Association between the T-381C polymorphism of the brain natriuretic peptide gene and risk of type 2 diabetes in human populations

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Brain natriuretic peptide (BNP/NPPB) is a member of the natriuretic family involved in the regulation of blood pressure and blood volume as well as lipolysis control in human fat cells. Thus BNP may play a role in energy metabolism and metabolic diseases. We therefore assessed the association between the BNP promoter T-381C polymorphism and risk of type 2 diabetes and metabolic and BNP expression traits in several population samples. In French population-based samples (n = 3216), we found that individuals bearing the -381CC genotype had lower (P = 0.005) fasting glucose levels than -381TC or -381TT individuals. Moreover, the -381CC genotype was less frequent in individuals with type 2 diabetes (n = 280, 13.6%) or with impaired fasting glucose (n = 248, 12.9%) compared with normoglycaemic individuals (n = 2485, 17.8%). The adjusted odds ratio (OR) (95% CI) of type 2 diabetes for -381CC individuals was 0.69 (0.47–1.00), P = 0.05, when compared with -381T allele bearers. We replicated this association in four additional case–control studies for type 2 diabetes. The overall OR (95% CI) of type 2 diabetes was 0.85 (0.76–0.96), P = 0.008, (under a recessive model) (3593 cases and 6646 controls in total). We also found that the -381C allele was associated with higher plasma BNP concentrations (P = 0.015, n = 634) and higher BNP promoter activity in reporter gene assays. Collectively, these data suggest that relatively high BNP expression may protect against type 2 diabetes in humans.

INTRODUCTION

Brain natriuretic peptide (BNP or NPPB) is a member of the natriuretic family that also includes the atrial natriuretic peptide (ANP/NPPA) and the C-type natriuretic peptide (CNP/NPPC). BNP, although first identified in the brain, is produced mainly by the cardiac ventricles in humans. ANP and BNP biological activities are mediated by a specific receptor bearing a guanylyl cyclase activity (NPR-A), which is...
DBP, diastolic blood pressure; SBP, systolic blood pressure; TG, triglycerides. Both a 3-genotype model and a recessive model (when indicated) were used.

aData were log-transformed to obtain normal distributions.

Table 1. Impact of the BNP T-381C polymorphism on clinical phenotypes in the French MONICA study

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TT 1147</th>
<th>TC 1529</th>
<th>CC 540</th>
<th>Crude $P$</th>
<th>Adjusted $P^a$</th>
<th>Adjusted $P^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>26.3 ± 4.6</td>
<td>26.3 ± 4.6</td>
<td>26.4 ± 4.5</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>90.1 ± 13.3</td>
<td>90.1 ± 13.6</td>
<td>90.1 ± 13.8</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>82.4 ± 11.7</td>
<td>82.6 ± 11.7</td>
<td>81.6 ± 11.5</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>131.1 ± 19.2</td>
<td>132.6 ± 19.2</td>
<td>131.5 ± 19.8</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.92 ± 1.06</td>
<td>5.90 ± 1.07</td>
<td>5.84 ± 0.97</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>1.32 ± 0.99</td>
<td>1.33 ± 1.08</td>
<td>1.21 ± 0.78</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose (mmol/l)$b$</td>
<td>5.59 ± 1.40</td>
<td>5.56 ± 1.34</td>
<td>5.42 ± 1.02</td>
<td>0.02</td>
<td>0.01</td>
<td>0.005</td>
</tr>
<tr>
<td>Insulin (µU/ml)$b$</td>
<td>11.05 ± 6.41</td>
<td>11.30 ± 7.41</td>
<td>10.99 ± 6.60</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Replication of the association between the T-381C polymorphism and risk of type 2 diabetes: meta-analysis

Given the suggestive evidence for statistical association in the MONICA study, we attempted to replicate the association between the BNP T-381C polymorphism and type 2 diabetes in four additional case–control studies [iNCA (iNsuffisance CAdiaque), Cambridgeshire case–control study (CCCS), EPIC (European Prospective Investigation into Cancer) and UK (United Kingdom)]. Details of genotypes by subject groups and studies are shown in Table 2. There was no evidence of departure from Hardy–Weinberg equilibrium in control participants. Figure 1 shows that the association was consistent among studies, although only approaching statistical significance in the individual studies. For this replication study set of 3313 cases and 4161 controls, and using a fixed-effects meta-analysis, the OR (95% CI) for risk of type 2 diabetes was 0.87 (0.77–0.98), overall $P = 0.027$ (under a
recessive model) with no detectable heterogeneity among studies \((P = 0.11)\). In a pooled analysis of all studies (including MONICA study), based on 3593 cases and 6646 controls, the OR (95% CI) for risk of type 2 diabetes was \(0.85 (0.76–0.96)\), overall \(P = 0.008\) (under a recessive model) with no detectable heterogeneity among studies \((P = 0.14)\) (Fig. 1).

