A G/T substitution in intron-1 of UNC13B gene is associated with increased risk of nephropathy in patients with type 1 diabetes.

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ABSTRACT

Objective: Genetic and environmental factors modulate the susceptibility to diabetic nephropathy (DN), either as initiating and/or progression factors. The objective of the EURAGEDIC study is to identify DN susceptibility genes. We report molecular genetic studies for 127 candidate genes for DN.

Research design and methods: Polymorphisms were identified through sequencing of promoter, exon and flanking intron gene regions and database search. Three-hundred-and-forty-four non-redundant SNPs and non-synonymous variants were tested for association with diabetic nephropathy (persistent albuminuria >300 mg/24h) in a large type 1 diabetes case/control (1176/1323) study from 3 European populations.

Results: Only one SNP, rs2281999 located in UNC13B gene, was significantly associated with DN after correction for multiple testing. Analyses of 21 additional markers fully characterizing the haplotypic variability of the UNC13B gene showed consistent association with SNP rs13293564 (G/T) located in intron 1 of the gene with DN in the 3 populations. The odds ratio (OR) for DN associated with the TT genotype was 1.68 [95%CI, 1.29 – 2.19], p = 1.0 10^{-4}). This association was replicated in an independent population of 412 cases and 614 controls, (combined OR of 1.63 [95%CI, 1.30 – 2.05], p = 2.3 10^{-5}).

Conclusions: We identified a polymorphism in UNC13B gene associated with DN. UNC13B mediates apoptosis in glomerular cells in the presence of hyperglycemia, an event occurring early in the development of DN. We propose that this polymorphism could be a marker for the initiation of DN. However, further studies are needed to clarify the role of UNC13B in DN.

Keywords: UNC13B, diabetic nephropathy, association, linkage disequilibrium, apoptosis, podocyte
Diabetic nephropathy (DN), characterised by persistent albuminuria, a relentless decline in glomerular filtration rate and raised arterial blood pressure, affects approximately one third of patients with diabetes (1). DN accounts for 40% of end-stage renal disease and is associated with high cardiovascular morbidity and mortality (2). Epidemiological and familial studies suggest that genetic factors influence the risk of diabetic nephropathy in both Type 1 and Type 2 diabetic patients (3-6). Despite rapid research progress, robust predictors of this complication are still lacking.

Phenotypic characterization of DN is more accurate in patients with type 1 diabetes than with type 2 diabetes where the kidney failure may often be caused by non-diabetic factors, mainly hypertension. Using a concerted effort including 2499 patients with type 1 diabetes from the Danish, Finnish and French populations, the EURAGEDIC consortium (European Rational Approach for the Genetics of Diabetic Complications) has established a large study for association with diabetic nephropathy (DN) that includes 1176 cases and 1323 controls (7). Single nucleotide polymorphisms (SNPs) located in 127 candidate genes selected through assessment of linkage studies, knowledge of metabolic pathways and animal models were sought for association with DN.

RESEARCH DESIGN AND METHODS

Patient populations Three European centers, from Denmark, Finland and France, contributed to the case/control study with a total of 2499 subjects with type 1 diabetes. Details for the recruitment of patients have previously been presented (7) and clinical characteristics of the patients are shown in Online Supplementary Table 1. Type 1 diabetes was considered present if the age at onset of diabetes was ≤ 35 years and the time to definitive insulin therapy ≤ 1 year. Patients in the initial phase of type 1 diabetes, that is, duration of diabetes less than 5 years, were not included. Established diabetic nephropathy (cases) was defined by persistent albuminuria (≥300 mg/24hours or ≥200 µg/min or ≥200 mg/l) in two out of three consecutive measurements on sterile urine. Patients with clinical or laboratory suspicion of non-diabetic renal or urinary tract disease were excluded. Absence of diabetic nephropathy (controls) was defined as persistent normoalbuminuria (urinary albumin excretion rate: <30 mg/24hours or <20 µg/min or <20 mg/l) after at least 15 years of diabetes duration in patients not treated with angiotensin converting enzyme inhibitors or angiotensin II receptor blockers.

Accordingly, for the initial study Denmark contributed 952 patients with type 1 diabetes including 489 cases and 463 controls for diabetic nephropathy, Finland 856 patients including 387 cases and 469 controls and France 691 patients including 300 cases and 391 controls adding up to a total of 2499 patients including 1176 cases and 1323 controls for DN.

