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SHORT COMMUNICATION

A genetic variation of the transcription factor 7-like 2 gene is associated with risk of type 2 diabetes in the Japanese population

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Abstract
Aims/hypothesis It has been suggested that transcription factor 7-like 2 protein (TCF7L2) plays an important role in glucose metabolism by regulating the production level of glucagon-like peptide-1, a hormone which modifies glucose-dependent insulin secretion. Recently, variants of *TCF7L2* gene were reported to confer an increased risk of type 2 diabetes in three different samples from European and European-origin populations. We studied whether the single nucleotide polymorphisms (SNPs) in *TCF7L2* were associated with type 2 diabetes in samples from a Japanese population.
Methods Five SNPs were genotyped in three different sample sets. Association with type 2 diabetes was investigated in each, as well as in combined sample sets.
Results The SNP rs7903146 was nominally associated with type 2 diabetes in the initial (p=0.08) and two replication sample sets (p=0.05 and 0.06). For the combined sample set, in which we successfully genotyped 1,174 type 2 diabetes patients and 823 control subjects, rs7903146 showed a significant association with type 2 diabetes (odds ratio=1.69 [95% CI 1.21–2.36], p=0.002) with the same direction as the previous reports in samples from European and European-origin populations. SNPs rs7903146 and rs7901695 were in complete linkage disequilibrium. The rest of the five SNPs (rs7895340, rs11196205 and rs12255372) did not show any significant associations with type 2 diabetes.
Conclusions/interpretation The consistent association between rs7903146 in *TCF7L2* and type 2 diabetes in different ethnic groups, including the Japanese population, suggests that *TCF7L2* is a common susceptibility gene for type 2 diabetes.

Keywords Association · Susceptibility gene · Type 2 diabetes

Abbreviations

GLP-1	glucagon-like peptide-1
HOMA	homeostasis model assessment
LD	linkage disequilibrium
OR	odds ratio
PAR	population attributable risk
SNP	single nucleotide polymorphism
TCF7L2	transcription factor 7-like 2

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Introduction

Transcription factor 7-like 2 protein (TCF7L2) regulates the production level of proglucagon, which is the precursor of the insulinotropic hormone glucagon-like peptide 1 (GLP-1) in enteroendocrine cells [1]. GLP-1 exerts critical effects on blood glucose homeostasis by increasing insulin secretion. TCF7L2 also has an essential role in the developmental and growth regulatory mechanisms of intestinal epithelial cells which secrete GLP-1, because *TCF7L2*-deficient mice lack an intestinal epithelial stem cell compartment [2]. TCF7L2 could influence susceptibility to type 2 diabetes by altering levels of GLP-1 and/or other hormones. Moreover, *TCF7L2* is located in a chromosomal region that has been reported to be linked to type 2 diabetes in the Icelandic population [3]. Therefore, *TCF7L2* is a plausible candidate gene for type 2 diabetes. Recently, the Icelandic group reported that genetic variations located in introns of *TCF7L2* were significantly associated with type 2 diabetes in samples from Icelandic, Danish and US populations [4].

In this study, we investigated whether the previously demonstrated genetic variations shown to be strongly associated with type 2 diabetes in the samples from European and European-origin populations are also associated with type 2 diabetes in samples from a Japanese population.

Subjects and methods

Subjects We performed association studies in three different sample sets (Table 1). In the initial and replication sample sets, diabetic patients were randomly recruited from among those attending the outpatient clinic of the Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo (Tokyo, Japan), and the non-diabetic subjects from among those undergoing annual health check-ups at the Hiroshima Atomic Bomb Casualty Council Health Management Center (Hiroshima, Japan). Another unrelated 356 diabetic patients and 192 control subjects were recruited from the same region and same facility in Hiroshima to avoid bias due to population stratification. The inclusion criteria for non-diabetic subjects were as follows: (1) >60 years of age; (2) HbA_{1c} values <5.8%; and (3) no family history of type 2 diabetes in first- and second-degree relatives. Diabetes was diagnosed in accordance with WHO criteria [5]. All participants gave informed consent, and the Ethics Committee of the University of Tokyo approved this study.

