

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/m² (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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Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome

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Adiponectin is an adipokine that is specifically and abundantly expressed in adipose tissue and directly sensitizes the body to insulin. Hypoadiponectinemia, caused by interactions of genetic factors such as SNPs in the *Adiponectin* gene and environmental factors causing obesity, appears to play an important causal role in insulin resistance, type 2 diabetes, and the metabolic syndrome, which are linked to obesity. The adiponectin receptors, AdipoR1 and AdipoR2, which mediate the antidiabetic metabolic actions of adiponectin, have been cloned and are downregulated in obesity-linked insulin resistance. Upregulation of adiponectin is a partial cause of the insulin-sensitizing and antidiabetic actions of thiazolidinediones. Therefore, adiponectin and adiponectin receptors represent potential versatile therapeutic targets to combat obesity-linked diseases characterized by insulin resistance. This Review describes the pathophysiology of adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome.

The prevalence of obesity has increased dramatically in recent years (1, 2). It is commonly associated with type 2 diabetes, coronary artery disease, and hypertension, and the coexistence of these diseases has been termed the metabolic syndrome (3-7). Insulin resistance is a key feature of these diseases and is defined as a state that requires more insulin to obtain the biological effects achieved by a lower amount of insulin in the normal state. Thus, any defects in the insulin signaling cascade can cause insulin resistance. Insulin stimulates a signaling network composed of a number of molecules, initiating the activation of insulin receptor tyrosine kinase and phosphorylation of the insulin receptor substrate (IRS) proteins (e.g., IRS-1 and IRS-2) (8). Among several components of the network, the signaling axis of IRS proteins and PI3K, which activates downstream serine/threonine kinases including Akt, regulates most of the metabolic actions of insulin, such as suppression of hepatic glucose production and activation of glucose transport in muscle and adipocytes (9). It is known that this pathway is impaired at the multiple steps through alterations in the protein levels and activities of the signaling molecules, enzymes, and transcription factors in insulin resistance caused by obesity, a state of increased adiposity (9).

White adipose tissue (WAT) is a major site of energy storage and is important for energy homeostasis: it stores energy in the form of triglycerides during nutritional abundance and releases it as FFAs during nutritional deprivation (10, 11). While WAT provides a survival advantage in times of starvation, excess WAT is now linked to obesity-related health problems in the current nutritionally rich environment. Regulated by multiple hormonal signals, nuclear hormone receptors (12, 13), and the CNS (14),

WAT has been increasingly recognized as an important endocrine organ that secretes a number of biologically active "adipokines" (15-19). Some of these adipokines have been shown to directly or indirectly affect insulin sensitivity through modulation of insulin signaling and the molecules involved in glucose and lipid metabolism (20). Of these adipokines, adiponectin has recently attracted much attention because of its antidiabetic and antiatherogenic effects and is expected to be a novel therapeutic tool for diabetes and the metabolic syndrome (21). Indeed, a decrease in the circulating levels of adiponectin by genetic and environmental factors has been shown to contribute to the development of diabetes and the metabolic syndrome. The thiazolidinedione (TZD) class of antidiabetic drugs, which also have pleiotropic effects on cardiovascular diseases and lipid metabolism, is known to exert its effects partly through increasing the levels of the active form of adiponectin, as described below.

In this Review, we describe recent progress in research on the pathophysiological role of adiponectin and adiponectin receptors in insulin resistance, type 2 diabetes, and the metabolic syndrome. Since the length of this Review is limited, we recommend that readers also consult other recent reviews on adiponectin research (21-23).

Association of hypoadiponectinemia with insulin resistance, diabetes, and the metabolic syndrome

Adiponectin, also termed Acrp30 (24), AdipoQ (25), apM1 (26), or GBP28 (27), was originally identified independently by 4 groups using different approaches. The *Adiponectin* gene encodes a secreted protein expressed exclusively in both WAT and brown adipose tissue. Adiponectin has a carboxyl-terminal globular domain and an amino-terminal collagen domain and is structurally similar to complement 1q (28, 29), which belongs to a family of proteins that form characteristic multimers (30, 31). Adiponectin exists in a wide range of multimer complexes in plasma and combines via its collagen domain to create 3 major oligomeric forms: a low-molecular weight (LMW) trimer, a middle-molecular weight (MMW) hexamer, and high-molecular weight (HMW) 12- to 18-mer adiponectin (32, 33). In contrast to the expression of adipokines such

Nonstandard abbreviations used: AdipoR, adiponectin receptor; AMPK, AMP-activated protein kinase; APPL, adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain, and leucine zipper motif; GPCR, G protein-coupled receptor; HMW, high molecular weight; IRS, insulin receptor substrate; LMW, low molecular weight; MMW, middle molecular weight; PR-5, pathogenesis related-5; TZD, thiazolidinedione; WAT, white adipose tissue.

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Table 1

Physiological and pathophysiological conditions and treatment modalities associated with either decrease or increase in plasma adiponectin levels

Pathophysiological conditions or treatment modality	Ref.
Hypoadiponectinemia is associated with:	
Genetic variation in <i>Adiponectin</i> gene	33, 72–77
Obesity	35–37
Insulin resistance	37, 38
Type 2 diabetes	37, 39–44
Metabolic syndrome	45
Dyslipidemia	45
Cardiovascular disease	46, 47
Hypertension	48, 49
Sex hormones (androgen, testosterone)	51, 52
Oxidative stress	57
Carbohydrate-rich diet	56
Increase in adiponectin levels is observed following:	
Administration of:	
TZDs	58, 79, 104–108
Angiotensin II receptor blocker (ARB)	110
Angiotensin-converting enzyme inhibitors (ACEIs)	111
Heart failure	114
Renal failure	115
Weight loss	112, 113
Dietary factors:	
Soy protein	53
Oils	54, 55

as TNF- α and resistin, which cause insulin resistance, adiponectin expression is reduced in obese, insulin-resistant rodent models (25). Plasma adiponectin levels are also decreased in an obese rhesus monkey model that frequently develops type 2 diabetes (34). Importantly, a decrease in plasma adiponectin levels preceded the onset of diabetes in these animals, in parallel with the observation of decreased insulin sensitivity (34). Plasma adiponectin levels have also been reported to be reduced in obese humans, particularly those with visceral obesity, and to correlate inversely with insulin resistance (35–38) (Table 1). Prospective and longitudinal studies (37, 39–44) have shown that lower adiponectin levels are associated with a higher incidence of diabetes. Adiponectin, but not inflammatory markers such as C-reactive protein and IL-6, has been shown to be significantly related to the development of type 2 diabetes in Pima Indians (44). Hypoadiponectinemia has also been demonstrated to be independently associated with the metabolic syndrome – indeed, more strongly than are any other inflammatory markers (45). Reduced plasma adiponectin levels are also commonly observed in a variety of states frequently associated with insulin resistance, such as cardiovascular disease (46, 47) and hypertension (48, 49).

How is the level of plasma adiponectin physiologically regulated? There is a sexual dimorphism in the circulating levels of adiponectin. Indeed, female humans and rodents have higher plasma adiponectin levels than males, suggesting that sexual hormones regulate the production of adiponectin, although it is controversial how these hormones, such as estrogen and testosterone, are involved in the regulation of plasma adiponectin level (50–52). Nevertheless, this may partly account for the fact that females are

more sensitive to insulin than males. Some dietary factors, such as soy protein (53), fish oils (54), and linoleic acid (55), are also suggested to increase plasma adiponectin levels, which is consistent with the fact that intake of these factors is thought to have a protective effect on the development of diabetes. On the other hand, a carbohydrate-rich diet appears to decrease plasma adiponectin level (56). Oxidative stress has also been suggested to inhibit the expression of adiponectin (57). Although the mechanism underlying this regulation is unclear, this may contribute to the decrease in plasma adiponectin in obesity, which is associated with increased oxidative stress in adipose tissue. Thus, the plasma adiponectin level is affected by multiple factors, including gender, aging, and lifestyle.

Discovery of the insulin-sensitizing action of adiponectin

The insulin-sensitizing effect of adiponectin was first identified by 3 independent groups in 2001 (58–60). We assessed whether adiponectin was able to improve insulin resistance in KKA^y mice (KK mice overexpressing the agouti protein), as a model of the metabolic syndrome and type 2 diabetes linked to obesity. Plasma adiponectin levels were decreased in KKA^y mice fed a high-fat diet. Replenishment of adiponectin significantly ameliorated high-fat diet-induced insulin resistance and hypertriglyceridemia, which led us to propose that adiponectin is an insulin-sensitizing adipokine (58). These data also strongly suggested that the high-fat diet-induced, obesity-linked decrease in adiponectin level is causally involved in obesity-linked insulin resistance and the metabolic syndrome. Scherer and colleagues reported that an acute increase in the level of circulating adiponectin triggers a transient decrease in basal glucose level by inhibiting both the expression of hepatic gluconeogenic enzymes and the rate of endogenous glucose production in both wild-type and type 2 diabetic mice, and they proposed that adiponectin sensitizes the body to insulin (59). A truncated form of adiponectin that includes the globular domain cleaved proteolytically from full-length adiponectin has been reported to exist in plasma, although in very small amounts (60). Lodish and colleagues reported that a proteolytic cleavage product of adiponectin, which structurally resembles globular adiponectin, increases fatty-acid oxidation in muscle, decreases plasma glucose, and causes weight loss in mice (60).

