer Muse, PowerMarker: new genetic data analysis software, version 3.0; free program distributed over the internet available from http://www.powermarker.net). Power calculations were completed using the Genetics Power Calculator (28). The pairwise r^2 values for SNPs in the HapMap were calculated by the Haploview (29).

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ARTICLES

Shugoshin collaborates with protein phosphatase 2A to protect cohesin

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Sister chromatid cohesion, mediated by a complex called cohesin, is crucial—particularly at centromeres—for proper chromosome segregation in mitosis and meiosis. In animal mitotic cells, phosphorylation of cohesin promotes its dissociation from chromosomes, but centromeric cohesin is protected by shugoshin until kinetochores are properly captured by the spindle microtubules. However, the mechanism of shugoshin-dependent protection of cohesin is unknown. Here we find a specific subtype of serine/threonine protein phosphatase 2A (PP2A) associating with human shugoshin. PP2A colocalizes with shugoshin at centromeres and is required for centromeric protection. Purified shugoshin complex has an ability to reverse the phosphorylation of cohesin *in vitro*, suggesting that dephosphorylation of cohesin is the mechanism of protection at centromeres. Meiotic shugoshin of fission yeast also associates with PP2A, with both proteins collaboratively protecting Rec8-containing cohesin at centromeres. Thus, we have revealed a conserved mechanism of centromeric protection of eukaryotic chromosomes in mitosis and meiosis.

Sister chromatid cohesion is carried out by a multisubunit complex, cohesin, consisting of two SMC (structural maintenance of chromosome) family proteins—a kleisin subunit Scc1/Rad21 and an accessory subunit Scc3 (also called stromal antigen (SA) in animal cells)[1–3]. Cohesion is maintained until metaphase when sister kinetochores attach to microtubules emanating from the opposite spindle poles. The cohesion at centromeres is especially important at this stage, because the establishment of bipolar spindle attachment depends on the tension generated by the pulling force of spindle microtubules and the counteracting force of centromeric cohesion of sister chromatids[4]. Indeed, in animal mitotic cells centromeric cohesin (and cohesion) persists until metaphase, whereas most cohesin dissociates from chromosome arms during prophase and prometaphase to resolve sister chromatids[3]. At the onset of anaphase, the anaphase promoting complex (APC)-dependent degradation of securin allows the activation of a specific endopeptidase, separase, which in turn cleaves and cleans off residual chromosomal Scc1/Rad21, allowing the separation of sister chromatids[5]. Thus, the dissociation of cohesin is regulated by at least two mechanisms in animal cells. During meiosis, the temporally staggered release of arm and centromeric cohesion is most striking. At the first meiotic division (meiosis I), Rec8—which replaces Scc1/Rad21 in meiosis—is cleaved along chromosome arms but is protected at centromeres, where it is only cleaved during the second division (meiosis II)[6,7].

In yeast and probably most eukaryotes, shugoshin (Sgo/MEI-S332) protects meiotic Rec8-containing cohesin from separase cleavage at meiosis I[6–12]. Human shugoshin (hSgo1; also called shugoshin-like 1 (SGOL1)), which is also expressed during proliferation, protects cohesin at centromeres for mitosis[13–15]. Phosphorylation of the cohesin subunit SA2 (an Scc3 homologue) by Polo-like kinase 1 (Plk1) is critical for prophase dissociation because the inactivation of Plk1, or the expression of a non-phosphorylatable form of SA2, substantially blocks dissociation of cohesin in early mitosis[16–18]. Moreover, the dissociation of sister chromatids in hSgo1-depleted cells is suppressed by expressing this mutant SA2 (ref. 15). In budding yeast and human cells, phosphorylation of the Scc1 subunit by Plk1 enhances its cleavability by separase[17,19,20], and this may similarly apply for the meiotic counterpart Rec8 (refs 21, 22). Therefore, a mechanism to protect cohesin at centromeres might be to inhibit its phosphorylation, but no evidence for this has been obtained as yet. It is also unknown whether shugoshin uses a similar mechanism to protect centromeric cohesin in both mitosis and meiosis. Therefore, we have investigated the mechanism by which shugoshin protects cohesin at the centromere.

