

Analysis of 14 Candidate Genes for Diabetic Nephropathy on Chromosome 3q in European Populations

Strongest Evidence for Association With a Variant in the Promoter Region of the Adiponectin Gene

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Linkage studies have mapped loci for diabetic nephropathy and associated phenotypes on chromosome 3q. We studied 14 plausible candidate genes in the linkage region because of their potential role in vascular complications. In a large-scale study of patients from Denmark, Finland, and France who have type 1 diabetes, 1,057 case and 1,127 control subjects, as well as 532 trios, were investigated for association with diabetic nephropathy. We analyzed 69 haplotype-tagging single nucleotide polymorphisms and nonsynonymous variants that were identified by sequencing. Polymorphisms in three genes, glucose transporter 2 (*SLC2A2*), kininogen (*KNG1*), and adiponectin (*ADIPOQ*), showed nominal association with diabetic nephropathy in single-point analysis. The T-allele of *SLC2A2*_16459CT was associated with a decreased risk of diabetic nephropathy (odds ratio 0.79 [95% CI 0.66–0.96], $P = 0.016$), whereas the T-allele of *KNG*_7965CT and the A-allele of *ADIPOQ*_prom2GA were associated with increased risk of nephropathy (1.17 [1.03–1.32], $P = 0.016$; 1.46 [1.11–1.93], $P = 0.006$, respectively). Analyses of the transmission disequilibrium test showed similar trends only for *ADIPOQ*_prom2GA with the overtransmission of the A-allele to patients with diabetic nephropathy (1.52 [0.86–

2.66], $P = \text{NS}$) and of the G-allele to patients without diabetic nephropathy (0.50 [0.27–0.92], $P = 0.026$). The overall significance for this variant (nominal $P = 0.011$) suggests that *ADIPOQ* might be involved in the development of diabetic nephropathy. *Diabetes* 55:3166–3174, 2006

Microvascular lesions and accelerated atherosclerosis are the major causes of morbidity and early mortality in diabetic patients. Diabetic nephropathy affects ~30–40% of all diabetic patients and represents a high risk factor for cardiovascular mortality (1,2). Epidemiological and familial studies suggest that genetic factors influence the risk of developing both micro- and macrovascular complications in patients who have type 1 and type 2 diabetes (3–7). Cases of nephropathy cluster in families, and a parental history of hypertension, cardiovascular disease, metabolic syndrome, obesity, and type 2 diabetes are more common in patients with than without diabetic nephropathy (8–11).

Several linkage studies for diabetic nephropathy and associated phenotypes have been performed. A region of special interest on chromosome 3q24–3qter has been identified, and several loci for vascular risk factors and vascular complications have been mapped to this ~60-Mb interval. These include loci for diabetic nephropathy in type 1 diabetes in Caucasians (12) and loci for diabetic nephropathy in type 2 diabetes in Pima Indians (13) and in African Americans (14). Loci have also been mapped for metabolic syndrome (15), type 2 diabetes (16), BMI (17), coronary heart disease in diabetic patients (18,19), and variability of renal function among hypertensive individuals (20).

A major challenge to identifying the underlying genes is the complexity of the genetic interactions, which necessitates large samples for association studies. The EURAGE-DIC (European Rational Approach for the Genetics of Diabetic Complications) consortium has established a powerful study that includes 3,665 type 1 diabetic patients and relatives from three European populations. Our research is based on large-scale, case-control, and intra-

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LD, linkage disequilibrium; SNP, single nucleotide polymorphism; TDT, transmission disequilibrium test.

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TABLE 1
Case/control and trio studies: sample size in each population

	Denmark	Finland	France	Combined
Case-control study	775	856	553	2,184
no. of probands				
Status				
Case subjects	390	387	280	1,057
Control subjects	385	469	273	1,127
Trio study				
no. of pedigrees (multiplex)	189 (21)	120 (0)	178 (22)	487 (43)
no. of probands	213	120	199	532
Status				
Case subjects	133	61	55	248
Control subjects	80	59	144	284

familial association studies of candidate genes. We hypothesized that genes located in the 3q24-qter region of chromosome 3q, whose products are potentially involved in the pathophysiology of vascular complications, are candidate genes for diabetic nephropathy. They include genes implicated in glucose modulation (adiponectin [ADIPOQ], protein phosphatase 1, regulatory [inhibitor] subunit 2 [PPP1R2], apolipoprotein D [APOD], solute carrier family 2 [facilitated glucose transporter], member 2 [SLC2A2]), cardiovascular risk (pentraxin 3 [PTX3], tumor necrosis factor [ligand] superfamily, member 10 [TNFSF10], thrombopoietin [THPO], histidine-rich glycoprotein [HRG], interleukin 12, subunit 35 [IL12A], endothelin converting enzyme 2 [ECE2]), and other kidney diseases (angiotensin II receptor, type 1 [AGTR1], kininogen [KNG1], somatostatin [SST], and enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase [EHHADH]). We report molecular genetic studies for 14 positional candidate genes located on the ~60-Mb region of linkage on chromosome 3q.