Subsequently, the chronic effects of adiponectin on insulin resistance in vivo were investigated by generation of adiponectin transgenic mice (61, 62) or adiponectin-deficient mice (63–66). Globular adiponectin transgenic *ob/ob* mice showed partial amelioration of insulin resistance and diabetes (61). Full-length adiponectin transgenic mice showed suppression of insulin-mediated endogenous glucose production (62). Our adiponectin-deficient mice showed mild insulin resistance with glucose intolerance while on a standard diet (63). The adiponectin-deficient mice examined by Maeda et al. exhibited a near-normal insulin sensitivity when fed a standard laboratory diet but developed severe insulin resistance, especially in skeletal muscle, in as few as 2 weeks on a high-fat, high-sucrose diet (64). Ma et al. reported that adiponectin-deficient mice displayed increased fatty-acid oxidation in skeletal muscle but showed no effect on either insulin sensitivity or glucose tolerance whether on a standard or a high-fat diet (65). Scherer's group reported that adiponectin-deficient mice showed mild insulin resistance in the liver while on a standard diet (66), and their phenotype was very similar to those of our adiponectin-deficient mice. On the other hand, some discrepancies in phenotypes that

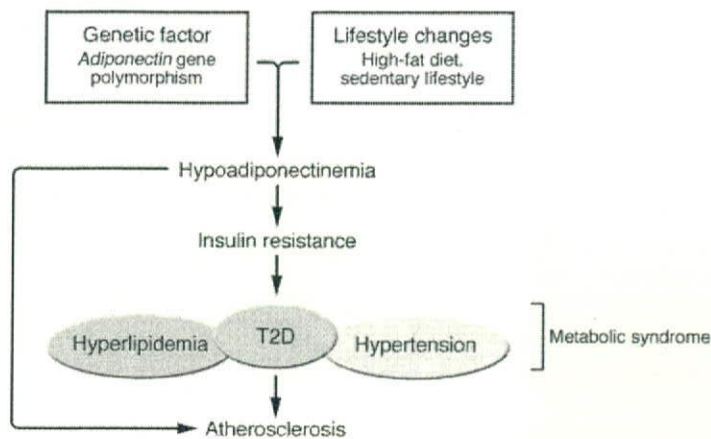


Figure 1

Adiponectin hypothesis for insulin resistance, the metabolic syndrome, and atherosclerosis. Reduced adiponectin levels can be caused by interactions of genetic factors such as SNP 276 in the *Adiponectin* gene itself and environmental factors, i.e., lifestyle changes that cause obesity, such as a high-fat diet and sedentary lifestyle. This reduction in adiponectin levels in turn appears to play an important causal role in the development of insulin resistance, type 2 diabetes (T2D), and metabolic disease, thereby indirectly causing atherosclerosis. Moreover, reduced adiponectin levels also directly play a causal role in the development of atherosclerosis.

have been described among adiponectin-deficient mice are most likely due to differences in genetic background. Adiponectin-deficient mice exhibited other features of the metabolic syndrome, such as hyperlipidemia and hypertension (48, 63).

With respect to the molecular mechanisms underlying the insulin-sensitizing action of adiponectin, we found that full-length adiponectin stimulated AMP-activated protein kinase (AMPK) phosphorylation and activation in the liver, while globular adiponectin did so in both skeletal muscle and the liver (67). Blocking AMPK activation by use of a dominant-negative mutant inhibited these effects of full-length or globular adiponectin, indicating that stimulation of glucose utilization and fatty-acid combustion by adiponectin occurs through activation of AMPK (67). Thus, an adipocyte-derived antidiabetic hormone, adiponectin, activates AMPK, thereby directly regulating glucose metabolism and insulin sensitivity (67). These data also suggested that there may be 2 distinct receptors for adiponectin in the liver and skeletal muscle, with different binding affinities for globular and full-length adiponectin. Lodish, Ruderman, and colleagues also showed that the adiponectin globular domain could enhance muscle fat oxidation and glucose transport via AMPK activation and acetyl-CoA carboxylase inhibition (68). Scherer et al. reported that in adiponectin transgenic mice (62), reduced expression of gluconeogenic enzymes such as phosphoenolpyruvate carboxylase and glucose-6-phosphatase is associated with elevated phosphorylation of hepatic AMPK, which may account for inhibition of endogenous glucose production by adiponectin (59, 67, 69).

Adiponectin also increased fatty-acid combustion and energy consumption, in part via PPAR α activation, which led to decreased triglyceride content in the liver and skeletal muscle, and thereby a coordinated increase of in vivo insulin sensitivity (61).

Adiponectin gene SNPs in human insulin resistance and type 2 diabetes

The *Adiponectin* gene is located on chromosome 3q27, which has been reported to be linked to type 2 diabetes and the metabolic syndrome (70–72). Therefore, the *Adiponectin* gene appears to be a promising candidate susceptibility gene for type 2 diabetes. Among the SNPs in the *Adiponectin* gene, 1 SNP located 276 bp downstream of the translational start site (SNP 276) was concomitantly associated with decreased plasma adiponectin level, greater insulin resistance, and an increased risk of type 2 diabetes (73). The subjects, both of whose 2 alleles of SNP 276 are the G (G/G

genotype), had an approximately doubled risk for developing type 2 diabetes as compared with those with the T/T genotype (73). It is noteworthy that more than 40% of Japanese individuals have the “at-risk” G/G genotype, which makes subjects prone to genetically decreased adiponectin levels and thus susceptible to type 2 diabetes (73). Subjects with an I164T missense mutation in the globular domain of adiponectin had significantly lower plasma adiponectin levels than those without, independently of BMI (74).

Similar associations of the *Adiponectin* gene with susceptibility to type 2 diabetes have also been reported in other ethnic groups. In white German and North American subjects, SNP 276, either independently or as a haplotype together with SNP 45 in exon 2, was shown to be associated with obesity and insulin resistance (75, 76). In white French subjects, 2 SNPs in the promoter region of the *Adiponectin* gene, SNP 11377 and SNP 11391, were significantly associated with hypoadiponectinemia and type 2 diabetes (72). Taken together, these data strongly support the hypothesis that adiponectin plays a pivotal role in the pathogenesis of type 2 diabetes. A recent haplotype analysis based on a dense SNP map in a large sample clarified a 2-block linkage disequilibrium structure of the *Adiponectin* gene, the first block including the promoter SNPs and the second spanning the exons and introns (77). It is noteworthy that neither block has more than 1 SNP significantly associated with the plasma adiponectin level. The haplotypes in the first block were associated with increased adiponectin level, whereas the haplotypes in the second block were associated with decreased adiponectin level. This result indicated the existence of at least 2 causal haplotypes or SNPs in the *Adiponectin* gene.

Based on the significant body of evidence discussed above, we have proposed the “adiponectin hypothesis,” in which reduced plasma adiponectin levels caused by interactions between genetic factors, such as SNPs in the *Adiponectin* gene itself, and environmental factors causing obesity, such as a sedentary lifestyle, may play a crucial role in the development of insulin resistance, type 2 diabetes, and the metabolic syndrome (21) (Figure 1).

Role of HMW adiponectin in insulin resistance and type 2 diabetes

Several observations support the hypothesis that HMW adiponectin is the more active form of the protein and has a more relevant role in insulin sensitivity and in protecting against diabetes. First, rare mutations — G84R and G90S — in the collagen domain are closely associated with type 2 diabetes (33, 73, 78). Subjects with

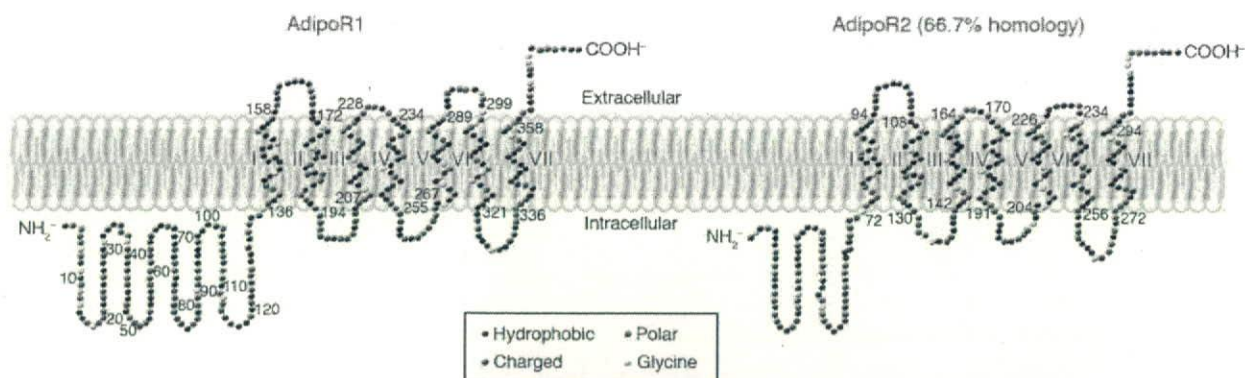


Figure 2

Structure of adiponectin receptors. AdipoR1 and AdipoR2 (66.7% amino acid identity with AdipoR1) are predicted to contain 7 transmembrane domains but are structurally and topologically distinct from GPCRs. Redrawn with permission from *Endocrine Reviews* (21); copyright 2005, The Endocrine Society.