Shugoshin associates with protein phosphatase 2A

To better understand shugoshin function, we sought to identify associating proteins by tagging hSgo1 with the Flag epitope and expressing the fusion protein in human embryonic kidney (HEK) 293T cells. Anti-Flag immunoprecipitates were analysed using liquid chromatography, followed by tandem mass spectrometry (LC–MS/MS)[23]. The majority of peptides identified in the analysis were those of serine/threonine protein phosphatase 2A (PP2A) (Fig. 1a). PP2A is known to act as a heterotrimeric complex consisting of a core dimer of the catalytic C subunit (PP2A-C) and the scaffold A subunit (PP2A-A), which recruits a third variable regulatory B subunit (PP2A-B/PR55/B55, PP2A-B′/PR61/B56, PP2A-B″ or PP2A-B‴) that controls substrate specificity or localization of PP2A[24]. Our MS analysis identified both core subunits PP2A-A and PP2A-C, and most isoforms of the regulatory B56 subunit, but not any isoforms of other B subunits, suggesting that hSgo1 specifically associates with PP2A containing the B56 subunit. Immunoprecipitation analysis of endogenous hSgo1 supported this conclusion (Fig. 1b) and yeast two-hybrid assays suggested direct association of hSgo1 with the PP2A-B56 subunit (Supplementary Fig. 1).

If PP2A functions together with hSgo1, PP2A would localize at the centromere during mitosis. Immunostaining experiments in HeLa cells indicated that the α isoform of PP2A-B56 (PP2A-B56α)

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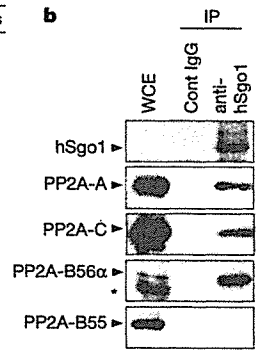
colocalizes with hSgo1 at centromeres from mitotic prophase to metaphase (Fig. 1c). The signals of both proteins decreased at the onset of anaphase. Immunostaining after chromosome spreading further indicated that PP2A-B56α localizes at the inner centromere between a pair of sister kinetochores (Fig. 1d), like hSgo1 (refs 14, 15, 25). When immunostaining for the core subunits, PP2A-A and PP2A-C, was performed in fixed cells, we found that the signals were dispersed throughout the cell (data not shown). However, by extracting mitotic cells before fixation, we could detect signals of PP2A-A and PP2A-C at centromeres in prometaphase cells but not in anaphase cells (Fig. 1e). Whereas PP2A-B56α localized only at the inner centromere, PP2A-A and PP2A-C were additionally found at spindle poles during mitosis (Fig. 1e and Supplementary Fig. 2b). These results suggest that the PP2A core complex localizes at various places within mitotic cells, including centromeres, but the PP2A complex containing the B56 subunit preferentially localizes at the inner centromere.

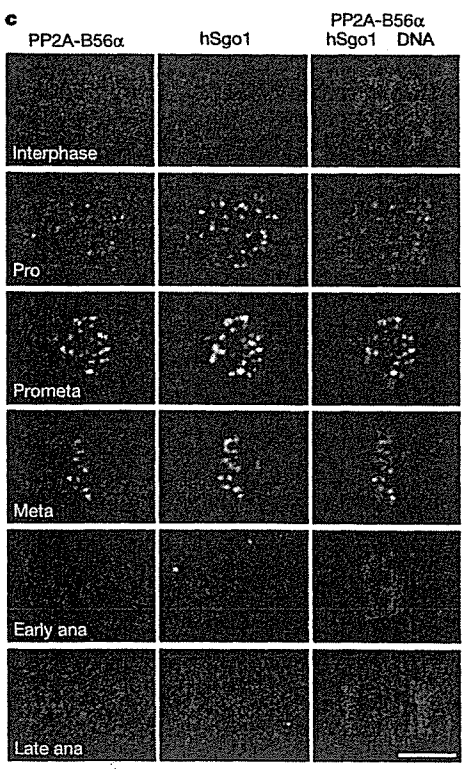
PP2A is required for the protection of centromeric cohesion

