Natriuretic peptide receptor 3 genotype modulates the relationship between B-type natriuretic peptide and left ventricular end-diastolic pressure

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Keywords: B-type natriuretic peptide, genetic polymorphisms, left ventricular end-diastolic pressure, natriuretic peptide receptor 3

Background: B-type natriuretic peptide levels are associated with left ventricular end-diastolic pressure (LVEDP) and patient outcomes. There is documented variation in the genetic sequence of natriuretic peptide receptor (NPR)3, which is a primary clearance mechanism for B-type natriuretic peptide (BNP). Methods: DNA was extracted from 147 patients, aged 60 years or over undergoing elective left-heart catheterization for any indication, excluding acute myocardial infarction or severe valvular disease. Genotype was determined at four loci in the NPR3 gene. The logBNP:LVEDP ratio was compared between genotype groups. Linear and logistic regression models of LVEDP were generated.

Results: The logBNP:LVEDP ratio was significantly different among NPR3 IVS2–84 A allele carriers compared with G allele homozygotes (p = 0.008), with the A allele carriers showing a higher BNP level for a given level of LVEDP. The other variants did not alter this relationship (all p > 0.4). When added to the optimal linear regression model for LVEDP, NPR3 IVS2–84 genotype incrementally added to the model (p = 0.024, model r = 0.54). This was also true of the optimal logistic regression model of LVEDP ≥ 20 mmHg (p = 0.026).

Conclusions: NPR3 IVS2–84 G>A genotype is associated with altered logBNP:LVEDP ratio and provides incremental value to predictive models of LVEDP. Further studies should address whether this or other variants in the BNP pathway modify the clinical importance of endogenous or exogenous BNP.

Heart failure is a modern epidemic and is one of few cardiovascular diseases that currently has increasing prevalence [1,2]. The importance of B-type natriuretic peptide (BNP) as a diagnostic and therapeutic modality in heart failure is well known [3,4]. Our group has demonstrated previously that BNP levels are predictive of left ventricular end-diastolic pressure (LVEDP) [5] and risk of hospitalization among elderly cardiac patients [6]. However, the optimal interpretation of native BNP is controversial [7,8]. BNP levels correlate imperfectly with cardiac filling pressures and the factors that govern BNP levels remain incompletely understood [8,9]. Common genetic sequence variants are increasingly being recognized as determinants of disease risk or drug response [10,11] and may help explain a portion of the interindividual variation in the physiology and pharmacology of the human BNP system.

It has been shown that approximately 40% of variation in native BNP levels is heritable [12]. Furthermore, our group and others have described common polymorphisms and/or haplotype structure of key genes in the BNP pathway [13-16], and some of these variants have already shown in vivo and in vitro functional activity [17-20]. Natriuretic peptide receptor 3 (NPR3) is a noncatalytic receptor that specifically binds atrial natriuretic peptide (ANP) and BNP. It is one of two primary clearance mechanisms for natriuretic peptides. Binding leads to peptide uptake and subsequent degradation with receptor recycling. The NPR3 gene is approximately 75 kbp long and is located on chromosome 5 (Figure 1). Several common variants have been documented previously [13,15], although associated phenotypes have not yet been described.

In order to begin to dissect the impact of genetic variation on the BNP system, we set out to assess the impact of sequence variants within NPR3 on the relationship between BNP and LVEDP. We hypothesized that these variants may be associated with an altered BNP:LVEDP ratio and may affect the predictive ability of measured BNP levels.

Methods

Subjects
The study was approved by the Washington University Human Studies Committee. Written informed consent was obtained for all
patients. Population data was obtained from 190 anonymous blood donors who were self-
identified as being of African or European
descent (95 of each). For the clinical study, 147
consecutive patients aged 60 years or older
referred for diagnostic left heart catheterization
at our institution were enrolled in the study.
Patients could be referred for any reason, the
most common being chest pain and/or abnor-
mal stress tests or shortness of breath. Exclu-
sion criteria included abnormal troponin
values, acute myocardial infarction or percuta-
neous coronary intervention within the prior
6 months, any previous cardiac surgery, pres-
ence of an implantable cardioverter defibrilla-
tor, history of known severe valvular heart
disease or hypertrophic cardiomyopathy, end-
stage renal or hepatic disease or unwillingness
to participate. Detailed clinical and demo-
graphic data were collected in all patients,
including catheterization data, standard
echocardiograms and BNP levels. Echocardiog-
graphic data assessed included early (E) and
late (A) mitral inflow velocity, corresponding
tissue Doppler measurements (EM and AM,
respectively), as well as other standard param-
eters. All clinical data were assessed by an inves-
tigator blinded to the genetic data. Patients
were self-identified in terms of race. A total of
122 were self-identified as European descent,
24 were self-identified as African descent and
one subject was from Sri Lanka.