either of these 2 mutations have extremely low levels of HMW adiponectin (Table 1). Moreover, the 2 mutant adiponectins recombinantly expressed in NIH-3T3 fibroblasts were not able to form the HMW form of adiponectin. Second, increases in the ratio of plasma HMW adiponectin levels to total adiponectin levels correlate with improvement in insulin sensitivity during treatment with an insulin-sensitizing drug, TZD, in both mice and human diabetic patients, whereas increases in total serum adiponectin levels do not show good correlations with improvement in insulin sensitivity during treatment with TZD at the individual level (79). Third, the level of plasma HMW adiponectin was reported to be associated with parameters related to glucose homeostasis in a cohort study (80). It is noteworthy that the ratio of plasma HMW adiponectin to total adiponectin correlated more significantly with glucose and insulin levels than did the total adiponectin level (80), suggesting that alterations in plasma HMW adiponectin level may be more relevant to the prediction of insulin resistance than are total plasma adiponectin alterations. Consistent with this, levels of total adiponectin, HMW adiponectin, LMW adiponectin, and the HMW-to-total adiponectin ratio all correlated significantly with key features of central obesity and the insulin-stimulated glucose disposal rate (81). However, HMW adiponectin levels, not total adiponectin levels, are primarily responsible for these relationships, suggesting that measurement of the HMW adiponectin level may be superior to measurement of total adiponectin (81). Using an ELISA system for selective measurement of HMW adiponectin (82), we also found HMW adiponectin and the HMW-to-total adiponectin ratio to have significantly better power for the prediction of insulin resistance and the metabolic syndrome in humans (83). Thus, HMW adiponectin level may be the superior biomarker for insulin resistance, the metabolic syndrome, and type 2 diabetes.

Cloning, function, and regulation of adiponectin receptors

In order to further determine the pathophysiological significance and molecular mechanism of adiponectin action, we isolated cDNA for adiponectin receptors mediating the antidiabetic effects of adiponectin from a human skeletal muscle cDNA library by screening for globular adiponectin binding (84). The cDNA ana-

lyzed encoded a protein designated human adiponectin receptor 1 (AdipoR1) (84). This protein is structurally conserved from yeast to humans (especially in the 7 transmembrane domains). Interestingly, the yeast homologue (YOL002c) plays a key role in metabolic pathways that regulate lipid metabolism, such as fatty-acid oxidation (85). Since at that time there may have been 2 distinct adiponectin receptors, as was described above (67), we searched for a homologous gene in the human and mouse databases. We found only 1 gene that was significantly homologous (67% amino acid identity) with AdipoR1, which was termed AdipoR2 (84). AdipoR1 is ubiquitously expressed, including abundant expression in skeletal muscle, whereas AdipoR2 is most abundantly expressed in the mouse liver. AdipoR1 and AdipoR2 appear to be integral membrane proteins; the N-terminus is internal and the C-terminus is external – opposite to the topology of all other reported G protein-coupled receptors (GPCRs) (84) (Figure 2). Expression of AdipoR1 and AdipoR2 or suppression of AdipoR1 and AdipoR2 expression supports our conclusion that AdipoR1 and AdipoR2 serve as receptors for globular and full-length adiponectin and mediate increased AMPK, PPAR α ligand activities, fatty-acid oxidation, and glucose uptake by adiponectin (Figure 3).

Lodish's group reported that T-cadherin was capable of binding adiponectin in C2C12 myoblasts; however, T-cadherin was not expressed in hepatocytes or the liver (86), the most important target organ (66, 69, 87). Moreover, T-cadherin by itself was thought to have no effect on adiponectin cellular signaling or function, since T-cadherin is without an intracellular domain. These data raised the possibility that T-cadherin may be one of the adiponectin-binding proteins.

Most recently, a 2-hybrid study revealed that the C-terminal extracellular domain of AdipoR1 interacted with adiponectin, whereas the N-terminal cytoplasmic domain of AdipoR1 interacted with APPL (adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain, and leucine zipper motif) (88). Moreover, interaction of APPL with AdipoR1 in mammalian cells was stimulated by adiponectin binding, and this interaction played important roles in adiponectin signaling and adiponectin-mediated downstream events such as lipid oxidation and glucose uptake. These data clearly indicated that adiponectin receptors directly interacted with adiponectin and mediated adiponectin effects. Furthermore,

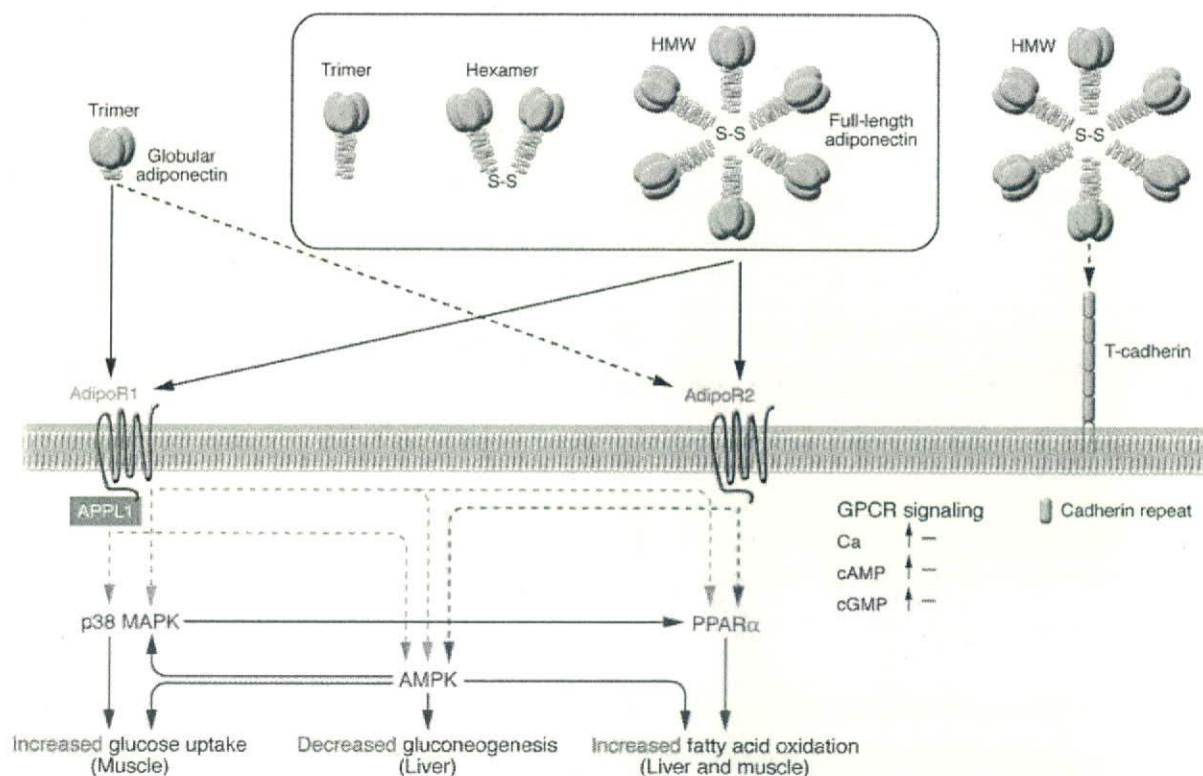


Figure 3

Signal transduction by adiponectin receptors. Globular adiponectin exists as a trimer, whereas full-length adiponectin exists as at least 3 species of multimers: an LMW trimer, an MMW hexamer, and an HMW multimer. Suppression of AdipoR1 by RNA interference markedly reduces globular adiponectin binding, whereas suppression of AdipoR2 by RNA interference largely reduces full-length adiponectin-specific binding (21, 84). The dotted line between AdipoR2 and globular adiponectin reflects that AdipoR2 is a relatively low-affinity receptor for globular adiponectin. AdipoR1 and AdipoR2 do not seem to be coupled with G proteins, since overexpression of AdipoR1/R2 has little effect on cAMP, cGMP, and intracellular calcium levels, but instead these receptors activate unique sets of signaling molecules such as PPAR α , AMPK, and p38 MAPK. In C2C12 myocytes overexpressing AdipoR1/R2, adiponectin stimulates PPAR α , AMPK, and p38 MAPK activation, glucose uptake, and fatty-acid oxidation (84). Suppression of AMPK or PPAR α partially reduces adiponectin-stimulated fatty-acid oxidation, and suppression of AMPK or p38 MAPK partially reduces adiponectin-stimulated glucose uptake. In hepatocytes overexpressing AdipoR1/R2, adiponectin stimulates PPAR α or AMPK and fatty-acid oxidation (84). Suppression of AMPK or PPAR α in these hepatocytes partially reduces adiponectin-stimulated fatty-acid oxidation. Moreover, treatment with adiponectin reduces plasma glucose levels and molecules involved in gluconeogenesis in the liver, and dominant-negative AMPK partly reduces these effects. These data support the conclusion that AdipoR1 and AdipoR2 serve as receptors for globular and full-length adiponectin and mediate increased AMPK, PPAR α ligand activities, p38 MAPK, and adiponectin-induced biological functions. T-cadherin is capable of binding adiponectin but is thought to have no effect on adiponectin cellular signaling, since T-cadherin lacks an intracellular domain (86). Interaction of APPL1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain, and leucine zipper motif 1) with AdipoR1 appears to play important roles in adiponectin signaling and adiponectin-mediated downstream events such as lipid oxidation and glucose uptake (88). S-S, disulfide bond. Adapted with permission from *Endocrine Reviews* (21); copyright 2005, The Endocrine Society.

these data strongly supported that the N-terminus of adiponectin receptors is internal and the C-terminus is external (88).