This association was consistent in men \([\text{OR (95\% CI)} = 0.86 (0.74–1.00), P = 0.051]\) and women \([\text{OR (95\% CI)} = 0.83 (0.69–1.00), P = 0.056]\) (Fig. 1). An association was also found in the INCA study between the -381 CC genotype and lower plasma glucose levels \((0.99 + 0.28 \text{ versus } 1.07 + 0.35 \text{ g/l for -381CC subjects and -381T allele bearers, respectively, } P = 0.05\) adjusted for age, gender and BMI).

As part of a sensitivity analysis, we also treated centers within the MONICA study as individual studies. We found no material difference in the magnitude of the pooled association using this approach (data not shown). A further sensitivity analysis, excluding the INCA study gave a similar pooled OR of \(0.88 (0.78–0.99), P = 0.033\), for men and women combined under a recessive model. This finding suggests that this study did not explain the overall pooled association.

### Functional studies for the BNP T-381C polymorphism

**Association between the T-381C polymorphism and plasma BNP levels.**

In the INCA study, the plasma mean BNP was \(409 + 619 \text{ pg/ml (median: 194, range 9–4460 pg/ml, } n = 208)\) with the fluorescence method and \(263 + 344 \text{ pg/ml (median: 129, range 1–2693 pg/ml, } n = 426)\) with the radioimmunoassay method. To increase statistical power, we combined BNP concentrations from both BNP dosage methods using 20-quantiles (see Materials and Methods section). As expected, plasma BNP levels were higher \((P < 0.02)\) in women than in men, increased \((P < 0.0001)\) with age, and were lower \((P < 0.0001)\) in obese compared with non-obese individuals (Table 3). No significant difference in plasma BNP levels could be detected between subjects with or without type 2 diabetes. We then assessed the impact of the BNP T-381C polymorphism on plasma BNP levels. We observed a trend towards higher plasma BNP levels in -381C allele bearers compared with -381T individuals for both BNP dosage methods (Table 3), with the homozygous CC subjects presenting the highest BNP levels. Using 20-quantiles, the -381C allele was significantly associated with higher plasma BNP levels \((P = 0.015)\) (Table 3). Moreover, there were more subjects carrying the -381C allele (TC + CC subjects) than the -381T genotype \((P < 0.005)\) in the highest BNP quartiles compared with the lowest quartiles (Fig. 2).

**Association between the T-381C polymorphism and luciferase reporter gene assays.** Finally, we evaluated the impact of the T-381C polymorphism on BNP promoter activity. We cloned the two allelic forms (-381T and -381C) of the proximal promoter (1027 bp) of BNP into the luciferase reporter pGL3-basic vector to create pGL3-BNP promoter vector.
Table 3. Plasma BNP levels in various subject groups in INCA