RESEARCH DESIGN AND METHODS

Three European centers in Denmark, Finland, and France contributed a total of 3,665 subjects to the case/control and trio studies. The contribution of patients from each country is shown in Table 1.

Details for the recruitment and clinical characteristics of patients are presented elsewhere (L.T., P.-H.G., S.H., G.K., F.C., M.M., C.F., H.-H.P., D.T., A.T., M.F., I.G., D.G., R.C., F.M., M.L., N.V., unpublished observations).

Type 1 diabetes was considered present if the age at onset of diabetes was ≤ 35 years and the time to definitive insulin therapy was ≤ 1 year. Patients in the initial phase of type 1 diabetes (duration of diabetes < 5 years) were not included.

Established diabetic nephropathy (in case subjects) was defined by persistent albuminuria (> 300 mg/24 h, > 200 μ g/min, or > 200 mg/l) in two of three consecutive measurements on sterile urine and the presence of retinopathy. Patients were excluded if they had clinical or laboratory suspicion of nondiabetic renal or urinary tract disease.

Absence of diabetic nephropathy (in control subjects) was defined as persistent normoalbuminuria (urinary albumin excretion rate < 30 mg/24 h, < 20 μ g/min, or < 20 mg/l) after at least 15 years of diabetes in patients not treated with ACE inhibitors or angiotensin II receptor blockers.

Single nucleotide polymorphism discovery. Genes selected for study (position on chromosome 3q shown in Fig. 1A) were examined for polymorphisms from databases and by single nucleotide polymorphism (SNP) discovery. All exons and flanking intron sequences, 5' and 3' untranslated regions, as well as promoter regions of each gene were screened by direct sequencing of 94 DNAs pooled two-by-two, including DNAs from 20–24 case and control subjects from each participating country plus 30 healthy Caucasians.

For each gene, primers were established to amplify by PCR the DNA fragments that contained exons and the promoters. The PCRs were performed

in a 15- μ l reaction mixture that contained 25 ng DNA. Primer sequences are available from the authors on request. Sequencing reactions were performed according to the dye-terminator method using an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). Alignment of experimental results, SNP discovery, and genotyping were performed with Genalys software (21).

SNP selection for genotyping. Haplotype structure and frequencies were determined from data concerning pooled DNA using the expectation maximization algorithm (22) in each population. SNPs tagging the most frequent haplotypes (at least 5% in one population) were selected for genotyping, and in addition, all nonsynonymous variants that were detected in at least one diseased population were systematically investigated. We also examined 94 SNP genomic control markers in nongenic regions spaced throughout the genome to control for possible stratification within each population (23).

Genotyping. The study design involved two phases for genotyping DNA variants, a first-line study (case/control) and a second-line study (532 trios from 487, possibly multiplex, families with a total of 3,665 DNAs: 2,184 and 1,481, respectively).

Genomic DNA was isolated from human leukocytes using standard methods. SNP genotyping was performed at the French National Genotyping Center using automated high-throughput methods including TaqMan, Amplifluor, MALDI-MS, and SNPlex. All liquid handling was performed robotically in 384-well plates with a BasePlate Robot (The Automation Partnership, Royston, U.K.). For SNP genotyping by mass spectrometry, the GOOD assay was applied as previously described (24). TaqMan (assay-by-design) was carried out in 5- μ l volume according to manufacturer's recommendations with probes and mastermix from Applied Biosystems (Courtaboeuf, France). For Amplifluor, primers were designed using AssayArchitect (<http://www.assayarchitect.com>). Primer sequences and conditions are available on request. End point fluorescence was detected for TaqMan and Amplifluor assays using an ABI7900HT reader (Applied Biosystems), and genotypes were assigned with the SDS 2.1 software. The genomic control markers were characterized using the SNPlex genotyping technology (Applied Biosystems).

The genotyping success rate was $> 85\%$ for all markers, and among 192 replicate samples genotyped blindly, no genotype differences were found. All markers were in Hardy-Weinberg equilibrium in both case and control subjects in all populations at the 5% significance level except for one, which was not considered in the case/control comparison.

For trios, microsatellite markers (Panel 16, LMSV2; Applied Biosystems) were genotyped to verify the family relationships. Trios that exhibited genotype patterns that were incompatible with the putative family structure were excluded from the analysis.