The expression levels of both AdipoR1 and AdipoR2 were significantly decreased in muscle and adipose tissue of insulin-resistant *ob/ob* mice, probably in part because of obesity-linked hyperinsulinemia (89). Moreover, adiponectin-induced activation of AMPK was impaired in the skeletal muscle of *ob/ob* mice. These data suggest that adiponectin resistance is present in *ob/ob* mice, presumably due to decreased expression of AdipoR1 and AdipoR2 (89). Thus, obesity decreases not only plasma adiponectin levels but also AdipoR1/R2 expression, thereby reducing adiponectin sensitivity and leading to insulin resistance, which in turn aggravates hyperinsulinemia, creating a "vicious cycle" (89). Adiponectin receptor expression in the skeletal muscle of type 2 diabetic patients has been reported to be

decreased (90). In addition, a correlation has been reported between adiponectin receptor gene expression and insulin sensitivity in non-diabetic Mexican Americans with or without a family history of type 2 diabetes (91). Moreover, AdipoR1 mRNA expression was positively correlated with *in vivo* insulin and C-peptide concentrations, first-phase insulin secretion, and plasma triglyceride and cholesterol concentrations before and after adjustment for sex, age, waist-to-hip ratio, and body fat. Expression of AdipoR2 mRNA was clearly associated only with plasma triglyceride concentrations. In multivariate linear regression models, mRNA expression of AdipoR1, but not AdipoR2, was a determinant of first-phase insulin secretion independently of insulin sensitivity and body fat (92). Since AdipoR1 and AdipoR2 are expressed in pancreatic β cells, these receptors may play a role in insulin secretion (93).

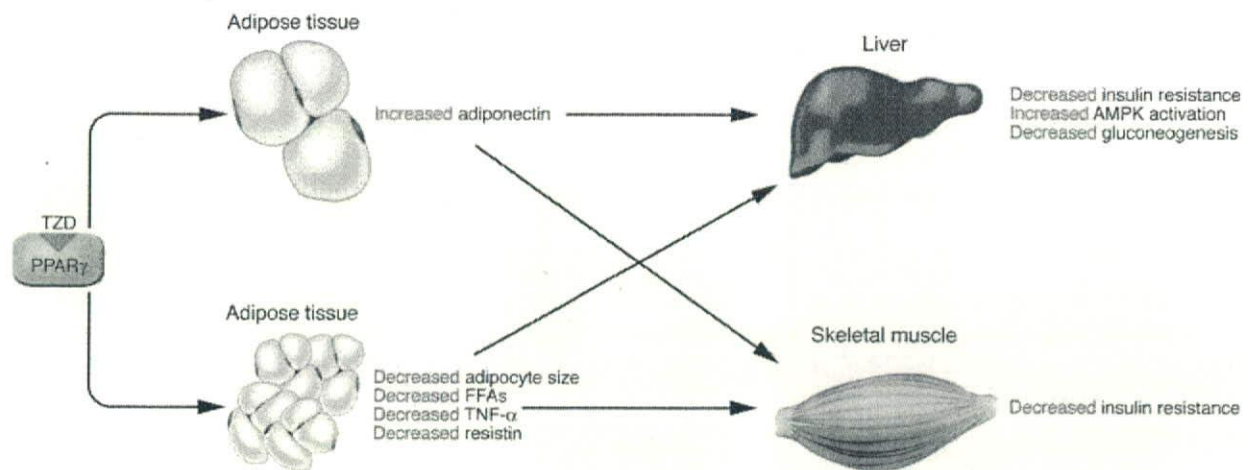


Figure 4

TZDs ameliorate insulin resistance and diabetes by both adiponectin-dependent and -independent pathways. We propose that there are 2 different pathways in the amelioration of insulin resistance induced by the PPAR γ agonists TZDs, such as pioglitazone and probably rosiglitazone. One involves an adiponectin-dependent pathway and the other an adiponectin-independent pathway. TZDs increase adiponectin levels, ameliorating insulin resistance, increasing AMPK activation, and decreasing gluconeogenesis in the liver. On the other hand, independently of adiponectin, TZDs decrease adipocyte size, serum FFA levels, and expression of TNF- α and resistin, thus contributing to amelioration of insulin resistance in skeletal muscle.

Adiponectin and adiponectin receptors as therapeutic targets

According to our adiponectin hypothesis (21), a therapeutic strategy for the treatment of insulin resistance, type 2 diabetes, the metabolic syndrome, and cardiovascular disease may include the upregulation of plasma adiponectin levels, the upregulation of adiponectin receptors, or the development of adiponectin receptor agonists.

TZD-mediated upregulation of plasma adiponectin level. TZDs are known to improve systemic insulin sensitivity in animal models of obesity-linked insulin resistance and diabetes, by enhancing glucose disposal in skeletal muscle and suppressing gluconeogenesis in the liver. TZDs have been widely used as therapeutic agents for the treatment of type 2 diabetes (94–99). TZDs have been proposed to ameliorate insulin resistance by binding to and activating PPAR γ in adipose tissue, thereby promoting adipocyte differentiation and increasing the number of small adipocytes that are more sensitive to insulin (100–103). Plasma adiponectin levels have been shown to be upregulated by TZDs (58, 79, 104–108) (Table 1), and HMW adiponectin is a predominant form of adiponectin upregulated by TZDs (89). TZDs may upregulate adiponectin by generating small adipocytes that abundantly express and secrete adiponectin (100, 102, 109) and/or directly activating *Adiponectin* gene transcription (106). TZDs may also directly facilitate the generation of HMW adiponectin. Since adiponectin is an insulin-sensitizing adipokine, it is reasonable to speculate that the action whereby TZDs increase insulin sensitivity is mediated, at least in part, by increased plasma adiponectin levels. However, whether the TZD-induced increase in plasma adiponectin level is causally involved in TZD-mediated insulin-sensitizing effects has not been addressed experimentally. Adiponectin-deficient (*Adipo*^{-/-}) *ob/ob* mice with a C57BL/6 background were used to investigate whether the PPAR γ agonist pioglitazone is capable of ameliorating insulin resistance in the absence of adiponectin (63, 87). The absence of adiponectin had no effect on either the obese-

ty or the diabetic phenotype of these mice. The severity of insulin resistance and diabetes observed in *ob/ob* mice was significantly reduced in association with significant upregulation of serum adiponectin levels by low-dose (10 mg/kg) pioglitazone treatment. Amelioration of insulin resistance in *ob/ob* mice was attributed to decreased glucose production and increased AMPK levels in the liver, but not to increased glucose uptake in skeletal muscle. In contrast, the severity of insulin resistance and diabetes was not reduced in *Adipo*^{-/-}/*ob/ob* mice (87). With high-dose pioglitazone treatment, the insulin resistance and diabetes of *ob/ob* mice were again significantly ameliorated; this was attributed not only to decreased glucose production in the liver but also to increased glucose uptake in skeletal muscle. Interestingly, *Adipo*^{-/-}/*ob/ob* mice also displayed significant amelioration of insulin resistance and diabetes. The serum FFA and triglyceride levels as well as adipocyte sizes in *ob/ob* and *Adipo*^{-/-}/*ob/ob* mice were unchanged after low-dose pioglitazone treatment but were significantly reduced to a similar degree after high-dose pioglitazone treatment. Moreover, the expression of TNF- α and resistin in adipose tissues of *ob/ob* and *Adipo*^{-/-}/*ob/ob* mice were unchanged after low-dose pioglitazone but were decreased after high-dose pioglitazone. Although both high and low doses of pioglitazone ameliorated insulin resistance and diabetes, the underlying mechanisms may be different (87). We propose that there are 2 different pathways in the amelioration of insulin resistance induced by TZDs such as pioglitazone, and probably rosiglitazone. One involves an adiponectin-dependent pathway and the other an adiponectin-independent pathway (Figure 4). TZDs increase adiponectin levels via activation of *Adiponectin* gene transcription without stimulating adipocyte differentiation (58, 106), thereby increasing AMPK activation, decreasing gluconeogenesis in the liver, and ameliorating insulin resistance and type 2 diabetes. On the other hand, independently of adiponectin, TZDs induce adipocyte differentiation, leading to an increase in the number of small adipocytes, which is



associated with decreased serum FFA levels and decreased TNF- α and resistin expression, together contributing to amelioration of insulin resistance in skeletal muscle (87).