<table>
<thead>
<tr>
<th></th>
<th>Radioimmunoassay method (n = 426)</th>
<th>Fluorescence method (n = 208)</th>
<th>Combined (n = 634)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude P Adjusted P</td>
<td>Crude P Adjusted P</td>
<td>Test on 20-quantiles</td>
</tr>
<tr>
<td>Men</td>
<td>272 ± 18 (347) 0.027 260 ± 16 0.85</td>
<td>404 ± 48 (164) 0.49 393 ± 43 1.0</td>
<td>0.018</td>
</tr>
<tr>
<td>Women</td>
<td>226 ± 39 (79) 0.04 282 ± 34</td>
<td>430 ± 94 (44) 0.46 469 ± 85 0.0003</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age &lt;56 yrs</td>
<td>224 ± 24 (198) &lt;0.0001 255 ± 24</td>
<td>408 ± 60 (106) 0.07 386 ± 61 0.0003</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age ≥56 yrs</td>
<td>299 ± 23 (227) &lt;0.0001 305 ± 23</td>
<td>410 ± 61 (102) 0.07 476 ± 65 0.05</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Non-obese</td>
<td>293 ± 19 (321) &lt;0.0001 292 ± 20</td>
<td>458 ± 48 (160) 0.015 484 ± 54 0.018</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Obese</td>
<td>175 ± 33 (104) 0.0064d 212 ± 33d</td>
<td>245 ± 89 (48) 0.015 300 ± 83 0.13</td>
<td>0.77</td>
</tr>
<tr>
<td>Without T2D</td>
<td>272 ± 20 (305) 0.91 273 ± 21</td>
<td>405 ± 49 (159) 0.88 429 ± 53 0.13</td>
<td>0.77</td>
</tr>
<tr>
<td>With T2D</td>
<td>238 ± 34 (103) 0.05 265 ± 33</td>
<td>398 ± 94 (43) 0.88 363 ± 93 0.04</td>
<td>0.77</td>
</tr>
<tr>
<td>-381TT</td>
<td>213 ± 28 (157) 0.09 237 ± 27</td>
<td>282 ± 76 (65) 0.09 337 ± 76 0.09</td>
<td>0.77</td>
</tr>
<tr>
<td>-381TC</td>
<td>281 ± 25 (193) 0.09 288 ± 24</td>
<td>439 ± 59 (108) 0.13 448 ± 60 0.28</td>
<td>0.015</td>
</tr>
<tr>
<td>-381CC</td>
<td>322 ± 39 (76) 0.21 294 ± 37</td>
<td>554 ± 104 (35) 0.13 547 ± 100 0.28</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Data are mean (pg/ml) ± SEM (n). T2D, type 2 diabetes. The age of 56 years corresponds to the median age of the sample. P-values were calculated on log-transformed BNP data to obtain normal distributions.

- Adjusted for age, BMI and LVEF (Left Ventricular Ejection Fraction).
- Mann–Whitney non-parametric test.
- Adjusted for age, gender, BMI and LVEF.
- Adjusted for age, gender, BMI and LVEF.
- Kruskal–Wallis non-parametric test.

Figure 2. BNP T-381C polymorphism distribution according to quartiles of plasma BNP concentrations in INCA (n = 634). Q: quartiles (number of individuals). *P < 0.005 (χ² test) when comparing the distribution of the BNP T-381C polymorphism (dominant model) between either quartile 3 or quartile 4 and pooled quartiles 1 and 2. Each histogram is divided into three colored groups corresponding to the three BNP genotypes: white, -381TT; gray, -381TC; black, -381CC.

In COS7 cells and in the basal state, the BNP promoter displayed 1.8-fold higher activity (P = 0.05) when carrying the -381C allele than when carrying the -381T allele (Fig. 3). Because the T-381C polymorphism is located in a putative binding site for the AREB6 transcription factor (assessed using the Genomatix - Eldorado tool), we also co-transfected the pGL3-BNP vector and an expression vector encoding the human AREB6 cDNA in COS7 cells. The presence of AREB6 increased BNP promoter activity despite whatever the allele at position -381 was (1.6- and 1.7-fold for the -381T and -381C alleles, respectively, P = 0.05 for both), suggesting the T-381C polymorphism did not modify AREB6 ability to enhance BNP promoter transcriptional activity (Fig. 3).

DISCUSSION

Consistent with recent data suggesting that BNP may be involved in energy metabolism, we observed that a functional BNP T-381C polymorphism was associated with lower plasma glucose levels and lower type 2 diabetes risk. Our data, based on 3593 cases and 6646 controls, indicate that individuals carrying the -381CC genotype may have about a 15% lower risk of type 2 diabetes compared with -381TT allele carriers. This association was similar in men and women. Population stratification is unlikely to explain this association because cases are compared with the corresponding controls within study and not between studies in ethnically homogeneous populations, and controls have been taken from a similar sampling frame to relevant cases. Study estimates were also broadly similar among studies. We also found that this polymorphism had a functional influence on BNP gene expression, the -381C allele being associated with higher BNP promoter activity in reporter gene assays. Finally, we discovered that the -381C allele was associated with higher plasma BNP levels in a sample of patients with stable CHF related to left ventricular systolic dysfunction, although this result should be double-checked in a general population-based sample, as it is known that this disease state influences BNP levels. Collectively, these data suggest that relatively higher BNP expression may protect against type 2 diabetes.