To directly assess the importance of PP2A for protecting sister chromatid cohesion at centromeres, we constructed short interfering (si)RNAs against *PP2A-A* and treated HeLa cells with them, which resulted in considerable reduction of PP2A-A protein (Fig. 2a). PP2A-A depletion resulted in an accumulation of mitotic cells, with the prometaphase population being particularly increased in number (Fig. 2b). Chromosomes were highly condensed and the number of spindle poles was often increased (Fig. 2c and Supplementary Fig. 2a), consistent with previous observations using okadaic acid, a potent PP2A inhibitor[26,27]. To examine centromeric cohesion, we spread the chromosomes of mitotic cells treated with *PP2A-A* siRNAs after incubation with nocodazole for 4 h. In control cells, only ~5% of mitotic cells showed separation of sister chromatids. In PP2A-A-depleted cells, however, ~15% of mitotic cells showed loosened or lost centromere cohesion, and ~30% showed sister chromatid separation (Fig. 2d, e). This separation occurred at prometaphase rather than anaphase, because most PP2A-A-depleted mitotic cells showed positive staining for cyclin B1 (Fig. 2b). Immunostaining of *PP2A-A* siRNA-treated cells with anti-Rad21 antibodies showed that cohesin localization was accordingly lost in prometaphase cells (Fig. 2f). The poor penetrance of the phenotype

a

Protein symbol	Protein name	Peptides
PPP2CA/PPP2CB	PP2A catalytic subunit C, α or β isoform	2
PPP2R1B	PP2A regulatory subunit A (PR65), β isoform	8
PPP2R5A	PP2A regulatory subunit B (B56), α isoform	7
PPP2R5B	PP2A regulatory subunit B (B56), β isoform	1
PPP2R5C	PP2A regulatory subunit B (B56), γ isoform	7
PPP2R5D	PP2A regulatory subunit B (B56), δ isoform	9
PPP2R5E	PP2A regulatory subunit B (B56), ε isoform	4
SET	SET translocation (leukaemia-associated)	5
PRO1855	Hypothetical protein PRO1855	1
RBM10/RBM5	RNA binding motif protein, 10 or 5	1
RPL10A	Ribosomal protein L10a	1
RPL28	Ribosomal protein L	1
RPL7	Ribosomal protein L7	1
RPL7A	Ribosomal protein L7a	1
RPLP1	Ribosomal protein, large, P1	1





