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Report

The G/G Genotype of a Resistin Single-Nucleotide Polymorphism at −420 Increases Type 2 Diabetes Mellitus Susceptibility by Inducing Promoter Activity through Specific Binding of Sp1/3

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Insulin resistance is a major cause of type 2 diabetes mellitus (T2DM). Resistin, an adipocyte-secreted hormone, antagonizes insulin. Transgenic mice that overexpress the resistin gene (*Retn*) in adipose tissue are insulin-resistant, whereas *Retn* (−/−) mice show lower fasting blood glucose, suggesting that the altered *Retn* promoter function could cause diabetes. To determine the role of *RETN* in human T2DM, we analyzed polymorphisms in its 5′ flanking region. We found that the −420G/G genotype was associated with T2DM (397 cases and 406 controls) (P = .008; adjusted odds ratio = 1.97 [by logistic regression analysis]) and could accelerate the onset of disease by 4.9 years (P = .006 [by multiple regression analysis]). Meta-analysis of 1,888 cases and 1,648 controls confirmed this association (P = .013). Linkage disequilibrium analysis revealed that the −420G/G genotype itself was a primary variant determining T2DM susceptibility. Functionally, Sp1 and Sp3 transcription factors bound specifically to the susceptible DNA element that included −420G. Overexpression of Sp1 or Sp3 enhanced *RETN* promoter activity with −420G in *Drosophila* Schneider line 2 cells that lacked endogenous Sp family members. Consistent with these findings, fasting serum resistin levels were higher in subjects with T2DM who carried the −420G/G genotype. Therefore, the specific recognition of −420G by Sp1/3 increases *RETN* promoter activity, leading to enhanced serum resistin levels, thereby inducing human T2DM.

Type 2 diabetes mellitus (T2DM [MIM #125853]), a common disease that affects ~5% of adults, is characterized by insulin resistance (DeFronzo et al. 1992). Major genetic factors for T2DM remain to be determined, although its association with some polymorphisms has been reported (Altshuler et al. 2000; Horikawa et al. 2000; McCarthy and Froguel 2002). Resistin (resistance to insulin) (MIM 605565), an adipocyte-secreted hormone, antagonizes insulin (Steppan et al. 2001; Pravenec et al. 2003; Rajala et al. 2003; Banerjee et al. 2004; Steppan and Lazar 2004). Transgenic mice that overexpress the resistin gene (*Retn*) in adipose tissue are insulin-resistant (Pravenec et al. 2003), whereas *Retn* (−/−) mice show lower fasting blood glucose (Banerjee et al. 2004), suggesting that altered *Retn* promoter function could cause diabetes. Despite the established role of *Retn* in rodents (Steppan et al. 2001; Pravenec et al. 2003; Rajala et al. 2003; Banerjee et al. 2004), a link between *RETN* and human T2DM remains to be elucidated (Engert et al. 2002; Ma et al. 2002; Cho et al. 2004; Steppan and Lazar 2004).

We initially sequenced an ~1-kb upstream region of *RETN* in 24 Japanese patients with T2DM (see online-only appendix C for a description of our methods), since we reported that SNPs in the exons and introns of *RETN* were not associated with T2DM (Osawa et al. 2002). We

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Table 1

Characteristics of the Study Populations

Panel and Subject Group	No. of Subjects (Male/Female)	No. of Subjects with Genotype			Mean Age ± SD (Years)		BMI (kg/m^2)	HbA1c (%)
		C/C	C/G	G/G	At Testing	At Onset of Diabetes[a]		
Panel 1:								
Patients with T2DM	200 (96/104)	86	84	30	60 ± 12	48 ± 12	23 ± 4	7.6 ± 1.7
Controls	200 (107/93)	97	88	15	54 ± 8	NA	23 ± 3	4.8 ± .3
Panel 2:								
Patients with T2DM	197 (119/78)	82	85	30	60 ± 11	50 ± 12	24 ± 4	8.6 ± 2.1
Controls	206 (108/98)	87	98	21	63 ± 6	NA	23 ± 3	5.0 ± .3
Panel 3:								
Patients with T2DM	149 (101/48)	48	85	16	56 ± 14	49 ± 13	25 ± 4	7.6 ± 1.7
Controls	158 (71/87)	63	83	12	67 ± 9	NA	24 ± 3	4.9 ± .3

NOTE.—Subjects in panels 1 and 2 were from the same region in Japan. Subjects in panel 3 were from a different area of Japan. See online-only appendix A for a description of subjects.