BNP and ANP are natriuretic peptides recently involved in lipolysis regulation in humans, independently of the activation of the sympathetic nervous system (7). Our results confirm that plasma BNP levels are inversely correlated with BMI (4–6). However, results on patients with type 2 diabetes are more controversial: some authors found that patients with type 2 diabetes had more circulating BNP (8) and Nt-proBNP (9) than non-diabetic individuals, whereas others found the opposite (5) or no difference at all (10). In the INCA study, individuals with type 2 diabetes had similar plasma BNP
levels than individuals without type 2 diabetes, even after adjustment for age, gender, BMI and left ventricular ejection fraction (LVEF). Because stable CHF is known to increase plasma BNP levels, this compensatory change in BNP levels may distort an association between BNP levels and risk of type 2 diabetes. Therefore, plasma BNP levels should be, in the future, measured in diabetic and normoglycaemic subjects issued from general population-based samples to clarify this point.

Nevertheless, in our study, we showed that -381CC individuals had a lower risk of type 2 diabetes and higher plasma BNP levels than individuals carrying the -381T allele, suggesting that high levels of BNP could protect against type 2 diabetes. Consistent with these observations, we also found that -381CC individuals had a lower risk of impaired fasting glucose and lower fasting glucose levels in the MONICA population-based study. This association might be due to relatively higher BNP levels leading to more efficient lipolysis in fat tissue, resulting in improved insulin sensitivity. Lipolysis efficiency experiments performed in individuals with contrasted genotypes for the T-381C polymorphism might help resolve the biological basis to this association.

We also compared BNP promoter activity when carrying the -381T or -381C allele, using luciferase reporter assays in COS7 cells. We found that the BNP promoter carrying the -381C allele had a 2-fold higher activity than the BNP promoter carrying the -381T allele, independently of the AREB6 transcription factor although the -381C allele disrupts a putative binding site (NNRCACC with the position of the T-381C polymorphism underlined) for AREB6. This suggests that AREB6 did not act via this particular putative binding site or that the presence of the -381C allele was not sufficient to alter the transactivation capability of AREB6 and that other transcriptional factors present in the cells are able to regulate BNP promoter activity in a manner depending on the allele at position -381. Other experiments are needed to determine which transcription factors are implicated in this allele-specific activity difference. There is the possibility that the BNP T-381C polymorphism might be in tight linkage disequilibrium with other nearby functional polymorphisms that could explain the present association with type 2 diabetes.

Only one other study had previously explored the impact of the BNP T-381C polymorphism on humans but not on metabolic phenotypes. Indeed, Kajita et al. showed that the T-381C polymorphism was associated with higher bone mineral density in Japanese postmenopausal women (11). It is worth noting that the frequency of the -381C allele in Japanese was 15%, whereas it ranged from 41 to 43% in our various Caucasian samples.

In conclusion, we have shown for the first time a potential role for BNP in the etiology of type 2 diabetes in several human populations. Other studies are needed to help clarify this association.

MATERIALS AND METHODS

Study subjects

MONICA study. Participants were recruited in the framework of the WHO-MONICA population survey conducted from 1995 to 1997 in three different regions of France: the Urban Community of Lille in the north (n = 1195), the Bas-Rhin county in the east (n = 1131) and the Haute-Garonne county in the south (n = 1182). The sample included individuals aged 35–65 randomly selected on electoral rolls after stratification by town size, gender and age to obtain 200 participants for each gender and 10-year age group (WHO-MONICA Project protocol) (12). To our knowledge, no individuals were related. The Ethical Committee of each center approved the protocol. All participants signed an informed consent. Details of the study have been described elsewhere (13).

From the 3216 individuals included in the French MONICA study, 280 individuals had type 2 diabetes based on a medical diagnosis and/or fasting glycaemia ≥7 mmol/l (1.26 g/l) and/or on the existence of a specific treatment or diet (14) and 2485 individuals had fasting glycaemia <6.1 mmol/l (1.10 g/l) and had no specific treatment or diet for type 2 diabetes. There were 203 individuals with unknown diabetes status and 248 individuals with intermediate fasting glucose levels (between 6.1 and 7 mmol/l).

