Am. J. Hum. Genet. 75:832–843, 2004

Association of the Gene Encoding Wingless-Type Mammary Tumor Virus Integration-Site Family Member 5B (*WNT5B*) with Type 2 Diabetes

Akio Kanazawa,[1] Syuuichi Tsukada,[1] Akihiro Sekine,[2] Tatsuhiko Tsunoda,[3] Atsushi Takahashi,[3] Atsunori Kashiwagi,[5] Yasushi Tanaka,[6] Tetsuya Babazono,[7] Masafumi Matsuda,[9] Kohei Kaku,[9] Yasuhiko Iwamoto,[7] Ryuzo Kawamori,[6] Ryuichi Kikkawa,[5] Yusuke Nakamura,[4,8] and Shiro Maeda[1]

Laboratories for [1]Diabetic Nephropathy, [2]Genotyping, and [3]Medical Informatics and [4]Research Group for Personalized Medicine, SNP Research Center, The Institute of Physical and Chemical Research, Kanagawa, Japan; [5]Department of Medicine, Shiga University of Medical Science, Shiga, Japan; [6]Department of Medicine, Metabolism & Endocrinology, School of Medicine, Juntendo University, [7]Diabetes Center, Tokyo Women's Medical University, and [8]Laboratory of Molecular Medicine, Human Genome Center, The Institute of Medical Science, University of Tokyo, Tokyo; and [9]Division of Endocrinology and Metabolism, Department of Internal Medicine, Kawasaki Medical School, Okayama, Japan

Recent reports have suggested that WNT signaling is an important regulator for adipogenesis or insulin secretion and might be involved in the pathogenesis of type 2 diabetes. To investigate possible roles of the *WNT* genes in conferring susceptibility to type 2 diabetes, we examined the association of the genes that encode members of the WNT family with type 2 diabetes in the Japanese population. First, 40 single-nucleotide polymorphism (SNP) loci within 11 *WNT* genes were analyzed in 188 subjects with type 2 diabetes (case-1) and 564 controls (control-1). Among them, six SNP loci exhibited a significant difference ($P < .05$) in the allele and/or genotype distributions between case and control subjects. These SNP loci were further analyzed in another set of case (case-2; $n = 733$) and control (control-2; $n = 375$) subjects to confirm their statistical significance. As a result, one SNP locus in the *WNT5B* gene was strongly associated with type 2 diabetes ($\chi^2 = 15.6$; $P = .00008$; odds ratio = 1.74; 95% confidence interval 1.32–2.29). Expression of the *WNT5B* gene was detectable in several tissues, including adipose, pancreas, and liver. Subsequent in vitro experiments identified the fact that expression of the *Wnt5b* gene was increased at an early phase of adipocyte differentiation in mouse 3T3-L1 cells. Furthermore, overexpression of the *Wnt5b* gene in preadipocytes resulted in the promotion of adipogenesis and the enhancement of adipocytokine-gene expression. These results indicate that the *WNT5B* gene may contribute to conferring susceptibility to type 2 diabetes and may be involved in the pathogenesis of this disease through the regulation of adipocyte function.

Introduction

Type 2 diabetes (MIM 125853) is one of the most common diseases of middle age, and its prevalence continues to increase steadily in many countries, including Japan. The pathogenesis of type 2 diabetes is characterized by the reduction of insulin secretion combined with peripheral insulin resistance (Kahn 1998), but the detailed mechanism is still not well known.

To date, several genes have been reported as candidates for conferring susceptibility to type 2 diabetes, including peroxisome proliferator-activated receptor γ (*PPARG* [MIM 601487]) (Altshuler et al. 2000), calpain-10 (*CAPN10* [MIM 605286]) (Horikawa et al. 2000), and adiponectin (*APM1* [MIM 605441]) (Hara et al. 2002), although these genes can account for the condition in only a small percentage of all diabetic patients; most of the susceptibility genes for type 2 diabetes remain to be identified.

The *WNT* genes have been reported to play a pivotal role in embryonic development and oncogenesis. Recently, one isoform of the WNT family has been reported to play an important role in adipogenesis (Ross et al. 2000; Bennett et al. 2002), and low-density lipoprotein receptor–related protein 5 (*LRP5* [MIM 603506]), which is known as a mediator for WNT signaling, has also been shown to be involved in glucose-induced insulin secretion (Fujino et al. 2003). Therefore, genes related to the WNT-signaling pathway could be considered as candidate genes for conferring susceptibility to type 2 diabetes. However, to our knowledge, no association studies that focus on the *WNT* genes have yet been reported.

In the present study, we demonstrate the results of a case-control association study for the *WNT* genes in Japanese subjects, and we provide evidence that the

Received May 26, 2004; accepted for publication August 24, 2004; electronically published September 21, 2004.

Address for correspondence and reprints: Dr. Shiro Maeda, Laboratory for Diabetic Nephropathy, SNP Research Center, The Institute of Physical and Chemical Research, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan. E-mail: smaeda@src.riken.jp

0002-9297/2004/7505-0009$15.00

Table 1

Association of the *WNT* Genes with Type 2 Diabetes and SNP Locations

Gene and JSNP ID[a]	Model[b]	χ^2	P[c]	Chromosome	SNP Location (Major Allele/Minor Allele)
WNT2:					
IMS-JST000659	R	3.1	.078	7	Intron 2+4921 (C/T)
IMS-JST007915	R	3.7	.054	7	Intron 3+340 (A/C)
IMS-JST011331	R	3.9	.049	7	Intron 3+5839 (T/C)
IMS-JST035778	R	3.2	.073	7	Intron 3+11329 (T/A)
IMS-JST035780	R	2.4	.120	7	Intron 3+6954 (A/C)
IMS-JST057756	D	2.2	.140	7	Intron 3+17103 (T/C)
IMS-JST111067	R	7.8	.0051	7	3′-flank+1629 (T/C)
IMS-JST160646	R	.1	.71	7	3′-flank+2696 (T/C)
WNT2B:					
IMS-JST068883	Allele	1.9	.31	1	Exon 6+870 (C/T)
IMS-JST147806	Allele	1.1	.29	1	Intron 2+13176 (C/A)
IMS-JST082135	Allele	1.6	.20	1	Intron 5+425 (A/G)
IMS-JST147811	D	1.7	.18	1	Intron 2+23287 (C/T)
IMS-JST147812	R	2.4	.12	1	Exon 6+224 (G/A)
WNT3:					
IMS-JST169568	Allele	.9	.34	17	Intron 2+2715 (C/A)
WNT3A:					
IMS-JST153590	R	.4	.52	1	Exon 4+665 (C/T)
WNT4:					
IMS-JST015559	D	.9	.32	1	Intron 2+1784 (C/T)
IMS-JST015560	D	.3	.59	1	Intron 2+2108 (A/G)
IMS-JST015561	D	.3	.56	1	Intron 2+2134 (G/C)
IMS-JST015562	Allele	.5	.45	1	Intron 2+2469 (G/A)
IMS-JST119480	D	.5	.45	1	Intron 4+388 (C/T)
WNT5A:					
IMS-JST086337	D	3.9	.049	3	Exon 4+2328 (G/A)
IMS-JST128940	D	.7	.40	3	Intron 3+1875 (T/C)
IMS-JST128941	R	4.9	.026	3	Intron 3+1798 (C/T)
WNT5B:					
IMS-JST024407	R	1.6	.20	12	Intron 4+1453 (T/G)
IMS-JST024409	R	5.0	.025	12	Intron 4+6763 (C/T)
IMS-JST024410	Allele	.5	.47	12	Intron 4+7770 (C/T)
IMS-JST024411	D	1.4	.24	12	Intron 4+7976 (T/C)
IMS-JST012095	R	.4	.53	12	Intron 5+905 (G/A)
IMS-JST012098	2 × 3	3.1	.22	12	Intron 5+4083 (G/T)
IMS-JST012099	R	1.2	.27	12	Intron 5+4148 (A/G)
IMS-JST024404	R	8.5	.0036	12	Intron 4+438 (C/G)
IMS-JST012100	R	1.7	.19	12	Intron 5+4214 (T/C)
IMS-JST161961	Allele	.6	.41	12	Intron 1+11798 (C/T)
IMS-JST168889	D	1.3	.25	12	Exon 1+234 (G/A)
WNT9A:					
IMS-JST153589	D	1.7	.19	1	Exon 2+58 (G/A)
WNT10B:					
IMS-JST092651	R	.7	.38	12	Exon 5+348 (G/A)
IMS-JST138743	Allele	1.4	.23	12	3′-flank+244 (G/A)
WNT11:					
IMS-JST091808	R	.2	.64	11	Exon 3+89 (G/A)
WNT16:					
IMS-JST159943	R	1.2	.26	7	Intron 3+5525 (A/G)
IMS-JST159946	D	.4	.51	7	Intron 3+2747 (T/C)