Single nucleotide polymorphism genotyping We genotyped all five *TCF7L2* single nucleotide polymorphisms (SNPs) previously described by Grant et al. [4]. rs7901695 and rs7895340 were genotyped by direct sequencing, performed with a BigDye terminator (Applied Biosystems, Foster City, CA, USA) and resolved using an ABI 3700 automated DNA sequencer (Applied Biosystems). rs7903146, rs11196205 and rs12255372 were genotyped using Taqman SNP Genotyping Assays by means of an ABI 7900HT (Applied Biosystems) according to the manufacturer's protocol. Genotyping success rates were 99% (rs7901695), 98% (rs7903146), 94% (rs7895340), 99% (rs11196205) and 99% (rs12255372). Concordance rate, based on duplicate comparisons in 192 control subjects and 192 type 2 diabetes, was 100%. All the SNPs were in accordance with Hardy–Weinberg equilibrium in type 2 diabetes subjects ($p>0.17$), control subjects ($p>0.5$) and the whole subject group ($p>0.13$).

Biological measurements Insulin resistance and beta cell function were quantified using homeostasis model assessment (HOMA-IR and HOMA-beta, respectively); HOMA-IR=(fasting insulin [pmol/l])×glucose [mmol/l])/22.5×6 and HOMA-beta=(fasting insulin [pmol/l])×20)/(glucose [mmol/l]−3.5)×6 as described elsewhere [6] (original equation modified to incorporate SI units, with mU/l converted to pmol/l). Data were expressed as means ± SD.

Table 1 Clinical characteristics (means ± SD) of the subjects

	Initial		Replication		Hiroshima		All subjects	
	Diabetes	Control	Diabetes	Control	Diabetes	Control	Diabetes	Control
n	192	272	657	360	356	192	1,205	824
Male	123	129	421	141	217	93	761	363
Female	69	143	236	219	139	99	444	461
Age at onset of diabetes (years)	48.4±11.2	NA	49.8±10.9	NA	57.5±11.0	NA	51.9±11.6	NA
Age at examination (years)	62.8±9.6	68.6±6.7	63.2±9.4	70.5±6.8	70.6±7.1	68.6±6.7	65.3±9.4	69.4±6.8
BMI (kg/m^2)	23.9±3.9	24.0±3.8	24.4±3.9	23.6±3.5	24.0±3.3	23.8±4.0	24.2±3.7	23.8±3.7
Fasting glucose (mmol/l)	8.9±3.0	5.2±0.5	8.9±3.0	5.0±0.8	8.0±3.2	5.2±0.9	8.6±3.1	5.1±0.7
HbA_{1c} (%)	7.4±1.4	5.2±0.3	7.6±1.7	5.2±0.2	6.9±1.2	5.2±0.2	7.4±1.6	5.2±0.2

NA Not applicable

Statistical analysis The proportions of genotypes or alleles were compared between type 2 diabetic and non-diabetic subjects using a multiple logistic regression analysis adjusted for age, sex and BMI. Differences in HOMA according to genotypes were determined by analysis of covariates in non-diabetic subjects, after adjustment for age, sex and BMI. The statistical analyses were performed using JMP for Windows version 4.00 software (SAS Institute, Cary, NC, USA). To examine the pairwise linkage disequilibrium (LD) structure, r^2 between the SNPs were estimated via the method of maximum likelihood from two-locus genotype data using the expectation–maximisation algorithm under the assumption of Hardy–Weinberg equilibrium. The calculations were performed with SNPAlyze v3.2 Pro software (Dynacom, Yokohama, Japan). We considered $p<0.05$ to be significant. The odds ratio (OR) was assessed by counting the number of risk alleles for each individual, and we used the number of risk alleles to predict case/control status using logistic regression. Population attributable risk (PAR) was calculated as $PAR=(p[OR-1])/(1+p[OR-1])$, where p is the prevalence of subjects with the risk allele.

Results

Genotype and allele frequencies of the five SNPs in *TCF7L2* are shown in Table 2. Genotype frequency of rs7903146 was nominally associated with type 2 diabetes in each sample set ($p=0.08$, 0.05, 0.06; initial, replication and Hiroshima sample sets, respectively). However, when all the samples were combined, both genotype and minor allele frequencies were significantly associated with type 2 diabetes (OR 1.69 [95% CI 1.21–2.36], $p=0.0075$ and $p=0.002$; genotype and minor allele frequencies, respectively). This significant association did not change when adjustment for BMI was omitted from the multiple logistic regression analysis (data not shown). The minor allele frequency of rs7903146 in the samples from a Japanese population (0.03–0.05) was substantially smaller than that of the previously reported three samples from European and European-origin populations, in which minor allele frequencies ranged from 0.27 to 0.39. Association between rs7903146 and type 2 diabetes in a dominant model was also examined, as its minor allele frequency was very low, but did not reach significance in two of our three sample sets. However, when all the samples were combined, we could confirm the significantly increased risk of type 2 diabetes in subjects with CT or TT (10.5 vs 6.4%, type 2 diabetes vs controls, respectively; OR 1.75 [95% CI 1.23–2.48], $p=0.0018$).

