Brief Genetics Report

Investigation of the Human ANP Gene in Type 1 Diabetic Nephropathy

Case-Control and Follow-up Studies

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The atrial natriuretic peptide gene (*PND*) is a candidate gene for diabetic nephropathy. We systematically analyzed five nonsynonymous *PND* polymorphisms and tested the association of genotype and haplotype distributions with diabetic nephropathy in a cross-sectional study and a 6-year follow-up study (489 and 301 type 1 diabetic patients, respectively). For this purpose, a new maximum-likelihood method dealing with haplotype-based association analysis for survival data was developed. None of the genotypes or haplotypes were associated with the disease in the case-control study. In the follow-up study, C708T and T2238C showed a weak association with disease progression, but T2238C was strongly associated with progression in poorly controlled subjects (mean HbA_{1c} during follow-up was more than the median value [8.5%]; log-rank [TC or CC versus TT], $P = 0.007$; adjusted hazard ratio, TC or CC versus TT, 2.28, 95% CI 1.10–4.74; $P = 0.027$). The raw effect of the 2238C allele (hazard risk ratio 1.93, 95% CI 1.15–3.24; $P = 0.012$) was further confirmed by the haplotype analysis, suggesting that the 2238C allele of PND may affect the course of nephropathy in inadequately controlled type 1 diabetic patients. *Diabetes* 53:1394–1398, 2004

Diabetic nephropathy results from the interaction of genetic factors with chronic hyperglycemia. Atrial natriuretic peptide (ANP) may affect the course of diabetic nephropathy by inducing afferent arteriolar dilatation and efferent constriction within the glomerulus (1–3). In type 1 diabetic subjects, high glomerular filtration rate and ANP concentration correlated with each other (4), and ANP infusion increases glomerular filtration rate, filtration fraction, and albuminuria (5). ANP favors diabetic hyperfiltration (1–3). Thus, the ANP gene is a candidate gene for diabetic nephropathy, but investigations (6,7) on this topic have been controversial.

We report here a systematic analysis of the ANP gene in large case-control and follow-up studies of type 1 diabetic subjects (8,9). We characterized genetic variability at the ANP gene locus and then investigated the association between polymorphisms and diabetic nephropathy. We carried out both genotype- and haplotype-based association analyses using newly elaborated statistical methods (10).

The characteristics of the 489 participants in the cross-sectional Genetique de la Nephropathie Diabetique (GENEDIAB) study (8) are given in Table 1. Allele frequencies for the nonsynonymous polymorphisms are available in the online appendix (available at http://diabetes.diabetesjournals.org). All of these single nucleotide polymorphisms (SNPs) were in Hardy-Weinberg equilibrium. No significant association was found between the distribution of any of these SNPs and the stages of nephropathy (online appendix). The pairwise linkage disequilibrium matrix is also available in the online appendix. Eight haplotypes had a frequency >1% and accounted for >94% of all haplotypes. No association was found between ANP gene haplotypes and nephropathy stages (online appendix).

The characteristics of the participants in the prospective follow-up study SURGENE (Survival Genetic Nephropathy) (9) are given in Table 2. Kaplan-Meier curves were generated for each polymorphism. The T2238C and C708T polymorphisms were weakly associated with renal disease progression, with the most common genotypes, TT and CC, respectively, conferring the best prognosis (T2238C: log-rank [TC or CC versus TT], $P = 0.06$; and C708T: log-rank [CT or TT versus CC], $P = 0.05$). The T2238C polymorphism was associated with progression of renal disease in poorly controlled subjects (log-rank [TC or CC versus TT], $P = 0.007$) but not in with well-controlled subjects ([TC or CC versus TT], $P = 0.74$) (Fig. 1). The other polymorphisms (G663A, T2332C, and ID2497) were not associated with renal event–free survival. After adjust-

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ANP, atrial natriuretic peptide; SNP, single nucleotide polymorphism.