NOTE.—In this study, 188 cases and 564 controls were examined.

[a] JSNP IDs selected from the IMS-JST Web site.

[b] D = dominant model; R = recessive model; Allele = allele frequency; 2 × 3 = genotype distribution (2 × 3).

[c] The smallest P value among the four models is presented.

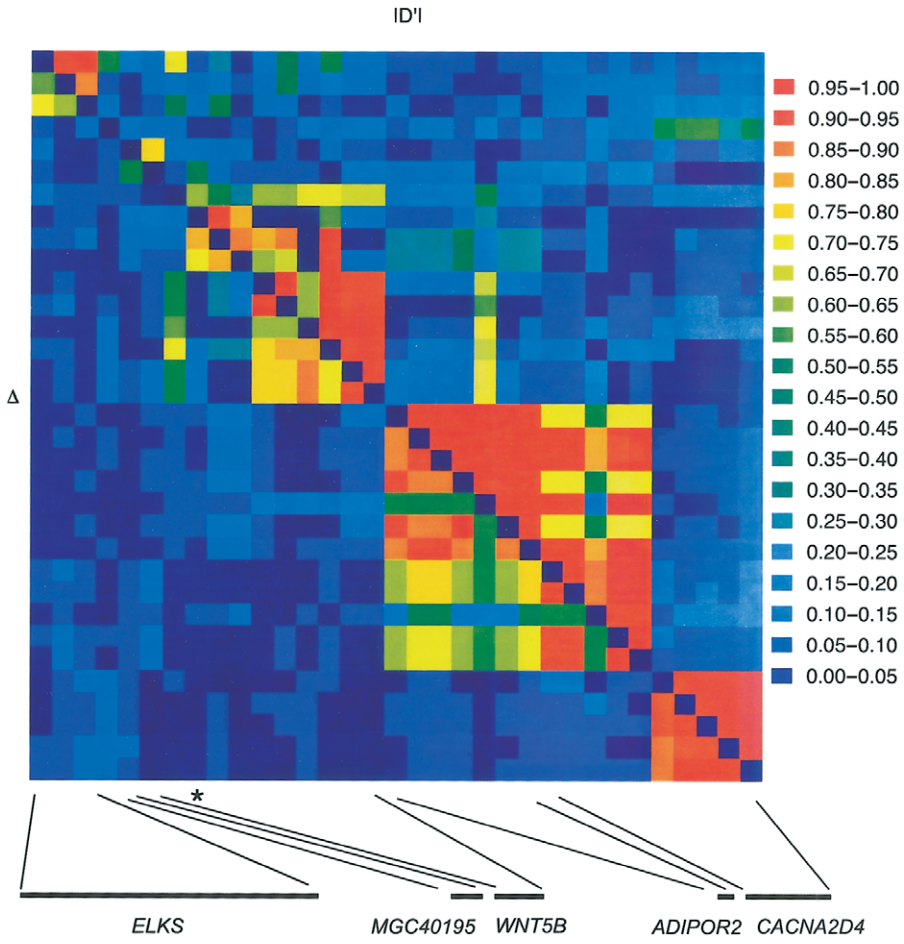

Figure 1 LD mapping around the *WNT5B* gene. LD coefficients (D' and Δ) between every two SNPs around the SNP in intron 4 (+438, C/G) were calculated. Minor allele frequencies of all SNPs used in this analysis are >10%. An asterisk (*) highlights the SNP in intron 4 (+438, C/G).

WNT5B gene (MIM 606361) is a good candidate for conferring susceptibility to type 2 diabetes.

Methods

DNA Preparation and SNP Genotyping

DNA samples were obtained from the peripheral blood of patients with type 2 diabetes who came regularly to the outpatient clinic of Shiga University of Medical Science, Tokyo Women's Medical University, Juntendo University, Kawasaki Medical School, or Chibanishi Medical Hospital. The diagnosis of diabetes was determined according to the criteria of the World Health Organization. Type 2 diabetes was clinically defined as "gradual, adult onset of the disease." The subjects who were positive for anti-glutamic acid decarboxylase antibody and the patients with mitochondrial disease (mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes [MELAS {MIM 540000}]) and maturity-onset diabetes of the young (MODY [MIM 606391]) were excluded. Control subjects consisted of members of the general population (control-1; $n = 564$) and another set of control subjects (control-2; $n = 375$) who were recruited through several medical institutes in Japan. DNA extraction was performed using a standard phenolchloroform procedure. Written informed consent was obtained from each patient, and the protocol was approved by the ethics committees of the Institute of Physical and Chemical Research and by those of each institute involved in the study.