Two independent datasets were used for replication, the first one being an additional case/control group from the FinnDiane study (8) including 412 cases and 614 controls that matched the criteria used in the initial study. The second set consisted of 674 patients with type 1 diabetes and microalbuminuria (urinary albumin excretion rate: [30-300] mg/24hours or [20-200] µg/min or [20-200] mg/l) from the Danish (n=60), Finnish (n=421) and French (n=193) populations. Clinical characteristics for the replication datasets are presented in Online Supplementary table 2.

Molecular screening and SNP selection The study is a systematic investigation of 127 candidate genes selected through studies of susceptibility loci from linkage studies, metabolic pathways known to be affected in
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A detailed list of genes and molecular analyses has been described elsewhere (7, 9). Single nucleotide polymorphisms (SNPs) in the genes selected for the study were identified through database searches and by direct SNP discovery. One hundred nineteen genes were screened by sequencing all exons, flanking intron sequences, 5’ and 3’ untranslated regions as well as promoter regions in at least 32 DNA samples. The sample consisted of healthy French Caucasian subjects from the Epidemiological study on the Genetics and Environment of Asthma (EGEA) (10). The sample size allowed us to detect SNPs with a minor allele frequency (MAF) of at least 5% with a probability of 96%. For 33 genes that were not initially included in the French National Genotyping Centre (CNG)’s re-sequencing effort of more than 15,000 human genes (http://www.cng.fr/en/teams/geneident/index.html), the screening was performed in 64 additional DNA samples from patients with type 1 diabetes. These included 24, 20 and 20 patients from Denmark, Finland and France, respectively, half of them (n=32) with DN and the other half (n=32) without. For sequencing, DNA samples of two individuals from the same population and with the same phenotype were pooled together. Accordingly, the screening was performed in 16 DNA pools for 86 genes and in 48 (16 + 32) DNA pools for 33 genes. For each gene, primers were defined for PCR amplification of the exon- and the promoters regions. PCR was performed in a 15 µl reaction mixture containing 25 ng of pooled genomic 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, USA). Alignment of experimental results, SNP detection and genotype calling were performed using the Genalys software (11) that allows for genotype calls obtained from pooled DNA.

For each gene, the haplotype structure and frequencies were determined from the genotypic data obtained from the control group and population groups using expectation maximization (EM) algorithm (12). Three hundred fifty variants were selected to account for all estimated haplotypes with frequencies > 5%. These tagSNPs represented a median genetic variation (haplotype diversity) by gene of 87% (range: 64-100%). All were retained for further genotyping in the case/control study. In addition, all non-synonymous variants that were detected in at least one diseased population were systematically investigated (n=19).

For two genes (\textit{RELA, TGFBR1}) for which no polymorphisms were identified, SNPs were selected using the SNPBrowser™ software v.2 (Applera Corporation). For 8 additional genes (\textit{CCR5, CNDP1, HNF4A, LTA, PON2, GCGR, INPPL1, PLA2G7}), polymorphisms were selected according to reported associations with phenotypes relevant for diabetic nephropathy (13-20). We also examined 94 SNP markers (genomic control markers) in non-genic regions spaced throughout the genome to control for possible stratification within each population (21, 22). Twenty-one additional SNPs in the \textit{UNC13B} gene (GenelD#10497; full name unc-13 homolog B (C. elegans)) were genotyped following the initial positive association results from the first step. These additional SNPs were selected from the Hapmap project (http://www.hapmap.org) so that more than 95% of the \textit{UNC13B} haplotypic variability was characterized.

\textbf{Genotyping}. Genomic DNA was isolated from human leukocytes using standard methods. SNP genotyping was performed at the French National Genotyping Center (CNG) using automated high throughput
methods including TaqMan, Amplifluor, MALDI-MS and SNPlex methods. All liquid handling was performed robotically in 384 well plates with a BasePlate Robot (The Automation Partnership, Royston, UK). For SNP genotyping by mass spectrometry the GOOD assay was applied as previously described (23). TaqMan (assay-by-design) was carried out in 5µl volume according to manufacturer’s recommendations with probes and mastermix from Applied Biosystems. For Amplifluor, primers were designed using “AssayArchitect” (http://www.assayarchitect.com). Primer sequences and conditions are available on request. Endpoint fluorescence was detected for TaqMan and Amplifluor assays using an ABI7900HT reader (Applied Biosystems, Courtaboeuf, France) and genotypes were assigned with the SDS 2.1 software. Genotyping with the SNPlex platform was performed according to manufacturer’s recommendations (Applied Biosystems, Courtaboeuf, France).