[a] NA = not applicable.

identified five common SNPs: −1093A→G, −638G→A, −537A→C, −420C→G, and −358G→A. When the regions encompassing these SNPs were sequenced in 176 additional cases and 200 controls, two additional SNPs, −1082C→T and −821G→A, were found. We sequenced the regions that included all these SNPs, in a total of 200 cases and 200 controls (see panel 1 in table 1). These SNPs were in Hardy-Weinberg equilibrium in controls. Of these SNPs, only the allele frequency of −420C→G tended to be increased in patients with T2DM compared with controls ($\chi^2 = 3.84$; $P = .05$) (table 2). When the frequency of the −420G/G genotype was compared with that of the C/C genotype, the G/G genotype was associated with T2DM ($P = .018$; odds ratio [OR] = 2.26; 95% CI 1.14–4.47) (see panel 1 in table 3). The frequencies of the C/G and C/C genotypes did not differ between cases and controls, suggesting that only homozygotes of −420G are associated with T2DM.

Table 2

Allele Frequencies of SNPs in the 5′ Flanking Region of *RETN* for 200 Patients with T2DM and 200 Control Subjects in Panel 1

SNP[a]	No. of Alleles (Frequency [%]) in			
	Patients with T2DM	Controls	χ^2	P
−1093A→G	41 (10.3)	29 (7.3)	2.25	.13
−1082C→T	0 (0)	1 (.3)	…	…
−821G→A	1 (.3)	0 (0)	…	…
−638G→A	89 (22.3)	77 (19.3)	1.10	.30
−537A→C	24 (6.0)	14 (3.5)	2.76	.10
−420C→G	144 (36.0)	118 (29.5)	3.84	.05
−358G→A	89 (22.3)	77 (19.3)	1.10	.30

NOTE.—Allele frequencies represent minor alleles that are different from the reference sequence. The χ^2 test was used for the statistical analysis.

[a] The nucleotide number of each SNP is counted from A of the start codon as 1.

To assess the possibility that −420C→G in *RETN* is a primary variant that determines susceptibility to T2DM, we first examined the pattern of linkage disequilibrium (LD) around *RETN* by typing 26 frequent SNPs selected from the ~70-kb region (fig. 1*A*). The LD between −420C→G and its nearby SNPs existed in a quite restricted area. In this area, the LD of −420C→G with its adjacent SNPs—namely, −638G→A or −358G→A—was strong, whereas that of −420C→G with distant SNPs—namely, −1093A→G or +299G→A—was weak. Thus, the LD of −420C→G did not extend beyond −1093A→G or +299G→A, suggesting that the association of −420C→G with T2DM is not caused by LD of an unidentified susceptibility variant around *RETN* with −420C→G.

We next calculated, using panel 1, the ORs of minor-allele homozygotes to major-allele homozygotes and those of heterozygotes to major-allele homozygotes for seven frequent SNPs that have minor-allele frequencies >5% and are located between −1093 and +299 (fig. 1*B*). The OR of minor-allele homozygotes to major-allele homozygotes was highest at −420C→G and was lower for SNPs distant from −420C→G. Of all the ORs, only the OR of −420G/G to −420C/C was significantly larger than 1 ($P = .018$). When haplotype frequencies defined by these seven SNPs were estimated for cases and controls, frequencies of any particular haplotypes including −420C→G were not significantly increased in patients with T2DM (table 4 [online only]). Collectively, all these findings suggest that the SNP −420C→G itself is the primary variant that determines susceptibility to T2DM.

To identify specific transcription factors that bind to the DNA element, we examined whether one base substitution (C→G at −420) affects the specific binding of proteins to the DNA element (fig. 2*A*). An electrophoretic mobility shift assay (EMSA), by use of nuclear ex-