SNP Genotyping

The SNPs for genotyping were selected from the IMS-JST (Institute of Medical Science–Japan Science and Technology Agency) Japanese SNP database (Hirakawa et al. 2002). First, each SNP locus within the *WNT* genes was genotyped by the invader assay, as described elsewhere (Ohnishi et al. 2001), for patients with type 2 diabetes

Table 2

Genotype and Allele Frequencies of the SNPs in *WNT5B*

	Analysis of Intron 4+438 (C/G)						
	No. (%) of Subjects			No. (%) of Chromosomes		HWE Test[a]	
Population	CC	CG	GG	C	G	χ^2	*P*
Case-1 (n = 188)	89 (47.3)	75 (40)	24 (12.7)	253 (67.3)	123 (32.7)	1.6	.20
Control-1 (n = 562)	199 (35.4)	280 (50.0)	83 (14.6)	678 (60.3)	446 (39.7)	.9	.32
Case-2 (n = 733)	304 (41.4)	331 (45.1)	98 (13.5)	939 (64.1)	527 (35.9)	.3	.60
Control-2 (n = 345)	100 (29.0)	192 (55.6)	53 (15.4)	392 (56.8)	298 (43.2)	6.2	.013
	Analysis of Intron 4+6763 (C/T)						
	No. (%) of Subjects			No. (%) of Chromosomes		HWE Test[a]	
	CC	CT	TT	C	T	χ^2	*P*
Case-1 (n = 188)	103 (54.8)	69 (36.7)	16 (8.5)	275 (73.1)	101 (26.9)	.8	.36
Control-1 (n = 558)	253 (45.3)	253 (45.3)	52 (9.4)	759 (68.0)	357 (32.0)	.9	.32
Case-2 (n = 696)	344 (50.0)	282 (40.5)	70 (9.5)	970 (69.7)	422 (30.3)	1.2	.28
Control-2 (n = 366)	147 (40.1)	183 (50.0)	36 (9.9)	477 (65.2)	255 (34.8)	3.8	.052

[a] Hardy-Weinberg equilibrium test.

(case-1; n = 188; male:female ratio 101:87; mean ± SD age [in years] 60.5 ± 11.4; duration [in years] of diabetes 17.5 ± 9.4; HbA1c [%] 7.5 ± 1.32; fasting plasma glucose [FPG] [mg/dl] 150.5 ± 46.2; mean ± SD BMI [kg/m^2] 23.5 ± 3.4) and in the general population (control-1: n = 564). After evaluation of the statistical data by use of four models (genotype distribution [2 × 3], allele frequency, dominant model, and recessive model), the SNPs that showed significant ($P < .05$) differences were further examined in another set of diabetic subjects (case-2; n = 733; male:female ratio 437:296; mean ± SD age [in years] 60.7 ± 11.0; mean ± SD duration [in years] of diabetes 12.8 ± 9.9; mean ± SD HbA1c [%] 7.2 ± 1.2; mean ± SD FPG [mg/dl] 159.5 ± 42.5; mean ± SD BMI [kg/m^2] 23.2 ± 3.4) and control subjects (control-2; n = 375).

SNP Discovery in the WNT5B Gene

On the basis of GenBank information about DNA sequence in the genomic region that contains the *WNT5B* gene (GenBank accession number AC005182), we designed PCR primers to amplify appropriate fragments of genomic DNA. PCR and DNA sequencing were performed as described elsewhere (Saito et al. 2001), by study of 48 diabetic subjects.

Cell Culture and Real-Time Quantitative RT-PCR

A mouse 3T3-L1 cell line was obtained from the Health Science Research Resources Bank. Cells were grown to confluence and were induced to differentiate into adipocytes, according to methods described elsewhere (Kishida et al. 2001). Total RNA was extracted from mouse 3T3-L1 cells with Trizol reagent (Invitrogen) at different stages of adipocyte differentiation, and reverse transcription was performed using an oligo-dT primer. Expression of the endogenous *Wnt5b* gene during adipogenesis and expression of the mouse PPAR-γ (*Pparg*), mouse C/EBP-α (*Cebpa*), mouse adiponectin (*apM1*), and mouse leptin (*Lep*) genes in *Wnt5b*-overexpressing 3T3-L1 cells were examined at the indicated days, after

Table 3

Association of the SNPS in *WNT5B* with Type 2 Diabetes

	Analysis of Intron 4+438 (C/G)				
Population	Model	χ^2	*P*	OR	95% CI
Case-1 versus control-1	CC vs. CG+GG	8.5	.0036	1.64	1.17–2.29
Case-2 versus control-2	CC vs. CG+GG	15.6	.00008	1.74	1.32–2.29
	Analysis of Intron 4+438 (C/G)				
	Model	χ^2	*P*	OR	95% CI
Case-1 versus control-1	CC vs. CT+TT	5.0	.025	1.46	1.05–2.04
Case-2 versus control-2	CC vs. CT+TT	8.3	.004	1.46	1.13–1.88

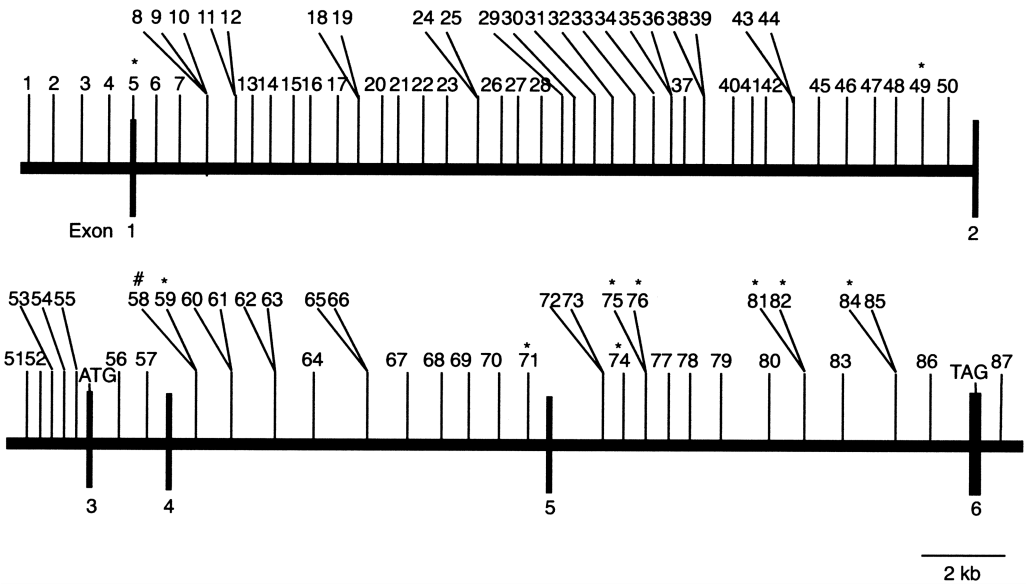

Figure 2 Polymorphisms identified in the *WNT5B* gene. The number sign (#) indicates the SNP in intron 4 (+438, C/G) that showed strong association. An asterisk (*) indicates SNPs selected from the IMS-JST Japanese SNP database.

induction of differentiation by real-time quantitative RT-PCR (Mx3000P Multiplex Quantitative PCR system [Stratagene]) with SYBR green detection. Results were normalized with mouse 36B4 (*Arbp*). Primers for the genes studied were as follows. For *Wnt5b,* sense 5′-CCA GTG CAG AGA CCG GAG ATG-3′, antisense 5′-GTT GTC CAC GGT GCT GCA GTT C-3′; for Arbp, sense 5′-CAA CGG CAG CAT TTA TAA CCC-3′, antisense 5′-CCC ATT GAT GAT GGA GTG TGG-3′; for *Pparg,* sense 5′-TTT GAA AGA AGC GGT GAA CCA C-3′, antisense 5′-ACC ATT GGG TCA GCT CTT GTG -3′; for *Cebpa,* sense 5′-AAA GCC AAG AAG TCG GTG GAC-3′, antisense 5′-GGT CAT TGT CAC TGG TCA ACT CC-3′; for *apM1,* sense 5′-GGG TGA GAC AGG AGA TGT TGG AAT G-3′, antisense 5′-GCC AGT AAA TGT AGA GTC GTT GAC G-3′; for *Lep,* sense 5′-TCC AGA AAG TCC AGG ATG ACA CCA A-3′, antisense 5′-TCA GCA TTC AGG GCT AAC ATC CAA C-3′. The amplifications were each performed in a 25-μl reaction volume that contained 1× EX *Taq* Buffer, 200 nM