The genotyping success rate was >85% for all markers (< 90% for 3% of the markers, between 90 and 95% for 17% and > 95% for 80% of the markers), and among 192 replicate samples genotyped blindly no genotype differences were found. Hardy-Weinberg (HW) equilibrium was checked in cases and in controls in all populations and markers showing deviation from HWE at the 0.001 significance level were not considered in the case/control comparison.

**Statistical analysis.** Allele frequencies were estimated by gene counting and deviation from HW equilibrium was tested by use of a \(\chi^2\) with 1 degree of freedom. Difference in allele frequencies between cases and controls were tested by a \(\chi^2\) test with 1 degree of freedom separately in each population and associated p-values were combined across populations using the Fisher's method (24) to produce an overall test of significance. Adjustment for multiple testing was carried out by correcting for the effective number of independent tests (25) in order to take into account the linkage disequilibrium (LD) between SNPs. Logistic regression analyses were performed to estimate genetic ORs, adjusted for age, smoking, diabetes duration and HbA1c. LD matrices were obtained using the Haploview software (26) and haplotype association analyses were carried out using the THESIAS software (27). Homogeneity of ORs across populations was investigated using the Mantel-Haenszel statistic (28).

**Expression studies**

**Cell culture.** Cell lines HepG2, MDCK I and II, MCF7, Cos7, HeLa, EAHy-926, SaOs-2, U2Os, SHSY5Y, and rat smooth muscle cells (rSMC) were maintained in Dulbecco’s Modified Eagle Medium (Sigma, Geissendorf, Germany) with 10% conditioned Fetal Calf Serum (PAA, Cölbe, Germany), penicillin (100 U/mL), streptomycin (100 ng/mL) and L-Glutamin (2 mM/mL). HEK293T cells received iron supplemented FCS (Cell Concepts, Umkirch, Germany). Suspension cell lines THP1, U937, K562, HL60, and RAW264.7 were maintained in RPMI-1640 medium (Sigma) with the same additions plus 1xMEM minimal amino acids (PAA). Differentiation of THP-1 monocytes into macrophages induced by stimulation with 10^{-8}M Phorbol 12-myristate 13-acetate (PMA), differentiation of SaOs-2 osteosarcoma cells was induced by stimulation with 100 mM glycerol-1-phosphate and 10 mM ascorbic acid.

**Isolation of total RNA and generation of cDNA.** Total RNA from cells was isolated from 10^6 cells each with the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. RNA from human brain was extracted from the left frontal cortex of a 75 year old male patient less than 24 hours post mortem, testis RNA was isolated one hour after surgical operation from a 62 year old patient who underwent orchidectomy for prostate cancer as described (29). RNA yield was controlled by TBE/agarose gel electrophoresis and adjusted
nanophotometrically. For first strand cDNA synthesis 5 µg total RNA were used (Fermentas, St. Leon-Rot, Germany). Efficiency was routinely controlled by diagnostic PCR for ribosomal protein RP27. Podocyte cDNA was generated from an immortalized human podocyte cell line (30).

**Diagnostic PCR.** Exon-spanning primers for nested diagnostic PCR were designed from **UNC13B** sequence NM_006377. (sense primers S1: GTGCACACTCTCATAACTT; S2: CAACCTACTGCTATGAGTGT; antisense primers A1: TGTGCAAGTCA GCAAAACTAAG, A2: AAGCCAAGGACAAAACAGGATC. PCR was conducted with GoTaq DNA-Polymerase (Promega) and 35 cycles of amplification. Integrity of the cDNA was controlled by diagnostic PCR for Ribosomal Protein 27 (rp27; sense-primer: 5’-CCAGGATAAGGAAGGAATTCCTCG-3’, antisense primer: 5’-CCAGCACCACATTCACTCAGAAGG-3’, not shown).