INCA study. The INCA population consisted of 646 Caucasian consecutive ambulatory patients with stable CHF related to left ventricular systolic dysfunction (LVEF ≤45%) (15). All patients had maximal tolerated doses of angiotensin converting enzyme inhibitors and beta-blockers. Patients were classified as diabetic (n = 156) if they had a previous history of elevated fasting glucose (>1.26 g/l) on two separate occasions or if they were receiving oral hypoglycaemic drugs or insulin. After informed consent was obtained, blood samples were drawn in the morning at rest in fasting condition for standard biological determination, BNP determination and genetic analysis. Because the BNP dosage method changed in the hospital in 2002, plasma BNP concentrations were measured either by radioimmunoassay (Shionoria BNP kit; Shionogi &

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**Figure 3.** Analysis of BNP promoter activity with luciferase reporter assays in COS7 cells. COS7 cells were transiently co-transfected with either empty basic-pGL3 or pGL3-BNP/-381T or pGL3-BNP/-381C promoter vector with or without AREB6 expression vector. Activity of pGL3-BNP promoter vector is expressed as fold activity compared with basic-pGL3 vector activity. White and black bars represent activities obtained for -381T and -381C alleles, respectively. Each bar represents the mean ± SD of three independent experiments. Firefly luciferase activities were normalized to the renilla luciferase activities. Data were compared with the Kruskal–Wallis non-parametric test. *P = 0.05.
Patients aged 45–76 with type 2 diabetes were randomly selected from general practitioner diabetes registers in Cambridgeshire, UK (16). Criteria for type 2 diabetes were: onset of diabetes after the age of 30 years without insulin treatment in the first year of diagnosis (n = 554). Controls individuals (n = 532) were matched for age, sex and geographical location. Diabetes was excluded in controls by medical record search, and by an HbA1c measurement <6%. The study received ethical approval from the Cambridge Local Research Ethics Committee and participants provided informed consent.

**UK study.** The samples examined in the UK study have all been previously described. For more details, see online appendix in (18). Briefly they include (a) 572 probands, all ascertained for positive family history, from the Diabetes UK Warren 2 sibpair collection (Warren 2 Sibpair Probands: W2SP); (b) 350 probands from the Diabetes UK Warren 2 Trio collection (Warren 2 Trio Probands: W2TP); (c) 1586 further T2D cases from the MRC/Diabetes UK Case resource, ascertained for type 2 diabetes diagnosed before age 65 (Warren2 cases: W2C); (d) 550 UK control subjects (HRC+): 480 from the Human Random Control (HRC) resource, with an additional 70 non-HRC control samples from the same source (ECACC, Salisbury, UK); and (e) 2024 UK control subjects from the British Birth Cohort of 1958 (58BC). All cases were ascertained using similar criteria for a diagnosis of diabetes (based on usage of oral agents and/or insulin, and/or biochemical evidence of hyperglycaemia), with subtypes other than type 2 excluded using clinical, genetic and/or immunological criteria (all are GAD-antibody negative). Glucose tolerance status is not known for any of the controls. All subjects in the case–control analysis are of known British/Irish European origin. Case and control subjects were pooled after first confirming heterogeneity of genotype frequencies between subgroups using standard contingency table methods (χ²).

**Genotyping**

The genotyping of the MONICA, INCA and CCCS population samples for the BNP T-381C polymorphism was done by the restriction fragment length polymorphism method as following. The oligonucleotides used for the PCR amplification were 5'-CTGTAAGTCACCCCGTGCTC-3' and 5'-GGGCAGGAACGGCTGGAGAC-3'. The PCR reaction was conducted with 25 mM MgCl₂ at an annealing temperature of 65°C, generating an amplicon of 188 bp. The PCR product was then digested with 2 U ofMspI (New England Biolabs, Hertfordshire, UK). The -381T allele was characterized by two fragments of 48 and 139 bp, whereas the -381C allele was characterized by three PCR fragments of 48, 68 and 71 bp, distinguished on a 4% agarose gel. Success rates were 96, 98 and 97% for the MONICA, INCA and CCCS studies, respectively.

