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Association of genetic variation, gene expression, and protein abundance within the natriuretic peptide pathway

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INTRODUCTION

Heart failure (HF) continues to be an enormous public health problem, despite the many advances in its pharmacotherapy over the past 25 years, with a prevalence of 5.7 million individuals affected and an incidence of over 500,000 new cases annually.[1] The relevance of the natriuretic peptide (NP) system, particular BNP, is well known in terms of HF pathophysiology,[2, 3] diagnosis,[4] prognosis,[5] and therapy.[6, 7] However, the full impact of testing, and particularly modulating, the NP pathway remains unclear. Part of this difficulty in how to harness this pathway for the benefit of patients is due to substantial inter-individual variability in function of the NP pathway. Not only are the optimal diagnostic and prognostic thresholds uncertain and varying,[8] but the response to extrinsic NP is highly variable with potential for adverse effects and unclear therapeutic range.[9-11] Better understanding of the variability in this important pathway is critical not only because it continues to be explored as a method for personalized therapy, but because there are numerous current (nesiritide, carperitide) and investigational therapeutics (ularitide,[12] CDNP[13]) targeting it.

Genetic variation may hold a key to better understanding this individual variability.[14] BNP levels are known to be heritable,[15] and specific genetic variants in NP pathway genes have been associated with hypertension,[16] BNP level and test performance,[17, 18] and intracardiac filling pressures,[19]. Despite these observations, a lack of systematic knowledge remains with respect to the effects of NP genetic variability on the production of relevant protein end-products. Better understanding of this variability may allow it to be used to personalize therapy by identifying differences in how patients metabolize and respond to NPs. Relevant to HF, NPs act primarily (Figure

1) by binding to two membrane-spanning receptors called natriuretic peptide receptor (NPR) A and NPRB, which are guanylate cyclases resulting in cyclic guanlyate mono phosphate (cGMP) production. The latter is thought to be the key second messenger mediating the NP effects. Active NP is broken down by neutral endopeptidase (aka membrane metallo-endopeptidase (MME)), and taken up by NPRC, another receptor which lacks guanylate cyclase function. These four proteins are produced by the genes *NPR1, NPR2, MME* and *NPR3* respectively. The purpose of this study was to systematically study sequence variants in these genes, quantify gene expression and protein abundance of each product in relevant human tissue samples in order to evaluate important associations.

METHODS

The study was approved by the Henry Ford Hospital Institutional Review Board. DNA, RNA, and tissue samples from human kidney (n=77) were obtained via the Alvin J. Siteman Cancer Center at Washington University School of Medicine and Barnes-Jewish Hospital in St. Louis, Mo., Tissue Procurement Core lab, under approval from Washington University Institutional Review Board and with informed consent. Kidney was chosen as target tissue because each of the four candidate genes and proteins (natriuretic peptide receptor (NPR1), NPR2, NPR3 and membrane metallo-endopeptidase (MME)) are expressed there. 100 samples were originally requested, to be distributed evenly among African Americans vs Whites and men vs. women. In total 103 suitable samples were tested.

Genotyping, Gene Expression, and Protein Quantitation

DNA samples were genotyped using a custom Illumina Goldengate 1536-plex array which contained candidate-gene coverage relevant to HF including focused attention on the four genes of interest. Single nucleotide polymorphisms (SNPs) were chosen for the array by attempting to include all coding variants, and then adding all non-coding variants to capture alleles with MAF >0.1 prevalence in Caucasians or Africans within the gene regions of interest. After processing requirements for the Goldengate technology and quality control of genotyping we were left with 118 SNPs in the four genes of interest for this study. Genotyping was auto clustered and then individual SNPs were reviewed manually. Call rates for all samples were >90%. mRNA

expression was quantified for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (comparator), NPR1, NPR2, NPR3, and MME using real-time reverse transcriptase polymerase chain reaction (RT-PCR) performed in duplicate for each sample. To determine the concentration of the protein targets, tissue samples were made into lysates and then assayed using double antibody sandwich Enzyme Linked Immunosorbant Assays (ELISA). The samples were homogenized by suspending in 1ml phosphate buffered saline solution and then sonicated. The resulting suspension was centrifuged for 5 minutes at 5000g. The supernatant was then removed and stored at -80°C for testing. The concentration of each marker was assayed using commercially available assay kits (Uscn Life Science Inc., Missouri City, Texas) according to manufacturer protocol and using standard curves and software. Total protein concentrations were determined by using a modified Lowry Protein Assay. The ratio of target protein to total protein was reported and tested for association with genotype or RNA quantity.