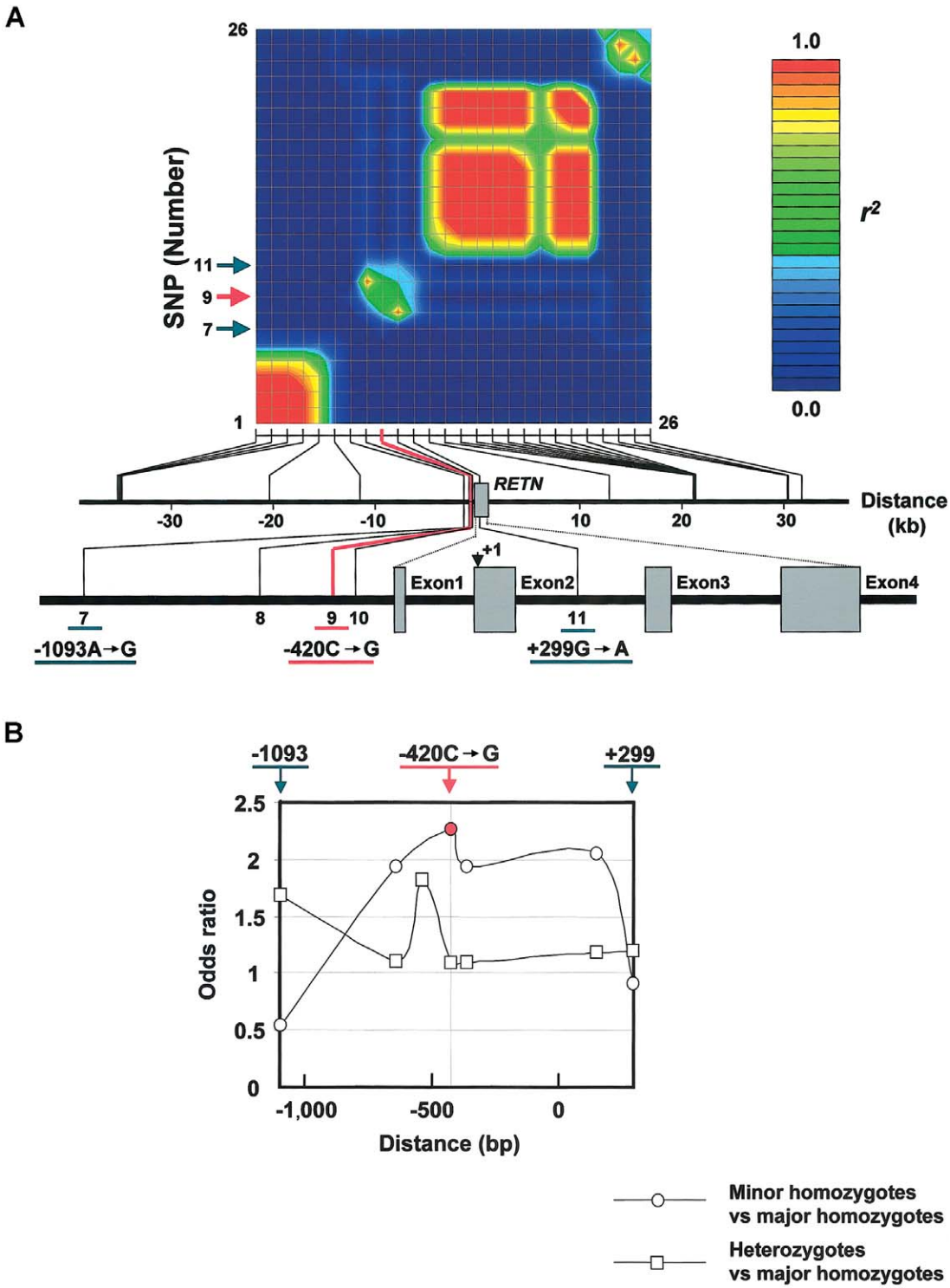

Figure 1 LD and OR results for *RETN* −420C→G, a primary variant associated with susceptibility to T2DM. *A,* The LD of −420C→G did not extend beyond −1093A→G or +299G→A. The pairwise LD of 26 frequent SNPs, as measured by r^2, is shown in the upper panel (see online-only appendix F for a description of our methods). The physical positions of these SNPs (counted from the translation start site of *RETN* as +1) are shown in the lower panel. Arrows indicate a restricted LD area around −420C→G. Five SNPs are labeled in the figure: −1093A→G (*7*), −638G→A (*8*), −420C→G (*9*), −358G→A (*10*), and +299G→A (*11*). The other SNPs are summarized in table C2 (online only). *B,* The OR of minor-allele homozygotes to major-allele homozygotes was highest at −420C→G, and this OR was significantly larger than 1 among the seven frequent SNPs that have minor-allele frequencies >5% and that are located between −1093 and +299 in *RETN*. The ORs of minor-allele homozygotes to major-allele homozygotes and of heterozygotes to major-allele homozygotes were calculated by use of panel 1 subjects, for each of the following variants: −1093A→G, −638G→A, −537A→C, −420C→G, −358G→A, +157C→T, and +299G→A. The distance was counted from the translation initiation site.