Table 4

Genotype Frequencies of SNPs in the *WNT5B* Gene

Position (Major/Minor)	Population	No. (%) of Subjects			
		Major Allele/ Major Allele	Major Allele/ Minor Allele	Minor Allele/ Minor Allele	Total
SNP 33	Case	232 (33)	327 (47)	139 (20)	698
Intron 1+6974 (C/T)	Control	102 (28)	200 (55)	64 (17)	366
SNP 51	Case	342 (49)	298 (42)	65 (9)	705
Intron 2+1079 (C/G)	Control	149 (41)	183 (50)	34 (9)	366
SNP 55	Case	341 (49)	297 (42)	63 (9)	701
Intron 2+1636 (C/G)	Control	142 (39)	183 (50)	38 (11)	363
SNP 57	Case	284 (40)	318 (45)	102 (15)	704
Intron 3+1016 (C/T)	Control	95 (28)	182 (54)	61 (18)	338
SNP 58	Case	304 (41)	331 (45)	98 (14)	733
Intron 4+438 (C/G)	Control	100 (29)	192 (56)	53 (15)	345
SNP 67	Case	291 (41)	313 (45)	99 (14)	703
Intron 4+5776 (G/C)	Control	106 (29)	194 (53)	66 (18)	366
SNP 71	Case	344 (50)	282 (41)	70 (9)	696
Intron 4+6763 (C/T)	Control	147 (40)	183 (50)	36 (10)	366
SNP 83	Case	587 (83)	114 (16)	4 (1)	705
Intron 5+4244 (A/C)	Control	319 (88)	41 (11)	3 (1)	363

Table 5

Analysis of SNPs in the *WNT5B* Gene with Type 2 Diabetes

	χ^2 FOR				*P* FOR			
POSITION	2 × 3[a]	Allele	Major/Major versus Others	Minor/Minor versus Others	2 × 3[a]	Allele	Major/Major versus Others	Minor/Minor versus Others
Intron 1+6974	5.9	.4	3.2	.92	.053	.516	.073	.34
Intron 2+1079	6.4	3.4	5.9	.001	.041	.063	.015	.97
Intron 2+1636	8.8	6.7	8.8	.61	.013	.0099	.0031	.43
Intron 3+1016	14.8	11.9	14.8	2.19	.0006	.0006	.0001	.14
Intron 4+438	15.8	10.4	15.6	.77	.0004	.0013	.00008	.38
Intron 4+5776	16.1	13.6	15.9	2.88	.0003	.0002	.00007	.09
Intron 4+6763	9.4	4.5	8.3	.01	.0092	.034	.004	.91
Intron 5+4244	4.8	3.1	4.0	.25	.092	.077	.046	.62

[a] Genotype distribution (2 × 3).

dNTP, 1/20,000 SYBR Green, 800 nM gene-specific primer, 0.05 U/ml EX *Taq* DNA polymerase, 2.75 ng/ml *Taq*Start antibody, and 5 ng of template DNA. The thermal profile was 50°C for 2 min, at 95°C for 10 min, followed by 40 cycles at 95°C for 30 s and at 60°C for 60 s.

Construction of Adenovirus Vector and Viral Infection

An adenovirus vector encoding the mouse *Wnt5b* gene was prepared using the Adenovirus Expression Vector Kit (TaKaRa). Forty-eight hours before the induction of differentiation, 3T3-L1 cells were transduced with a multiplicity of infection of 50 plaque-forming units/cell for 6 h, and samples were obtained at the indicated days after induction of differentiation.

Oil Red O Staining

At the indicated number of days after induction of differentiation, the cells were washed twice with PBS and then were fixed for 2 h with 3.7% formaldehyde. Fixed cells were incubated with oil red O for 15 min at room temperature. After the cells were washed once with water, the stained lipid droplets in the cells were visualized by light microscopy. For quantification, the dye was extracted with isopropyl alcohol, and the absorbance was measured at 540 nm.

Statistical Analysis

The statistical analysis used in the association study to calculate the Hardy-Weinberg equilibrium (Nielsen et al. 1998), and the linkage disequilibrium (LD) coefficients (D' and Δ) (Devlin and Risch 1995) were as described elsewhere. The analysis of haplotype structure was performed by estimation of haplotype phasing by use of the EM algorithm (Excoffier and Slatkin 1995) and by construction of haplotype blocks, as described elsewhere (Gabriel et al. 2002; Tanaka et al. 2003). To analyze interaction between SNPs, logistic regression analysis was performed. The probability (P_c) of an individual being a case subject rather than a control subject is assumed to be affected by a set of SNPs, according to the logistic model; for example, $\text{logit}(P) = a_0 + a_1x_1 + a_2x_2$ for a single SNP. Here, we use a coding scheme $x_1 = -1,0,1$, and $x_2 = -0.5,0.5,-0.5$ for genotypes 1/1, 1/2, and 2/2, respectively, for representing an additive effect by x_1 and a dominance/recessive effect by x_2 (Cordell and Clayton 2002). The weights are estimated by the maximum-likelihood method and are tested by comparison with the null hypothesis $\text{logit}(P_c) = a_0$ (constant). For

Table 6

Analysis of SNPs in the *WNT5B* Gene with Type 2 Diabetes

	OR (95% CI) OF		
POSITION	Allele	Major/Major versus Others	Minor/Minor versus Others
Intron 1+6974	1.06 (.89–1.27)	1.29 (.98–1.70)	.85 (.61–1.18)
Intron 2+1079	1.20 (.99–1.45)	1.37 (1.06–1.77)	1.01 (.65–1.56)
Intron 2+1636	1.28 (1.06–1.55)	1.47 (1.14–1.91)	1.18 (.77–1.81)
Intron 3+1016	1.39 (1.15–1.67)	1.73 (1.31–2.29)	1.30 (.92–1.84)
Intron 4+438	1.35 (1.13–1.63)	1.74 (1.32–2.29)	1.18 (.82–1.69)
Intron 4+5776	1.41 (1.17–1.69)	1.73 (1.32–2.27)	1.34 (.95–1.89)
Intron 4+6763	1.23 (1.01–1.48)	1.46 (1.13–1.88)	.98 (.64–1.49)
Intron 5+4244	.73 (.52–1.04)	.69 (.47–1.00)	1.46 (.33–6.56)