Statistical analyses. All selected variants were tested in the first-line study, and polymorphisms that were potentially associated with disease were then tested in the trio study. To increase the power of the first-line study, a number of probands from Danish and French trios were included (177 and 138, respectively). When markers are selected to be genotyped in the second-line study, those probands are included solely in the trio analyses.

Association studies. In the first-line study, analyses were performed in each population separately. Allele frequencies were estimated by gene counting, and departure from Hardy-Weinberg equilibrium was tested using a χ^2 with 1 d.f. (degree of freedom). Logistic regression analyses were performed to assess the association of each polymorphism with the disease status. Pairwise linkage disequilibrium (LD) was estimated using THESIAS software (www.genecanvas.org) (25) and was expressed as the standardized D' coefficient. THESIAS software was also used for haplotype analysis. A global test for difference in the haplotype frequency distributions between case and control subjects was performed by means of a likelihood ratio test (χ^2 with $m-1$ d.f. in the case of m haplotypes), and haplotype effects (95% CI) were expressed as haplotypic odds ratios (ORs) under the assumption of additive effects. All analyses were performed while adjusting for sex, diabetes duration, HbA_{1c}, and smoking (L.T., P.-H.G., S.H., G.K., F.C., M.M., C.F., H.-H.P., D.T., A.T., M.F., I.G., D.G., R.C., F.M., M.L., N.V., unpublished observations). The homogeneity of allelic and haplotypic effects across populations was investigated by the Mantel-Haenszel statistic (26). In addition, the Fisher's method (27) was used to combine the P values of the global haplotypic test obtained in each population to produce an overall test of significance. Any suggestive ($P < 0.10$) association found to be homogeneous in the first-line study was tested further by performing a transmission disequilibrium test (TDT)-style analysis using the Transmit program in trios with and without diabetic nephropathy (28). Transmit implements a score test that captures the robust (with respect to admixture) information on association and linkage in nuclear families; it extends the classic TDT (29) to include information from incompletely typed and multiplex families. If results of the association (excluding overlapping Danish and French trio probands) and TDT analyses in trios with diabetic nephropathy were homogeneous, they were then combined as previously described (30) to provide an overall estimate of the allelic OR (31). If results