**Polymorphism selection & genotyping**
Polymorphisms were selected using the pro-
gram PolyMAPr (Polymorphism Mining and
Annotation Programs) [21] due to their presence
in publicly available databases and the plausi-
bility of functional consequence based on their
position (i.e., regulatory sequence, coding

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**Table 1. Polymerase chain reaction and Pyrosequencing® primers and conditions.**

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primer</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter -2664 GA</td>
<td>Forward 5'-Bioteg-gtccaaagctgcggagagg-3'</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-cgcgtgtgtgtggtggttta-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Internal 5'-acgcctgtcccatc-3'</td>
<td></td>
</tr>
<tr>
<td>Intron 2 -84 GA</td>
<td>Forward 5'-cgtgtgccatcataactaagtgc-3'</td>
<td>69</td>
</tr>
<tr>
<td>(IVS2–84 G&gt;A)</td>
<td>Reverse 5'-Bioteg-ttattacacgtaccaccacc-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Internal 5'-tgccaaagtaacatgc-3'</td>
<td></td>
</tr>
<tr>
<td>Intron 2 -79 CT</td>
<td>Forward 5'-cgtgtgccatcataactaagtgc-3'</td>
<td>69</td>
</tr>
<tr>
<td>(IVS2–79 G&gt;A)</td>
<td>Reverse 5'-Bioteg-ttattacacgtaccaccacc-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Internal 5'-tgccaaagtaacatgc-3'</td>
<td></td>
</tr>
<tr>
<td>Non-Syn Coding 1561 AG</td>
<td>Forward 5'-Bioteg-ggcactctttgaagaaaaccatg-3'</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-ggggcttcctttaagctactga-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Internal 5'-cgtgttttcccaaggt-3'</td>
<td></td>
</tr>
</tbody>
</table>

*Bioteg: 5'-biotin tag required for Pyrosequencing®.*
sequence or within intron/exon splice sites). Specifically the -2664 G>A variant was chosen because it was in the 5´ flanking region possibly representing a regulatory sequence; two variants within intron 2 were chosen owing to their proximity to the 30´ splice site (84 and 79 bp upstream, respectively); the 1561 A>G variant was chosen because it was the only common nonsynonymous coding variant identified. Genomic DNA was isolated using the PureGene® extraction kit (Gentra). Genotyping was accomplished using Pyrosequencing® (Biotage). DNA containing the region of interest was amplified with the polymerase chain reaction (PCR). PCR primers were designed using Primer Express® version 1.5 (ABI). Pyrosequencing primers were designed using the Pyrosequencing SNP Primer Design Version 1.01 software [101]. Pyrosequencing was carried out as previously described using the PSQ hs96A instrument and software [22]. Primers and reaction conditions are shown in Table 1. Genotype calls were made by the Pyrosequencing software with review by an investigator unaware of the clinical data. Pairwise linkage (D´) and haplotype analysis was carried out using the Polymorphism and Haplotype Analysis Suite [23,102] among African–Americans and European–Americans separately.

**Statistics**

Hardy–Weinberg equilibrium and population genotype frequencies were assessed using the chi square statistic. The logBNP:LVEDP ratio was compared between genotype groups using the Mann–Whitney or Kruskall–Wallace tests, as appropriate. This ratio was used (as opposed to BNP level alone) due to the strong influence LVEDP is known to have on BNP levels and since it would serve to adjust for differences in loading conditions between patients. Log transformation of BNP was used because it provided a more normal distribution and a stronger bivariate association with LVEDP in the entire cohort than raw BNP level. Baseline characteristics were compared between IVS2–84 genotype groups using the Mann–Whitney test or Fisher exact test, as appropriate.