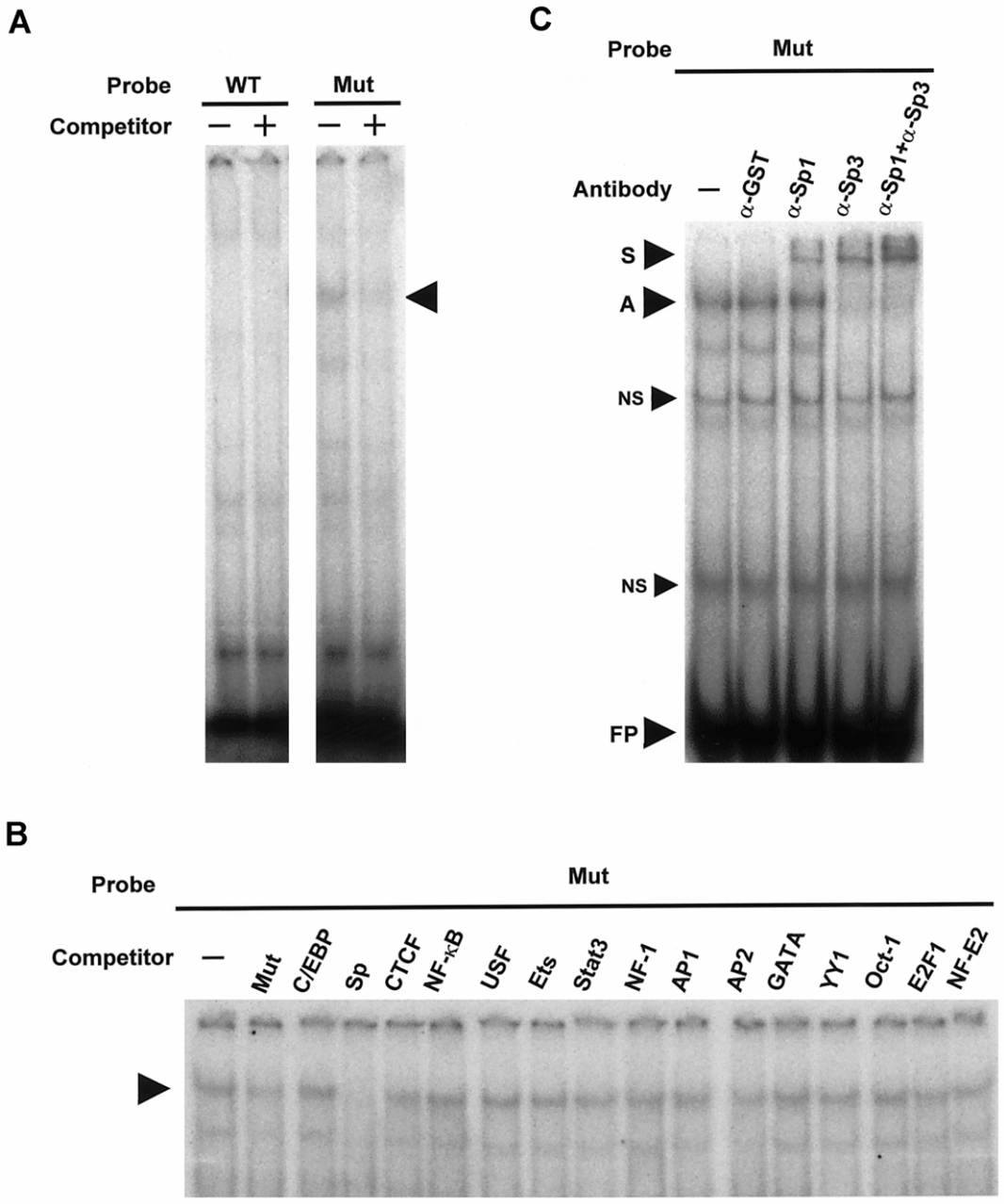

Figure 2 Identification of Sp1 and Sp3 as major transcription factors binding only to the diabetes-susceptibility DNA element with −420G in *RETN*. *A,* Specific protein binding to the diabetes-susceptibility DNA element was analyzed. For the EMSA, nuclear extracts prepared from differentiated 3T3-L1 adipocytes were incubated with ^{32}P-labeled double-stranded oligonucleotide probes that corresponded to the region (−434/−406) of *RETN*, as described in appendix D (online only). The wild-type (WT) probe includes C at −420, and the mutant (Mut) probe includes G at −420. Unlabeled double-stranded oligonucleotides for WT and Mut sequences (a 200-fold molar excess) were used as competitors for WT and Mut probes, respectively. An arrow indicates complexes with specific nuclear factors. *B,* Only an Sp transcription factor consensus binding site competes with the diabetes-susceptibility DNA element for specific protein binding. For a competition analysis, a 200-fold molar excess of unlabeled double-stranded oligonucleotides for consensus binding sites for a variety of transcription factors was added. Cold oligonucleotides for the mutant (Mut) DNA element (positive control [*second lane from the left*]) and the Sp consensus binding site (*fourth lane*) competed with the protein binding. An arrow indicates complexes with specific nuclear factors. *C,* Sp1 and Sp3 are major transcription factors binding specifically to the diabetes-susceptibility DNA element. Antibodies against Sp1, Sp3, and GST (negative control) were added to the reaction. S = supershifted complex with specific nuclear factors and antibodies; A = complex with specific nuclear factors; NS = complex with nonspecific binding nuclear proteins; FP = free probes. These data represent at least three independent experiments.

Table 3

Association of the *RETN* −420G/G Genotype with T2DM

Panel(s) (No. of Cases/Controls) and Genotype Comparison[a]	χ^2	*P*	OR	95% CI
Panel 1 (200/200):				
G/G vs. C/C	5.59	.018	2.26	1.14–4.47
C/G vs. C/C	.12	.728	1.08	.71–1.63