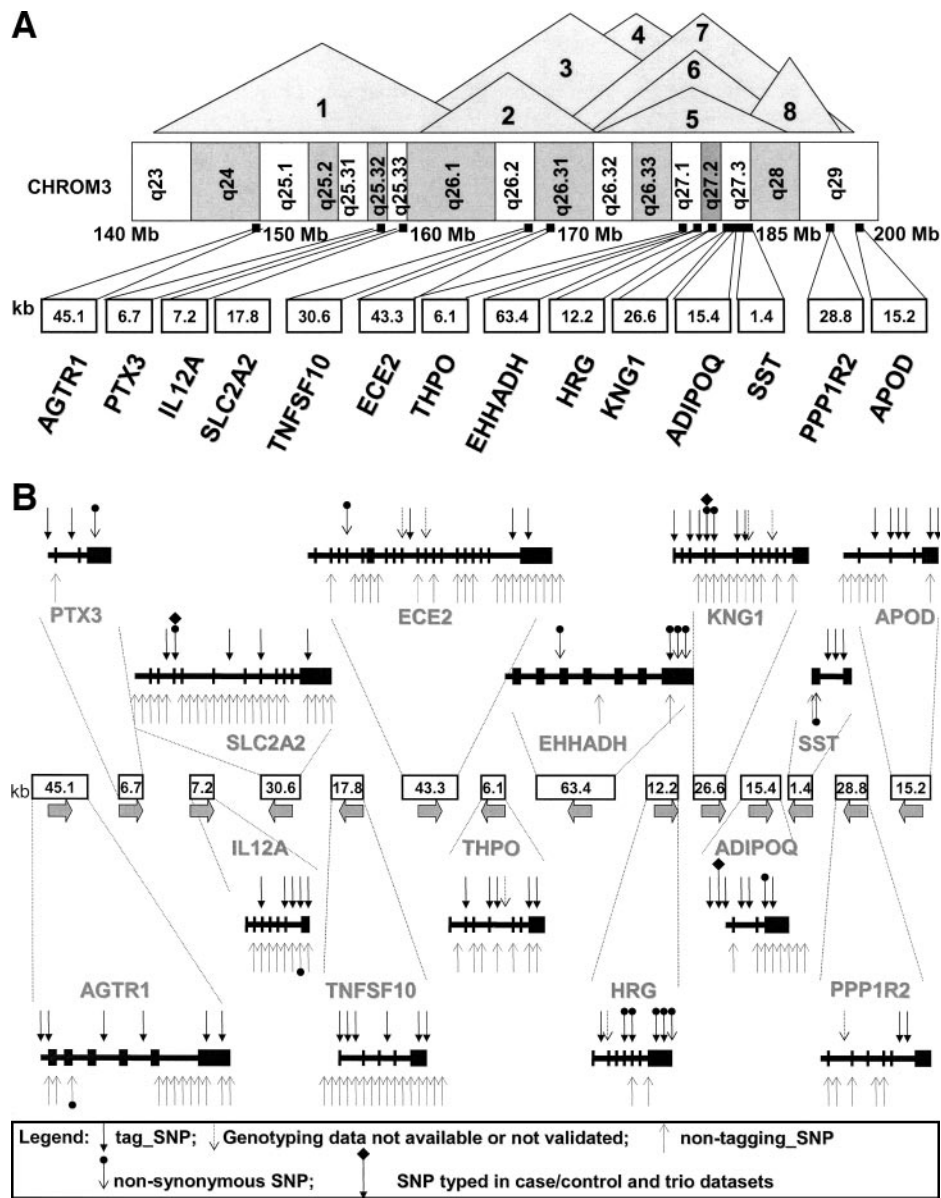


FIG. 1. A: Diagram of the chromosome 3q region. Depicted is the linkage intervals reported in eight different genome scan analyses for phenotypes related to vascular complications, as follows: 1) diabetic nephropathy in type 1 diabetes (ref. 12), 2) diabetic nephropathy in type 2 diabetes (ref. 13), 3) coronary heart disease (ref. 19), 4) obesity (ref. 17), 5) coronary heart disease in type 2 diabetic patients (ref. 18), 6) type 2 diabetes (ref. 16), 7) metabolic syndrome (ref. 15), and 8) renal function in hypertensive patients (ref. 20). The respective positions for the 14 genes selected for this study along the long arm of chromosome 3 are also shown. **B:** Structure of the 14 candidate genes and results of SNP discovery. The figure shows the respective sizes and orientations of the genes and the positions of the polymorphisms identified after sequencing of the exons and flanking regions in 188 chromosomes. Tag-SNPs and nonsynonymous variants selected for genotyping are shown above and the nontagging SNPs below the gene structure schema. The three markers that met the criteria to be further investigated in the trio dataset are indicated.

of the TDT analyses in trios without diabetic nephropathy were consistent with results of the combined analyses described above (e.g., risk allele for diabetic nephropathy is protective in trios without diabetic nephropathy), both results were then combined using the Fisher's method to obtain the overall significance of the association.

RESULTS

SNP discovery and selection. A total of 197 polymorphisms were identified, of which 59% were newly discovered at the time of study. They include 11 insertion-deletions and 186 SNPs. In addition, 33 were located in coding regions, of which 18 resulted in amino acid change. A total of 120 haplotypes with a frequency >5% in at least one population were determined. They represented 64–

100% of the haplotype diversity found in the three diseased populations and in the healthy control subjects. A total of 76 polymorphisms were selected for genotyping, including 69 haplotype-tagging SNPs (of which 8 were nonsynonymous) and 7 additional nonsynonymous variants identified in one diseased population (Fig. 1B). We were not able to obtain data for six markers because it was impossible to obtain a genotyping assay, and one marker was excluded because it showed significant departure from Hardy-Weinberg equilibrium in case and control subjects from the three populations. The 69 SNPs available for the case/control analysis were distributed across all 14 genes, and the number of markers per gene ranged from two to seven

