The -318 C>G Single-Nucleotide Polymorphism in GNAI2 Gene Promoter Region Impairs Transcriptional Activity through Specific Binding of Sp1 Transcription Factor and Is Associated with High Blood Pressure in Caucasians from Italy

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Inhibiting Ga subunit 2 protein, which is encoded by the GNA2 gene, is suggested to be pathogenic for essential hypertension and/or insulin resistance. The aim of this study was to determine whether GNAI2 variations modulate the risk for these abnormalities. Seven single-nucleotide polymorphisms (SNP) at the GNAI2 locus were identified. Because of either low allelic frequency or unlikely biologic relevance (i.e., synonymous or intronic), six SNP were not studied further. The -318C>G SNP (allelic frequency 6%) in the promoter region was studied for association with adiposity, systolic BP (SBP) and diastolic BP, fasting insulin and glucose, and lipids levels in 655 nondiabetic Caucasians from Italy. As compared with individuals who carry the C/C genotype, G carriers (i.e., individuals who carry either the G/G or the C/G genotype) had higher SBP (117.8 ± 16 versus 113.6 ± 12.6 mmHg; \( P = 0.010 \)) and were at increased risk for hypertension (odds ratio 2.2; 95% confidence interval 1.1 to 4.5). Compared with C, the G allele had a 2.5-fold reduced transcriptional activity in transfected HEK293 cells. As predicted by the TRANSFAC database, competition with YY1 or Sp1 transcription factors specifically reduced the binding of HeLa cell nuclear proteins to -318C or -318G allele, respectively, as indicated by shifted electrophoretic mobility. A “supershift” of the nuclear proteins/−318G allele complex was observed after anti-Sp1 was added but not anti-YY1 antibody. The GNAI2 −318 C>G SNP impairs transcriptional activity through specific binding of Sp1 and is associated with high SBP in Caucasians from Italy.


GTP-binding guanine nucleotide regulatory proteins (G proteins; either stimulatory [Gs] or inhibitory [Gi]) are heterotrimeric, composed of α-, β-, and γ-subunits (1). Three distinct isoforms of G\(_{\text{ia}}\) (G\(_{i1\alpha}\), G\(_{i2\alpha}\), and G\(_{i3\alpha}\)) are encoded by three distinct genes, are implicated in adenylyl cyclase inhibition and in the activation of atrial K\(^+\) channels (2). The adenylyl cyclase/cAMP system plays a role in a variety of cellular functions that are related to BP control, including vascular permeability (3), salt and water transport (4), catecholamine release (5), heart contractility (6), and vascular smooth tone (7). In addition, abnormal G protein signal transduction, with much emphasis on the G\(_{i\alpha}\) isoform, has been reported in both hypertension and diabetes (8–15). Increased G\(_{i2\alpha}\) mRNA expression and protein activation have been reported in hearts and aortas from spontaneously hypertensive rats and in hearts from deoxycorticosterone acetate–salt hypertensive rats (9,10). At variance, human studies have shown a reduction of G\(_{i2\alpha}\) expression in platelets, erythrocytes, and coronary arteries of hypertensive patients (11–14) and in fibroblasts of hypertensive insulin-resistant patients (15). In mice, cells that so not express G\(_{i2\alpha}\) are insulin resistant (16); conversely, overexpression of a constitutively active mutant of G\(_{i2\alpha}\) has an insulin-mimetic effect (17). Taken together, these data suggest that altered G\(_{i2\alpha}\) expression and/or function may be involved in the pathogenesis of hypertension and/or insulin resistance (IR). The aim of this study was to determine whether variations in the GNAI2 gene, encoding for the G\(_{i2\alpha}\) protein, modulate the risk for these abnormalities.

Materials and Methods

The association between the −318 GNAI2 variant and metabolic traits was evaluated in 655 nondiabetic white residents of the Gargano area (Center East Coast of Italy). Participants had fasting plasma glucose <7 mmol/L at screening and were not taking any medications. The study protocol and informed consent procedures were approved by the local research ethics committee. The recruitment criteria and the clinical characteristics of these participants have been described previously (18).