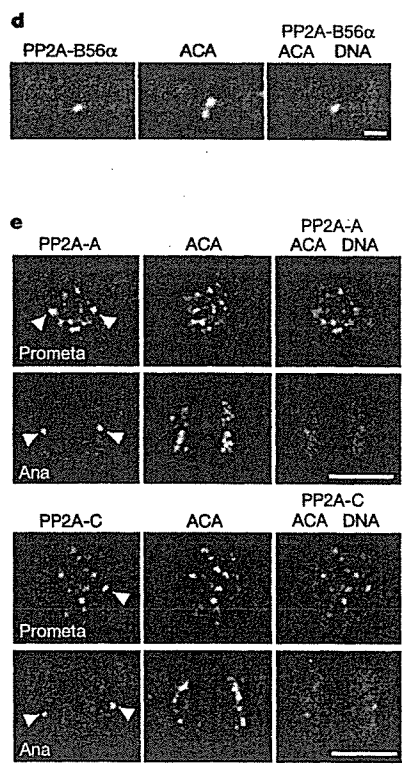


Figure 1 | PP2A associates and colocalizes with hSgo1. **a**, Proteins reproducibly detected in two independent hSgo1 immunoprecipitations by MS analysis are listed. The number of identified peptides of each protein is also shown. **b**, Immunoprecipitates (IP) from an extract of asynchronously growing HeLa cells, obtained using an anti-hSgo1 antibody or control (Cont) IgG, were analysed by western blotting using antibodies against the indicated proteins. An asterisk indicates a cross-reaction. WCE, 0.05% of the whole cell extract. **c**, HeLa cells were stained with anti-PP2A-B56α (green) and anti-hSgo1 (red) antibodies. DNA was counterstained using Hoechst 33342 (blue). PP2A-B56α colocalizes with Sgo1 at centromeres from mitotic prophase to metaphase in almost all cells ($n > 50$). Pro, prophase; Prometa, prometaphase; Meta, metaphase; Ana, anaphase. **d**, A single mitotic chromosome stained with anti-PP2A-B56α (green) and anti-centromere antibodies (ACA) (red). **e**, Mitotic cells were spun onto glass slides using a cytospin and pre-extracted before fixation. The cells were immunostained with anti-PP2A-A or anti-PP2A-C (green) and ACA (red). DNA was stained with Hoechst 33342 (blue). Signals on the spindle poles are indicated by arrowheads. Scale bars, 10 μm (**c**, **e**) and 1 μm (**d**).

after exposure to the *PP2A-A* siRNA can be explained by the residual amount of PP2A-A in the siRNA-treated cells (Fig. 2a), as a more severe phenotype was obtained by treating HeLa cells with okadaic acid (Supplementary Fig. 3). Taken together, we conclude that, like hSgo1, PP2A is required for centromeric protection of sister chromatid cohesion during prophase and prometaphase.

hSgo2 is required for the localization of PP2A at centromeres

Given that hSgo1 associates with PP2A, we examined the possibility that hSgo1 and PP2A require each other for their localization to centromeres (Fig. 3, and see also Supplementary Fig. 4). When PP2A-A was depleted by siRNA, centromeric hSgo1 signals became weakened (Fig. 3a), suggesting that PP2A has a role in facilitating hSgo1 localization to centromeres. In contrast, PP2A-B56 and PP2A-A localization was preserved at centromeres in the hSgo1-depleted cells (Fig. 3c, d), indicating that PP2A can associate with centromeres independently of hSgo1.

Human cells contain another shugoshin-like protein, hSgo2 (also known as SGOL2 and TRIPIN)[8], which has not been studied as yet. To gain a thorough understanding of the relationship between shugoshin and PP2A, we included hSgo2 in our analysis. We found that hSgo2 localizes at centromeres throughout prophase until metaphase, and disappears at anaphase (Supplementary Fig. 5), which is very similar to the localization of hSgo1 (refs 13–15, 25). Likewise, the depletion of hSgo2 using siRNA caused precocious dissociation of centromeric cohesin and separation of sister chromatids (Fig. 2), indicating that hSgo2 also functions in the centromeric protection of cohesin. The depletion of either hSgo1 or hSgo2 did not influence the localization of the other, indicating that they can independently localize to centromeres (Fig. 3a, b). Notably, the depletion of hSgo2 abolished the localization of PP2A (both the regulatory PP2A-B56 and core PP2A-A subunits) at centromeres (Fig. 3c, d). Consistent with this, PP2A coprecipitates with hSgo2; however, the association may occur through the core subunit PP2A-A