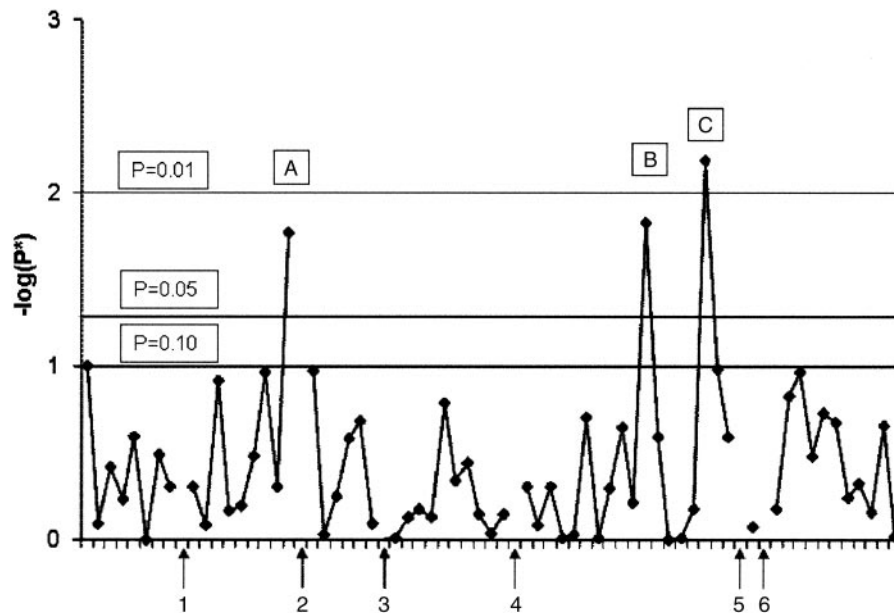


FIG. 2. Results of the univariate association analyses. **P* value of the Mantel-Haenszel statistic testing for the significance of the combined OR across the three populations. The polymorphisms have been ordered according to their relative position along the chromosome 3 (as given in online appendix Table 1). A, *SLC2A2*_16459CT; B, *KNG*_7965CT; and C, *ADIPOQ*_Prom2GA. *P* values are not given for rare polymorphisms (1, *PTX3*_5902 and 3, *ECE2*_4032) and for those showing heterogeneity across the three populations (2, *SLC2A2*_12567CT; 4, *EHHADH*_62837CA; 5, *ADIPOQ*_4096GT; and 6, *ADIPOQ*_5839).

(online appendix Table 1 [available at <http://diabetes.diabetesjournals.org>]). Allele frequencies in case and control subjects from the three populations are shown in online appendix Table 2.

The pattern of LD along chromosome 3q was similar in the three populations (data not shown). Although strong LD was restricted to SNPs within the same gene, we did observe some nearby SNP pairs from different genes that had statistically significant LD. These pairs were in the *ECE2* and *THPO* genes, which are located within a 100-kb interval, the *HRG*, *KNG1*, and *ADIPOQ* genes, which are located within a 200-kb interval, and the *PPP1R2* and *APOD* genes, which are located within a 70-kb interval.

Case/control analysis results. The statistical results from the case/control comparisons of the 69 SNP frequen-

cies are given in Fig. 2. Two nonsynonymous polymorphisms were too rare to be analyzed (allele frequency <1%), and for four polymorphisms, results were statistically heterogeneous across the three populations. Among the 63 remaining polymorphisms, 3 were found to have statistically consistent association evidence with nephropathy in all three populations according to our study criteria ($P < 0.1$) (Table 2). These were the T-allele of the *SLC2A2*_16459CT (Thr110Ile; rs5400) polymorphism associated with a decreased risk of nephropathy (OR 0.79 [95% CI 0.66–0.96], $P = 0.016$); the T-allele of the *KNG*_7695CT (Thr178Met; rs1656922) polymorphism, which showed an increased risk of nephropathy (1.17 [1.03–1.32], $P = 0.016$); and the A-allele of the *ADIPOQ*_Prom2GA (rs17300539) polymorphism associated with an increased risk of ne-

TABLE 2
Main results of the association analysis of chromosome 3 SNPs with nephropathy status

	Denmark		Finland		France		Combined*
	Control subjects	Case subjects	Control subjects	Case subjects	Control subjects	Case subjects	
<i>n</i>	463	489	469	387	391	300	
<i>SLC2A2</i> _16459CT†							
Allele frequencies	0.876/0.124	0.895/0.105	0.847/0.153	0.883/0.117	0.861/0.139	0.867/0.133	
OR (95% CI)	0.76 (0.54–1.07)		0.76 (0.56–1.03)		0.88 (0.62–1.24)		0.79 (0.66–0.96)
<i>P</i>	0.112		0.074		0.463		0.016
<i>KNG1</i> _7965CT†							
Allele frequencies	0.521/0.479	0.498/0.502	0.525/0.475	0.486/0.514	0.542/0.458	0.514/0.486	
OR (95% CI)	1.11 (0.90–1.36)		1.22 (0.99–1.50)		1.18 (0.94–1.48)		1.17 (1.03–1.32)
<i>P</i>	0.351		0.069		0.150		0.016
<i>ADIPOQ</i> _Prom2GA‡							
Allele frequencies	0.931/0.069	0.909/0.091	0.974/0.026	0.974/0.026	0.933/0.067	0.903/0.097	
OR (95% CI)	1.70 (1.13–2.55)		0.91 (0.47–1.77)		1.52 (0.97–2.38)		1.46 (1.11–1.93)
<i>P</i>	0.011		0.784		0.071		0.006

*Pooled OR according to the Mantel-Haenszel method after having checked for the homogeneity across populations: *SLC2A2*, solute carrier family 2 (facilitated glucose transporter), member 2; *KNG1*, kininogen; and *ADIPOQ*, adiponectin. Association tests were performed while adjusting for sex, diabetes duration, HbA_{1c}, and smoking, assuming either †additive or ‡dominant effects of the polymorphisms.