We found a significant interaction between SNP rs7903146 and BMI ($p=0.031$) in the logistic regression analysis, suggesting that the effect of SNP rs7903146 on the risk of type 2 diabetes was different according to BMI. When we restricted the subjects to those with BMI lower than the median (BMI<23.8 and <23.5 kg/m^2, for type 2 diabetes patients and control subjects, respectively), rs7903146 showed a higher OR (2.02 [95% CI 1.28–3.21], $p=0.0027$; Electronic supplementary Table 1). The association was negative in subjects with BMI higher than the median (OR 1.32 [95% CI 0.81–2.17], $p=0.27$).

rs7901695 showed a significant association with type 2 diabetes in the initial sample set (minor allele frequency: diabetes/control; 0.07/0.04, $p=0.04$), and because it was in complete LD ($r^2=1.0$; Electronic supplementary Fig. 1) with rs7903146, we did not conduct further genotyping in the replication and Hiroshima sample sets. There were no significant differences in genotype or allele frequencies between type 2 diabetes patients and control subjects regarding rs7895340, rs11196205 and rs12255372 (Table 2).

We tested the SNP rs7903146 for quantitative trait association in non-diabetic subjects. rs7903146 did not show any association with age, sex, BMI and other clinical parameters related to type 2 diabetes such as HbA_{1c}, fasting glucose, fasting insulin, HOMA-IR (CC vs CT/TT; 1.83±1.2 vs 1.65±0.8, $p=0.25$), and HOMA-beta (CC vs CT/TT; 100.0±65.7 vs 97.6±49.8, $p=0.79$) in the non-diabetic subjects.

Discussion

In this study, we found a significant association in samples from a Japanese population between the variation in *TCF7L2* and type 2 diabetes, an association similar to that previously reported in samples from European and European-origin populations [4, 7–13]. It is noteworthy that the association between the SNP in *TCF7L2* and type 2 diabetes has consistently been observed in different ethnic groups [14, 15], which supports the reliability of both previous studies as well as our present study. The mechanism of action of TCF7L2 in glucose metabolism and the pathogenesis of type 2 diabetes has yet to be elucidated, but it is possible that TCF7L2 has a role in regulating glucose-sensitive insulin secretion from beta cells. The prevalence of type 2 diabetes in the Japanese population is as high as in the USA [16], although the prevalence of obesity is much lower than that seen in Western countries [17]. One of the possible explanations is that fewer Japanese than European subjects are able to secrete enough insulin to compensate adequately for insulin resistance due to obesity [18]. Therefore, it is important to clarify the genetic components of susceptibility to insulin deficiency. Based on the present results, subjects having an at-risk allele account for 4% of the population, and the corresponding PAR is 3%, a value much lower than that in samples from European and European-origin populations