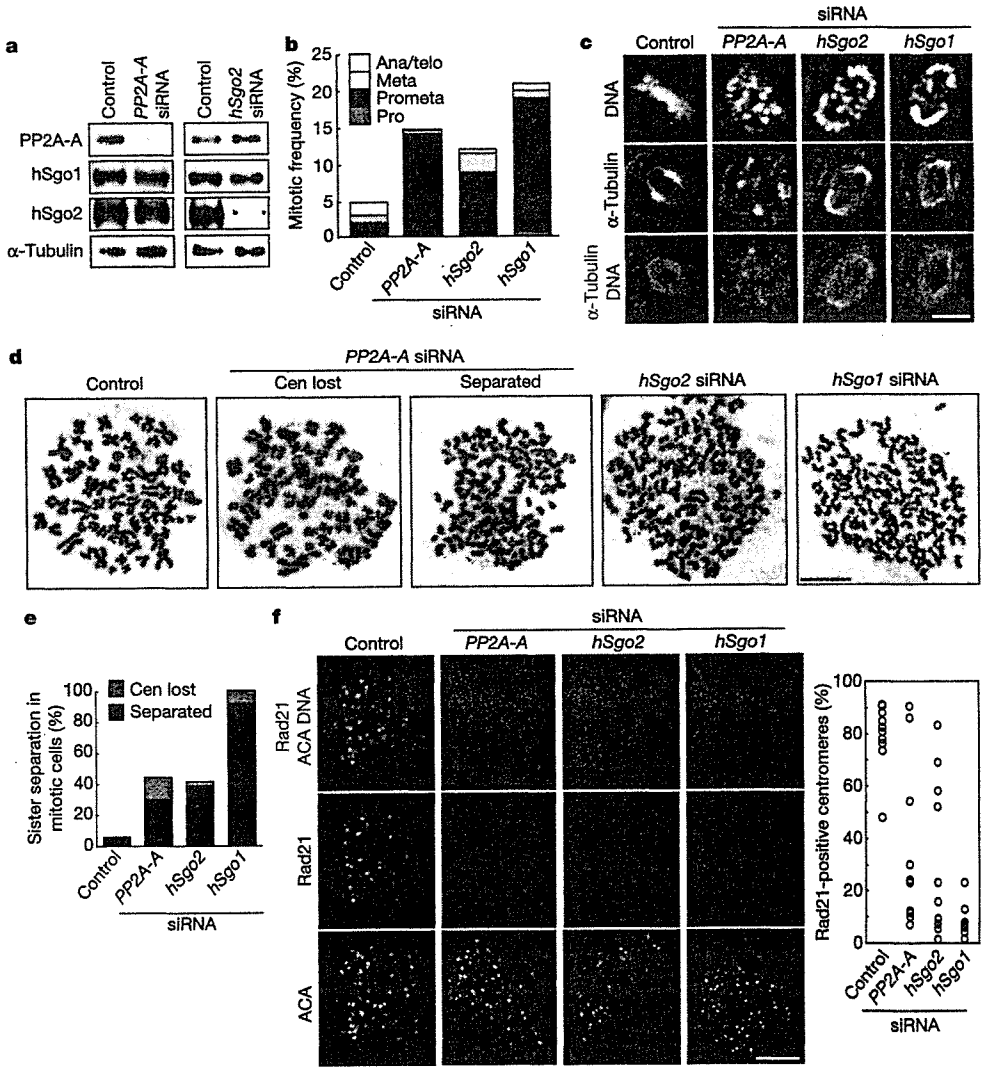


Figure 2 | PP2A is required for centromeric protection. **a**, Extracts from mitotic HeLa cells after exposure to siRNA were immunoblotted with the indicated antibodies. **b**, Mitotic index after siRNA treatment was determined by observing cell shape in living cells ($n > 560$). The mitotic phase was determined by staining for cyclin B1 and DNA in fixed cells ($n > 100$). **c**, Representative prometaphase or metaphase cells stained with anti-α-tubulin (green) and Hoechst 33342 (purple) are shown. **d**, **e**, Mitotic cells after siRNA exposure were treated with nocodazole for 4 h, and chromosomes were spread and stained with Giemsa (**d**). The frequency of cells showing sister chromatid separation ('Separated') or loss of centromeric cohesion ('Cen lost') was determined ($n > 100$) (**e**). **f**, Mitotic cells treated with the indicated siRNAs were spread by cytospin and stained with ACA (red) and anti-Rad21 (green). DNA was counterstained with Hoechst 33342 (blue). Average percentage of Rad21-positive centromeres are shown (one dot represents the average of positive centromeres within one cell). Scale bars, 10 μm (**c**, **d**, **f**).