TABLE 3
Results of the TDT analyses in trios with and without diabetic nephropathy

	TDT in trios with diabetic nephropathy			TDT in trios without diabetic nephropathy		
	<i>n</i> *	T†	OR‡	<i>n</i> *	T†	OR‡
SLC2A2_16459CT						
Denmark	56.43	0.513	1.05 (0.63–1.78)	26.72	0.385	0.63 (0.29–1.37)
Finland	24.01	0.622	1.64 (0.72–3.77)	29.01	0.480	0.92 (0.45–1.92)
France	24.84	0.596	1.48 (0.66–3.29)	76.53	0.419	0.72 (0.46–1.14)
Test for homogeneity§			<i>P</i> = 0.605			<i>P</i> = 0.74
Pooled			1.26 (0.86–1.85)			0.74 (0.52–1.04)
KNG_7965CT						
Denmark	94.78	0.496	0.98 (0.66–1.47)	78.41	0.396	0.66 (0.42–1.03)
Finland	58.00	0.396	0.66 (0.39–1.11)	60.00	0.500	1.00 (0.60–1.66)
France	55.61	0.561	1.28 (0.75–2.17)	137.36	0.472	0.89 (0.64–1.25)
Test for homogeneity§			<i>P</i> = 0.208			<i>P</i> = 0.42
Pooled			0.95 (0.72–1.25)			0.84 (0.66–1.07)
ADIPOQ_Prom2GA						
Denmark	28.43	0.686	2.18 (0.99–4.83)	11.00	0.350	0.54 (0.16–1.88)
Finland	6.00	0.333	0.50 (0.09–2.73)	3.00	0.333	0.49 (0.04–5.45)
France	19.03	0.563	1.29 (0.52–3.18)	32.25	0.333	0.49 (0.24–1.04)
Test for homogeneity§			<i>P</i> = 0.274			<i>P</i> = 0.991
Pooled			1.52 (0.86–2.66)			0.50 (0.27–0.92) (<i>P</i> = 0.026)¶

*Number of informative transmissions (in terms of heterozygous parent transmitting to an affected child). †Proportion of transmitted allele to diseased offspring (probands with or without diabetic nephropathy) in TDT analysis. ‡OR = T/(1 - T) is an estimate of the allelic OR under the assumption of additive effect (on a logistic scale). §Test for homogeneity of the combined OR across populations according to Mantel-Haenszel statistic. ||Combined OR across populations according to Mantel-Haenszel statistic. ¶Associated *P* value.

phropathy (1.46 [1.11–1.93], *P* = 0.006). Although no heterogeneity across the populations was found for the ADIPOQ_Prom2 variant, the association was mainly observed in Denmark and France but not in Finland, where the allele frequency of the variant was rarer (twice lower) than that observed in the other two populations (Table 3). Multiple testing error was corrected using the effective number of independent tests after taking into account LD between markers (32). This number was estimated to be ~49 in our SNPs dataset, which corresponds to an experiment-wise significance level at *P* ≤ 0.001. Applying this correction, the effect of the three polymorphisms was no longer significant.

In contrast, the association for the 94 genomic control markers was compatible with expectations under the null hypothesis of no association (results not shown), which indicates that stratification within one or more of the

populations is an unlikely source of positive association results.

Analyses of haplotypes for markers within genes are summarized in Fig. 3. The most significant result was obtained with *SLC2A2* (Fisher's *P* value = 0.064 for test of association with diabetic nephropathy). Examination of the alleles carried by the *SLC2A2* haplotypes showed agreement with the patterns observed for single SNPs (online appendix Table 3). The ADIPOQ_Prom2 A-allele was carried by a unique haplotype that was associated with increased risk of nephropathy (online appendix Table 4), which is also consistent with the results of the single SNP analyses. The haplotype analyses for *EHHADH* are not shown because only one tagging SNP had been identified for this gene, and the three additional nonsynonymous polymorphisms studied were too rare (minor allele frequency <2%) to contribute to haplotype analyses.