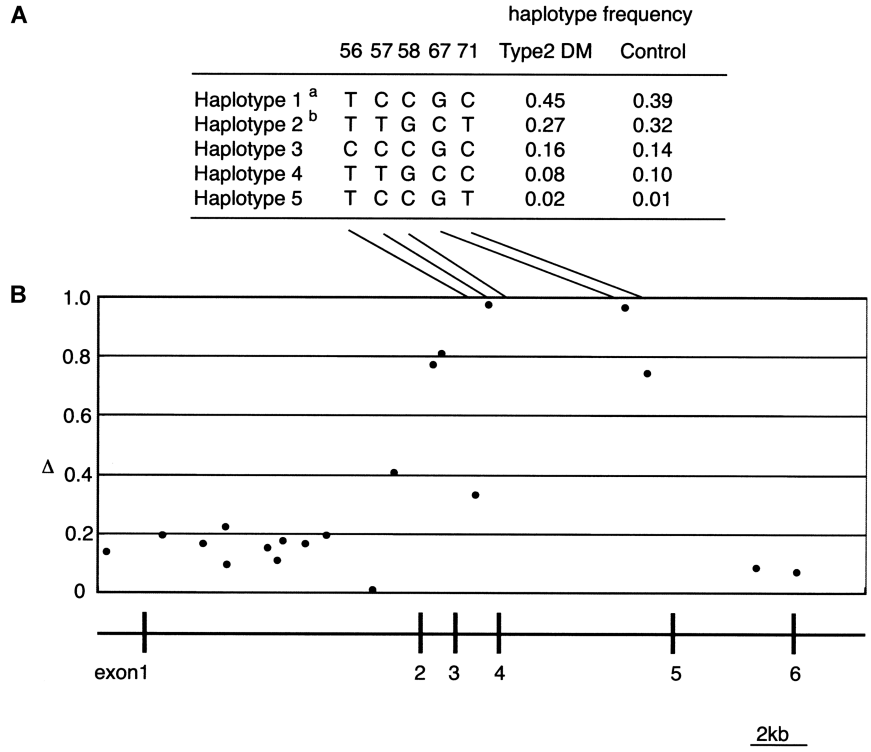

Figure 3 *A*, Analysis of haplotype structure and estimated haplotype frequencies within the *WNT5B* gene. 56 = intron 3+439; 57 = intron 3+1016; 58 = intron 4+438; 67 = intron 4+5776; 71 = intron 4+6763. [a], Haplotype 1/others: $\chi^2 = 4.41$; $P = .036$; OR 1.24, 95% CI 1.01–1.52. [b], Haplotype 2/others: $\chi^2 = 5.59$; $P = .018$, OR .77, 95% CI .62–.96. *B*, Plots of LD coefficients (Δ) between the SNP at intron 4+438 and other SNPs within the *WNT5B* gene.

multiple SNPs, interaction effects are also added, in addition to the main effects of additional SNPs, and are tested stepwise, to determine whether their effects are significant. The tests were performed using R. To evaluate population structures, Wright's *F* statistics were calculated for 66 randomly selected SNPs. A single statistic (F_{ST}) between each group was calculated as described elsewhere (Weir 1996). To analyze the results of the oil red O staining, the statistical significance of the difference between the two groups was analyzed using the unpaired *t* test. *P* values <.05 were considered significant.

Results

Association Study

A total of 45 SNPs within 11 *WNT* genes were found in the JSNP database. Among them, 40 SNP loci within 11 *WNT* genes were successfully genotyped with the invader assay. As shown in table 1, six SNP loci (two in *WNT2*, two in *WNT5A*, and two in *WNT5B*) showed a significant ($P < .05$) association with type 2 diabetes, and these SNP loci were further analyzed in another set of case ($n = 733$) and control ($n = 375$) subjects. The results indicated that only two SNP loci in the *WNT5B* gene at chromosome 12p13.3 were associated with type 2 diabetes (intron 4+438, C/G, CC vs. CG+GG: $\chi^2 = 15.6$, $P = .00008$, OR 1.74, 95% CI 1.32–2.29; intron 4+6763, C/T, CC vs. CT+TT: $\chi^2 = 8.3$, $P = .004$, OR 1.46, 95% CI 1.13–1.88) (tables 2 and 3).

To exclude the possibility that this result instead reflected the association of other genes located near the *WNT5B* gene with type 2 diabetes, we performed LD mapping around this locus, using 34 SNPs with allele frequencies of >0.1. The result indicated that the LD in this region extended to ~20 kb upstream and 10 kb downstream of this SNP site (fig. 1). Hence, the crucial region for susceptibility to type 2 diabetes seemed to exist within this 30-kb LD block, which contained only the *WNT5B* gene. Therefore, we concluded that the *WNT5B* gene itself could explain the association of this locus with type 2 diabetes.

Next, we sought genetic polymorphisms in an entire region of the *WNT5B* gene. We identified 76 additional SNPs, for a total of 87 SNPs in this gene (fig. 2). We could not identify any variations within the coding region of the *WNT5B* gene. Among these 87 SNP loci, 45 SNP loci were successfully genotyped and were analyzed in the second set of case (case-2) and control (control-2)

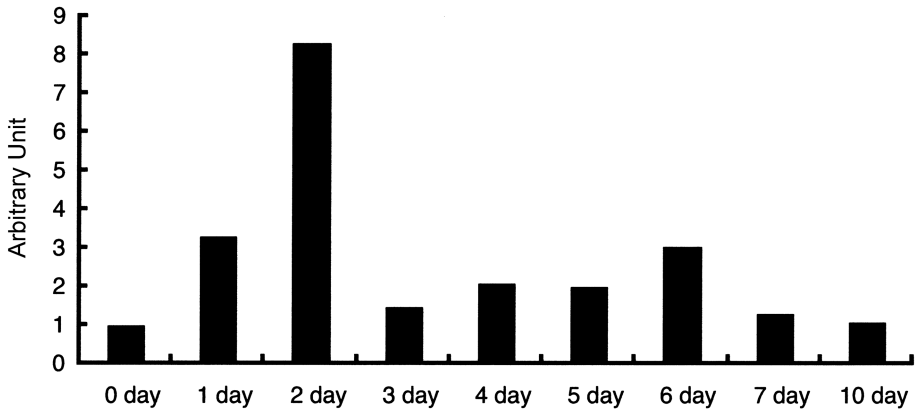

Figure 4 Expression pattern of the *Wnt5b* gene in 3T3-L1 cells during differentiation. Quantitative real-time PCR was used to evaluate the amount of *Wnt5b* mRNA. Total RNA was extracted from 3T3-L1 cells at 0, 1, 2, 3, 4, 5, 6, 7, and 10 d after induction of differentiation.

subjects. The result indicated that several of them were significantly associated with type 2 diabetes (tables 4–6); the association of SNPs at intron 3 and intron 4 with type 2 diabetes was especially strong (intron 3+1016: $\chi^2 = 14.8$, $P = .0001$, OR 1.73, 95% CI 1.31–2.29; intron 4+438: $\chi^2 = 15.6$, $P = .00008$, OR 1.74, 95% CI 1.32–2.29; intron 4+5776: $\chi^2 = 15.9$, $P = .00007$, OR 1.73, 95% CI 1.32–2.27).

We then examined haplotype structure using the EM algorithm, which indicated that five SNPs with the allelic frequency of >0.15 in the *WNT5B* gene, including intron 4+438 C/G, constituted one haplotype block and that five common haplotypes could cover >90% of the population (fig. 3*A*). Subsequent association studies for each haplotype with type 2 diabetes identified a significant association of haplotypes 1 and 2 with type 2 diabetes. However, the associations of these haplotypes were not stronger than those found at the single locus.