TABLE 1
Characteristics of patients in the GENEDIAB study, according to nephropathy stage

Stage of nephropathy	All patients	No nephropathy	Incipient	Established	Advanced	*P*
n	489	155	103	125	106	—
Age (years)*	44 ± 12	46 ± 12	43 ± 13	42 ± 12	44 ± 11	0.0066
Sex (M/F)†	275/214	84/71	61/42	65/60	65/41	0.45
Age at diabetes onset (years)*	15 ± 9	16 ± 9	16 ± 9	15 ± 8	15 ± 8	0.74
Diabetes duration (years)*	29 ± 10	31 ± 10	27 ± 9	27 ± 9	29 ± 9	0.0017
Time to retinopathy onset (years)*	24 ± 8	25 ± 9	23 ± 8	23 ± 8	23 ± 8	0.0139
BMI (kg/m^2)*	24 ± 3	24 ± 3	24 ± 3	24 ± 3	23 ± 3	0.71
HbA_{1c} (%)‡	8.6 ± 1.8	8.4 ± 1.6	8.6 ± 1.9	9.1 ± 2.1	8.5 ± 1.6	0.0492
Systolic/diastolic blood pressure (mmHg)*	138 ± 19/79 ± 11	129 ± 14/74 ± 0	137 ± 18/79 ± 12	141 ± 18/82 ± 11	148 ± 20/85 ± 11	$<10^{-4}/<10^{-4}$
Plasma creatinine (μmol/l)‡§	96 (35–1,047)	77 (35–134)	82 (59–144)	98 (35–148)	186 (78–1,047)	$<10^{-4}$

Data are means ± SD or medians (ranges). HbA_{1c} data were obtained from only 462 participants for technical reasons. Plasma creatinine concentrations are those measured in patients on treatment at the time of study. *ANOVA; †χ^2 test; ‡Kruskal-Wallis test; §some of the participants in the advanced nephropathy group had undergone successful renal transplantation and therefore displayed plasma creatinine <150 μmol/l at the time of the study.

ment for other prognostic factors (sex, HbA_{1c} during follow-up, systolic blood pressure, diabetes duration, nephropathy stage, and age at baseline), ANP T2238C remained a significant determinant of nephropathy progression for poorly controlled patients (adjusted hazard ratio [TC or CC versus TT] 2.28, 95% CI 1.10–4.74; P = 0.027).

The results of the haplotype-based hazard model are given in Table 3. Five common haplotypes were inferred, accounting for 99% of all chromosomes. The most frequent (reference) haplotype (~76%) combined the major alleles at each site. The 2238C allele was carried by two different haplotypes. Global association between haplotypes and nephropathy progression was not significant (P = 0.28). By comparison with the reference haplotype, the effects of the two haplotypes carrying the 2238C allele (GCCTT and GTCGT) (Table 3) were homogenous on the progression of nephropathy (test for heterogeneity, P = 0.79), and the common haplotypic hazard risk ratio (HRR) associated with the 2238C allele was significant (1.76, 95% CI 1.08–2.87; P = 0.023). After adjustment for other promoting factors, the HRR associated with the two 2238C haplotypes remained significant (1.93, 1.15–3.24; P = 0.012). This was especially true regarding the poorly controlled subjects, but not the well-controlled subjects (Table 3). The two single haplotypes carrying the 663A and 2332C alleles were not associated with the progression of nephropathy.

Thus, haplotypes carrying the 2238C allele in the ANP gene were associated with the progression of diabetic nephropathy in the follow-up study after adjustment for other confounding factors. To our knowledge, this is the first study to investigate the association between candidate gene polymorphisms and a survival outcome by means of haplotype-based analysis.