G/G vs. C/C + C/G	5.63	.018	2.18	1.13–4.19
Panels 1 and 2 (397/406):				
G/G vs. C/C	6.59	.010	1.83	1.15–2.90
C/G vs. C/C	.00	.974	1.00	.74–1.34
G/G vs. C/C + C/G	7.44	.006	1.83	1.18–2.84
Panels 1, 2, and 3 (546/564):				
G/G vs. C/C	8.38	.004	1.81	1.21–2.71
C/G vs. C/C	.36	.548	1.08	.84–1.39
G/G vs. C/C + C/G	8.18	.004	1.74	1.19–2.55

NOTE.—The χ^2 test was used for the statistical analysis. The nucleotide position is counted from A of the start codon as 1.

[a] See table 1 for the number of subjects with each genotype.

tracts from 3T3-L1 adipocytes, indicated that proteins bound specifically to the susceptible DNA element that included G at −420 (mutant probe) (third lane from the left in fig. 2*A*) but not to the wild-type element that included C (wild-type probe) (first lane in fig. 2*A*). A mutant competitor inhibited protein binding to the mutant probe (fourth lane in fig. 2*A*).

To determine which consensus DNA element shares binding proteins with this susceptible DNA element, we added oligonucleotides of each binding site for various transcription factors as competitors (fig. 2*B*). Only oligonucleotides for Sp binding sites reduced specific protein binding (fourth lane from the left in fig. 2*B*), suggesting that the susceptible DNA element binds specifically to Sp transcription factors. A positive-control competitor also reduced the specific protein binding (second lane in fig. 2*B* [Mut]).

To identify which Sp factors bind to the susceptible DNA element, we examined the effects of anti-Sp1 and anti-Sp3 antibodies on protein-DNA binding (fig. 2*C*). Whereas the anti-Sp1 antibody weakly affected protein binding (third lane from the left in fig. 2*C*), the anti-Sp3 antibody strongly supershifted the complex (fourth lane in fig. 2*C*). When the anti-Sp1 and anti-Sp3 antibodies were added together, the complex was completely supershifted (fifth lane in fig. 2*C*). A negative-control anti-GST antibody had no effect (second lane in fig. 2*C*). Therefore, we provisionally identified Sp1 and Sp3 as major transcription factors binding specifically to the susceptible DNA element.

