Hypoadiponectinemia Is Associated With Progression Toward Type 2 Diabetes and Genetic Variation in the *ADIPOQ* Gene Promoter

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OBJECTIVE — Adiponectin encoded by the *ADIPOQ* gene modulates insulin sensitivity and glucose homeostasis. The aim of the current study was to investigate whether *ADIPOQ* gene variants in the promoter region predict adiponectin levels and type 2 diabetes progression.

RESEARCH DESIGN AND METHODS — A total of 550 subjects with increased risk of type 2 diabetes were investigated; they underwent a 75-g oral glucose tolerance test, repeated after 3 years. Adiponectin levels were analyzed, and two *ADIPOQ* promoter variant single nucleotide polymorphisms, −11391G>A and −11377C>G, were genotyped.

RESULTS — Tertiles of the adjusted adiponectin levels were associated with single nucleotide polymorphism −11391G>A and −11377C>G haplotypes ($P < 0.0001$). Carriers of the intermediate/high-level haplotype combination showed a bisected diabetes risk at the 3-year follow-up and were characterized by a "regression" of glucose tolerance. Evolution of disease status correlates with preexisting low adiponectin levels at inclusion rather than with variation in adiponectin levels.

CONCLUSIONS — We present data that gene variants in the *ADIPOQ* promoter region are associated with variations in adiponectin levels and thus with future type 2 diabetes and disease progression.

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In conjunction with environmental and behavioral factors, genetic susceptibility is an integral part in the pathogenesis of type 2 diabetes (1). There is evidence that adipose tissue not only functions as an energy reservoir but also produces and secretes several cytokines that modulate energy metabolism and glucose homeostasis (2–5).

The *ADIPOQ* gene encoding adiponectin has been mapped to chromosome 3q27 (6) and is expressed in adult adipocyte tissue (7). Both reduced expression of adiponectin and lower serum adiponectin levels were detected in patients with obesity, type 2 diabetes, and coronary artery disease (8,9). Moreover, treatment of diabetic mice with adiponectin was shown to induce a marked improvement in insulin sensitivity (10). Clinical studies showed that an altered serum adiponectin level is an independent risk factor for progression to type 2 diabetes (11,12).

Screening for mutation within the adiponectin gene in the French, African-American, and Swedish populations (13–15) revealed an association of haplotypes −11391G>A and −11377C>G single nucleotide polymorphisms (SNPs) comprising promoter and coding variants of the adiponectin gene with insulin resistance and type 2 diabetes (13,14,16–18) as well as adiponectin levels (16,19). These data provide evidence that genetic variation within the *ADIPOQ* gene may be part of the genetic determinants of risk of type 2 diabetes and insulin resistance (13,18) via modulation of adiponectin levels (16,17,20).

By using a prospective cohort study, our objective in this investigation was to test the hypothesis that adiponectin promoter variants predict adiponectin levels and that adiponectin promoter variants and/or adiponectin serum levels predict type 2 diabetes progression. Because of the previously reported associations in the French Caucasian population (13), we analyzed SNPs −11391G>A and −11377C>G in the *ADIPOQ* gene promoter.

RESEARCH DESIGN AND METHODS

— Five hundred and fifty subjects from German families with a family history of type 2 diabetes, obesity, or dyslipoproteinemia were investigated. The mean ± SD age of the subjects was 56.9 ± 8.1 years and BMI 27.2 ± 4.1 kg/m^2. All subjects were from the city of Dresden and adjoining areas. Exclusion criteria were known diabetes, severe renal disease, disease with a strong impact on life expectancy, and therapy with drugs known to influence glucose tolerance (thiazide diuretics, β-blockers, and steroids). All individuals underwent a 75-g oral glucose tolerance test (OGTT) after an overnight period of fasting (10 h minimum) with measurements of plasma glu-

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Abbreviations: IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; SNP, single nucleotide polymorphism.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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Table 1—*Basic data on subjects during the baseline visit according to the four glucose tolerance classes*