Genotyping

Genomic DNA was isolated from whole blood using a commercial DNA isolation kit (Roche Diagnostics, GmbH, Mannheim, Germany). Oligonucleotide primers were designed to amplify all GNAI2 exons, intron-exons boundaries, 1.5 kb of the promoter, and the 3′ untranslated region by PCR in a screen of 50 unrelated individuals. The PCR
products then were sequenced bidirectionally using an ABI Prism dye terminator cycle sequencing ready reaction kit and an ABI Prism 310 automated sequencer (Applied Biosystems, Foster City, CA). Genotyping of the newly detected promoter single-nucleotide polymorphism (SNP) was achieved by restriction fragment length polymorphism analysis, because a BstUI restriction site is created by the −318G variant. Primers sets and PCR conditions are available from the authors.

**Subcloning and DNA Sequencing**

Fragments of the promoter region (440 bp) that contained the −318C/G polymorphisms was subcloned into pGL2-Basic (5.6 kb; Promega, Madison WI) and sequenced by the direct sequencing method (ABI PRISM 310; Applied Biosystem) using the universal M13 and GLP primers and specific primers.

**Cell Culture**

Human embryonic kidney cell line HEK293 was obtained from Sigma-Aldrich (St. Louis, MO). Cells were grown in DMEM Nutrient Mix F12 supplemented with 10% FBS at 37°C.

**Transfection and Luciferase Assays**

Transient HEK293 transfection with 4 mg of reporter plasmid and co-transfection with 4 mg of control plasmid that contained the β-gal reporter gene (pSV-β-Galactosidase 6.8 kb; Promega) was performed using the CellPect Tranfection Kit Sterile (Amersham Biosciences, Piscataway, NJ). Luciferase assays were performed 72 h after transfection. Protein concentration was determined by the Bradford Standard Curve method, and transfection efficiency was evaluated by β-Galactosidase Enzyme Assay System (Promega). Protein and β-gal content were used for normalization of luciferase activity.

**Electrophoretic Mobility Shift Assay**

Electrophoretic mobility shift assay was performed using the Gel Shift Assay System (Promega) according to manufacturer’s instructions with HeLa Nuclear Extract and using γ[^32]P]ATP (Perkin Elmer, Wellesley, MA). For supershift assays, 4 mg of affinity-purified rabbit polyclonal antibody against YY1 (sc-281X) and Sp1 (sc-059X; Santa Cruz Biotechnologies, Santa Cruz, CA) were added to the binding reactions and incubated using the CellPect Tranfection Kit Sterile (Amersham Biosciences, Piscataway, NJ). Luciferase assays were performed 72 h after transfection. Protein concentration was determined by the Bradford Standard Curve method, and transfection efficiency was evaluated by β-Galactosidase Enzyme Assay System (Promega). Protein and β-gal content were used for normalization of luciferase activity.

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**Statistical Analyses**

All statistical analyses were performed using the SPSS statistical package (version 10; Chicago, IL). Continuous variables (expressed as mean ± SD) were compared by univariate ANOVA when adjusted for potential confounders. The association between hypertension and genotypes was analyzed by logistic regression analysis to adjust for potential confounders. As a descriptive measure of association between genotypes and outcomes, odds ratios were calculated along with 95% confidence intervals. P < 0.05 was considered as significant.