Scherer's group demonstrated that *ob/ob* mice showed significantly improved glucose tolerance after rosiglitazone treatment, whereas *Adipo^{-/-}ob/ob* mice responded only partially to this treatment and remained severely glucose intolerant (66), suggesting that rosiglitazone ameliorated glucose intolerance via both adiponectin-dependent and -independent pathways. Moreover, rosiglitazone significantly increased AMPK activity in the livers of wild-type mice, whereas it had no effect on *Adipo^{-/-}* mice. In skeletal muscle, AMPK activity was also significantly increased in wild-type mice, while no increase was detectable in *Adipo^{-/-}* mice. These data are in complete agreement with our data. Other pharmacological agents as well as lifestyle changes have also been reported to be associated with upregulation of plasma adiponectin levels (53, 55, 110–115) (Table 1).

Upregulation of adiponectin receptors and development of adiponectin receptor agonists. Since AdipoR1 and AdipoR2 are downregulated in obesity-linked insulin resistance and diabetes, both upregulation of AdipoR1 and AdipoR2 expression and agonism of AdipoR1 and AdipoR2 may be a logical approach to providing a novel treatment modality for insulin resistance and type 2 diabetes (84, 89). Previously, Staels's group reported that adiponectin receptors are expressed in human macrophages and that *adiponectin receptor* expression levels may be regulated by agonists of the nuclear receptors PPAR α , PPAR γ , and liver X receptor (116). We have recently shown that, in KKA γ mice, a PPAR α agonist reversed decreases in AdipoR1 and AdipoR2 expression, which was lower in white and brown adipose tissue of KKA γ mice than in that of wild-type control KK mice (117). These data suggested that dual activation of PPAR γ and PPAR α enhanced the action of adiponectin by increasing both total and HMW adiponectin level and adiponectin receptor number, which can ameliorate obesity-linked insulin resistance.

Osmotin is a member of the pathogenesis related-5 (PR-5) family of plant defense proteins (24 members in *Arabidopsis thaliana*) that induce apoptosis in yeast. It is ubiquitous in fruits and vegetables, etc., and the genes encoding the PR-5 protein sequenced from many different species are about 50–95% identical. PR-5 family proteins are also extremely stable and may remain active even when in contact with the human digestive or respiratory system. Bressan's group isolated a yeast clone that exhibited hypersensitivity to osmotin, sequenced the cDNA inserts, and found that PHO36/YOL002c, the yeast homologue of AdipoR1, is a receptor for osmotin (118). X-ray crystallographic studies revealed that both globular adiponectin and osmotin consist of antiparallel β -strands arranged in the shape of a β -barrel. Domain I (lectinlike

domain) of osmotin showed similarity to globular adiponectin in 3D structure, suggesting that these 2 proteins share the lectin-like domain (118). Interestingly, osmotin activates AMPK via adiponectin receptors in mammalian C2C12 myocytes (118). These data raise the possibility that further research examining similarities in adiponectin and osmotin may facilitate the development of potential adiponectin receptor agonists (118). Although further studies will be needed to determine the physiological and pathophysiological roles of AdipoR1 and AdipoR2, the enhancement or mimicking of adiponectin action through modulation of expression and/or function of AdipoR1 and AdipoR2 can be a novel therapeutic strategy for the treatment of insulin resistance, the metabolic syndrome, and type 2 diabetes.

In summary, adiponectin is an adipokine that exerts a potent insulin-sensitizing effect by binding to its receptors such as AdipoR1 and AdipoR2, leading to activation of AMPK, PPAR α , and presumably some other unknown signaling pathways. Indeed, circulating levels of adiponectin, especially HMW adiponectin, are positively correlated with insulin sensitivity and altered by various genetic and environmental factors, pathological conditions, and medications. Thus, monitoring the levels of HMW adiponectin is a good predictable marker for type 2 diabetes and the metabolic syndrome. Moreover, methods to increase adiponectin levels, such as TZD administration, are expected to be effective for the treatment of these diseases. In the future, enhancing or mimicking adiponectin action through modulation of expression and/or function of the adiponectin receptors may be a novel and promising therapeutic strategy for insulin resistance, type 2 diabetes, and the metabolic syndrome.

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Brief Genetics Report

Hepatocyte Nuclear Factor-4 α P2 Promoter Haplotypes Are Associated With Type 2 Diabetes in the Japanese Population

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Hepatocyte nuclear factor (HNF)-4 α is a transcription factor known as a key molecule in the development and functions of the β -cells. In a previously performed genome-wide scan of Japanese type 2 diabetic sibpairs, we observed linkage of type 2 diabetes to chromosome 20q12-q13, a region in which the *HNF4A* gene is located. Recent studies have reported associations between type 2 diabetes and polymorphisms in the P2 promoter region specific to β -cells. In this study, we attempted to assess whether the *HNF4A* gene plays a role in the genetic susceptibility to type 2 diabetes in the Japanese population by analyzing polymorphisms and haplotypes of the *HNF4A* gene. Linkage disequilibrium across the P2 promoter region was preserved in the Japanese population, consistent with previous reports. Although none of the individual polymorphisms examined showed any significant association with type 2 diabetes, we found very strong evidence of the association between type 2 diabetes and the haplotype consisting of two polymorphisms in the P2 promoter region of the *HNF4A* gene ($P = 3.82 \times 10^{-4}$). In contrast, there was no association between type 2 diabetes and haplotypes consisting of polymorphisms not located in the P2 promoter region, suggesting that the type 2 diabetes susceptibility loci are localized in the P2 promoter region of the *HNF4A* gene. The association was replicated using two additional cohorts ($P = 1.51 \times 10^{-4}$ and 0.019, respectively). The results of the present analysis revealed that the *HNF4A* gene might be a type 2 diabetes susceptibility gene common to different ethnic groups. The study also

suggested the possible existence of an as-yet-unidentified but functional polymorphism in the P2 promoter region of the *HNF4A* gene that directly influences susceptibility to type 2 diabetes. *Diabetes* 55:1260–1264, 2006

We previously conducted a 10-cM genome-wide scan for regions linked to type 2 diabetes in 224 affected Japanese sibpairs and found one suggestive linked region and seven potentially linked regions, including 20q12-q13 (the logarithm of odds [LOD] in a multipoint analysis was 2.32 [$P = 0.00102$] at D20S119 in a population subset whose maximum BMI was $<30 \text{ kg/m}^2$), near the gene for hepatocyte nuclear factor (HNF)-4 α (1). Evidence for a type 2 diabetes locus in chromosome 20q12-q13 (OMIM no. 603694) has also been reported from studies in several Caucasian and Chinese populations (2–5), suggesting that this region may harbor a susceptibility gene for type 2 diabetes common to these ethnic groups. The transcription factor HNF-4 α (*HNF4A*) gene, the gene for maturity-onset diabetes of the young (MODY) type 1 (6), a dominantly inherited, early-onset type 2 diabetes characterized by defective glucose-dependent insulin secretion (7), is located in this region. The *HNF4A* has a complex expression pattern, in part due to alternative splicing, and is expressed in many tissues, including the liver and pancreas. Three of the isoforms are transcribed by an alternative P2 promoter, located about 46 kb upstream of the P1 promoter and the coding exons. Transcripts from both the P1 and P2 promoters have been detected in pancreatic β -cells, but the P2 promoter is suggested to be the major transcription start site in these cells (8–10). Mutations of *HNF4A* have been identified in families of MODY type 1 diabetic families, in both the coding and the regulatory regions of the gene, including the P2 promoter region (11). In the β -cells, HNF4A regulates the expression of genes involved in glucose metabolism and insulin secretion (12,13). Therefore, *HNF4A* is currently one of the most attractive candidates as the type 2 diabetes susceptibility gene. Indeed, significant associations between single nucleotide polymorphisms (SNPs) in the P2 promoter region of *HNF4A* and type 2 diabetes have been shown in three Caucasian populations (14–16). However, the precise SNPs directly influencing the susceptibility to type 2 diabetes remain to be identified.

In this study, we conducted a haplotype analysis to investigate whether SNPs in the *HNF4A* gene influence the susceptibility to type 2 diabetes in the Japanese population.

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HNF, hepatocyte nuclear factor; LD, linkage disequilibrium; MODY, maturity-onset diabetes of the young; SNP, single nucleotide polymorphism.
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TABLE 1.