	NGT	IGT /IFG	Type 2 diabetes
n (male/female)	254 (111/143)	220 (104/116)	76 (41/35)
Age (years)	56.8 ± 7.3	59.1 ± 8.1	60.2 ± 6.5
BMI (kg/m^2)	25.9 ± 3.7	27.8 ± 4.5	29.3 ± 3.9
Fasting plasma glucose (mmol/l)	5.4 ± 0.3	6.2 ± 0.3	7.4 ± 1.5
A1C (%)	5.5 ± 0.4	5.7 ± 0.6	6.5 ± 1.1
Insulin fasting (pmol/l)	68 ± 40	93 ± 47	119 ± 89
Proinsulin fasting (pmol/l)	1.7 ± 1.1	2.8 ± 2.8	4.9 ± 4.8
Free fatty acids (mmol/l)	0.42 ± 0.2	0.49 ± 0.22	0.56 ± 0.32
HOMA*	16.26 ± 10.0	26.34 ± 15.99	39.94 ± 30.85

Data are means ± SD. *The insulin resistance index was calculated by homeostasis model assessment (HOMA).

cose, insulin, and free fatty acids at fasting and at 30, 60, 90, and 120 min after glucose challenge. After a 3-year period, 496 subjects again underwent an OGTT using the same protocol.

The cohort was divided into three glucose tolerance groups according to the results of the baseline and follow-up OGTTs: normoglycemic (normal glucose tolerance [NGT]), impaired glucose tolerance (IGT) including those with impaired fasting glucose (IFG), and type 2 diabetes based on the World Health Organization /American Diabetes Association criteria of 1997/1999. As patients underwent OGTT analyses both at inclusion and after the 3-year interim, five groups were defined according to the evolution of their diabetes status: those whose disease status remained unchanged as NGT, IGT/IFG, and type 2 diabetes, those presenting a regression of the disease, and those presenting a progression of the disease. The detailed procedure of subject recruitment and the methods used have been described previously (21,22). Informed consent was obtained from all subjects, and the study was approved by the local ethics committee.

Laboratory procedures

Plasma glucose was measured using the hexokinase method (interassay coefficient of variation [CV] 1.5%). Analysis of insulin levels was performed by enzyme immunoassay (BioSource EUROPE, Nivelles, Belgium) (interassay CV 7.5%, no cross-reactivity with human proinsulin). Adiponectin was measured using a Human Adiponectin ELISA Kit (BioCat, Heidelberg, Germany). HbA_{1c} (A1C) values were detected using high-performance liquid chromatography.

Genetic analysis

Real-time PCR was performed on a LightCycler (Roche Diagnostics, Mannheim, Germany). The *ADIPOQ* promoter SNPs, −11391G>A (rs17300539) and −11377C>G (rs266729), were genotyped. To analyze SNP −11391G>A, the primers F, 5′-acttgccctgcctctgtctg-3′, and R, 5′-gcctggagaactggaagctg-3′, as well as the hybridization probes, 5′-gcaggatct gagccggttct-x-3′ and 5′-LCRed640-gcaagccacacattctgatgaattaaattacgaccc-ph-3, were used; for SNP −11377C>G analysis, the primers F, 5′-acttgccctgc ctctgtctg-3′, and R, 5′-gcctggagaactg gaagctg-3′, and the hybridization probes, 5′-ctcagatcctgcccttcaaaaac-x-3′ and 5′-LCRed640-acatgagcgtgccaagaaagtccaag gtgttg-ph-3′, were used. PCR was performed in a total volume of 8 μl within glass capillaries. The reaction mixture used in each PCR consisted of 25 ng of genomic DNA, 0.5 μl of each primer (5 μmol/l), 0.5 μl of the DNA Master Hybridization Probes Kit, 0.5 μl of each hybridization probe (1.5 μmol/l), and 0.6 μl of $MgCl_2$ (25 mmol/l). After initial denaturation at 94°C for 3 s, 40 PCR cycles were performed with 1 s of denaturation at 94°C; 10 s of annealing at 58°C for SNPs −11391G>A, −11377C>G, and +45T>G, and extension at 72°C for 5 s. Genotyping was carried out via melting curve analysis. Quality control was performed by direct sequencing of 11% randomly chosen samples.