Regression models for LVEDP (continuous) and LVEDP 20 mmHg or more (dichotomous) were generated using linear and logistic regression, respectively. Variables with bivariate associations with LVEDP with a p value of less than 0.1 were considered for regression modeling. Possible input variables were grouped according to subtype (echo, clinical, demographic and laboratory) and the multivariate predictors from each subgroup were then identified for inclusion into regression models. The final regression models were constructed from multivariate predictors with p values of 0.05 or less. All analyses were carried out using SAS version 9.1.3.

**Results**

The polymorphisms under consideration were first validated in population samples of 95 African Americans and 95 European Americans. Resulting genotype frequencies are shown in Table 2. All four polymorphisms had minor allele frequencies of 4% or higher in both populations. There were significant differences in genotype frequency between races for the -2664 G>A and 1561 A>G variants (p = 0.037 and p = 0.045, respectively). The 1561 A>G and IVS2–79 C>T polymorphisms were in strong linkage disequilibrium among European–Americans (D´ = 0.84, p < 0.001), but only weakly among African–Americans (D´ = 0.19, p = 0.044). The IVS2–79 C>T polymorphism was outside Hardy–Weinberg equilibrium in African–Americans only (p < 0.001).

Baseline and demographic description of the overall clinical cohort (and divided by NPR3 IVS2–84 G>A genotype) is summarized in Table 3. Genotype was obtained for 98–100% of subjects for each polymorphism studied.

### Table 2. NPR3 genotype frequencies among 190 population samples.

<table>
<thead>
<tr>
<th>Polymorphism (dbSNP number if listed)</th>
<th>Genotype</th>
<th>Freq (%) AA</th>
<th>Freq (%) EA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promotor</td>
<td>G/G</td>
<td>74</td>
<td>53</td>
</tr>
<tr>
<td>-2664 G&gt;A*</td>
<td>G/A</td>
<td>26</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Intron 2</td>
<td>G/G</td>
<td>89</td>
<td>91</td>
</tr>
<tr>
<td>IVS2–84 G&gt;A (rs2292025)</td>
<td>G/A</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Intron 2</td>
<td>C/C</td>
<td>83</td>
<td>70</td>
</tr>
<tr>
<td>IVS2–79 G&gt;A (rs2292026)</td>
<td>C/T</td>
<td>11</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Non-syn coding</td>
<td>A/A</td>
<td>80</td>
<td>57</td>
</tr>
<tr>
<td>1561 A&gt;G* (rs2270915)</td>
<td>A/G</td>
<td>19</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>G/G</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

95 AA and 95 EA subjects were used in the study.

*p-value < 0.05 for comparison between races.

In order to take into account other factors that may affect the BNP–LVEDP relationship and to assess the clinical utility of this genotype (for noninvasive determination of volume status), we assessed the incremental value of IVS2–84 G>A genotype in regression models for prediction of LVEDP. Optimal noninvasive models were generated using all collected data, excluding the genetic data [5]. LogBNP was one of the strongest predictors of LVEDP. The optimal linear regression model for LVEDP included echocardiographic mitral E:A ratio, body mass index and logBNP. The optimal logistic regression model for predicting LVEDP 20 mmHg or over included body mass index, heart rate and logBNP as input variables. When added to these linear and logistic models, logBNP:LVEDP ratio by race (European American vs African–American, p = 0.98, 0.39 and 0.35, respectively). The NPR3 IVS2–84 genotype groups differed only in their assessment of left ventricular (LV) systolic function, with A allele carriers tending to have slightly worse LV function on average (LV function grade 1.4 vs 1.9; p = 0.03).

Of the four polymorphisms examined, only the IVS2–84 G>A variant was significantly associated with logBNP:LVEDP ratio (p = 0.008; all others p > 0.4) (Figure 2). No A allele homozygotes were identified in the study population. As indicated above, this variant was not significantly linked to any of the other variants in either African–Americans or European–Americans.
IVS2–84 G>A genotype incrementally added to both models (p-values 0.025 and 0.026, respectively) (Table 4). Since the severity of left ventricular dysfunction was associated with genotype (p = 0.03) (Table 2), we also tested models that included this variable (in addition to the other independent variables above). This did not significantly alter the results (p = 0.036 and 0.044 for linear and logistic models, respectively).