Table 2 Genotype and allele frequencies (*n* [%]) of *TCF7L2* SNPs

	Initial			Replication			Hiroshima			All subjects			Minor allele frequency		OR (95% CI)[c]
	Diabetes	Control	*p* value[a]	Diabetes	Control	*p* value[a]	Diabetes	Control	*p* value[a]	Diabetes	Control	*p* value[a]	Diabetes/control	*p* value[b]	
rs7901695[d]															
CC	165 (87)	251 (92.3)													
CT	22 (12)	21 (7.7)													
TT	2 (1)	0 (0)	0.08												
rs7903146															
CC	165 (87)	251 (92.3)		584 (89.8)	338 (94)		302 (90)	181 (95)		1,051 (89.5)	770 (93.6)				
CT	22 (12)	21 (7.7)		64 (9.8)	20 (5.5)		33 (10)	10 (5)		119 (10.2)	51 (6.2)		0.05/0.03	0.002	
TT	2 (1)	0 (0)	0.08	2 (0.4)	2 (0.5)	0.05	0 (0)	0 (0)	0.06	4 (0.3)	2 (0.2)	0.0075			1.69 (1.21–2.36)
rs7895340															
GG	148 (85)	226 (90.4)		559 (87.6)	306 (90)		308 (91.7)	162 (90.5)		1,015 (88.4)	694 (90.2)				
GA	23 (13)	23 (9.2)		75 (11.8)	32 (9.4)		27 (8)	17 (9.5)		125 (10.9)	72 (9.4)		0.06/0.05	0.16	
AA	3 (2)	1 (0.4)	0.15	4 (0.6)	2 (0.6)	0.53	1 (0.3)	0 (0)	0.65	8 (0.7)	3 (0.4)	0.37			1.18 (0.88–1.58)
rs11196205															
GG	161 (83.9)	244 (90)		578 (88)	319 (88.6)		310 (89)	172 (90.7)		1,049 (87.6)	739 (89.6)				
GC	28 (14.6)	27 (10)		76 (11.6)	38 (10.6)		35 (10)	18 (9.3)		139 (11.6)	83 (10)		0.06/0.05	0.13	
CC	3 (1.5)	0 (0)	0.03	3 (0.4)	3 (0.8)	0.67	3 (1)	0 (0)	0.40	9 (0.8)	3 (0.4)	0.29			1.21 (0.92–1.56)
rs12255372															
GG	175 (93)	254 (94)		615 (93.6)	343 (95.3)		330 (95..1)	185 (96.4)		1,120 (93.7)	782 (95.0)				
GT	16 (7)	17 (6)		41 (6.2)	16 (4.4)		16 (4.6)	7 (3.6)		73 (6.1)	40 (4.9)		0.03/0.02	0.21	
TT	0 (0)	0 (0)	0.46	1 (0.2)	1 (0.3)	0.43	1 (0.3)	0 (0)	0.64	2 (0.2)	1 (0.1)	0.44			1.21 (0.82–1.81)

[a] *p* values are based on genotype frequencies.

[b] *p* values are based on allele frequencies.

[c] ORs were calculated using an additive genetic model that in logistic regression is multiplicative on the OR scale. OR for each SNP was adjusted simultaneously for age, sex and BMI.

[d] Replication and Hiroshima sample sets were not genotyped owing to the complete LD between rs7901695 and rs7903146.

(21%) [4]. It is possible that *TCF7L2* plays a substantial role in genetic susceptibility to insulin deficiency in the Japanese population. Florez et al. [19] reported that *TCF7L2* polymorphisms were associated with increased risk of developing type 2 diabetes in samples from a population of European origins. In that study, associations between *TCF7L2* polymorphisms and type 2 diabetes in other ethnic groups including Asians were also investigated. However, no significant associations were identified, possibly due to the small sample size. The present study provides important information suggesting *TCF7L2* is a type 2 diabetes susceptibility gene common to various ethnic groups including Japanese.

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Duality of interest There is no duality of interest.