**In silico analyses.** For the prediction of putative transcription factor binding sites, a sequence of 25 bases to either side of the SNP was submitted for each SNP individually to a net-based search tool (Alibaba2.1, Transfac7.0; http://www.gene-regulation.com). Settings for core and pair similarities, matrix conservation, and factor class levels were adjusted according to factors predicted. Five polymorphisms, rs10081672, rs10972356, rs13288912, rs12377498 and rs10972333 were in complete association with rs13293564 located in intron 1 of the **UNC13B** gene associated with DN. They were detected on the NCBI B35 (http://www.ncbi.nlm.nih.gov/) at the respective nucleotide positions 35145908, 35143435, 35143123, 35140841 and 35126146, the beginning of the 5’-UTR within exon 1 residing at nucleotide position 35151989. This start site was confirmed in all reference sequences in UCSC genome browser, with no indication of alternative upstream exons or presence of alternative promoters. Hence, the variants rs10081672, rs10972356, rs13288912, rs12377498 and rs10972333 are located respectively -6081, -8554, -8866, -11148, and -25843 bp upstream of the transcription start site of the human **UNC13B** gene. Sequence homology scans and chromosomal neighbourhood analyses were performed using the UCSC genome browser (http://genome.ucsc.edu) covering chromosomal region 9:35,101,909-35,160,332. Special emphasis was put on placental mammal conserved elements in a 28-way multiz alignment. Results were cross-checked using the rVista 2.0 software (http://rvista.dcode.org/). There was no noticeable sequence conservation this far upstream of **UNC13B** exon 1 in either species.

**RESULTS**

**SNP discovery, selection and genotyping.** One hundred nineteen genes were re-sequenced and 1833 sequence variants were detected, including 1673 SNPs and 160 insertion/deletion polymorphisms. Seven hundred and seventy three (42.2%) of these variants were not present in the dbSNP (build 126), and therefore represent novel polymorphisms. All data have been cataloged in the dbSNP database and are available online at http://genecanvas.ecgene.net. They were located in the 5’flanking region (53), 5’UT (31), intron (1166), non synonymous coding (139), synonymous coding (182), slice site (1), 3’UT (221) and the 3’flanking region (40). The proportion of SNPs detected in exons was not different between the 773 newly discovered polymorphisms compared with the 1060 variants in dbSNP build 126 (32.4 versus 30.3%, chi-squared test: P = 0.35). As expected, newly discovered SNPs were mainly rare, 66.1% with MAF < 5% compared to 12.3% of SNPs in dbSNP (P <
The same held true for insertions/deletions (17.2% new versus 2.5% in dbSNP, P < 10^{-4}). A total of 532 haplotypes with a frequency > 5% in at least one population were determined. For these 119 genes, a total of 369 polymorphisms, including haplotype-tagging SNPs and non synonymous variants were selected for genotyping. For two genes with no variant identified through re-sequencing (RELA, TGFBR1) 4 SNPs were selected with SNPBrowser\textsuperscript{TM}. In addition, 15 SNPs were selected in 8 genes from previously reported associations with phenotypes relevant for diabetic nephropathy. We were not able to obtain data for 28 markers due to the impossibility of obtaining a genotyping assay, and 16 markers were excluded because they showed significant deviation from HW in cases and controls from the three populations.

**Association studies.** A total of 344 SNPs were investigated for association with DN in the EURAGEDIC study. Allele frequencies in case and control groups from three populations are shown in Supplementary Table 3 (Online Appendix). Nominally significant association across the three populations (p<0.05) was observed for 33 SNPs out of 344 with p-values ranging from p = 1.79 \times 10^{-5} to p = 0.050 (Supplementary Table 3 Online Appendix). Out of the 15 polymorphisms in the 8 genes selected from the literature, only one, rs1799987 located in the CCR5 gene showed nominal significant association across the three populations (p=0.025). However, this association did not remain significant after correction for multiple testing. For the 119 remaining genes, the number of independent tests was estimated to be $N_{\text{eff}} = 317$, with a corresponding significance threshold of p= 1.58 \times 10^{-4} (= 0.05/317). Only one SNP, rs2281999 located in UNC13B, remained significantly associated with DN (p=1.79 \times 10^{-5}) after correction. This association was mainly observed in the Finnish sample with still a trend in the Danish but not in the French samples (Supplementary Table 3 Online Appendix, Table 1). Another UNC13B SNP (rs661712) also showed nominal evidence for association with DN (p= 4 \times 10^{-4}) but did not remain significant after correction for multiple-testing. The association for the 94 genomic control markers was compatible with expectations under the null hypothesis of no association indicating that stratification within one or more of the populations is an unlikely source of positive association results. Furthermore, correction of the association for the two significant eigenvectors identified by performing principal components analysis (using Eigenstrat) on the genomic control markers had no effect on the results.