Comparison of genotypic and allelic distribution of SNPs in *HNF4A* between subjects with type 2 diabetes and those without diabetes

Polymorphisms	Genotypes			<i>P</i>	Allele		<i>P</i>
rs6065723 (SNP1)	11	12	22		1	2	
Non-diabetes	136 (70.8)	49 (25.5)	7 (3.7)	0.805	321 (83.6)	63 (16.4)	0.551
Type 2 diabetes	140 (72.9)	47 (24.5)	5 (2.6)		327 (85.2)	57 (14.8)	
rs1884612 (SNP2)	11	12	22		1	2	
Non-diabetes	122 (63.5)	59 (30.7)	11 (5.7)	0.457	303 (78.9)	81 (21.1)	0.416
Type 2 diabetes	126 (65.6)	60 (31.3)	6 (3.1)		312 (81.2)	72 (18.8)	
rs4812822 (SNP3)	11	12	22		1	2	
Non-diabetes	72 (37.5)	90 (46.9)	30 (15.6)	0.451	234 (61.0)	150 (39.0)	0.213
Type 2 diabetes	61 (31.8)	95 (49.5)	36 (18.7)		217 (56.5)	167 (43.5)	
rs4810424 (SNP4)	11	12	22		1	2	
Non-diabetes	52 (27.1)	101 (52.6)	39 (20.3)	0.497	205 (53.4)	179 (46.6)	0.469
Type 2 diabetes	62 (32.3)	91 (47.4)	39 (20.3)		215 (56.0)	169 (44.0)	
rs1884613 (SNP5)	11	12	22		1	2	
Non-diabetes	53 (27.6)	98 (51.0)	41 (21.4)	0.198	204 (53.1)	180 (46.9)	0.718
Type 2 diabetes	59 (30.7)	81 (42.2)	52 (27.1)		199 (51.8)	185 (48.2)	
rs1884614 (SNP6)	11	12	22		1	2	
Non-diabetes	50 (26.0)	96 (50.0)	46 (24.0)	0.470	196 (51.0)	188 (49.0)	0.219
Type 2 diabetes	59 (30.7)	95 (49.5)	38 (19.8)		213 (55.5)	171 (44.5)	
rs2144908 (SNP7)	11	12	22		1	2	
Non-diabetes	53 (27.6)	96 (50.0)	43 (22.4)	0.728	202 (52.6)	182 (47.4)	0.426
Type 2 diabetes	59 (30.7)	95 (49.5)	38 (19.8)		213 (55.5)	171 (44.5)	
rs4812829 (SNP8)	11	12	22		1	2	
Non-diabetes	51 (26.6)	100 (52.1)	41 (21.3)	0.782	202 (52.6)	182 (47.4)	0.515
Type 2 diabetes	57 (29.7)	97 (50.5)	38 (19.8)		211 (54.9)	173 (45.1)	
rs6073418 (SNP9)	11	12	22		1	2	
Non-diabetes	124 (64.6)	61 (31.8)	7 (3.6)	0.798	309 (80.5)	75 (19.5)	0.579
Type 2 diabetes	130 (67.7)	55 (28.6)	7 (3.6)		315 (82.0)	69 (18.0)	
rs4812831 (SNP10)	11	12	22		1	2	
Non-diabetes	80 (41.7)	93 (48.4)	19 (9.9)	0.102	253 (65.9)	131 (34.1)	0.202
Type 2 diabetes	77 (40.1)	82 (42.7)	33 (17.2)		236 (61.5)	148 (38.5)	
rs2273618 (SNP11)	11	12	22		1	2	
Non-diabetes	69 (35.9)	86 (44.8)	37 (19.3)	0.086	224 (58.3)	160 (41.7)	0.344
Type 2 diabetes	52 (27.1)	107 (55.7)	33 (17.2)		211 (54.9)	173 (45.1)	
rs3818247 (SNP12)	11	12	22		1	2	
Non-diabetes	75 (39.1)	93 (48.4)	24 (12.5)	0.685	243 (63.3)	141 (36.7)	0.414
Type 2 diabetes	67 (34.9)	98 (51.0)	27 (14.1)		232 (60.4)	152 (39.6)	

Data are *n* or *n* (%).

RESEARCH DESIGN AND METHODS

The inclusion criteria used for diabetic and nondiabetic subjects in this study have been previously described (17). Diabetes was diagnosed according to the criteria of the World Health Organization (WHO) (18). All subjects enrolled in

this study were of full Japanese ancestry. SNPs were genotyped in 192 nondiabetic subjects (mean age 69.1 ± 6.93 years and mean BMI 23.9 ± 2.77 kg/m²) and type 2 diabetic subjects (mean age 63.1 ± 4.16 years and mean BMI 24.6 ± 2.78 kg/m²) (first case-control cohort). We confirmed the results

TABLE 2

Haplotype frequencies consisting of rs1884614 (SNP6) and rs2144908 (SNP7) in the P2 promoter region of *HNF4A* between subjects with type 2 diabetes and those without diabetes

rs1884614	rs2144908	Frequency (without diabetes)	Frequency (with diabetes)	<i>P</i>	OR (95% CI)	<i>P</i> (overall)
First cohort						
C	G	0.502	0.503	0.98	1.00 (0.75–1.33)	3.82 × 10 ⁻⁴
C	A	0.008	0.051	4.3 × 10 ⁻⁴	6.66 (2.67–29.8)	
T	G	0.024	0.052	0.04	2.23 (1.06–5.35)	
T	A	0.466	0.394	0.05	0.75 (0.56–1.00)	
Second cohort						
C	G	0.507	0.498	0.74	0.96 (0.79–1.18)	1.51 × 10 ⁻⁴
C	A	0.019	0.041	0.02	2.21 (1.15–4.05)	
T	G	0.012	0.042	3.6 × 10 ⁻⁴	3.50 (1.67–7.11)	
T	A	0.462	0.419	0.09	0.84 (0.69–1.03)	
Third cohort						
C	G	0.538	0.485	0.142	0.81 (0.60–1.07)	0.019
C	A	0.020	0.019	0.92	0.95 (0.30–2.45)	
T	G	0.007	0.039	0.003	5.83 (1.69–22.4)	
T	A	0.435	0.457	0.55	1.10 (0.82–1.45)	

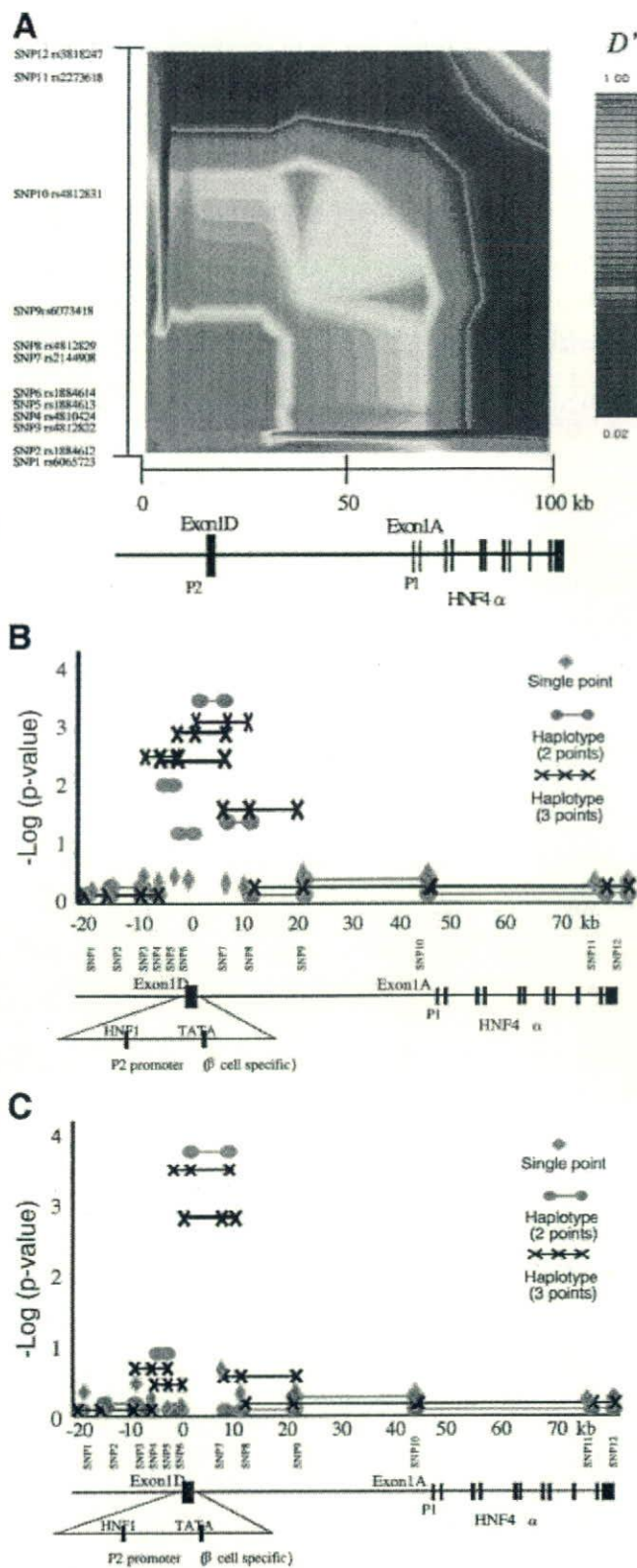


FIG. 1. Association of a haplotype in the *HNF4A* region with type 2 diabetes. A: The pairwise marker LD between SNPs in the *HNF4A* region. The axes are scaled by distance markers (kb). The LD structure in the *HNF4A* region was plotted using GOLD software. B: $-\log_{10} P$ values of the differences in haplotype frequencies between the type 2 diabetic and nondiabetic subjects were plotted against the physical distance in the first cohort (192 diabetic and 192 nondiabetic sub-

jects). At the bottom, the two promoters and exons of *HNF4A* are described. C: The result obtained from the first cohort was confirmed in the second cohort (384 diabetic and 384 nondiabetic subjects). HNF1 refers to the HNF1 binding site, and TATA refers to the TATA box in the P2 promoter region.

derived from our first case-control cohort in a second cohort (384 type 2 diabetic subjects with a mean age of 62.5 ± 9.78 years and a mean BMI of 24.4 ± 3.92 kg/m² and 384 nondiabetic subjects with a mean age of 68.4 ± 9.80 years and a mean BMI of 23.4 ± 5.87 kg/m²) and a third cohort (192 type 2 diabetic subjects with a mean age of 63.7 ± 8.31 years and mean BMI of 23.8 ± 1.39 kg/m² and 192 nondiabetic subjects with a mean age of 69.1 ± 8.32 years and a mean BMI of 22.9 ± 2.7 kg/m²). The three cohorts of subjects (first, second, and third cohorts) were recruited during different times (in the order of first, second, and third cohorts). The type 2 diabetic subjects of the first and second cohorts were recruited from the outpatient clinic of University of Tokyo Hospital; they were all residents of the Tokyo metropolitan area. The type 2 diabetic subjects of the third cohort and the nondiabetic subjects of all the three cohorts were recruited from the Hiroshima Atomic Bomb Casualty Council Health Management Center; they were all residents of Hiroshima, a city 500 miles west of Tokyo (note that all of the nondiabetic subjects were recruited from Hiroshima). Informed consent was obtained from all of the subjects enrolled in the study, and the study was approved by the Ethics Committee of the University of Tokyo.