The genotyping of the EPIC study for the BNP T-381C polymorphism was done by Taqman using the oligonucleotides 5'-GTCTCCCGCTTTCTTCTTCC-3' and 5'-CAGGAAAGAACGCCTCAACTA-3' and the probes 5'-CAAATGTCCAGGTGTCC-3' (for the -381T allele, Vic labeled) and 5'-AAATGTCGGGTTCC-3' (for the -381C allele, Fam labeled). Success rate was 99%.

The individuals of the UK study were genotyped at KBiosciences (Hoddesdon, UK), using a fluorescence-based competitive allele-specific PCR system (KASPar). Assay details are available from the authors on request. Success rate was 94% with an estimated error rate of 0.87% (based on 576 duplicate pairs with complete genotypes).

**Statistical analyses**

Analyses were performed with the SAS statistical software release 8 (SAS Institute Inc., Cary, NC, USA). The MONICA and control samples were in Hardy–Weinberg equilibrium using the χ² test (1 df). Pearson χ² was used to compare genotype and allele distributions between individual groups. We calculated the association between BNP T-381C class genotype and risk of type 2 diabetes (ORs and 95% CIs) using unconditional logistical regression. In the primary analysis, we used a co-dominant genetic model, assuming a linear relationship between the number of C alleles of the BNP T-381C genetic variant. In a secondary analysis, we made a preliminary assessment of the shape of the association by visually comparing ORs under a general model. Comparison means among genotype groups was tested with a general linear model followed by Tukey’s test (comparison procedure for all pairwise groups) which led to the a posteriori use of a recessive model.

For the meta-analysis, fixed-effect summary estimates were calculated for a recessive model—based on the shape of the model found in MONICA study—as the inverse-variance weighted average of the log ORs. Heterogeneity among studies was assessed by the heterogeneity Q statistic. Meta-analyses were done using Stata version 8.2 software (Stata Corp., TX, USA).

Plasma BNP concentrations were measured using two dosage methods (see above). To combine information from both methods and to increase statistical power, BNP levels were divided into 20 quantiles for each method, and quantiles were then pooled to form a unique ordinal variable. Non-parametric tests (Mann–Whitney or Kruskal–Wallis tests when appropriate) were used to perform the analysis. The graphical representation of the influence of the BNP T-381C
polymorphism on BNP levels was based on quartiles. When appropriate, covariables were age, gender, BMI and LVEF, as levels of BNP increase with the severity of the left ventricular impairment.

Data from in vitro luciferase assays were compared with the Kruskal–Wallis non-parametric test.

Cell culture

COS7 cells were maintained in DMEM medium containing 10% FCS, 2 mM glutamine and 20 IU/ml penicillin/20 µg/ml streptomycin (Gibco, Invitrogen, Paisley, UK). The cells were maintained in a humidified incubator with 5% CO₂ at 37°C.

Plasmids

The BNP promoter luciferase reporter vectors (pGL3-BNP) containing proximal BNP promoter (1027 bp) with either the -381T or the -381C allele were obtained from genomic DNA of individuals with known contrasted genotypes. They were generated by subcloning the PCR product amplified using the oligonucleotides: 5'-GAC GTT ACC CCA TCC TGT GTT GGC TTG GTG-3' (containing a KpnI restriction site) and 5'-CTG CTC GAG CTC TCT GGA GGG ACT TGT GTT GGC TTG GTG-3' (containing a XhoI restriction site) into the KpnI and XhoI restriction sites of the basic luciferase reporter vector pGL3 (Promega, Madison, WI, USA). The insert identity was verified by sequencing and only the T-381C change was present. The expression vector (pCMV-flag2) for human AREB6 was a kind gift from Dr Kiyoshi Kawakami (Tochigi, Japan).

Transient transfections

For luciferase promoter activity assays, COS7 cells were grown to 70% confluence in 24-well plates. Transfections were carried out in serum-free DMEM with FuGENE™ transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions, using 300 ng luciferase reporter vector (pGL3 basic or pGL3-BNP/T or pGL3-BNP/C) with or without 300 ng AREB6 expression vector per dish. The renilla luciferase reporter vector (Promega) (20 ng) was used to normalize for transfection efficiency. Forty hours after transfection, firefly and renilla luciferase assays were performed as recommended by the manufacturer. Transfections were realized in triplicates and data of three independent experiments are presented.

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Conflict of Interest statement. None declared.

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