Statistics

Clinical data are expressed as means ± SD and adiponectin levels as means ± SEM. The proportions of genotypes or alleles were compared by χ^2 analysis. Differences in continuous parameters among genotypes were evaluated by the Wilcoxon-Kruskal-Wallis test. Statistical analysis was performed using the StatView 5.0 software package (SAS Institute, Cary, NC). Diploid haplotype combinations for every individual were assigned using both expectation maximum and Stephens-Smith-Donnelly algorithms. Haplotype frequencies were determined using the PM+EH+ software package and compared with a permutation procedure, performing Monte Carlo tests of significance (23). Effects of haplotypes on quantitative variables were assessed using haplotype regression software (24). The diploid configuration of haplotypes were inferred by two different algorithms, expectation maximum and Stephens-Smith-Donnelly, as implemented in the GENECOUNTING and PHASE software packages, respectively (25–27). Adiponectin levels were adjusted for confounding variables, age, sex, and BMI. Scheffe's procedure was used for multiple testing corrections. CLUMP software (27) was used to assess the significance of the departure of observed values in $2 \times N$ contingency tables. The significance was obtained using a Monte Carlo approach with repeated simulations. Moreover, the software clumps the columns in two groups (2×2 contingency table) to maximize the χ^2 value. Receiver operating characteristic curves were obtained using SPSS 11.0 Software. The odds ratio (OR) was calculated using multivariate logistic regression. The linkage disequilibrium between SNPs was calculated using Haploview software (28) and the PM+EH+ software package. Individuals with IGT and IFG were analyzed as a combined group for disease evolution. A value of $P < 0.05$ was assumed to indicate significance unless otherwise stated.

RESULTS

— Of the 550 subjects initially collected, 46.2% were found to have NGT, 40.0% presented with IGT including IFG, and 13.8% had newly diagnosed type 2 diabetes (Table 1).

Analysis of *ADIPOQ* SNPs

Allelic frequencies of the common alleles in the SNPs were 0.89 for −11391G>A and 0.73 for −11377C>G, respectively. Both SNPs were determined to be in Hardy-Weinberg equilibrium and were in complete linkage disequilibrium ($D' = 0.99$). The wild-type G allele at SNP −11391 and the variant G allele at SNP −11377 were associated with lower adi-

Table 2—*Adiponectin levels at inclusion of the patients*

		n	Mean adiponectinemia (μg/ml)
Variant			
−11391G>A,	GG	447	9.76 ± 0.34
−11391G>A,	GA	97	11.55 ± 0.37*
−11391G>A,	AA	6	9.81 ± 0.62
−11377C>G	CC	309	10.53 ± 0.83†
−11377C>G	CG	202	9.69 ± 0.47
−11377C>G	GG	39	9.10 ± 0.65
Haplotype combination			
I/H	G/C; A/C	70	12.23 ± 0.56
H/H	A/C; A/C	6	11.18 ± 1.93
L/H	G/G; A/C	27	10.53 ± 0.91
I/I	G/C; G/C	233	10.01 ± 0.31
I/L	G/C; G/G	175	9.59 ± 0.36
L/L	G/G; G/G	39	9.43 ± 0.76

Data are means ± SE. *Significant difference to −11391GG; $P = 0.0006$, †Significant difference to −11377CG; $P = 0.04$. H, high adiponectin level haplotype (−11391A and −11377C); I, intermediate adiponectin level haplotype (−11391G and −11377C); L, low adiponectin level haplotype (−11391G and −11377G).

ponectin levels ($P = 0.0001$ and $P = 0.02$, respectively) (Table 2). The −11391GA and the −11377CC genotypes were associated with higher adiponectin levels (Table 2). None of the SNPs were associated separately with type 2 diabetes. Analysis using haplotype regression software ascertained the relationship between SNPs −11391G>A and −11377C>G and decreased adiponectin levels ($P = 0.0012$). Mean adiponectin levels associated with the A_C (SNP −11391A_-SNP −11377C) haplotype (10.96 μg/ml) were found to be higher than those associated with either allele on its own.

Adiponectin levels and *ADIPOQ* haplotypes

As reported by Vasseur et al. (13), after stratification according to the tertiles of adjusted adiponectin levels and comparison of haplotype frequencies in the first and third tertiles, a significant association between promoter haplotypes and adiponectin levels was disclosed ($P < 0.0001$) (Table 3), defining a low adiponectin level (L, SNP −11391G_11377G), a high adiponectin level (H, SNP −11391A_11377C), and an intermediate adiponectin level (I, SNP −11391G_11377C) haplotype based on the frequency changes between the first, second, and third tertiles of adjusted adiponectin level at the baseline visit, respectively. A similar result was obtained at the follow-up visit ($P = 0.0036$) (Table 3).