Screening and selection of SNPs in HNF4A. To establish an SNP map encompassing *HNF4A*, SNPs were ascertained by both direct sequencing and a search of databases. We conducted a screening for SNPs by direct sequencing at all the exons of *HNF4A* and their exon-intron boundaries and along a 1,500-bp region 5' upstream of exon D, which has been reported to be a β -cell-specific promoter region. The conditions and sequences of the primers used in the PCR shall be made available upon request. The SNPs were identified based on the sequences reported in GenBank that contain the *HNF4A* gene (accession no. NT_086910). From the public database, rs6065723, rs1884612, rs4812822, rs4810424, rs1884613, rs1884614, rs2144908, rs2273618, and rs3818247 were selected and validated in 30 type 2 diabetic subjects. The SNPs were genotyped in type 2 diabetic subjects and nondiabetic subjects by direct sequencing. PCR was performed under standard conditions, and the sequencing reactions were performed using the BigDye terminator kit (Applied Biosystems, Foster City, CA) and resolved using an ABI 3700 automated DNA sequencer (Applied Biosystems). The results were integrated using a Sequencher (Gene Codes Corporation, Ann Arbor, MI) and individual SNPs were manually genotyped. Ambiguous base callings were eliminated from further analysis. There was no genotyping error based on blind replicates for two SNPs in 192 samples.

Statistical analysis. The characteristics of the populations were described as means \pm SD. The proportions of specific genotypes or alleles in subjects with and without type 2 diabetes were compared using the χ^2 test. The differences among subjects with different SNP genotypes were statistically tested using ANOVA. The statistical analyses, except for the haplotype estimation, were performed using JMP for Windows, Version 4.00 (SAS Institute, Cary, NC).

Haplotype analysis. The frequencies of each haplotype were estimated, and the differences in the haplotype frequencies between nondiabetic and diabetic subjects were assessed using a software based on the expectation-maximization (EM) algorithm, SNPalyze (Dynacom, Tokyo, Japan), and PHASE, Version 2 (<http://www.stat.washington.edu/stephens/software.html>). The differences in the haplotype frequencies were then analyzed using the χ^2 and permutation tests. The linkage disequilibrium (LD) structure in the *HNF4A* region was plotted using GOLD software.

RESULTS AND DISCUSSION

Genomic DNAs from 192 unrelated type 2 diabetic and nondiabetic Japanese individuals were genotyped at an average of one SNP every 4.1 kb across an ~78-kb region harboring the *HNF4A* gene and its alternative upstream promoter, P2. All the genotypic distributions of the SNPs that were identified were in Hardy-Weinberg equilibrium in both the nondiabetic and type 2 diabetic subjects ($P > 0.05$). Among those identified, SNPs with a minor allele frequency >0.10 were investigated for LD in *HNF4A* and for their association with type 2 diabetes. As shown in Fig. 1A, SNPs were used to determine the pattern of LD in the chromosomal region of *HNF4A*. The LD plot illustrates that the chromosomal region beginning with rs6065723,

-27 kb upstream of the P2 promoter, and rs6073418, 17kb downstream of the P2 promoter, represents one block of strong LD, which is consistent with previous reports (15,16,20). No difference in the distributions of either the genotypes or the SNPs in *HNF4A* were observed between the nondiabetic and type 2 diabetic subjects (Table 1). We then tested the association of haplotypes with susceptibility to type 2 diabetes and found very strong evidence of an association between type 2 diabetes and a certain haplotype in the promoter region of the *HNF4A* gene. This highly significant association was seen with the haplotype consisting of two SNPs, namely rs1884614 and rs2144908, in the P2 promoter region of the *HNF4A* gene (overall P value = 3.82×10^{-4}) (Table 2). In contrast, there was no significant association between type 2 diabetes and haplotypes consisting of polymorphisms not located in the P2 promoter region. Indeed, the extent of association between haplotypes and type 2 diabetes decreased with increasing distance from the P2 promoter region (Fig. 1B). This result supports the idea that a type 2 diabetes susceptibility polymorphism might be located in the P2 promoter region but not in any other region of the *HNF4A* gene. Martin et al. (19) reported that haplotype analysis allowed more accurate mapping of the Alzheimer's disease susceptibility locus to the APOE gene than analysis of individual SNPs, similar to our findings. The association of type 2 diabetes with the rs1884614-rs2144908 haplotype was replicated in two other case-control cohorts. The second cohort (overall P value = 1.51×10^{-4}) consisted of 384 diabetic and 384 nondiabetic subjects. The third cohort (overall P value = 0.019) (Table 2) included both type 2 diabetic and nondiabetic subjects recruited from the same area of Japan to exclude the possibility of false haplotype associations as a result of population stratification between samples enrolled from different areas of Japan (Tokyo and Hiroshima). The T-G rs1884614-rs2144908 haplotype was found consistently more frequently in the type 2 diabetic subjects than in the nondiabetic subjects of the first (0.052 vs. 0.024, haplotype-specific P value = 0.04, odds ratio [OR] 2.23 [95% CI 1.06-5.35]), second (0.042 vs. 0.012, haplotype-specific P value = 3.6×10^{-4} , OR 3.50 [95% CI 1.67-7.11]), and third cohorts (0.039 vs. 0.007, haplotype-specific P value = 0.003, OR 5.83 [95% CI 1.69-22.4]) (Table 2).

We found no association between individual SNPs in the P2 promoter region and type 2 diabetes, which differs from the results reported by Silander et al. (15) in a Finnish population and by Love-Gregory et al. (16) in Ashkenazi Jews. However, our result was consistent with that reported in two recent articles published by Bagwell et al. (20) and Winckler et al. (21), who reported no evidence of any association of single SNPs with type 2 diabetes in Scandinavians and American Caucasians. These discrepant results among the studies may be attributable to the different genetic backgrounds of the subjects. In contrast, we found significant and consistent association between certain haplotypes and type 2 diabetes, which is consistent with the report of Bagwell et al. in which haplotypes consisting of SNPs in the P2 promoter region, but not any individual SNPs, were significantly associated with type 2 diabetes. The haplotype found to be associated with type 2 diabetes in the present study is relatively uncommon. However, it is noteworthy that the association between this haplotype and type 2 diabetes was observed consistently in all the three cohorts, suggesting the existence of an as-yet-unidentified polymorphism that might be rela-

tively uncommon but associated with susceptibility to type 2 diabetes.

The results of the present study revealed that the P2 region of the *HNF4A* gene might be a susceptibility region for type 2 diabetes common to different ethnic groups, including the Japanese. Given that specific haplotypes, but not individual SNPs, in the P2 promoter region were significantly associated with type 2 diabetes, there might exist an as-yet-unidentified but functional polymorphism in the P2 promoter region of the *HNF4A* gene directly influencing the susceptibility to type 2 diabetes. It is also possible that SNPs constituting the haplotype in the P2 promoter region of the *HNF4A* gene associated with type 2 diabetes may coordinately affect the susceptibility to type 2 diabetes.