We next tested whether the C→G substitution at −420 affects *RETN* promoter activity through the specific binding of Sp1 and Sp3 (fig. 3). To assess isolated effects of Sp1 and Sp3, we employed *Drosophila* Schneider line 2 (SL2) cells, which lack endogenous Sp family transcription factors. When Sp1 or Sp3 was overexpressed in SL2 cells, the *RETN* promoter activity with G at −420 (mutant) was significantly enhanced, compared with the activity with C at −420 (wild type). Thus, Sp1 and Sp3 are major transcription factors enhancing *RETN* promoter activity by binding specifically to the DNA element with −420G.

To evaluate the association between the −420G/G genotype and T2DM in a larger sample size, we further sequenced only the regions containing −420C→G for the 197 cases and 206 controls in panel 2 (table 1), which was collected from the same geographic area as panel 1. When the G/G genotype was compared with the C/C genotype in the combined data of panels 1 and 2, the G/G genotype was associated with T2DM ($P = .010$; OR = 1.83; 95% CI 1.15–2.90) (table 3). The adjusted ORs of the G/G and C/G genotypes were estimated by use of multiple logistic regression analysis, adjusted for age, sex, and maximum BMI (see online-only appendix F for a description of our methods). The adjusted OR of G/G was significantly high (two-sided $P = .008$; OR = 1.97; 95% CI 1.19–3.26). Since the adjusted OR of C/G was 1.08 ($P = .65$; 95% CI 0.78–1.49), we conclude that the −420G/G genotype increases the risk of T2DM. Multiple regression analysis, adjusted for sex and maximum BMI, of 397 subjects with T2DM revealed that the age

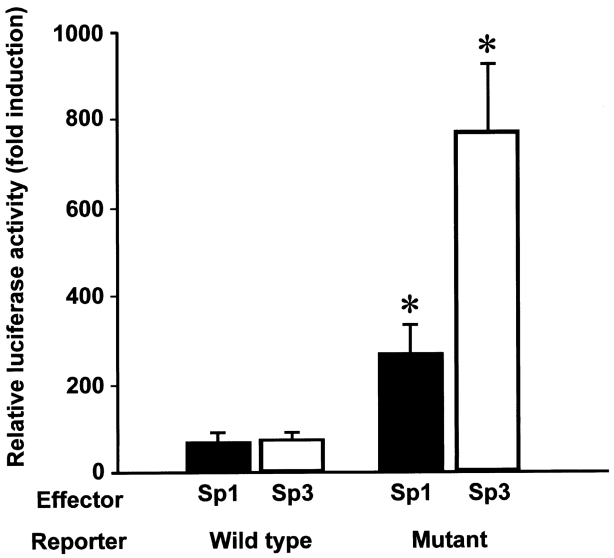

Figure 3 Activation of the *RETN* promoter with the diabetes-susceptibility SNP −420G binding specifically to Sp1/3. Either the wild-type (with −420C) or mutant (with −420G) *RETN* promoter reporter was transiently transfected into SL2 cells with effectors—namely, pPac (control), pPac-Sp1, or pPac-USp3, and the internal control pPac-βGal. Luciferase activity was measured as described in appendix E (online only). Relative luciferase activities are shown as the mean fold induction ± SE, relative to the activity of each reporter with a control effector. The data represent four independent experiments with duplicate wells for each condition. The asterisk (*) indicates $P < .05$, compared with the wild-type promoter activity with the same effector (ANOVA).