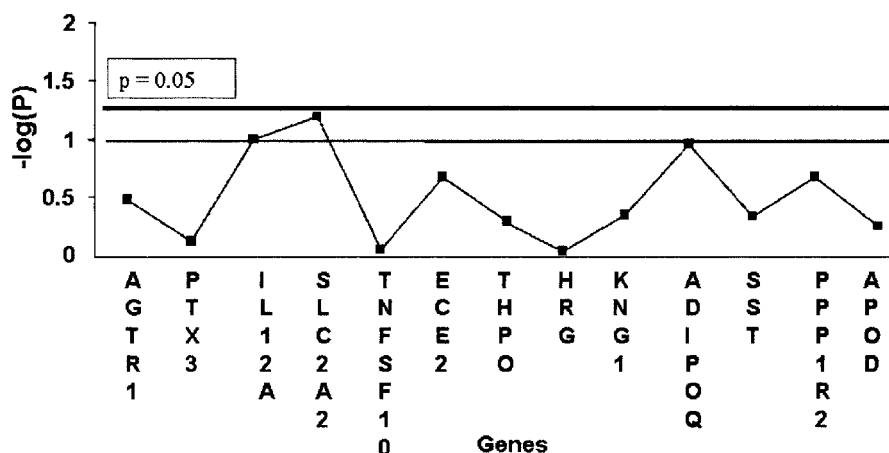


FIG. 3. Results of the haplotype analyses. Results were combined across the populations with the Fisher's method. The haplotype analysis for *EHHADH* is not shown, as only one haplotype-tagging SNP had been genotyped for this gene.

TDT analyses. Three SNPs met our criteria for being further investigated using trios analysis: SLC2A2_16459CT, KNG_7965CT, and ADIPOQ_Prom2GA. Results of TDT analyses for these markers in trios are reported in Table 3. Although the TDT results for diabetic nephropathy in the combined dataset across the three populations did not reach significance at the 0.05 level, they were not statistically different from the case/control results for KNG_7965CT and ADIPOQ_Prom2GA (test of homogeneity: $P = 0.26$ and $P = 0.46$, respectively). When case/control and trio data were combined, the association of KNG_7965CT polymorphism with nephropathy failed to reach significance (1.09 [95% CI 0.98–1.22], $P = 0.098$), but the result for ADIPOQ_prom2GA suggested a marginal association between the A-allele and nephropathy (1.25 [0.99–1.58], $P = 0.06$). The latter was reinforced by TDT analyses in trios without nephropathy, where a consistent pattern of undertransmission of the A-allele to probands without nephropathy was found in all three populations (0.50 [0.27–0.92], $P = 0.026$). A combined analysis of the case/control and the two trio panels for this marker showed a significant association overall ($P = 0.011$). In contrast, for SLC2A2_16459CT, the results of case/control (0.79 [0.67–0.95]) and TDT (1.26 [0.86–1.85], $P = \text{NS}$) analyses were statistically heterogeneous ($P = 0.029$), preventing us from combining both analyses. However, the TDT (transmission to diabetic nephropathy) was not significant ($P > 0.05$), implying that the case/control result has not been replicated.

DISCUSSION

Linkage of diabetic nephropathy or traits associated with vascular complications to a region on chromosome 3q has been repeatedly replicated (12–20). Among the ~250 genes located in the region of interest, we selected 14 for study because of their plausible involvement in the pathogenesis of vascular complications, and we investigated their role in the genetic susceptibility to diabetic nephropathy. Among these, the adiponectin gene encodes a cytokine synthesized in the adipose tissue with substantial anti-inflammatory properties and is a major modulator of insulin resistance and dyslipidemia. We found nominal evidence for association between one polymorphism in the promoter region of the adiponectin gene, rs17300539 (ADIPOQ_prom2/rs17300539 G>A), and diabetic nephropathy. The A-allele increased the risk of diabetic nephropathy, and conversely the G-allele was protective against diabetic nephropathy. To our knowledge, this is the first report on the genetic implication of the adiponectin gene with respect to microangiopathy in type 1 diabetes.

Molecular strategy. We systematically resequenced all informative intragenic sequences (exons and flanking intron sequences, 5' flanking regions) in healthy Caucasian individuals (to provide a catalog of common SNPs), as well as in case and control subjects from each of the three populations investigated, in order to identify any potentially causative or protective marker. Haplotype structure and frequencies in each population were then inferred from the sequencing data to allow selection of the nonredundant SNPs to obtain a cost-effective genotyping strategy. The number of SNPs and haplotypes per gene we observed is in accordance with the figures described in a recent survey that resequenced 100 genes in their entirety (33). The fact that we performed SNP discovery in 64 additional diseased individuals has dramatically increased

the haplotype diversity and therefore the number of haplotype-tagging SNPs (120 haplotypes [8.6/gene] and 68 htSNPs [4.8/gene]) as compared with that observed in the 30 healthy Caucasians only (60 haplotypes [4.3/gene] and 40 htSNPs [2.8/gene]). On average, 85% of the chromosomes were “tagged” with the SNPs selected (range 64–100%). Yet, we have to acknowledge the limitations of our large-scale genotyping strategy because some of the haplotype-tagging SNPs could not be typed using different high-throughput technologies. Therefore, it is possible that assessment of 5 of the 14 genes studied as candidates for diabetic nephropathy was not exhaustive. However, because the utility of haplotype-tagging SNP strategy and the use of common SNPs in mapping complex diseases are still largely debated (34–36), in an attempt to more exhaustively investigate the variability of each gene, nonsynonymous markers were also studied, as they might have a functional role.