Furthermore, at the baseline visit, subjects with NGT presented a higher frequency of the H haplotype than type 2 diabetic patients (0.098 vs. 0.079; $P > 0.05$); at the 3-year follow-up, the difference was similar (0.110 vs. 0.054) and significant ($P = 0.029$, as determined by PM+EH+ software).

Haplotype combinations and adiponectin levels

A strong association between haplotype combination and adjusted adiponectin levels was detected ($P = 0.0006$). The highest adiponectin levels were in the subgroup of individuals harboring at least one H haplotype (Table 2). Scheffe's correction for multiple testing revealed a significant difference between subjects harboring one H haplotype compared with those presenting two wild-type haplotypes ($P = 0.001$), one L haplotype ($P = 0.001$), or two L haplotypes ($P = 0.049$). Because the remaining configurations were underrepresented, no significant difference was detected.

Evolution of the disease and adiponectin levels

The second set of analyses done after 3 years produced similar results as those presented above (Table 3). Adiponectin levels were highly associated with the subsequent evolution of the disease ($P < 0.0001$) (Table 4). Notably, there was no significant variation in adiponectin levels for the single individual in the period between inclusion and after the 3-year follow-up, whatever the evolution of the disease ($P = 0.5$). As presented in Table 4, adiponectin levels were highest in patients remaining normoglycemic or presenting with a regression of the disease and lowest in patients with type 2 diabetes in a stable condition or after the disease had progressed. It is tempting to associate these physiological data with genetic variation within the *ADIPOQ* promoter haplotypes.

To investigate the evolution of the disease, we opted for measuring the area

Table 3—*Haplotype frequencies of SNPs −11391G>A and −11377C>G for the 1st, 2nd, and 3rd tertiles of adjusted adiponectin levels at baseline and follow-up visit*

Haplotype		Haplotype frequency						Haplotype association
		Adiponectin at baseline visit			Adiponectin at follow-up visit			
Allele at SNP −11391	Allele at SNP −11377	1st tertile	2nd tertile	3rd tertile	1st tertile	2nd tertile	3rd tertile	
G	C	0.651	0.649	0.634	0.637	0.680	0.617	I
G	G	0.285	0.261	0.201	0.266	0.251	0.231	L
A	C	0.040	0.079	0.163	0.071	0.056	0.151	H
A	G	0.023	0.009	0.000	0.024	0.012	0.000	

χ^2 statistic for heterogeneity model for comparison of haplotype frequencies between 1st and 3rd tertiles of adjusted adiponectin levels at baseline visit = 34.26 ($P < 0.0001$) and follow-up visit = 13.52 ($P = 0.0036$).

Table 4—*Adiponectin levels and haplotype frequencies according to the evolution of the disease*

Evolution of the disease	L haplotype	H haplotype	Adiponectin level at inclusion (μg/ml)	Adiponectin level after 3 years (μg/ml)	*n*
Remain NGT	0.239	0.103	11.52 ± 0.35	11.39 ± 0.36	171
Regression of the disease	0.231	0.126	10.00 ± 0.44	9.66 ± 0.50	104
Remain IGT/IFG	0.264	0.076	9.11 ± 0.47	9.06 ± 0.56	91
Progression of the disease	0.272	0.079	8.55 ± 0.50	8.60 ± 0.55	82
Remain diabetic	0.283	0.054	8.49 ± 0.68	8.46 ± 0.78	44

Data are means ± SEM.

under the curve of glycemia during an OGTT rather than simple fasting glycemia. The variation of the area under the curve for glycemia during the 3-year period calculated for every subject was significantly different among the subjects with the I/H haplotype combination compared with the remaining configurations (−59.7 vs. + 5.3, $P = 0.01$). Indeed, paired *t* tests displayed a significant ($P = 0.03$) reduction for the I/H haplotype subgroup without any significant variation for the remaining genotypes ($P = 0.7$). The risk of type 2 diabetes at the end of the 3-year follow-up was determined to be bisected for subjects harboring the I/H haplotype combination (OR 0.42 [95% CI 0.17–0.94], $P = 0.04$). Moreover, only 7.9% of patients presenting with disease progression showed an H haplotype combination compared with the increase of 12.6% for the subgroup of individuals presenting with disease regression.