Statistical Analysis

Following log transformation of the protein and RNA expression data, linear regression was used to test for the association of each SNP with RNA and protein quantity under an additive genetic model. A principal components (PC) based method was also used to capture the underlying correlation structure within each locus and test the association of overall gene variation with RNA and protein quantity. We selected top PCs that explain at least 80% of the variation as the gene representation and the PCs were used

as covariates in the linear regression to test for association of SNPs with RNA and protein expression. All models were adjusted for gender and race. P values <0.05 were considered of possible interest in this exploratory study. To account for multiple comparisons we also utilized the method of Hochberg[20] and considered findings with false discovery rate (FDR) <0.05 significant.

RESULTS

Genotype was obtained in 7 loci in *NPR1*, 18 loci in *NPR2*, 53 loci in *NPR3*, and 40 loci in *MME*. Each site was tested individually for association with RNA and protein quantity with summary results shown in Figure 1. In terms of gene expression, several variants in *MME* and *NPR3* showed crude associations with unadjusted $p<0.05$. For there were four SNPs in *MME* (rs1025192, rs1436630, rs10513469 and rs1816558) and one in *NPR3* (rs696831) that showed suggestive associations with RNA levels ($p \leq 0.05$). However, none of these met significance once adjusted for FDR. There were no significant associations of genotype with gene expression for *NPR1* or *NPR2*.

Considering protein quantitation, we assayed each sample for the ratio of specific protein of interest (NPR1, NPR2, NPR3, and MME) to total protein, and then tested the association of this ratio (i.e. the target protein abundance) with genotypes within the corresponding gene. There were no significant associations of genotype with NPR1 and protein abundance. There were two sequence variants in *NPR3* (rs696836, rs2062708) and one in *MME* (rs3773895) with significant associations of genotype with protein quantity; however these did not withstand adjustment for multiple comparisons. Interestingly, eleven SNPs in *NPR2* were significantly associated with protein expression ($p<0.05$) and this association persisted after controlling for FDR at 0.05. Boxplots of protein abundance by genotype for each significant loci is shown in Figure 2. There were no SNPs associated with both RNA and protein expression in any of the candidate genes.

RNA and Protein quantity poorly correlated with each other; NPR1 and MME showed weak but statistically significant positive correlations (Pearson's correlation coefficient=0.23 and 0.26, p = 0.04 and 0.03, respectively) while NPR2 and NPR3 did not (Figure 3). The PC analyses were broadly consistent with the individual SNP analysis above. PC1 of *NPR2* (which accounted for 71% of genetic variability) was the only significant association of genetic variation with protein abundance (p=0.04). The factor loadings for PC1 (data not shown) suggest that it is mainly determined by the same 11 SNPs above, which each had equally high weight. We also found an association between PC5 of *NPR3* and its gene expression (p=0.0084). The loadings of SNPs indicates that PC5 is highly contributed by SNPs rs764124, rs1847018, rs10057069, rs6889608, rs696831, and rs2302954.

DISCUSSION

Our systematic interrogation of genotype, gene expression, and protein quantity correlations reveals that genetic variation may play a role in determining protein abundance for NPRB. Interestingly, these associations did not seem to occur via changes in gene expression, which did not correlate to either protein quantity or genotype for *NPR2*. The other genes tested did not show indications of genetic variation importantly effecting gene expression or protein abundance in kidney.

Although there have been numerous studies examining the relationship of NP pathway genetic polymorphisms to clinical phenotypes, corresponding functional data is less available. While our study is exploratory and descriptive in nature, these data add to the existing knowledgebase by describing the potential physiologic impact of candidate variants on gene and protein expression, and prioritizing these for future investigation. These data could be used to buttress the biologic plausibility of previously described clinical phenotypes, and in terms of prioritizing variants for subsequent clinical interrogation *NPR2* appears to be the best target. While there were some interesting genotype: gene expression associations for other pathway candidate genes, these did not meet significance and did not correlate to protein abundance.

There are several limitations of this study that should be considered when evaluating these data. First is the relatively small sample size, limiting the power somewhat; for example we estimate 90% power to detect at least 2.5-fold variation and minor allele frequency of 0.2. While high-throughput methods are available for DNA sequencing, real-time PCR, and more importantly protein quantification, remain labor

intensive and impractical for very large sample sizes. Our sample size was designed to accommodate this limitation and indentify robust variation, not very subtle changes in protein or gene expression. Second is that we examined only kidney tissue, and cannot deduce information about gene and protein expression in other tissues that may be of interest such as cardiac tissue. However, kidney was felt to be the best choice when considering the NP pathway physiologically, and as it pertains to pharmaceuticals (such as recombinant NPs or endopeptidase inhibitors), because it is a key location for both clearance and effect of NPs, and there is expression of all the candidate genes. Another potential concern is that renal tissue is not homogenous; how whether and how this impacted on our findings is unknown. Finally, we have focused on protein quantitation and have not tested protein function. This remains important investigation for follow up studies.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Graphical presentation of p-values for testing SNP association with gene expression and protein expression for *NPR1, NPR2, NPR3, MME*

Figure 2. Target protein abundance by genotype in the 11 statistically significant SNPs of *NPR2* (FDR≤0.05).