A careful systematic search for contributive SNPs in the coding and noncoding regions of the ANP gene led us to genotype five SNPs in both the case-control and follow-up

TABLE 2
Characteristics of the patients in the SURGENE study at baseline and during follow-up, according to progression and no progression of baseline nephropathy stage

Progression/no progression of nephropathy	All patients	Progression	No progression	*P*
Baseline				
n	301	53	248	—
Age (years)	34 ± 13	35 ± 14	33 ± 13	0.67
Sex (M/F)†	177/124	36/17	141/107	0.14
Age at diabetes onset (years)	19 ± 10	18 ± 9	19 ± 10	0.43
Diabetes duration (years)	15 ± 11	17 ± 11	15 ± 11	0.23
BMI (kg/m^2)	23 ± 3	23 ± 4	23 ± 3	0.84
HbA_{1c} (%)‡	9.5 ± 2.4	9.7 ± 2.6	9.4 ± 2.3	0.36
Systolic/diastolic blood pressure (mmHg)	127 ± 16/73 ± 11	135 ± 19/77 ± 13	125 ± 15/72 ± 10	$<10^{-4}$/0.003
Stage of nephropathy† (absent/incipient/established/advanced)	244/33/18/6	38/4/8/3	206/29/10/3	0.002
During follow-up				
HbA_{1c} (%)‡	8.6 ± 1.3	8.8 ± 1.5	8.6 ± 1.2	0.14
Systolic/diastolic blood pressure (mmHg)*	130 ± 13/77 ± 8	136 ± 14/78 ± 7	129 ± 12/73 ± 8	$<10^{-4}/10^{-4}$

Data are means ± SD. *ANOVA; †χ^2 test; ‡Kruskal-Wallis test.

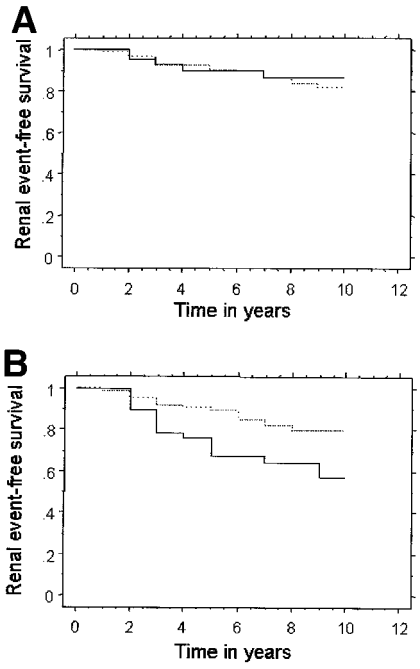

FIG. 1. Kaplan-Meier curves for renal event–free survival according to ANP T2238C genotype for the SURGENE subjects. *A*: Subjects with well-controlled glycemia during follow-up (mean HbA_{1c} less than the median value, i.e., 8.5%). Log-rank test (TC or CC versus TT), $P = 0.74$. *B*: Subjects with poorly controlled glycemia during follow-up (mean HbA_{1c} more than the median value, i.e., 8.5%). A renal event was defined as progression to a higher stage of diabetic nephropathy. Log-rank test (TC or CC versus TT), $P = 0.007$. The time to first renal event was calculated and plotted to generate the Kaplan-Meier curve, according to the patient's genotype. —, TT; - - -, TC or CC.

studies. The association of the 2238C allele with the progression of diabetic nephropathy was significant only in poorly controlled subjects in the SURGENE study, suggesting that the interaction might only be detectable under certain circumstances. Such a stress-the-genotype interaction is classical in complex traits and has been characterized for the ACE I/D polymorphism (11). However, because the cross-sectional study was negative, we cannot exclude a stratification effect, even though the interaction with glycemic control was defined to be tested a priori in our analysis (9).

One major advantage of haplotype analyses is a considerable reduction in the number of statistical tests required to investigate several polymorphisms; the number of haplotypes is usually much smaller than the number of tests involving all possible interactions between genotypes. Therefore, such analyses do not necessarily inflate the number of tests. Besides, it allows for differentiation of the true effect of a polymorphism from those due to its linkage disequilibrium with other "functional" variants. This was especially true here; the effect of the C708T polymorphism on renal disease progression observed in univariate analysis was in fact the consequence of its linkage disequilibrium with the T2238C polymorphism. However, even though we found that the observed effect of the T2238C polymorphism was consistent on the two haplotypes by which it was carried, replication in other populations and studies would be required because it cannot be excluded that the observed association is artificial and, for instance, due to multiple testing.