Discussion
Our data indicate a gene–environment interaction, where NPR3 IVS2–84 G>A genotype is associated with changes in the BNP-LVEDP relationship and incrementally adds to noninvasive prediction of LVEDP. This is the first report of a genetic variant affecting this relationship or helping to predict LVEDP noninvasively. Noninvasive prediction of LVEDP is important because LVEDP is an important indicator of cardiac physiological state and pathology, and currently it can only be measured invasively. The most common surrogate measure, the pulmonary capillary wedge pressure, also requires an invasive procedure. Noninvasive data, such as echocardiographic parameters and BNP levels, have been previously correlated with cardiac filling pressures, but our data are the first to demonstrate incremental value from genetic analyses for predicting elevations in left ventricular filling pressures.

The fact that the A allele carriers demonstrated higher BNP levels for each level of LVEDP could suggest impaired clearance receptor function in these subjects relative to G allele homozygotes. The possible functional consequences of this variant have not, to our knowledge, been explored and, hence, the mechanism of this effect remains unexplained. Since this variant is intronic, one could hypothesize that it is in linkage disequilibrium with the true functional variant, in which case the true functional variant remains to be identified. Alternatively, the IVS2–84 GA genotype may alter mRNA splicing or stability. However, despite lying relatively close to a splice junction, it does not alter a consensus splice donor/acceptor sequence and is not predicted to be a functionally significant variant by the commonly used computer algorithms JASPAR or ESEfinder (24,25,103,104).

Our study has significant limitations. Not all factors affecting BNP levels or ventricular pressure could be accounted for, but we have attempted to characterize our population in terms of the commonly accepted clinical and physiological factors that may affect BNP LVEDP and their relationship. Given the size of our cohort, the fact that it is an experience from a single center, and that only patients aged over 60 years were studied, one must use caution when interpreting the findings and applying them to other patients.

Our findings have potential implications for BNP-based diagnostic testing and therapeutic applications. The fact that a variant in a BNP pathway gene is associated with alterations in BNP’s predictive ability for LVEDP suggests that this variant, along with others in relevant genes, may improve the predictive abilities of BNP for diagnosis and prognosis in heart failure. For example, currently, there is a range of BNP values that don’t clearly indicate the presence or absence of heart failure. If the genetic variation in the BNP pathway is better understood it may allow clinicians to more clearly interpret the significance of BNP levels since the individual’s genetic make-up will inform what values are expected for that person. In addition, since the IVS2–84 variant we identified lies within the clearance receptor, it could impact dosing and adverse events when using BNP infusions as heart failure therapy. This is especially important in light of the recent reports highlighting the potential for adverse impact of exogenous BNP therapy, such as hypotension and renal dysfunction (26). Further study...
of the functional impact of this variant, as well as broader genetic characterization of the entire BNP pathway, is required in order to optimize the clinical use of BNP levels and improve targeting of exogenous BNP therapy.

Acknowledgements
All authors contributed to this research and preparation of the manuscript. The authors would like to thank Derek Van Booven for extraordinary informatics support. Genotype data has been submitted to PharmGKB (submission PS204841; www.PharmGKB.org).

Conflict of interest
This work was supported, in part, by a Society of Geriatric Cardiology Clinical Research Grant, a NIH Pharmacogenetics research network (U01 GM63340) and a Heart Failure Society of America Research Fellowship Grant.

Table 4. Linear and logistic regression models of LVEDP.

<table>
<thead>
<tr>
<th>Factor</th>
<th>p-value</th>
<th>Factor</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDP (linear)</td>
<td></td>
<td>LVEDP ≥ 20 mmHg (logistic)</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>0.0011</td>
<td>BMI</td>
<td>0.0009</td>
</tr>
<tr>
<td>Echocardiographic E:A ratio</td>
<td>0.0051</td>
<td>Heart Rate</td>
<td>0.0251</td>
</tr>
<tr>
<td>logBNP</td>
<td>0.0001</td>
<td>logBNP</td>
<td>0.0038</td>
</tr>
<tr>
<td>NPR3 IVS2–84 genotype</td>
<td>0.0246</td>
<td>NPR3 IVS2–84 genotype</td>
<td>0.0262</td>
</tr>
</tbody>
</table>

Model r = 0.54 (Linear)
BMI: Body mass index; BNP: B-type natriuretic peptide; E:A ratio: Early to late ventricular filling ratio; LVEDP: Left ventricular end-diastolic pressure; NPR: Natriuretic peptide receptor.

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