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Brief Genetics Report

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

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Hepatocyte nuclear factor (HNF)-4α 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 kg/m^2), near the gene for hepatocyte nuclear factor (HNF)-4α (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-4α (*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			*P*	Allele		*P*
rs6065723 (SNP1)	11	12	22		1	2	
Non-diabetes	136 (70.8)	49 (25.5)	7 (3.7)		321 (83.6)	63 (16.4)	
Type 2 diabetes	140 (72.9)	47 (24.5)	5 (2.6)	0.805	327 (85.2)	57 (14.8)	0.551
rs1884612(SNP2)	11	12	22		1	2	
Non-diabetes	122 (63.5)	59 (30.7)	11 (5.7)		303 (78.9)	81 (21.1)	
Type 2 diabetes	126 (65.6)	60 (31.3)	6 (3.1)	0.457	312 (81.2)	72 (18.8)	0.416
rs4812822 (SNP3)	11	12	22		1	2	
Non-diabetes	72 (37.5)	90 (46.9)	30 (15.6)		234 (61.0)	150 (39.0)	
Type 2 diabetes	61 (31.8)	95 (49.5)	36 (18.7)	0.451	217 (56.5)	167 (43.5)	0.213
rs4810424 (SNP4)	11	12	22		1	2	
Non-diabetes	52 (27.1)	101 (52.6)	39 (20.3)		205 (53.4)	179 (46.6)	
Type 2 diabetes	62 (32.3)	91 (47.4)	39 (20.3)	0.497	215 (56.0)	169 (44.0)	0.469
rs1884613 (SNP5)	11	12	22		1	2	
Non-diabetes	53 (27.6)	98 (51.0)	41 (21.4)		204 (53.1)	180 (46.9)	
Type 2 diabetes	59 (30.7)	81 (42.2)	52 (27.1)	0.198	199 (51.8)	185 (48.2)	0.718
rs1884614 (SNP6)	11	12	22		1	2	
Non-diabetes	50 (26.0)	96 (50.0)	46 (24.0)		196 (51.0)	188 (49.0)	
Type 2 diabetes	59 (30.7)	95 (49.5)	38 (19.8)	0.470	213 (55.5)	171 (44.5)	0.219
rs2144908 (SNP7)	11	12	22		1	2	
Non-diabetes	53 (27.6)	96 (50.0)	43 (22.4)		202 (52.6)	182 (47.4)	
Type 2 diabetes	59 (30.7)	95 (49.5)	38 (19.8)	0.728	213 (55.5)	171 (44.5)	0.426
rs4812829 (SNP8)	11	12	22		1	2	
Non-diabetes	51 (26.6)	100 (52.1)	41 (21.3)		202 (52.6)	182 (47.4)	
Type 2 diabetes	57 (29.7)	97 (50.5)	38 (19.8)	0.782	211 (54.9)	173 (45.1)	0.515
rs6073418 (SNP9)	11	12	22		1	2	
Non-diabetes	124 (64.6)	61 (31.8)	7 (3.6)		309 (80.5)	75 (19.5)	
Type 2 diabetes	130 (67.7)	55 (28.6)	7 (3.6)	0.798	315 (82.0)	69 (18.0)	0.579
rs4812831 (SNP10)	11	12	22		1	2	
Non-diabetes	80 (41.7)	93 (48.4)	19 (9.9)		253 (65.9)	131 (34.1)	
Type 2 diabetes	77 (40.1)	82 (42.7)	33 (17.2)	0.102	236 (61.5)	148 (38.5)	0.202
rs2273618 (SNP11)	11	12	22		1	2	
Non-diabetes	69 (35.9)	86 (44.8)	37 (19.3)		224 (58.3)	160 (41.7)	
Type 2 diabetes	52 (27.1)	107 (55.7)	33 (17.2)	0.086	211 (54.9)	173 (45.1)	0.344
rs3818247 (SNP12)	11	12	22		1	2	
Non-diabetes	75 (39.1)	93 (48.4)	24 (12.5)		243 (63.3)	141 (36.7)	
Type 2 diabetes	67 (34.9)	98 (51.0)	27 (14.1)	0.685	232 (60.4)	152 (39.6)	0.414

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/m^2) and type 2 diabetic subjects (mean age 63.1 ± 4.16 years and mean BMI 24.6 ± 2.78 kg/m^2) (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)	*P*	OR (95% CI)	*P* (overall)
First cohort						
C	G	0.502	0.503	0.98	1.00 (0.75–1.33)	
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)	3.82×10^{-4}
Second cohort						
C	G	0.507	0.498	0.74	0.96 (0.79–1.18)	
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)	1.51×10^{-4}
Third cohort						
C	G	0.538	0.485	0.142	0.81 (0.60–1.07)	
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)	0.019