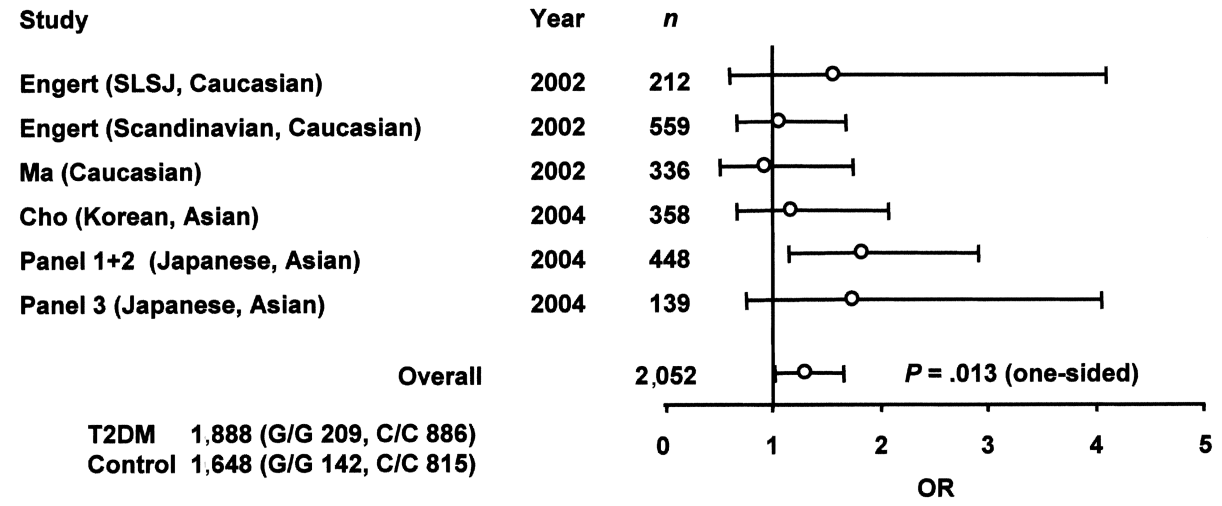

Figure 4 Meta-analysis confirming association of the −420G/G genotype with T2DM. The data from Engert et al. (2002), Ma et al. (2002), and Cho et al. (2004) were combined with data from the present study. Samples from different geographic locations were analyzed separately (panels 1 and 2 and panel 3). The total number of subjects typed was 1,888 patients with T2DM and 1,648 controls. ORs of G/G genotypes to C/C genotypes were estimated, as described in appendix F (online only). Circles indicate ORs; bars represent 95% CIs; *n* = number of subjects with G/G and C/C genotypes. SLSJ = Saguenay–Lac-Saint-Jean region of Quebec.

at onset of disease for patients with the G/G genotype was 4.9 years younger than for patients with the C/C genotype ($P = .006$; 95% CI 1.39–8.41). When panel 3 (table 1), which was collected from another area in Japan, was added, the association of the G/G genotype with T2DM was consistent ($P = .004$; OR = 1.81; 95% CI 1.21–2.71) (see panels 1, 2, and 3 in table 3). Therefore, the −420G/G genotype is associated with T2DM in a large number of Japanese subjects.

Although the association of −420C→G with T2DM has been examined in several ethnic populations (Engert et al. 2002; Ma et al. 2002; Cho et al. 2004), individual studies did not have sufficient power, probably because of limited sample size or a lower frequency of −420C→G than that found in Japanese subjects. To obtain evidence that was more conclusive (Lohmueller et al. 2003), we conducted a meta-analysis of results from previous studies (Engert et al. 2002; Ma et al. 2002; Cho et al. 2004) and the present study, in which the OR was used as the metric of association (fig. 4). The random-effects OR estimate for the risk of developing T2DM was significantly higher in subjects with −420G/G compared with subjects with −420C/C (one-sided $P = .013$; OR = 1.31; 95% CI 1.03–1.66), and no evidence of between-study heterogeneity was found in the meta-analysis ($P = .470$). Therefore, the G/G genotype is associated with susceptibility to T2DM, which supports a common-disease/common-variant hypothesis (Lander 1996). Since the OR of the G/G genotype appears to be higher in Japanese subjects, racial differences may exist.

Finally, to examine whether the enhanced *RETN* promoter activity with −420G is associated with enhanced *RETN* expression in humans, we measured fasting serum resistin levels by use of 93 samples available from subjects with T2DM in panels 1 and 2 (fig. 5). Consistent with our genetic and molecular data, subjects with the G/G genotype had higher serum resistin levels than subjects with the C/C genotype ($P = .0018$). Subjects with the C/G genotype also had higher resistin levels than those with the C/C genotype, although this difference was smaller. All these findings suggest that the G/G genotype of a functional *RETN* promoter SNP at −420 determines human T2DM susceptibility—probably through enhanced *RETN* expression.