Figure 3. Scatter plot showing the relation between RNA and protein quantity for each gene.

Figure 1.

Figure 2. Target protein abundance by genotype for *NPR2* (loci with FDR≤0.05). (0,1,2) represents the additive coding of the number of copies of the minor allele.
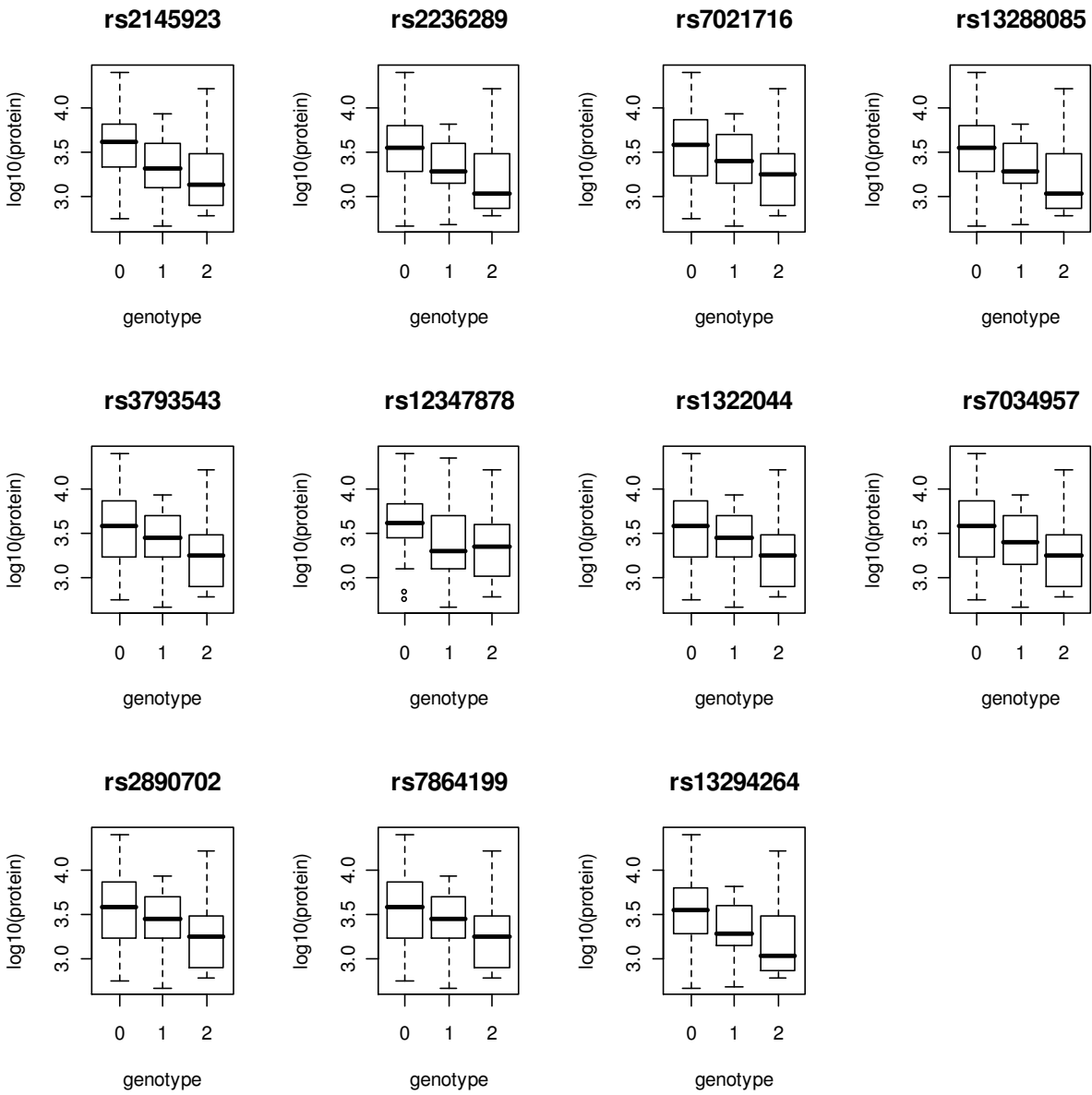
Figure 3. Scatter plot showing the relation between RNA and protein quantity for each gene. Solid curve represents the smooth fit to better visualize the trends. The fit was generated using locally weighted scatter plot smoother (LOWESS).