Our initial sequencing of the 39 exons of UNC13B has identified a total of 13 SNPs that could be tagged by 4 SNPs. These 4 SNPs, together with a non synonymous variant located in exon 28 (R1124Q) were genotyped in the whole EURAGEDIC sample. However, analysis of the available HapMap data revealed that these 4 SNPs were not sufficient to correctly characterize the haplotypic variability of the gene which spans over ~240kb on chromosome 9p12-p11. Therefore, 21 additional tagging SNPs spanning the whole gene were further genotyped in order to clarify the observed association of UNC13B SNPs with DN (Figure 1). The results of all UNC13B SNPs (apart from two rare variants shown in Supplementary Table S3) with DN are summarized in Table 1. While most of the SNPs were associated with DN in the whole study, none showed significant allelic association in the three populations and only one, rs13293564, showed nominal allelic association in two populations, Denmark and Finland. In these two populations, homozygous carriers of the T allele were more frequent in cases than in controls (0.18
association of UNC13B with diabetic nephropathy

vs 0.12 and 0.25 vs 0.17, respectively), and were then at higher risk of DN (OR=1.60 [1.10 – 2.32], p = 0.013; OR= 1.57 [1.11 – 2.22], p = 0.011; respectively) (Supplementary Table S4; Online Appendix). Interestingly, French homozygous carriers of this allele also tended to be more frequent in cases than in controls (0.18 vs 0.15) but the association failed to reach nominal significance (OR= 1.26 [0.83 – 1.92], p = 0.278). These 3 ORs were not statistically different from each other (p = 0.663) and were therefore combined, leading to an increased risk of DN associated with the TT genotype of 1.49 [1.20 – 1.85] (p = 0.0003). Further adjustment for smoking and HbA1c did not modify these associations (Supplementary Table S4).

One feature of the French patients with diabetes is that 76% of them had proliferative retinopathy while this percentage was 49% and 58% in Denmark and Finland, respectively (7, Supplementary Table S1). Further adjustment for retinopathy status strengthened the observed association, in particular in France where the OR associated with the TT genotype was then similar to that observed in Denmark (Fig. 2), leading to a common OR for DN associated with the TT genotype of 1.68 [1.29 – 2.19] (p = 0.0001). This was explained by the slightly more pronounced difference in TT genotype frequencies between cases and controls observed in patients without proliferative retinopathy (0.25 vs 0.15) than in patients with (0.19 vs 0.13) (Supplementary Table S5; Online Appendix). No heterogeneity was however detected according to the retinopathy status (p = 0.48).

A two-locus association analysis (Table 2) on the rs2281999 and rs13293564 SNPs showed that the difference in the genotype distribution between cases and controls mainly came from the rs13293564-TT genotype, suggesting that the initial association observed between the rs2281999 and DN was due to its LD with the rs13293564. All further LD, multi-locus and haplotype analyses converge to a unique recessive effect of the rs13293564 polymorphism (Supplementary Tables S6-S8, Supplementary Fig.1; Online Appendix), homogeneously in men and women (data not shown), and across the 3 EURAGEDIC populations.

The rs13293564 SNP was further investigated in an independent Finnish sample from the FinnDiane study (8) including 412 cases and 614 controls for DN. In this population, the TT genotype was also associated with an increased risk of DN, OR=1.45 [1.06 – 1.98] (p = 0.020) that was hardly modified by further adjustment for smoking, HbA1c and proliferative retinopathy (OR=1.51 [0.97 – 2.36]; p = 0.070). Finally, in the combined sample from EURAGEDIC and FinnDiane studies, the adjusted OR for DN associated with the TT genotype was 1.63 [1.30 – 2.05] (p = 2.3 10^{-5}) (Fig. 2).

The rs13293564 SNP was further investigated in 674 patients with type 1 diabetes and microalbuminuria from the three populations. The frequency of the TT genotype in patients with microalbuminuria was significantly higher than the frequency of this genotype in patients with normoalbuminuria (0.22 vs 0.15, p <10^{-4}) (Supplementary Table S9; Online Appendix). The frequency of the TT genotype was similar whatever the stage of diabetic nephropathy, incipient nephropathy (microalbuminuria) (0.22), macroalbuminuria (0.21) or end-stage renal disease (ESRD) (0.23) (Supplementary Table S9; Online Appendix).