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Brief Genetics Report

A Polymorphism in the AMPK α 2 Subunit Gene Is Associated With Insulin Resistance and Type 2 Diabetes in the Japanese Population

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AMP-activated protein kinase (AMPK) acts as a fuel gauge for glucose and lipid metabolism. The gene encoding the α 2 isoform of the catalytic subunit of AMPK (*PRKAA2*) is located at one of the Japanese type 2 diabetes loci mapped by our previous genome scan (1p36-32). *PRKAA2* is, therefore, a good candidate gene for insulin resistance and type 2 diabetes. We screened all nine exons, their exon-intron boundaries, and the 5' and 3' flanking regions of *PRKAA2* to identify single nucleotide polymorphisms (SNPs), and we genotyped 192 type 2 diabetic patients and 272 nondiabetic subjects to assess possible associations between genotypes or haplotypes and type 2 diabetes. None of the 10 SNPs genotyped was associated with type 2 diabetes, but the haplotype analysis, consisting of six representative SNPs, revealed one haplotype, with the A (minor) allele for rs2051040 and a major allele for the other five SNPs, to be associated with type 2 diabetes ($P = 0.009$). This finding was confirmed in two larger replication samples (657 case and 360 control subjects, $P = 0.021$; and 356 case and 192 control subjects from the same area in Japan, $P = 0.007$) and a significant P value was obtained in the joint haplotype analysis of all samples (1,205 case and 824 control subjects, $P = 0.0001$). Furthermore, insulin resistance was associated with rs2051040 in nondiabetic subjects, and those with the A (minor) allele had a higher homeostasis model assessment of insulin resistance index than those who did not (initial control subjects [$n = 272$], $P = 0.002$; and joint replication control subjects [$n = 552$], $P = 0.037$). We speculate that the *PRKAA2* gene influences insulin resistance and susceptibility to type 2 diabetes in the

Japanese population. *Diabetes* 55:919–923, 2006

Insulin resistance has been well demonstrated to be a fundamental element in the etiology of type 2 diabetes. The molecular mechanisms and genetic factors involved in insulin resistance have been extensively investigated. AMP-activated protein kinase (AMPK) is an enzyme that is activated by physiological and pharmacological stimuli, including muscle contraction (1), the antidiabetic agent metformin (2), and adipokines, such as leptin (3) or adiponectin (4). It is a heterotrimeric protein composed of a catalytic subunit (α) and two regulatory subunits (β and γ). The catalytic subunit α has two isoforms (α 1 and α 2), and while the α 1 subunit isoform is ubiquitously expressed, the α 2 subunit isoform, *PRKAA2*, is predominantly found in skeletal muscle and liver. Activation of AMPK in skeletal muscle leads to increased glucose uptake and enhanced insulin sensitivity (5), whereas in the liver AMPK activation inhibits glucose production (6). These characteristics of AMPK make this enzyme one of the key regulators of insulin sensitivity and glucose homeostasis. While *AMPK α 1^{-/-}* mice have no apparent metabolic defects, *AMPK α 2^{-/-}* mice exhibit insulin resistance (7). Moreover, *PRKAA2* is located on 1p36-32 (8), which is reportedly linked to type 2 diabetes in the Japanese population (9). *PRKAA2* is therefore a good candidate for the susceptibility gene to insulin resistance and type 2 diabetes.

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AMPK, AMP-activated protein kinase; HOMA, homeostasis model assessment; LD, linkage disequilibrium; SNP, single nucleotide polymorphism.

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RESEARCH DESIGN AND METHODS

To examine the association between SNPs in *PRKAA2* and type 2 diabetes, we first recruited 192 type 2 diabetic patients (123 men and 69 women, aged 61.3 ± 0.6 years, and BMI 24.1 ± 0.2 kg/m²) and 272 nondiabetic subjects (129 men and 143 women, aged 69.1 ± 0.5 years, and BMI 23.9 ± 0.2 kg/m²) as the initial study populations. To confirm these initial results, we also recruited 657 diabetic patients (421 men and 236 women, aged 63.1 ± 0.3 years, and BMI 24.6 ± 0.2 kg/m²) and 360 nondiabetic subjects (141 men and 219 women, aged 69.6 ± 0.3 years, and BMI 23.4 ± 0.2 kg/m²) to obtain replication samples. In both the initial and the replication sample sets, diabetic patients were randomly recruited among those attending the outpatient clinic of the Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo (Tokyo, Japan), and the nondiabetic subjects from those undergoing routine health check-ups at the Hiroshima Atomic Bomb Casualty Council Health Management Center (Hiroshima, Japan). To prevent stratification bias, another unrelated 356 diabetic patients (217 men and 139 women, aged 63.1 ± 0.5 years, and BMI 23.8 ± 0.5 kg/m²) and 192 control subjects (93 men and 99 women, aged 69.9 ± 0.4 years, and BMI 24.6 ± 0.2 kg/m²) were recruited from the same region and same facility in Hiroshima, Japan. The inclusion criteria for this study were described previously (10). All participants gave informed

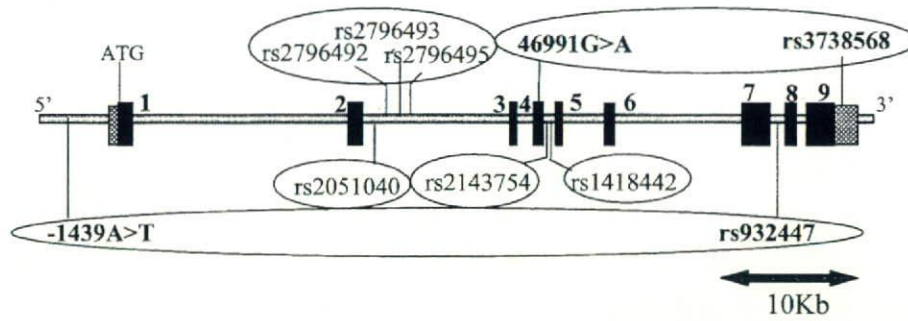


FIG. 1. *PRKAA2* gene structure and haplotype blocks. Structures of *PRKAA2* and SNPs analyzed in this study. Coding and untranslated regions of *PRKAA2* are represented by ■ and □, respectively. SNPs identified by sequencing are shown in boldface. Each circle indicates a cluster of SNPs with $r^2 > 0.8$.

consent, and the ethics committee of the University of Tokyo approved this study.

Screening and genotyping of *PRKAA2*. We screened all nine exons, including their exon-intron boundaries, 5' untranslated region, 3' untranslated region, and 2,000-bp upstream region of *PRKAA2* in 32 diabetic patients to detect new single nucleotide polymorphisms (SNPs). The primer sequences are available from the authors. SNPs were genotyped by direct sequencing. Sequencing reactions were performed with the BigDye terminator (Applied Biosystems, Foster City, CA) and resolved using an ABI 3700 automated DNA

sequencer (Applied Biosystems). The results were integrated using a Sequencer (Gene Codes, Ann Arbor, MI), and individual SNPs were manually genotyped. Ambiguous base identifications were genotyped twice. SNPs were identified based on the sequence reported in GenBank (*PRKAA2*: NT_032977). **Statistical analysis.** Genotype or allele associations for each SNP with type 2 diabetes were tested by Fisher's exact test. Homeostasis model assessment (HOMA) of insulin resistance was calculated as described previously for the nondiabetic subjects (11) in the initial samples because insulin therapy or treatment with oral hypoglycemic agents for type 2 diabetes would presum-

TABLE 1
Genotype and allele frequencies for SNPs in *PRKAA2* in case and control samples (initial samples)

SNP ID*	Distance from ATG†	Location	Genotype	Genotype frequency			Minor allele frequency		
				Case (n = 192)	Control (n = 272)	P value‡	Case/Control	P value§	Odds ratio (95% CI)
-1439A>T	-1439	Promoter	AA	114 (0.59)	149 (0.55)	0.57	0.22/0.25	0.31	0.85 (0.62-1.16)
			AT	71 (0.37)	110 (0.40)				
			TT	7 (0.04)	13 (0.05)				
SNP1 rs2051040	31695	Intron 2	GG	64 (0.33)	106 (0.39)	0.38	0.41/0.37	0.16	1.21 (0.92-1.58)
			GA	95 (0.50)	129 (0.47)				
			AA	33 (0.17)	37 (0.14)				
	rs2796492	Intron 2	CC	77 (0.40)	85 (0.31)	0.14	0.37/0.42	0.11	0.80 (0.61-1.05)
			CT	87 (0.45)	143 (0.53)				
			TT	28 (0.15)	44 (0.16)				
	rs2796493	Intron 2	GG	76 (0.39)	83 (0.31)	0.12	0.37/0.43	0.07	0.78 (0.60-1.04)
			GA	88 (0.46)	142 (0.52)				
			AA	28 (0.15)	47 (0.17)				
SNP2 rs2796495	31919	Intron 2	GG	75 (0.39)	82 (0.30)	0.13	0.37/0.43	0.09	0.80 (0.60-1.04)
			GA	90 (0.47)	146 (0.54)				
			AA	27 (0.16)	44 (0.16)				
	46991G>A	Exon 4	GG	134 (0.70)	176 (0.65)	0.50	0.17/0.20	0.24	0.81 (0.58-1.14)
			GA	52 (0.27)	85 (0.31)				
			AA	6 (0.03)	11 (0.04)				
SNP3 rs2143754	47801	Intron 4	TT	70 (0.36)	83 (0.30)	0.15	0.40/0.46	0.06	0.77 (0.59-1.01)
			TC	92 (0.48)	128 (0.47)				
			CC	30 (0.16)	61 (0.23)				
SNP4 rs1418442	48077	Intron 4	AA	110 (0.57)	144 (0.53)	0.60	0.24/0.26	0.48	0.89 (0.66-1.21)
			AG	73 (0.38)	116 (0.43)				
			GG	9 (0.05)	12 (0.04)				
SNP5 rs932447	59169	Intron 7	AA	113 (0.59)	155 (0.57)	0.81	0.22/0.22	0.83	1.04 (0.76-1.42)
			AG	73 (0.38)	110 (0.40)				
			GG	6 (0.03)	7 (0.03)				
SNP6 rs3738568	62531	3' UTR	TT	153 (0.80)	198 (0.73)	0.22	0.11/0.15	0.12	0.73 (0.49-1.08)
			TC	35 (0.18)	68 (0.25)				
			CC	4 (0.02)	6 (0.02)				

*Newly identified SNPs are shown in boldface. †A of the start codon is counted as 1. P values for ‡genotype frequency and §allele frequency. UTR, untranslated region.