The GENEDIAB multicenter cross-sectional study (8) aimed to assess the association between genetic variations and the severity of diabetic nephropathy. Because all subjects had past or present proliferative diabetic retinopathy, they had expressed their risk of renal complications due to diabetes duration and control. In the SURGENE study (9), the same issue was investigated on a follow-up basis with Caucasian participants recruited at a single center in the Angers region. End-stage renal disease was the only exclusion criterion at baseline (12 patients), so survival bias is unlikely. The baseline characteristics of the participants and their rate of progression are very similar to those reported elsewhere (12).

The first work (6) investigating ANP gene polymorphisms and type 1 diabetic nephropathy found that the 2238C allele was associated with lower severity of the disease. This result was not reproduced elsewhere (7). Our case-control study was also inconclusive. However, ANP haplotypes carrying the 2238C allele accelerated the course of renal disease in SURGENE. The reasons for this discrepancy are unclear. The participants of the Italian and German studies were also Caucasian, and the reported genotype frequencies were similar (6,7). Survival bias and population stratification cannot be ruled out in case-control studies. We believe that differences in study design were probably a major cause of the differences in the results obtained.

The substitution of a T for a C at position 2238 eliminates the regular stop codon. A new stop codon arises six

TABLE 3
Analysis of the association between the main haplotypes of the ANP gene and progression of nephropathy in the SURGENE study ($n = 301$) for well-controlled patients (mean HbA_{1c} during follow-up <8.5%) and for poorly controlled patients (mean HbA_{1c} during follow-up >8.5%)

Polymorphisms					Whole SURGENE population			Patients with mean HbA_{1c} less than median (adjusted)	Patients with mean HbA_{1c} more than median (adjusted)
G663A	C708T	T2238C	G2311T	T2332C	Haplotype frequency	Nonadjusted	Adjusted		
G	C	T	G	T	0.756	—	—	—	—
G	C	T	G	C	0.037	0.73 (0.18–3.03)	0.52 (0.12–2.24)	0.94 (0.19–4.49)	—†
G	C	C	T	T	0.069	1.64 (0.80–3.38)	2.14 (1.04–4.41)	0.43 (0.05–3.33)	2.80 (1.27–6.19)
G	T	C	G	T	0.082	1.84 (1.01–3.36)	1.80 (0.96–3.37)	1.78 (0.64–4.87)	2.09 (0.87–5.08)
A	C	T	G	T	0.047	1.19 (0.49–2.93)	1.23 (0.49–3.11)	1.14 (0.35–3.65)	1.10 (0.27–4.47)

Data are haplotypic HRR (95% CI) for each haplotype, adjusted for confounding factors or not. *Reference haplotype; †undefined HRR due to too few events.

nucleotides further on, and translation results in a polypeptide with two additional arginines (13). Nannipieri et al. (6) found plasma ANP levels and albumin transcapillary escape rates to be lower in type 1 diabetic patients carrying the 708T and 2238C alleles. However, another study (14) found no association between ANP T2238C polymorphism and ANP levels in Japanese subjects. Finally, the functional effect of ANP T2238C polymorphism was evaluated three times after low-, normal, and high-salt diets in 105 Polish subjects. Following all three diets, plasma ANP levels were strongly associated with T2238C polymorphism and were lowest in TT subjects (15) (A. Ciechanowicz, personal communication). Thus, the functional significance of this polymorphism remains to be determined.

RESEARCH DESIGN AND METHODS

The case-control study, GENEDIAB, was a cross-sectional, multicenter, binational (France and Belgium) study designed to evaluate the genetic components of diabetic nephropathy in patients with type 1 diabetes (8). The main selection criteria were age ≤35 years at diabetes onset and past or present proliferative retinopathy (8). Of 494 participants, only 489 were analyzed here for technical reasons (incomplete genotyping due to poor-quality DNA). Their characteristics are given in Table 1.