Our in vitro data are supported by previous studies (Smith et al. 2003; Cho et al. 2004). A DNA element that includes −420G binds to unidentified factors in nuclear extracts of 3T3-L1 adipocytes (Cho et al. 2004). The *RETN* promoter activity with −420G (described as −180) is enhanced to 400% of that with −420C in 3T3-L1 adipocytes, without manipulation of expression of Sp transcription factors (Smith et al. 2003). In humans, obese subjects with the G/G genotype have higher resistin mRNA levels in their abdominal subcutaneous fat (Smith et al. 2003). Cho et al. (2004) reported that subjects with the G/G genotype have higher serum resistin levels, and our results support this finding. Taken together, these results lead us to propose that one base substitution from C to G at −420 activates *RETN* transcription by specific Sp1/3 binding, which could induce insulin resistance associated with T2DM (fig. 6 [online only]). By recognizing specific sequences in regulatory

regions, ubiquitous factors could affect disease susceptibility. In the *RETN* promoter with −420G, Sp3 acted as a stronger activator than Sp1, whereas Sp3 has been shown to act as a transcriptional activator or an Sp1-mediated transcription repressor (Bouwman and Philipsen 2002).

Controversy exists about whether an increase in serum resistin levels is associated with human T2DM and obesity (Lee et al. 2003; McTernan et al. 2003; Cho et al. 2004; Fujinami et al. 2004; Steppan and Lazar 2004). Serum resistin probably exists as a hexamer (major form) or a trimer (a more biologically active form) (Patel et al. 2004), which may affect the assay results. Serum resistin levels were higher in subjects with T2DM who had the −420G/G genotype than in those who had other genotypes, a finding that is supported by Cho et al. (2004), suggesting that the discrepancy may be resolved by considering the different genotypes at −420. It should be noted that the main source of human serum resistin remains unknown, because it is most highly expressed in macrophages (Nagaev and Smith 2001; Savage et al. 2001; Wellen and Hotamisligil 2003; Banerjee et al. 2004).

In summary, the −420G/G genotype in *RETN* is associated with susceptibility to T2DM. Sp1 and Sp3 bind specifically to the DNA element with −420G and enhance the promoter activity. This provides evidence for a link between an *RETN* promoter SNP and human T2DM, encouraging further detailed studies in rodents (Steppan et al. 2001; Pravenec et al. 2003; Rajala et al. 2003; Banerjee et al. 2004). Functional SNPs in regulatory regions could represent promising candidates for susceptibility genes in other common diseases as well; in fact, a similar mechanism has been recently reported in an organic cation transporter gene, *SLC22A4*, and in the gene encoding Runt-related transcription factor 1 (*RUNX1*) for rheumatoid arthritis (Tokuhiro et al. 2003).

Descriptions of the methods used in this study are available in appendices A–F (online only).

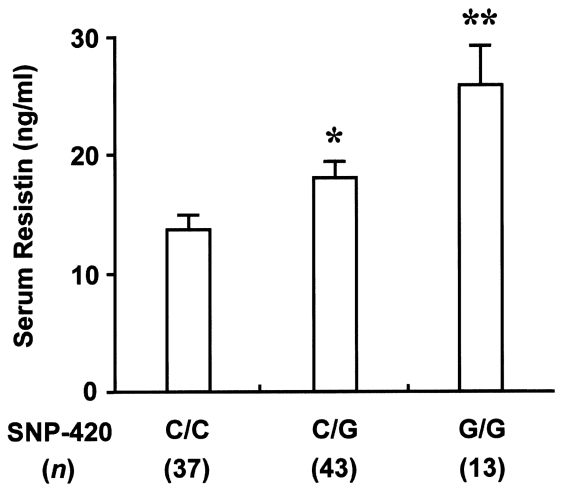

Figure 5 Increased fasting serum resistin levels in subjects with T2DM and a −420G/G genotype. Serum resistin levels were measured by use of a human resistin ELISA kit (LINCO Research), as described in appendix B (online only). We analyzed 93 fasting serum samples available from subjects with T2DM in panels 1 and 2. Data represent mean ± SE. Student's *t* test was used for statistical analysis. The asterisk (*) indicates $P = .012$; two asterisks (**) indicate $P = .0018$, compared with subjects with the C/C genotype.

Acknowledgments

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Electronic-Database Information

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

dbSNP Home Page, http://www.ncbi.nlm.nih.gov/SNP/ (for −1093A→G [accession number rs3760678], −420C→G [accession number rs1862513], −358G→A [accession number rs3219175], +157C→T [accession number rs3219177], and +299A→G [accession number rs3745367])

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for genomic structure of the *RETN* gene [accession number NT_077812] and *RETN* cDNA [accession number NM_020415])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for T2DM and resistin)

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