Candidate gene results. We found the strongest evidence for association between one polymorphism in the promoter region of the gene coding for adiponectin (ADIPOQ_prom2GA) and nephropathy: the A-allele increasing the risk for nephropathy and conversely the G-allele is protecting against nephropathy. It should be noted that when the populations are examined individually, the association was significant in Denmark (1.70 [95% CI 1.13–2.55], $P = 0.01$) and marginal in France (1.52 [0.97–2.38], $P = 0.07$) but was not significant in Finland (0.91 [0.47–1.77], $P = 0.784$), probably because the frequency of the A-allele is much lower in this population. Similar differences in allele frequencies of ADIPOQ genetic variants across populations have already been reported (37). Despite the difference in trend in Finland, the results for the case/control analyses are not statistically heterogeneous across the three populations, validating the calculation of the combined OR and the test of association using the Mantel-Haenszel statistic (1.46 [1.11–1.93], $P = 0.006$). When we applied a correction for multiple testing, this result was no longer significant. Nevertheless, the fact that the association in the case/control analysis was consistent in two of three populations and was independently replicated in the trio analyses (although the trio dataset was less powered than the case/control) strongly suggests that this adiponectin variant is associated with diabetic nephropathy at least in the Danish and French populations.

Haplotypes of the ADIPOQ gene have previously been reported to be associated with the risk of type 2 diabetes and with reduced adiponectin levels in type 2 diabetic patients (37). Interestingly, the ADIPOQ_prom2 variant has been found to be associated with higher adiponectin levels both in healthy individuals and type 2 diabetic patients in an earlier study, where this marker is labeled SNP –11391G/A (38). We found that several other ADIPOQ alleles that were also reported to be associated with adiponectin levels in this report are found on the single haplotype bearing the ADIPOQ_prom2 A-allele in our populations. This haplotype is characterized by the presence of the C-allele of ADIPOQ_prom3CG (rs266729/–11377), the T-allele of the ADIPOQ_4096GT (rs1501299/+276), and the delA-allele of the ADIPOQ_5839AX (+2019 delA), all of which were associated with higher adiponectin levels in reports by Vasseur and colleagues (37,38). Low adiponectin levels are associated with type 2 diabetes, obesity, insulin resistance, and macrovascular complications (39). Features known as risk factors for

microvascular complications and genetic associations between these risk factors and adiponectin variants have been described (40).

In contrast, in type 1 diabetic patients, adiponectin levels are higher than in healthy subjects. We recently reported that in the Danish, Finnish, and French populations, in type 1 diabetes, increased adiponectin levels are associated with microangiopathy in cross-sectional and prospective studies (41–43). The relationship between diabetic nephropathy and adiponectin concentration is not yet fully understood, but our data suggest that genetic variation in the promoter region of the *ADIPOQ* gene may contribute to an increased risk of developing nephropathy partly through the increase in adiponectin levels. The recent characterization of the *ADIPOQ* promoter region has shown that the promoter region from –676 to +41 relative to the transcription site was sufficient for basal promoter activity (44,45). *ADIPOQ*_prom2GA variant is located upstream of this region (position –1003), and the biological role of this particular genetic variant in the expression of the adiponectin gene has not been documented. It is also possible that this variant is not the functional variant associated with the susceptibility to diabetic nephropathy but that it is in LD with another variant not yet identified. Our molecular screening did not encompass the intronic regions of the gene, and recent studies have shown that DNA encoding the first intron of the human adiponectin gene contains an intronic enhancer that regulates adiponectin gene expression in an adipose tissue-specific manner (46). This could also explain why the association was not found in the Finnish population.

Our study strategy was based on candidate genes and was informed by linkage studies. We selected 14 plausible genes for vascular complications that were more or less evenly distributed on the 40- to 60-Mb terminal end of the long arm of chromosome 3q. Although the *ADIPOQ* gene is located ~40 Mb distal from the original linkage peak for diabetic nephropathy in type 1 diabetes (12), it is a positional candidate gene because of the large variation of locations found in linkage studies of complex traits. However, based on our data, the role of *ADIPOQ* in susceptibility to diabetic nephropathy appears to be rather modest, with a risk attributable to 3% of the associated variant. We therefore cannot exclude that another susceptibility gene for diabetic nephropathy could be located in this region. Under the linkage peak for diabetic nephropathy, *AGTR1* was the most plausible candidate gene, but we failed to find any association between the gene variants and diabetic nephropathy in our study, thus confirming previous reports (47–49). However, our screening did not include the 4-Mb region located upstream of the *AGTR1* gene, where Chistiakov et al. (50) recently reported association with diabetic nephropathy in Russian type 1 diabetic patients.

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