We further evaluated interactions among the SNPs used in this study by logistic regression analysis, as described in the "Methods" section, and we identified significant interaction between the SNP at intron 4+438 of the *WNT5B* gene and the SNP at 3′-flanking+1629 of the *WNT2* gene (recessive for the *WNT5B* SNP, plus additive for the *WNT2* SNP with interaction; $P = .007$, combined empirical $P = .000035$; OR 2.22; 95% CI 1.58–3.11). We could not identify any significant interaction between other combinations of the *WNT* genes.

Expression of Wnt5b *during Adipogenesis*

To learn the possible involvement of the *WNT5B* gene in the pathogenesis of type 2 diabetes, we first investigated the expression pattern of the mouse *Wnt5b* gene during adipogenesis, by quantitative real-time PCR. As shown in figure 4, expression of the *Wnt5b* gene was most abundant at 2 d after induction of differentiation, followed by a rapid decrease in response as a result of further cell differentiation into adipocytes.

Effect of Overexpression of the Mouse Wnt5b *Gene on Adipogenesis and Adipocyte Gene Expression*

We next examined the effect of overexpression of the mouse *Wnt5b* gene on adipocyte differentiation. 3T3-L1 preadipocytes were infected with adenovirus vectors encoding mouse *Wnt5b* or *lacZ* 48 h before induction of differentiation, and cells then were induced to differentiate into adipocytes, as described in the "Methods" section. As shown in figure 5*A* and 5*B*, the cells overexpressing *Wnt5b* significantly accelerated differentiation of 3T3-L1 cells compared with control cells (the content of lipid droplets was 1.6-fold, 1.9-fold, and 1.7-fold at 4, 7, and 10 d, respectively; $P < .05$ vs. *lacZ*).

Expression of the *Cebpa* and *Pparg* genes, which are thought to play a key role in the adipogenesis process, was also elevated in the *Wnt5b*-overexpressing cells (fig. 6*A* and 6*B*). We further examined the expression of several adipocytokine genes in the same cells and identified that the expression of *apM1* and *Lep* was significantly increased in *Wnt5b*-overexpressing cells compared with control cells (*apM1* is expressed 2.0-fold and 1.5-fold, and *Lep* is expressed 1.6-fold and 1.7-fold, at day 3 and day 7, respectively [fig. 6*C* and 6*D*]), whereas the expression of *Il-6* and *Rstn* was unchanged between these two cell lines (data not shown).

Discussion

In this study, we examined the association of the WNT genes with type 2 diabetes, and we identified the *WNT5B* gene at chromosome 12p13.3 as a new candidate for conferring susceptibility to type 2 diabetes. Recent cumulative evidence suggested that some members of the

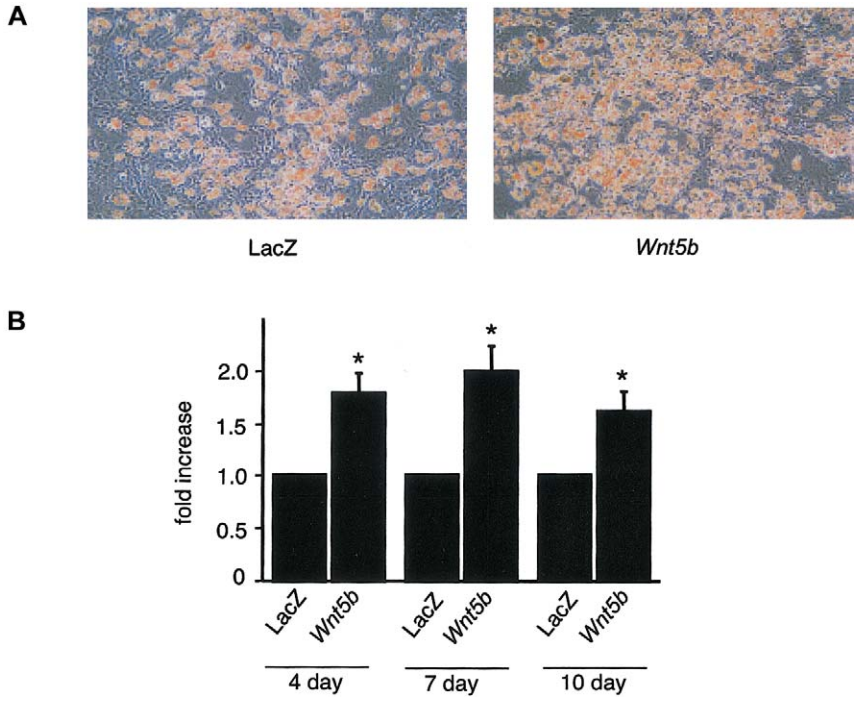

Figure 5 Effects of *Wnt5b* gene overexpression in 3T3-L1 cells on adipogenesis. An adenovirus vector encoding the *Wnt5b* gene was infected into 3T3-L1 cells 48 h before induction of differentiation at a multiplicity of infection of 50. *A,* Lipid droplets stained with oil red O at 5 d after induction of differentiation. *B,* Quantitative analysis of oil red O staining. Values are mean ± SE of five independent experiments. An asterisk (*) indicates $P < .05$ versus control cells (LacZ).

WNT family had remarkable functions in relation to the pathogenesis of type 2 diabetes. To date, the WNT family consists of 19 genes, including the *WNT5B* gene (details can be viewed on the Wnt Web site). Most WNT proteins are considered to be secreted from cells and to act through cell-surface receptors, namely Frizzled receptors and their coreceptors *LRP5* and *LRP6* (MIM 603507). The *WNT1* gene (MIM 164820) has been reported to inhibit adipogenesis through the β-catenin pathway (Ross et al. 2000), and the *WNT10B* (MIM 601906) gene has also been shown to play an important role in an early stage of adipogenesis (Bennett et al. 2002). In addition, it has been reported that *LRP5* plays some role in glucose-induced insulin secretion in the pancreatic islets and in cholesterol metabolism in the liver (Fujino et al. 2003). On the basis of these observations, the *WNT* genes seem to be worthy candidates for conferring susceptibility to type 2 diabetes, but, until now, the association of these genes with type 2 diabetes had not, to our knowledge, been examined.

In this study, we identified a significant association of SNP loci within the *WNT5B* gene with type 2 diabetes. Because we examined the association between 40 SNP loci in 11 *WNT* genes and type 2 diabetes, by use of four models for each locus, type 1 error due to multiple testing should be included. To eliminate the possibility of type 1 error, we adopted Bonferroni's method to our overall analytical process, according to the following calculation: overall *P* values (*P* test-1 × *P* test-2) were multiplied by the number of tests (40 × 4 + 6 × 4). The corrected *P* value obtained for a particular SNP within the *WNT5B* gene was .00005 (.0036 × .00008 × 46 × 4); thus, the association of this locus with type 2 diabetes was thought to be statistically significant.