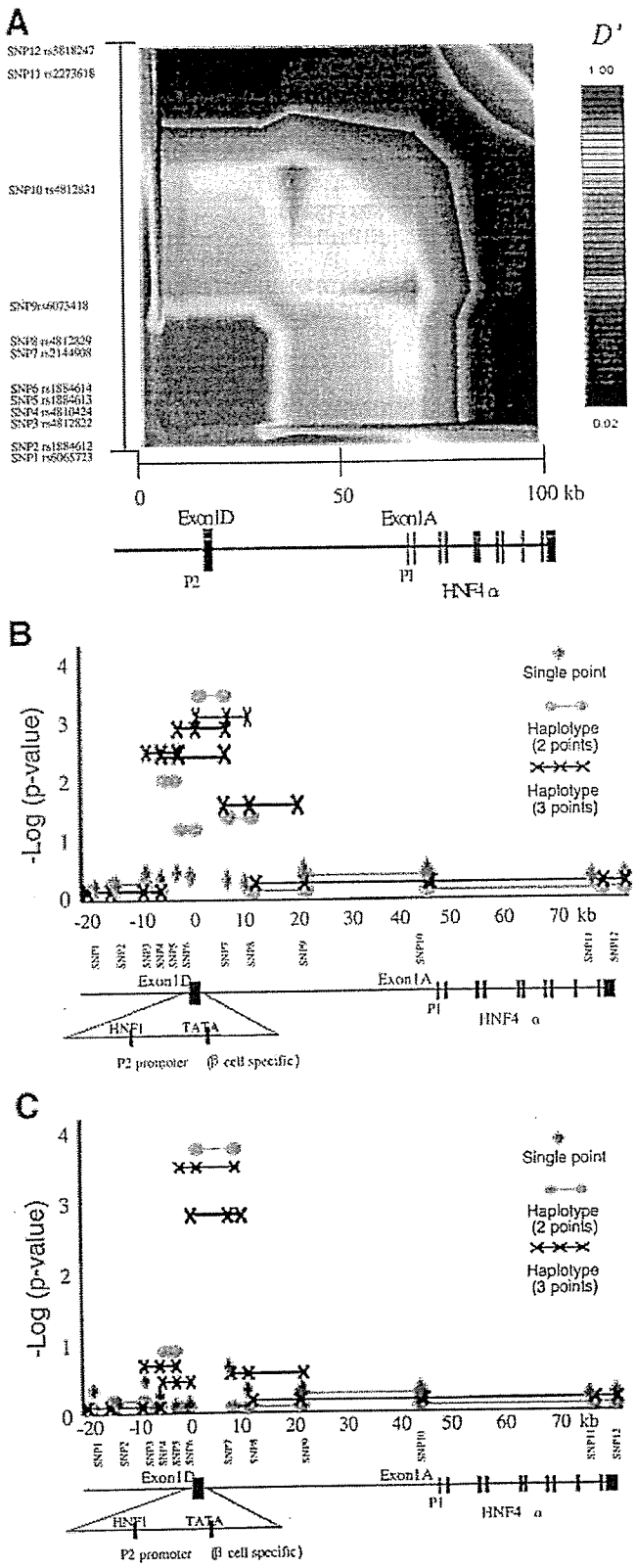

FIG. 1. Association of a haplotype in the *HNF4A* region with type 2 diabetes. *A*: The pairwise marker LD between SNPs in the *HNF4A* region. The axes are scaled by distance markers (kb). The LD structure in the *HNF4A* region was plotted using GOLD software. *B*: $-\text{Log}_{10}$ *P* values of the 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 subjects). 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/m^2 and 384 nondiabetic subjects with a mean age of 68.4 ± 9.80 years and a mean BMI of 23.4 ± 5.87 kg/m^2) 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/m^2 and 192 nondiabetic subjects with a mean age of 69.1 ± 8.32 years and a mean BMI of 22.9 ± 2.7 kg/m^2). 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 ± 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. 1*A*, 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 *HFN4A* 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. 1*B*). 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 relatively 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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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 *Adiponectin* 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

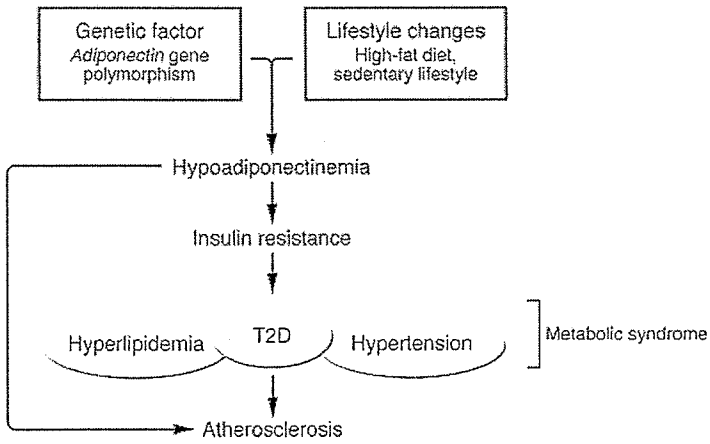

Figure 1
Adiponectin hypothesis for insulin resistance, the metabolic syndrome, and atherosclerosis. Reduced adiponectin levels can be caused by interactions of genetic factors such as SNP 276 in the *Adiponectin* gene itself and environmental factors, i.e., lifestyle changes that cause obesity, such 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 PPARα 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