CONCLUSIONS — The data presented in this investigation were gathered from a German population and thus replicate the previously reported association of the −11391G>A and −11377C>G *ADIPOQ* promoter haplotypes with adiponectin levels (13). This investigation provides evidence that progression toward type 2 diabetes is associated with altered serum adiponectin levels and that altered adiponectin levels are associated with a genetic variation in the adiponectin gene promoter region in a German Caucasian population. We present data supporting the idea that this effect is associated with the protective H haplotype rather than with the "at-risk" L haplotype as reported by Vasseur et al. in 2002 (13). This apparent discrepancy indicates an unknown functional variant in linkage disequilibrium with the promoter haplotype, a hypothesis that is further supported by the association of SNP −11377 C>G in type 2 diabetes reported in the Japanese population, with a C allele presenting the risk as opposed to the French Caucasian population with the G allele presenting the risk (29).

The follow-up study done here presents novel findings supporting the hypothesis that preexisting high adiponectin levels seem to prevent the development of type 2 diabetes rather than the variation in adiponectin levels during the development of the disease. Daimon et al. (11) reported that a decrease in serum adiponectin levels over a 5-year follow-up is a risk factor for diabetes progression but not associated with the progression to diabetes from IGT. In our study the lowest adiponectin levels were found in patients remaining diabetic or those presenting with disease progression during the follow-up period. Conversely, the highest adiponectin levels were associated with subjects displaying a regression of the disease or remaining normoglycemic and thus should confirm a favorable prognosis for disease development (12,30). This finding emphasizes the impact that these results have on the potential use of adiponectin levels as a biomarker of future disease or disease progression. If low adiponectin levels predict diabetes development, the diagnosis of hypoadiponectinemia in normoglycemic subjects may become a diagnostic criterion for new therapeutic or preventive interventions.

Furthermore, the follow-up study supports the idea that genetic variation in the *ADIPOQ* promoter is associated with evolution of glucose tolerance. During the 3-year follow-up, subjects with the I/H haplotype combination were characterized by regression of the disease. This was significantly different from the evolution of the remaining genotypes ($P = 0.01$) and confirmed the protective effect of the I/H haplotype combination in the evolution of the disease. With the inclusion of clinical and genetic data in this prospective follow-up, our study seems to present a more universal finding in the Caucasian population.

Several limitations of this study warrant consideration. First, we have analyzed only promoter variants, whereas other studies showed associations either for coding variants such as the +276 polymorphism (13,14,16) or variants in the 3′ untranslated region (31). Second, we measured total adiponectin and did not separate the trimere or hexamere structure of adiponectin, limiting the clinical interpretation of our results (32,33). Furthermore, it seems to be inconsistent that the SNP −11391AA homozygotes have lower adiponectin levels than the −11391GA heterozygotes, but, indeed, there were only six subjects in this group, and this observation could also be consistent with the existence of additional variations at the adiponectin locus. Finally, only the most common haplotype combinations have sufficient numbers to be confident of the effects presented. Thus, the interpretation of homozygous haplotype combinations is limited because most are rare.

Our investigation has shown that adiponectin gene variants predict adiponectin levels, as we observed an association between the SNPs −11391G>A and −11377C>G, haplotypes composed of these SNPs, and the diploid haplotype combinations in the *ADIPOQ* promoter with adiponectin levels. Furthermore, we have shown that adiponectin levels play a predictive role in progression toward type 2 diabetes. Additionally, we show an association of genetic variants in the *ADIPOQ* promoter with evolution of glucose tolerance. This means that a genetic variation in the *ADIPOQ* promoter may be among the genetic determinants involved in the pathogenesis of diabetes with adiponectin levels being at least partly genetically determined. In conclusion, decreased adiponectin levels seem to predict future diabetes progression in the

Caucasian population, whereas elevated adiponectin levels may be considered as protective in diabetes.

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