Assuming a minor allele frequency of 0.39 at the rs13293564 locus in patients with type 1 diabetes and an increased risk of 1.6 in homozygous carriers of the T allele, the population attributable risk for rs13293564 would be 8.3%.

Expression studies
association of *UNC13B* with diabetic nephropathy

**Diagnostic PCR for *UNC13B* transcripts in various cell lines and tissues (Supplementary Fig2; Online Appendix).** Strongest expression of *UNC13B* was detectable in human tissues from brain, testis, and podocytes, as well as the human immortalized podocyte cell line SHSy Kidney cell lines COS7, and to a minor extent MDCK I and II express *UNC13B*, but not embryonic kidney cell line HEK293T. Osteosarcoma cell lines (SaOs2, U2Os), Liver (HepG2) and breast cancer (MCF7) show noticeable expression whereas in monocyctic cell lines, either differentiated or not, expression is strictly cell line dependent. Choriocarcinoma cells HeLa do not express *UNC13B*.

**In silico analyses**

There was no feature to suggest that rs13293564 located in intron 1 of the *UNC13B* is the functional variant. Analyses of the 5 polymorphisms in complete association with rs13293564 (rs10081672, rs10972356, rs13288912, rs12377498 and rs10972333) located in the putative promoter region showed that the rs10081672 and rs10972333 SNPs, located respectively at positions -6081 bp and -25843 bp upstream of the transcription start site of the human *UNC13B* gene, affect potential Sp1 and USF binding sites respectively, suggesting that those SNPs could be the functional variants. It is possible that regulatory elements are located this far upstream of the proximal regulatory regions within the core promoter. Functional molecular analyses are needed to clarify their impact. It should also be stressed that an expressed repetitive element NM_001039797 of 3.032 kbp is located within *UNC13B* intron 1, 20 kb downstream of rs13293564, the function of which is unknown and strong LD spans over this region. This opens up the possibility that genetic variation affects a regulatory element for *UNC13B* within the intronic region. Such mechanisms have recently been shown to be involved in susceptibility to different diseases (32-34).

The human *UNC13B* gene product (also called Hmunc13) has been cloned from human kidney library (35) and is homologous to rat munc13 proteins, which are members of the protein kinase C (PKC) superfamily that lack a kinase domain. Human UNC13B has one C1 diacylglycerol (DAG) and three C2 (Ca++)-binding domains. It is both upregulated and activated in the presence of hyperglycemia in renal cortical tubular cells and in glomerular mesangial cells (36). Using reverse transcription PCR to further assess the expression of *UNC13B* in human tissues, we found that *UNC13B* was also highly expressed in podocytes (Supplemental Figure 2). It has been shown that DAG activation of *UNC13B*-expressing cells induces apoptosis. As hyperglycemia increases intracellular DAG levels and is associated with apoptosis in various tissues including human kidney, it only 5 SNPs (rs10081672, rs10972356, rs13288912, rs12377498 and rs10972333) located in the putative promoter region. While there is no feature to suggest that rs13293564 is the functional variant, the rs10081672 and rs10972333, located respectively at positions -6081 bp and -25843 bp upstream of the transcription start site of the human *UNC13B* gene, affect potential Sp1 and USF binding sites respectively, suggesting that those SNPs could be the functional variants. It is possible that regulatory elements are located this far upstream of the proximal regulatory regions within the core promoter. Functional molecular analyses are needed to clarify their impact. It should also be stressed that an expressed repetitive element NM_001039797 of 3.032 kbp is located within *UNC13B* intron 1, 20 kb downstream of rs13293564, the function of which is unknown and strong LD spans over this region. This opens up the possibility that genetic variation affects a regulatory element for *UNC13B* within the intronic region. Such mechanisms have recently been shown to be involved in susceptibility to different diseases (32-34).