**Results**

By sequencing all exons, intron-exon boundaries, 1500 bp of the promoter region, and the 3’ untranslated region of the GNAI2 gene in 50 unrelated Caucasians from Gargano, seven SNP were identified (−318C>G, 14867C>T, rs762707, 19027C>T, 19777A>G, 20289A>G, and 20305T>C). Of these, only one (the rs762707) had been reported previously in the National Center for Biotechnology Information dbSNP database (http://www.ncbi.nlm.nih.gov/SNP). Other SNP (rs1050067, rs11557219, and rs12721539), previously reported in the same database, were not confirmed in our population. Because of either low allelic frequency or unlikely biologic relevance (i.e. synonymous or intronic) six SNP were not studied further. At variance, the −318C>G SNP (allelic frequency 6%) in the promoter region, which, as inferred from HapMap genotype data from white populations, lies in a 14-kb linkage disequilibrium block that covers the 5’ half of the gene and is informative of all of this area (r² = 0.8), was analyzed further for association with features related to IR, including body mass index, waist circumference, systolic (SBP) and diastolic BP (DBP), fasting serum insulin and glucose, and lipids levels in 655 nondiabetic Caucasians from Gargano, Italy. Genotype frequencies were in Hardy-Weinberg equilibrium (P = 0.71 by χ² test). Data from individuals who were homozygous (n = 2) and heterozygous

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CC (%)</th>
<th>XG (%)</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td>N (%)</td>
<td>593 (90.5)</td>
<td>62 (9.5)</td>
<td>0.87</td>
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<tr>
<td>Male/female</td>
<td>225/368</td>
<td>24/38</td>
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<tr>
<td>Age (yr)</td>
<td>36.6 ± 11.6</td>
<td>38.2 ± 12.4</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>25.5 ± 4.5</td>
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</tr>
<tr>
<td>SBP (mmHg)</td>
<td>113.6 ± 12.6</td>
<td>117.8 ± 16.0</td>
<td>0.11</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>75.9 ± 8.6</td>
<td>77.6 ± 10.9</td>
<td>0.80</td>
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<tr>
<td>Glucose (mmol/L)</td>
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<td>5.0 ± 0.51</td>
<td>0.97</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>57.4 ± 27.3</td>
<td>55.2 ± 32.3</td>
<td>0.74</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.26 ± 0.29</td>
<td>1.37 ± 0.34</td>
<td>0.77</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.05 ± 0.66</td>
<td>1.04 ± 0.65</td>
<td>0.010</td>
</tr>
</tbody>
</table>

*BMI, body mass index; DBP, diastolic BP; SBP, systolic BP.*
(n = 60) for the −318G allele were combined, named as G carriers, and compared with those of homozygous individuals for the −318C allele (C/C, n = 593; Table 1). As compared with individuals who carried the C/C genotype, G carriers had higher SBP (117.8 ± 16 versus 113.6 ± 12.6 mmHg; P = 0.010; Table 1). Also, DBP tended to be higher in G carriers as compared with C/C individuals, although the difference did not reach a statistical significance with the present sample size (Table 1). At variance, similar levels of all other features related to IR were observed across the two genotype groups (Table 1). G carriers had a significantly increased risk for hypertension (SBP ≥130 mmHg and/or DBP ≥85 mmHg; odds ratio 2.2; 95% confidence interval 1.1 to 4.5; P = 0.031, after adjustment for age, gender, body mass index, and smoking status).

To investigate the biologic relevance of the promoter −318G SNP, we tested both variants by reporter gene assay in transfected HEK293 cells. After normalization of luciferase activities to the corresponding transfection efficiencies, the −318G promoter had a 2.5-fold reduced transcriptional activity (P = 0.035; n = 6) as compared with the −318C promoter (Figure 1).

Figure 1. GNAI2/−318G has reduced transcriptional activity in reporter assay. HEK293 cells were transiently transfected with −318C/luc, −318G/luc, and pGL 2-Basic. Luciferase activity was normalized for transfection efficiency using a control pSV-β-Galactosidase plasmid and protein concentration. Results are reported as fold increase in relative luciferase activity of the GNAI2 reporter constructs compared with empty control vector pGL2-Basic. Data were obtained from six independent experiments and are expressed as means ± SEM. *P = 0.035 using unpaired t test.