We further compared population structures among the case and control groups to confirm that our replication samples were appropriately collected for the study. The average values of F_{ST} between each group suggested that there was no population stratification within each group (average F_{ST} = 0.0018, 0.0032, 0.0089, and 0.0014, calculated by comparison between case-1 and case-2, control-1 and control-2, case-1 and control-1, and case-2 and control-2, respectively).

Subsequent LD mapping revealed that the LD block of this locus contained only the *WNT5B* gene (fig. 1). Therefore, the *WNT5B* gene itself should be a candidate for conferring susceptibility to type 2 diabetes. Because the gene encoding *APM1* type 2 receptor (*ADIPOR2* [MIM 607946]), which was recently identified and considered to be a candidate gene for type 2 diabetes, was located next to the *WNT5B* gene, we also analyzed

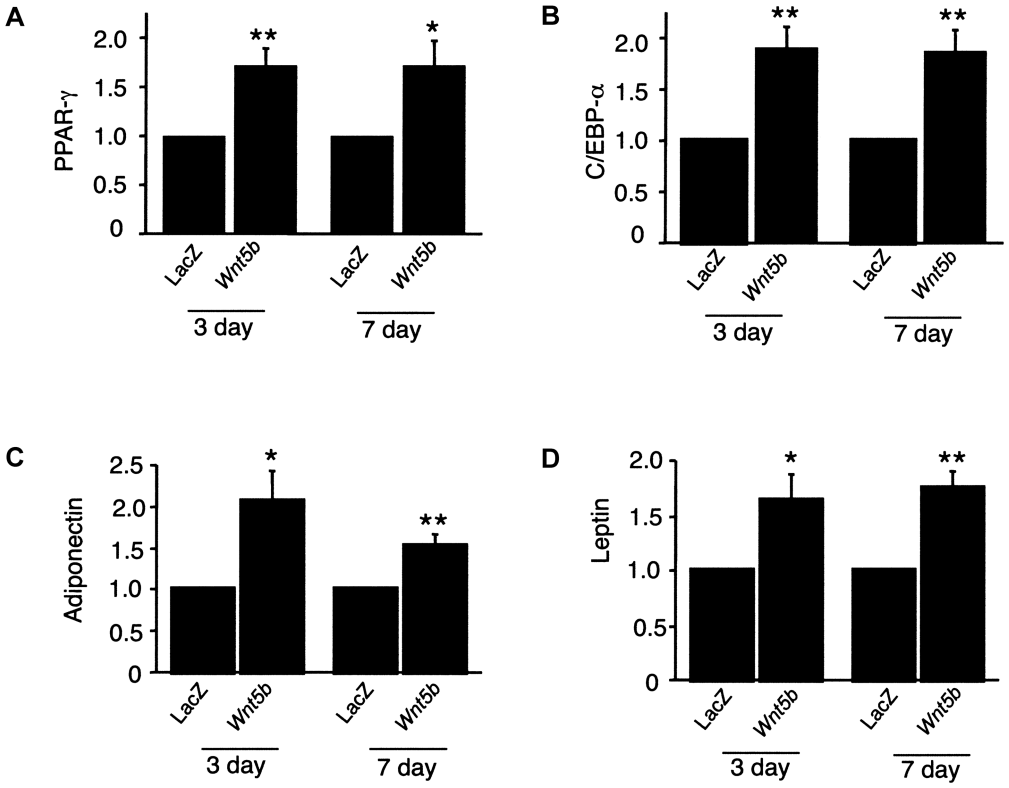

Figure 6 Effects of *Wnt5b* gene overexpression in 3T3-L1 cells on adipocyte gene expression: the expression of *Pparg* (*A*), *Cebpa* (*B*), *apM1* (*C*), and *Lep* (*D*) in *Wnt5b* overexpressing 3T3-L1 cells at 3 and 7 d after induction of differentiation. The results obtained from four independent experiments are presented. An asterisk (*) indicates that $P < .05$. A double asterisk (**) indicates $P < .01$ versus control cells (LacZ).

seven SNPs in *ADIPOR2,* but we could not identify any association between them and type 2 diabetes ($P > .05$; data not shown).

On the basis of previous reports, expression of the *WNT5B* gene appears to be moderately detectable in adult prostate and fetal brain and weakly detectable in adult liver and kidney (Saitoh et al. 2001). It was also reported that the *Wnt5b* gene is involved in the differentiation and proliferation of several types of cells, such as chondrocytes (Yang et al. 2003), and that expression of the *Wnt5b* gene is transiently increased during myogenesis (Jamali et al. 2001). However the involvement of this gene in the pathogenesis of type 2 diabetes has not emerged thus far. To our knowledge, the present study is the first to demonstrate clear expression of the *Wnt5b* gene in 3T3-L1 adipocytes. It is interesting that expression of the *Wnt5b* gene was transiently increased at an early phase of adipogenesis, which suggests some role for this gene in the regulation of adipocyte differentiation.

In a previous report, other members of the WNT family, *WNT1* and *WNT10B,* were shown to be capable of inhibiting adipocyte differentiation (Ross et al. 2000). We therefore examined a possible role of the *Wnt5b* gene in adipogenesis, using 3T3-L1 cells overexpressing the *Wnt5b* gene. The present study clearly demonstrates that overexpression of the *Wnt5b* gene in preadipocytes results in the promotion of adipocyte differentiation, when compared with the control cells. The expression of *Pparg* and *Cebpa,* which are essential transcription factors for adipogenesis, was also increased in *Wnt5b*-overexpressing 3T3-L1 cells. Therefore, it appears that the *Wnt5b* gene has an opposite effect on adipogenesis, when compared with *WNT1* or *WNT10B*. We also identified that the expression of several anti-apoptotic genes, such as insulinlike growth factor-1 (*Igf-1*) and *WNT1*-inducible secreting protein-1 (*Wisp1*), was decreased in *Wnt5b*-overexpressing 3T3-L1 cells (*Igf-1,* 36% and 44%; *Wisp1,* 30% and 44% at day 0 and day 2, respectively; authors' unpublished data). Because these genes are thought to be a downstream target of the WNT-1/β-catenin–signaling pathway—which has an anti-apoptotic action by activation of Akt kinase (Su et al. 2002) and is reported to be upregulated by overexpression of the *WNT1* gene in 3T3-L1 cells (Longo et al. 2002)—*Wnt5b* has the opposite effect of *WNT1,* in terms of the regulation of adipocyte function.

The mechanism by which the *WNT5B* gene can affect

the susceptibility to type 2 diabetes remains uncertain. Differentiated adipocytes have been known to act as endocrine cells and are known to be capable of secreting several cytokines, so-called "adipocytokines." These cytokines, which are produced and secreted by adipocytes, were thought to be involved in systemic insulin sensitivity. Therefore, we examined the expression of several adipocytokine genes in *Wnt5b*-overexpressing 3T3-L1 cells and identified that the overexpression of the *Wnt5b* gene resulted in significant increases in the expression of *apM1* and *Lep* in 3T3-L1 cells. From these observations, we hypothesized that the difference in the expression or function of the *WNT5B* gene among the individuals could contribute to the susceptibility to type 2 diabetes through, at least in part, affecting adipocyte function, including the production of adipocytokines, although the elucidation of the precise mechanism requires further study.