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DISCUSSION

We have shown for the first time that a common variation in the *UNC13B* gene was reproducibly associated with DN. A recent study that assessed 115 candidate genes for DN in 82 trios did not find significant results for any of the 6 *UNC13B* markers analyzed (31). This might be explained by the insufficient number of markers studied and a study of limited power. The rs13293564 lies within the first intron of the *UNC13B* gene and is in strong LD with many other SNPs all along the gene (see Supplementary data and HapMap data). However, it is in complete association with
association of UNC13B with diabetic nephropathy

is plausible that UNC13B plays a role in mediating renal complications of diabetes (36). Apoptosis of glomerular cells occurs quite early in the natural history of diabetic nephropathy and it is now recognized that it could be an inciting event rather than a late consequence caused by increasing proteinuria (37-41). Interestingly, TT carriers of UNC13B_rs13293564 tended to be at slightly higher risk of DN if they had not yet developed proliferative retinopathy, a clinical control for longstanding diabetes, emphasizing the role of UNC13B at the early stage of the disease. This is further strengthened by our observation that the frequency of the UNC13B_rs13293564 TT genotype in patients with incipient diabetic nephropathy, an early stage of the renal complication characterised by the presence of microalbuminuria, was significantly higher than the frequency of this genotype in controls. The similar distribution of the UNC13B_rs13293564 polymorphism genotype in three groups of patients with different stages of diabetic nephropathy also suggests that this polymorphism is implicated in the initiation rather than in the progression of the disease to more severe stages. However, due to the cross-sectional design of this study, we cannot exclude a survival bias in the ESRD group that would artificially modify the allele frequency patterns of susceptibility genes in this group.

The molecular analyses of the other 118 candidate genes identified no further variant associated with DN. However, the SNPs genotyped in this study do not tag the haplotype architecture of these loci, but rather they tag a subset of the haplotypes across these genes, based on the SNPs identified around exons, flanking intronic sequence, untranslated regions and promoters. To fully characterize the haplotypic variability of these genes, as would tagging SNPs selected from the Hapmap project, the genotyping of ~1300 SNPs would be required while 344 have been typed in this study. Therefore, we cannot exclude that these candidate genes contribute to the development of diabetic nephropathy.

Our strategy based on the genetic assessment of many candidate genes in a large case/control study for diabetic nephropathy including material from 3 different European populations provided evidence of replicated association between UNC13B and DN, and allowed us to establish for the first time the involvement of UNC13B variants in DN. To assess whether or not these variants are clinically relevant to predict the initiation of DN or the progression to more advanced stages of nephropathy will require further investigations in cohorts of patients with follow-up data.

URLs.


ACKNOWLEDGEMENTS

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REFERENCES
association of UNC13B with diabetic nephropathy


association of *UNC13B* with diabetic nephropathy


Table 1 Association analysis between UNC13B gene polymorphisms and diabetic nephropathy in the EURAGEDIC study