Figure 2. Identification of Sp1 as major transcription factors that bind differently to the hypertension-susceptibility DNA element GNAI2/−318G. (A) Specific protein binding to the hypertension-susceptibility DNA element was analyzed by electrophoretic mobility shift assay in HeLa cell nuclear extracts. The wild-type probe includes C at −318, and the mutant probe includes G at −318. The YY1 and Sp1 transcription factor consensus binding sites specifically compete with the −318 C and −318G DNA element, respectively. For competition analysis, a 200-fold molar excess of unlabeled double-stranded oligonucleotides was added. Cold oligonucleotides for YY1 DNA element (lanes 3 and 7) and the Sp1 consensus-binding site (lanes 4 and 8) specifically competed with protein binding. (B) Sp1 is a major transcription factor that binds specifically to the hypertension-susceptibility DNA element. Antibody against Sp1 and YY1 (negative control) were added to the reaction (lanes 2 and 3). S, supershifted complex with specific nuclear factors and antibodies; A, complex with specific nuclear factors; NS, complex with nonspecific binding nuclear proteins; FP, free probes. These data represent at least three independent experiments.
Electrophoretic mobility shift assay showed a shift of DNA elements that harbored either the C or the G nucleotide at −318 incubated with nuclear protein extracts from HeLa cells (Figure 2A, lanes 2 and 6). Worth noting, analysis of this promoter sequence using the TRANSFAC database (http://www.gene-regulation.com/pub/databases.html#transfac) revealed that transcription factors YY1 and Sp1 are specific binders to −318C and −318G alleles, respectively. In fact, competitor oligonucleotides of YY-1 or Sp1 specifically reduced the binding of nuclear proteins to −318G or −318G allele, respectively (Figure 2A, lanes 3 and 8). Finally, a “supershift” of the nuclear proteins/−318G allele complex was observed after rabbit polyclonal antibody against SP1 but not against YY1 was added (Figure 2B, lane 3).

Discussion

To the best of our knowledge, this is the first report to indicate that a common variation in the GNAI2 gene (the −318G variant in the promoter region) is associated with higher SBP and increased risk for hypertension in the general population. This association is likely to be related to the decreased promoter activity shown by the −318G variant. Regulation of BP involves systemic mechanisms that are related to the renin-angiotensin-aldosterone system (19). The angiotensin II receptor–Gi protein complex stimulates several intracellular signaling pathways, including adenylyl cyclase, leading to decreased cAMP production. An impairment of this system is believed to play a significant role in hypertension both in humans (8,11) and in animals (9,10). It could be hypothesized, therefore, that, as a result of reduced GNAI2 gene transcription and, consequently, decreased Gi2 expression, −318G carriers are characterized by reduced cAMP levels that, in turn, may increase BP. Our functional studies on transfected cells clearly suggest that reduced GNAI2 gene transcription shown by the −318G variant is due to specific binding with Sp1 transcription factor. The role of Sp1 in transcriptional regulation of human genes is well established (20). A deeper understanding of the molecular mechanisms of Sp1 in adversely modulating GNAI2 promoter activity certainly requires additional studies and is beyond the scope of this initial report. Human studies have shown a reduction of G_{i2α} expression in cells from hypertensive insulin-resistant patients (15). Also, studies on genetically modified mice have suggested that G_{i2α} expression is correlated directly with insulin sensitivity (16). Reduced G_{i2α} expression might be expected, therefore, to be associated with IR. In fact, we could not find any association of the −318 G allele with IR and related traits, suggesting a lack of effect of this variant on these specific phenotypes. It is also possible that the effect on IR induced by this SNP is a small one and is modulated by interaction with other genetic and/or environmental factors; larger and more adequately powered samples are needed to unravel it.

Conclusion

In nondiabetic Caucasians from Italy, the GNAI2 −318 C>G SNP is associated with higher BP and with a 2.2-fold increased risk for hypertension. The risk allele decreases promoter transcription activity most likely through DNA binding of Sp1 transcription factor. These data provide evidence for a link between reduced GNAI2 promoter activity and higher SBP. If the association is confirmed in other populations, the GNAI2 −318 C>G SNP may help to identify patients who are at increased risk for hypertension so that preventive programs could be targeted specifically at these individuals early in the course of their life.

Acknowledgments

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References