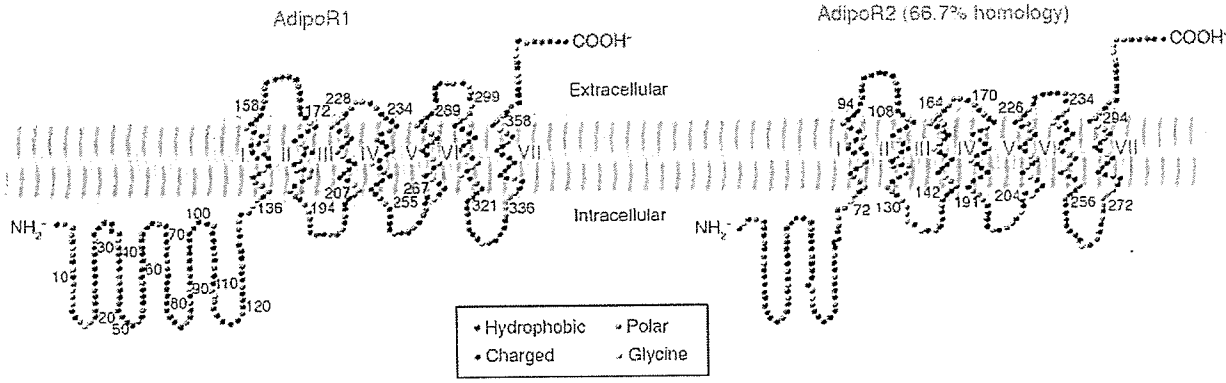

Figure 2
Structure of adiponectin receptors. AdipoR1 and AdipoR2 (66.7% amino acid identity with AdipoR1) are predicted to contain 7 transmembrane domains but are structurally and topologically distinct from GPCRs. Redrawn with permission from *Endocrine Reviews* (21); copyright 2005, The Endocrine Society.

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 analyzed 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, PPARα 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,