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Denmark</th>
<th>Finland</th>
<th>France</th>
<th>Whole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Allele frequency in controls</td>
<td>Allele frequency in cases</td>
<td>Allele frequency in cases</td>
<td>P(*)</td>
</tr>
<tr>
<td></td>
<td>0.584 / 0.416</td>
<td>0.633 / 0.367</td>
<td>0.624 / 0.376</td>
<td>p = 0.0282</td>
</tr>
<tr>
<td>rs13285401 (C/T)</td>
<td>0.599 / 0.401</td>
<td>0.624 / 0.376</td>
<td>0.619 / 0.381</td>
<td>p = 0.3046</td>
</tr>
<tr>
<td>rs13293564 (G/T)</td>
<td>0.653 / 0.357</td>
<td>0.624 / 0.376</td>
<td>0.605 / 0.395</td>
<td>p = 0.5974</td>
</tr>
<tr>
<td>rs10972365 (T/C)</td>
<td>0.663 / 0.347</td>
<td>0.624 / 0.376</td>
<td>0.736 / 0.264</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs4879877 (A/G)</td>
<td>0.599 / 0.401</td>
<td>0.624 / 0.376</td>
<td>0.624 / 0.376</td>
<td>p = 0.3046</td>
</tr>
<tr>
<td>rs4111859 (A/T)</td>
<td>0.622 / 0.178</td>
<td>0.799 / 0.201</td>
<td>0.686 / 0.131</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs3904435 (A/G)</td>
<td>0.643 / 0.357</td>
<td>0.624 / 0.376</td>
<td>0.624 / 0.376</td>
<td>p = 0.3046</td>
</tr>
<tr>
<td>rs12685290 (A/G)</td>
<td>0.579 / 0.421</td>
<td>0.797 / 0.203</td>
<td>0.736 / 0.264</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs17360668 (G/A)</td>
<td>0.748 / 0.252</td>
<td>0.797 / 0.203</td>
<td>0.736 / 0.264</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs10972396 (G/T)</td>
<td>0.748 / 0.252</td>
<td>0.797 / 0.203</td>
<td>0.736 / 0.264</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs10972397 (A/G)</td>
<td>0.672 / 0.178</td>
<td>0.814 / 0.186</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs7851161 (G/A)</td>
<td>0.653 / 0.357</td>
<td>0.624 / 0.376</td>
<td>0.624 / 0.376</td>
<td>p = 0.3046</td>
</tr>
<tr>
<td>rs10758301 (T/G)</td>
<td>0.672 / 0.178</td>
<td>0.814 / 0.186</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs12102009 (C/T)</td>
<td>0.711 / 0.289</td>
<td>0.784 / 0.216</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs10114937 (T/C)</td>
<td>0.711 / 0.289</td>
<td>0.784 / 0.216</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs10758303 (A/G)</td>
<td>0.672 / 0.178</td>
<td>0.814 / 0.186</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs10972397 (A/G)</td>
<td>0.711 / 0.289</td>
<td>0.784 / 0.216</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs661712 (C/T)</td>
<td>0.672 / 0.178</td>
<td>0.814 / 0.186</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs17296428 (C/G)</td>
<td>0.672 / 0.178</td>
<td>0.814 / 0.186</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs12684897 (T/C)</td>
<td>0.672 / 0.178</td>
<td>0.814 / 0.186</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs2282001 (G/C)</td>
<td>0.672 / 0.178</td>
<td>0.814 / 0.186</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs2281999 (C/T)</td>
<td>0.672 / 0.178</td>
<td>0.814 / 0.186</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs1927962 (T/C)</td>
<td>0.672 / 0.178</td>
<td>0.814 / 0.186</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs12339582 (G/T)</td>
<td>0.672 / 0.178</td>
<td>0.814 / 0.186</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs12726 (G/A)</td>
<td>0.672 / 0.178</td>
<td>0.814 / 0.186</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>rs10814234 (G/C)</td>
<td>0.672 / 0.178</td>
<td>0.814 / 0.186</td>
<td>0.861 / 0.139</td>
<td>p = 0.0000</td>
</tr>
</tbody>
</table>

(*) Difference in allele frequencies between cases and controls were tested by a χ² test with 1 degree of freedom, separately in each population.

§) For each tested SNP, the p-values of the association tests obtained in the three populations were combined by the Fisher's method.
association of **UNC13B** with diabetic nephropathy

**Table 2** Genotype distribution derived from rs2281999 and rs13293564 according to diabetic nephropathy status in the whole EURAGEDIC study.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>rs13293564</th>
<th>Cases</th>
<th>rs13293564</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2281999</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG (36%)</td>
<td>GT (50%)</td>
<td>TT (14%)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>112 (9%)</td>
<td>261 (22%)</td>
<td>147 (12%)</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>219 (18%)</td>
<td>323 (27%)</td>
<td>17 (2%)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>115 (9%)</td>
<td>15 (1%)</td>
<td>1 (1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2281999</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG (32%)</td>
<td>GT (48%)</td>
<td>TT (20%)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>94 (9%)</td>
<td>241 (22%)</td>
<td>201 (19%)</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>177 (17%)</td>
<td>263 (24%)</td>
<td>11 (1%)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>81 (7%)</td>
<td>10 (1%)</td>
<td>0 (1%)</td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE LEGENDS**

**Figure 1:** Schematic representation of the **UNC13B** gene.
The structure of UNC13B gene on chromosome 9 is presented with the respective positions of the 39 exons and of the 24 SNPs genotyped (rsID are given in Table 1), as well as the HapMap haplotype blocks (in D'). Arrows at both ends: SNPs selected through sequencing; single arrow: haplotype tagging SNPs selected from Hapmap; arrow with bullet to begin: position of rs13293564, the SNP associated with DN; dashed arrow with bullet to begin: SNPs in complete association with rs13293564 (not typed).
Figure 2: Odds Ratio for diabetic nephropathy associated with the TT genotype at the rs13293564 polymorphism

Number of controls / Number of cases
Empty square : OR adjusted for age and gender.
Full square : OR adjusted for age, gender, smoking, HbA1c and proliferative retinopathy