The follow-up cohort, SURGENE, is a prospective single-center study of 310 type 1 diabetic patients (Angers, University Hospital, France) selected on diabetes duration >3 years, diagnosis before age 40 years, and no other chronic disease (whatever their retinopathy or nephropathy stages), except end-stage renal disease at baseline (9). The median follow-up duration was 6 years (range 2–10). For technical reasons, only 301 patients were analyzed (characteristics in Table 2). A renal event was defined as progression to a further stage of nephropathy, thereby defining the progressors against the nonprogressors during the study.

All participants gave written informed consent, and the studies were approved by the ethics committee of Angers University Hospital (Angers, France).

Diabetic nephropathy classification. Diabetic nephropathy stages (8) were defined as follows: no nephropathy, normal urinary albumin (<30 mg/24 h, 20 μg/min, or 20 mg/l) and plasma creatinine <150 μmol/l without antihypertensive treatment in two or three of three consecutive assessments; incipient nephropathy, microalbuminuria (30–300 mg/24 h, 20–200 μg/min, or 20–200 mg/l) without antihypertensive treatment and plasma creatinine <150 μmol/l; established nephropathy, past or present macroalbuminuria (>300 mg/24 h, 200 μg/min, or 200 mg/l) in patients on antihypertensive treatment or macroalbuminuria without antihypertensive treatment and plasma creatinine <150 μmol/l; and advanced nephropathy, past or present macroalbuminuria with or without antihypertensive treatment and plasma creatinine >150 μmol/l or renal replacement therapy.

Genotyping. Several SNPs were already described (14) for the ANP gene. We carried out a systematic search for new SNPs by direct sequencing of the DNA of a series of 48 subjects. The promoter region was not sequenced because previous results (14) showed a single, uncommon polymorphism. We used direct sequencing to genotype the two cohorts. Three PCR products were analyzed for the case-control study and two for the follow-up study (see technical conditions in the online appendix). Thus, we genotyped known variations (nucleotide numbering according to genomic position, GenBank accession number K02043): G663A, C708T, G1837A, A1869G (these two SNPs for only the case-control study), T2238C, T2325C, T2332C, and T2455C, and we also identified a new SNP at position 2311 (G2311T) and a new insertion/deletion polymorphism at position 2497 (2497 I/D). The polymorphisms G1837A and A1869G were in strong linkage disequilibrium with G663A and T2238C, respectively. We therefore did not genotype them in SURGENE subjects.

Statistical analysis. Statistical analysis was carried out with Statview software (SAS Institute, Cary, NC). Data are presented as means ± SD or medians (ranges) for skewed distributions. Groups were compared using parametric (ANOVA or Student's t test) or nonparametric (if not normally distributed, Kruskal-Wallis or Mann-Whitney U tests) tests for continuous variables. For survival analysis (with progression to a higher stage of diabetic nephropathy, i.e., defining a renal event, as the outcome variable) time to first renal event curves were generated by Kaplan-Meier estimation and compared, using the log-rank test, both on all participants and separately in those having poor (HbA_{1c} higher than the median, i.e., 8.5%) versus good (HbA_{1c} at median or less) glycemic control (9). Cox's proportional hazards model was used to investigate the relationship between several candidate prognostic variables and the outcome variable in the genotype-based analyses.

Association between ANP gene haplotypes and nephropathy status (no nephropathy versus nephropathy [incipient, established, and advanced pooled]) was investigated by means of a maximum likelihood method that we recently proposed (10) for haplotype-based association analysis. This method allows us to simultaneously estimate haplotype frequencies and covariate-adjusted haplotypic odds ratios by comparison with a reference haplotype.

Here this method was extended to take into account survival data analysis by use of a parametric Weibull model to describe the association between haplotype and survival outcome, progression to a further stage of nephropathy. This allowed us to estimate the haplotype effects, expressed as HRRs (see the online appendix for details) (10).

By setting appropriate constraints on parameters, we were also able to use these models to test for homogeneity in certain haplotype effects. For example, we investigated whether the effects of the GCCTT and GTCGT haplotypes could be assumed to be equal by means of the likelihood-ratio criterion (10).

Finally, differences in haplotype frequency distributions according to the stages of nephropathy were investigated with Arlequin software (16).

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