Finally, it should be mentioned that the OR for the SNP associated with type 2 diabetes in this study was not very high, even when the association of the SNP was shown to be strong by χ^2 test. This discrepancy is probably explained by the fact that multiple genetic factors are involved in susceptibility to type 2 diabetes in a complex manner, and effects of individual genes in a complex genetic and environmental background are often very small on their own. Therefore, evaluation of interactions among individual genes with and without main effects is necessary for full understanding of the involvement of genetic background for conferring susceptibility to type 2 diabetes. In the present study, we examined interaction between the SNPs among genes encoding the WNT family, and we identified significant interaction between *WNT5B* and *WNT2* (MIM 147870). However, interaction with other gene families should be examined in the future, and those kinds of studies will contribute to further understanding of the mechanism of the genetic susceptibility to type 2 diabetes.

In conclusion, we have identified the *WNT5B* gene as a new candidate for conferring susceptibility to type 2 diabetes. Our results also suggest that the *WNT5B* gene plays an important role in the regulation of adipocyte function and might contribute to the pathogenesis of type 2 diabetes.

Acknowledgments

This work was supported by a grant from the Japanese Millennium Project. We thank Ms. Ohashi for her technical assistance.

Electronic-Database Information

The accession number and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *WNT5B* [accession number AC005182])

Institute of Medical Science–Japan Science and Technology Agency (IMS-JST) Japanese SNP database, http://snp.ims.u-tokyo.ac.jp/ (for SNPs and primers)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for type 2 diabetes, *PPARG, CAPN10, APM1, LRP5, WNT5B,* MELAS, MODY, *LRP6, WNT1, WNT10B, ADIPOR2,* and *WNT2*)

Wnt Gene Web site, http://www.stanford.edu/~rnusse/wntwindow.html

References

Altshuler D, Hirschhorn JN, Klannemark M, Lindgren CM, Vohl MC, Nemesh J, Lane CR, Schaffner SF, Bolk S, Brewer C, Tuomi T, Gaudet D, Hudson TJ, Daly M, Groop L, Lander ES (2000) The common PPARγ Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes. Nat Genet 26:76–80

Bennett CN, Ross SE, Longo KA, Bajnok L, Hemati N, Johnson KW, Harrison SD, MacDougald OA (2002) Regulation of Wnt signaling during adipogenesis. J Biol Chem 277: 30998–31004

Cordell HJ, Clayton DG (2002) A unified stepwise regression procedure for evaluating the relative effects of polymorphisms within a gene using case/control or family data: application to *HLA* in type 1 diabetes. Am J Hum Genet 70:124–141

Devlin B, Risch N (1995) A comparison of linkage disequilibrium measures for fine-scale mapping. Genomics 29:311–322

Excoffier L, Slatkin M (1995) Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. Mol Biol Evol 12:921–927

Fujino T, Asaba H, Kang MJ, Ikeda Y, Sone H, Takada S, Kim DH, Ioka RX, Ono M, Tomoyori H, Okubo M, Murase T, Kamataki A, Yamamoto J, Magoori K, Takahashi S, Miyamoto Y, Oishi H, Nose M, Okazaki M, Usui S, Imaizumi K, Yanagisawa M, Sakai J, Yamamoto TT (2003) Low-density lipoprotein receptor-related protein 5 (LRP5) is essential for normal cholesterol metabolism and glucose-induced insulin secretion. Proc Natl Acad Sci USA 100:229–234

Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D (2002) The structure of haplotype blocks in the human genome. Science 296:2225–2229

Hara K, Boutin P, Mori Y, Tobe K, Dina C, Yasuda K, Yamauchi T, Otabe S, Okada T, Eto K, Kadowaki H, Hagura R, Akanuma Y, Yazaki Y, Nagai R, Taniyama M, Matsubara K, Yoda M, Nakano Y, Tomita M, Kimura S, Ito C, Froguel P, Kadowaki T (2002) Genetic variation in the gene encoding adiponectin is associated with an increased risk of type 2 diabetes in the Japanese population. Diabetes 51:536–540

Hirakawa M, Tanaka T, Hashimoto Y, Kuroda M, Takagi T, Nakamura Y (2002) JSNP: a database of common gene variations in the Japanese population. Nucleic Acids Res 30:158–162

Horikawa Y, Oda N, Cox NJ, Li X, Orho-Melander M, Hara

M, Hinokio Y, et al (2000) Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. Nat Genet 26:163–175

Jamali M, Rogerson PJ, Wilton S, Skerjanc IS (2001) Nkx2–5 activity is essential for cardiomyogenesis. J Biol Chem 276: 42252–42258

Kahn BB (1998) Type 2 diabetes: when insulin secretion fails to compensate for insulin resistance. Cell 92:593–596

Kishida K, Shimomura I, Kondo H, Kuriyama H, Makino Y, Nishizawa H, Maeda N, Matsuda M, Ouchi N, Kihara S, Kurachi Y, Funahashi T, Matsuzawa Y (2001) Genomic structure and insulin-mediated repression of the aquaporin adipose (AQPap), adipose-specific glycerol channel. J Biol Chem 276:36251–36260

Longo KA, Kennell JA, Ochocinska MJ, Ross SE, Wright WS, MacDougald OA (2002) Wnt signaling protects 3T3-L1 preadipocytes from apoptosis through induction of insulin-like growth factors. J Biol Chem 277:38239–38244

Nielsen DM, Ehm MG, Weir BS (1998) Detecting marker-disease association by testing for Hardy-Weinberg disequilibrium at a marker locus. Am J Hum Genet 63:1531–1540

Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y (2001) A high-throughput SNP typing system for genome-wide association studies. J Hum Genet 46:471–477

Ross SE, Hemati N, Longo KA, Bennett CN, Lucas PC, Erickson RL, MacDougald OA (2000) Inhibition of adipogenesis by Wnt signaling. Science 289:950–953

Saito S, Iida A, Sekine A, Miura Y, Sakamoto T, Ogawa C, Kawauchi S, Higuchi S, Nakamura Y (2001) Identification of 197 genetic variations in six human methyltranferase genes in the Japanese population. J Hum Genet 46:529–537

Saitoh T, Katoh M (2001) Molecular cloning and characterization of human WNT5B on chromosome 12p13.3 region. Int J Oncol 19:347–351

Su F, Overholtzer M, Besser D, Levine AJ (2002) WISP-1 attenuates p53-mediated apoptosis in response to DNA damage through activation of the Akt kinase. Genes Dev 16:46–57

Tanaka N, Babazono T, Saito S, Sekine A, Tsunoda T, Haneda M, Tanaka Y, Fujioka T, Kaku K, Kawamori R, Kikkawa R, Iwamoto Y, Nakamura Y, Maeda S (2003) Association of solute carrier family 12 (sodium/chloride) member 3 with diabetic nephropathy, identified by genome-wide analyses of single nucleotide polymorphisms. Diabetes 52:2848–2853

Weir BS (1996) Genetic Data Analysis II. Sinauer Associates, Sunderland, Massachusetts

Yang Y, Topol L, Lee H, Wu J (2003) *Wnt5a* and *Wnt5b* exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. Development 130:1003–1015