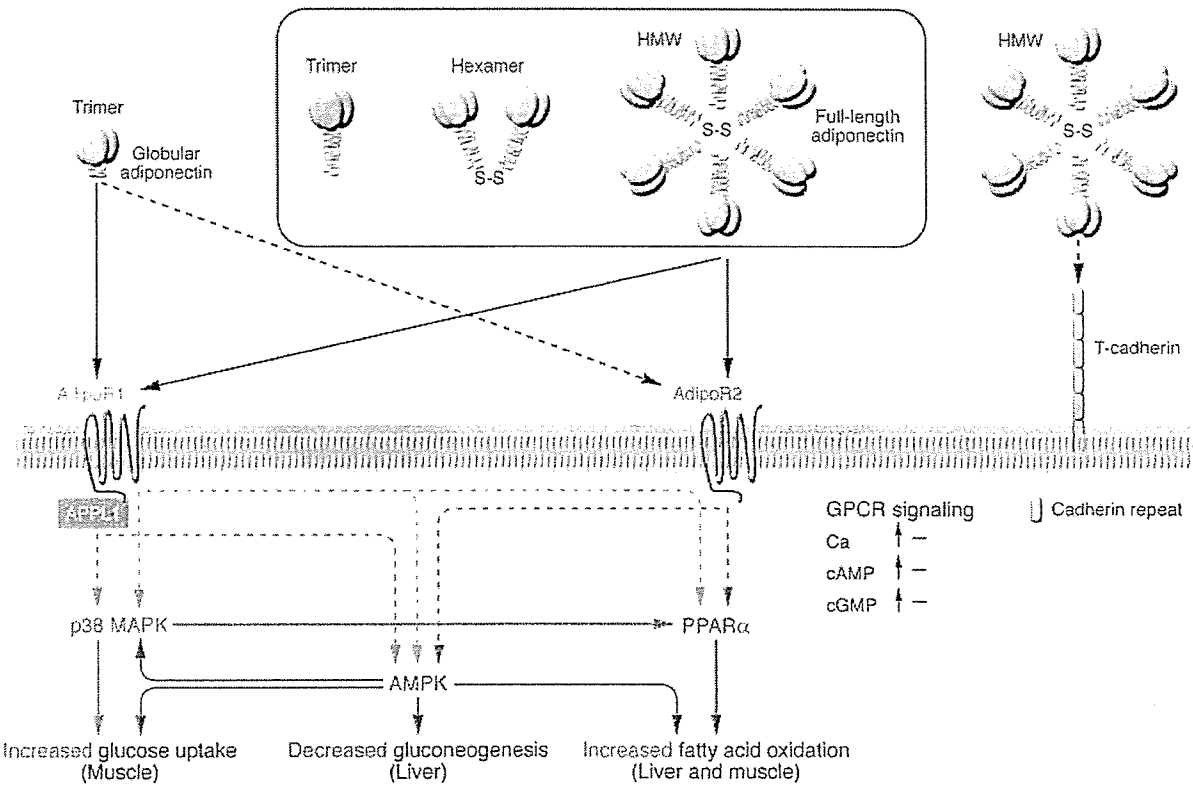

Figure 3

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 PPARα, AMPK, and p38 MAPK. In C2C12 myocytes overexpressing AdipoR1/R2, adiponectin stimulates PPARα, AMPK, and p38 MAPK activation, glucose uptake, and fatty-acid oxidation (84). Suppression of AMPK or PPARα 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 PPARα or AMPK and fatty-acid oxidation (84). Suppression of AMPK or PPARα 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, PPARα 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 nondiabetic 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).

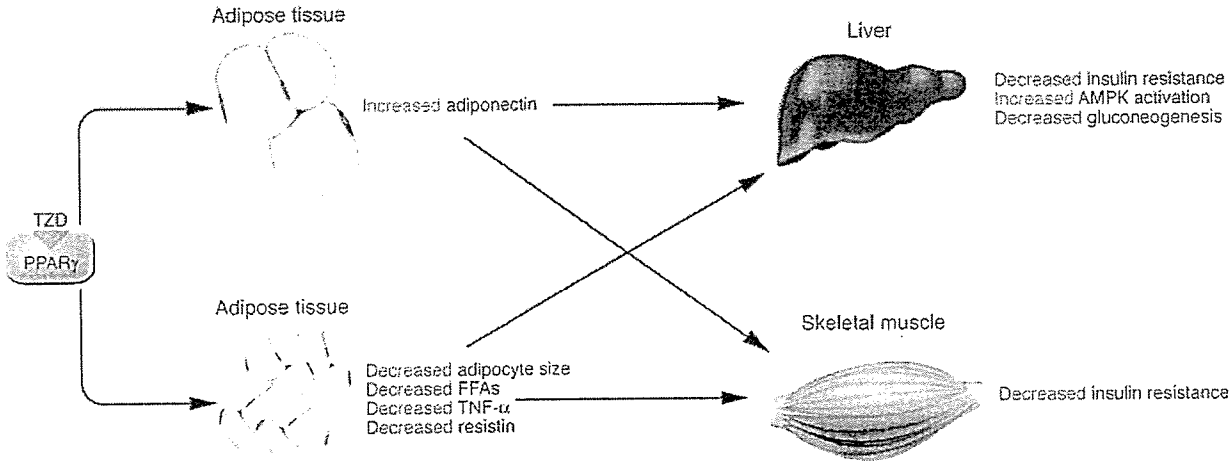

Figure 4
TZDs ameliorate insulin resistance and diabetes by both adiponectin-dependent and -independent pathways. We propose that there are 2 different pathways in the amelioration of insulin resistance induced by the PPARγ agonists TZDs, such as pioglitazone and probably rosiglitazone. One involves 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 PPARγ 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 PPARγ 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 obesity 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 PPARα, PPARγ, and liver X receptor (116). We have recently shown that, in KKA^{y} mice, a PPARα agonist reversed decreases in AdipoR1 and AdipoR2 expression, which was lower in white and brown adipose tissue of KKA^{y} mice than in that of wild-type control KK mice (117). These data suggested that dual activation of PPARγ and PPARα 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, PPARα, 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

A Polymorphism in the AMPKα2 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 α2 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 (α1 and α2), and while the α1 subunit isoform is ubiquitously expressed, the α2 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 AMPKα1$^{-/-}$ mice have no apparent metabolic defects, AMPKα2$^{-/-}$ 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.

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/m^2) and 272 nondiabetic subjects (129 men and 143 women, aged 69.1 ± 0.5 years, and BMI 23.9 ± 0.2 kg/m^2) 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/m^2) and 360 nondiabetic subjects (141 men and 219 women, aged 69.6 ± 0.3 years, and BMI 23.4 ± 0.2 kg/m^2) 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/m^2) and 192 control subjects (93 men and 99 women, aged 69.9 ± 0.4 years, and BMI 24.6 ± 0.2 kg/m^2) 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

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