European Journal of Human Genetics (2007) 15, 878–883

www.nature.com/ejhg

ARTICLE

Genotypic and haplotypic associations of the *DBH* gene with plasma dopamine β-hydroxylase activity in African Americans

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Several variants at *DBH* are significantly associated with plasma DβH activity (pDβH). However, the overwhelming majority of data on this genotype–phenotype relationship has been gathered in samples from Europeans and European Americans (EAs). In this study, we examined the relationship between *DBH* polymorphisms and pDβH in samples from African-American (AA) subjects. Genotypes were determined at a 19-bp insertion/deletion polymorphism (ins/del) and four single-nucleotide polymorphisms (SNPs) at *DBH* in 109 samples. Analyses were performed using analyses of variance (ANOVAs) (for individual SNPs) and regression procedures (to assess the joint effects and the specific SNP-based haplotypes). We found: (1) single-variant analysis of all polymorphisms revealed apparent associations to pDβH, with rs1611115 accounting for the largest proportion of the variance in pDβH (28.7%) and ins/del the smallest (6.5%); (2) modest but significant linkage disequilibrium (LD) existed between ins/del and rs1611115; (3) LD between all other pairs of variants was not observed; (3) stepwise regression showed that a model containing rs1611115, rs2519152 and rs6271 accounted for 37.6% of the variance in pDβH, with rs6271 showing additional 7.6% above the effect of rs1611115, and rs2519152 showing additional 2% above rs1611115 and rs6271; (4) two common haplotypes, C-T-C and T-C-C at rs1611115-rs2519152-rs6271 were significantly associated with pDβH ($P = 0.0025$ and 0.0036, respectively). The data support the validity of prior reported associations and underscore the importance of analyzing multiple SNPs across *DBH* in future association studies examining disease and biochemical phenotypes.
European Journal of Human Genetics (2007) **15**, 878–883; doi:10.1038/sj.ejhg.5201838; published online 25 April 2007

Keywords: dopamine β-hydroxylase; African American; linkage disequilibrium; genetic association; haplotype

Introduction

Dopamine (DA) β-hydroxylase (DβH) catalyzes the conversion of DA into norepinephrine (NE) and is localized within vesicles of central noradrenergic and adrenergic neurons as well as peripheral noradrenergic (sympathetic) neurons and adrenomedullary neurosecretory cells.[1] DβH enzyme activity and DβH immunoreactive protein are measurable in the serum (or plasma) because the enzyme is released from vesicles during sympathetic activity.[2–4]

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Received 18 October 2006; revised 23 February 2007; accepted 22 March 2007; published online 25 April 2007

Plasma DβH activity (pDβH) is a genetic trait controlled by only a few genes.[1,5] Most of the variance in pDβH is explained by variation in plasma levels of DβH protein, as shown by strong correlations ($r \geqslant 0.80$) between pDβH and plasma levels of DβH immunoreactive protein.[3,4,6]

pDβH is a highly heritable trait. Abundant evidence indicates that the structural gene encoding the protein, *DBH*, regulates much of the genetic variation in the trait (reviewed by Cubells and Zabetian[16]). Prior work has shown several polymorphisms at *DBH* to be associated with pDβH.[7–14] These polymorphisms include an insertion/deletion polymorphism located approximately 3 kb 5′ to the DBH transcriptional start site.[7] Another polymorphism is −1021C→T (rs1611115), a single-nucleotide polymorphism (SNP) located approximately 1 kb upstream of the transcriptional initiation codon of *DBH* that accounts for 31–52% of the variance in pDβH in populations from diverse geographic origins.[11,15] In European Americans (EAs) and ethnic Germans, rs1611115 resides within a haplotype block (defined as described by Zabetian *et al*[11]) extending from approximately −2200 bp through intron 4 (~9.9 kb), but the boundaries of the block remain imprecisely mapped. Rs1611115 accounted for a greater proportion of the variance in pDβH than any of 10 other common diallelic variants at *DBH* examined in that study.[12] Linkage disequilibrium (LD) between each of those variants and rs1611115 appears to be responsible for the association of those variants to pDβH.[12] Another SNP, rs6271, is located in exon 11 and encodes a nonconservative change in predicted primary amino-acid sequence (arg535cys). Rs6271 is not in LD with rs1611115, and appears to be the only one of four common nonsynonymous SNPs at *DBH* that independently accounts for additional variance in pDβH after accounting for the effect of rs1611115.[11] This observation is consistent with computer modeling suggesting that of the four nonsynonymous SNPs, only rs6271 predicts a substantial change in protein tertiary structure.[16] Besides the above three polymorphisms, another SNP, rs2519152, located in intron 5 of *DBH* and easily genotyped by restriction digest with the endonuclease *Taq* I[17,18] is also of interest because several studies have observed nominally significant associations between this SNP and attention-deficit/hyperactivity disorder (ADHD).[19–21] In addition, this SNP was found to associate significantly with pDβH in EAs, even after accounting for the associations of rs1611115 and rs6251 to the phenotype.[14]

Although we have previously demonstrated a robust association between rs1611115 and pDβH in African Americans (AAs),[11] data on independent associations of other variants with pDβH in AAs have not been reported. This paper investigates the association between the four polymorphisms with pDβH, the haplotype and LD structure of *DBH* in AA subjects and the possible specific haplotype associated with pDβH. We examined LD among the four SNPs, and used stepwise regression to evaluate whether polymorphisms other than rs1611115 account for significant variance in pDβH after controlling for the effects of rs1611115.

Methods and materials

Samples

The samples for this study were 109 self-identified AA subjects, recruited in the New Haven, CT area, who met criteria for a variety of substance use or psychiatric disorders. As discussed elsewhere, it is unlikely that differences in psychiatric diagnosis or drug use[9] substantially alter the relationship between *DBH* genotype and pDβH. Some of the participants had psychiatric symptoms at the time of assessment, but none were impaired to the point of being unable to provide informed consent for genetic studies, as approved by the Human Investigations Committee of Yale University, or the Human Subjects Subcommittee of the VA Connecticut Health Care System. On average, the subjects were 40.9 (SD = 8.6)-years old, and 71 (65.1%) were male. Some of the genotypes for rs1611115 and rs6271 (51% of the sample) had been generated already for a previous study, and the association results for rs1611115 (not for rs6271) were reported previously,[11] but all of the data for ins/del and rs2519152, about half of the data for rs1611115 and rs6271 are newly reported here. In addition, results from the stepwise regression analyses, as well as the haplotype-based regression analyses, are new to this report.

Biochemical, bioinformatic and molecular analysis

Heparinized plasma was separated from the cellular fraction and assayed for DβH activity as described in detail elsewhere.[9]

The *DBH* gene contains 12 exons that span approximately 23 kb of human chromosome 9 (Figure 1). A schematic representation of the *DBH* gene, showing the relative positions of the SNPs, is shown as Figure 1 below.

We extracted DNA as described previously.[22] Genotypes at rs2519152 were determined by PCR amplification followed by digestion with *Taq* I and agarose gel electrophoresis, as described elsewhere.[19] The primers were: 5′-CTGTATTTG GAACTTGGCATC-3′ (forward) and 5′-AGG CATTTTACTACCCAGAGG-3′ (reverse). Methods for genotyping the other SNPs have been published previously[7,9,11] and are available from the corresponding author (JFC).

Statistical analysis

LD measures D' and r^2 were calculated for all possible combinations of the SNPs analyzed using Haploview.[23]

We used analyses of variance (ANOVAs) to examine the individual effects of the four SNPs on pDβH and used stepwise linear regression to consider the joint effects of

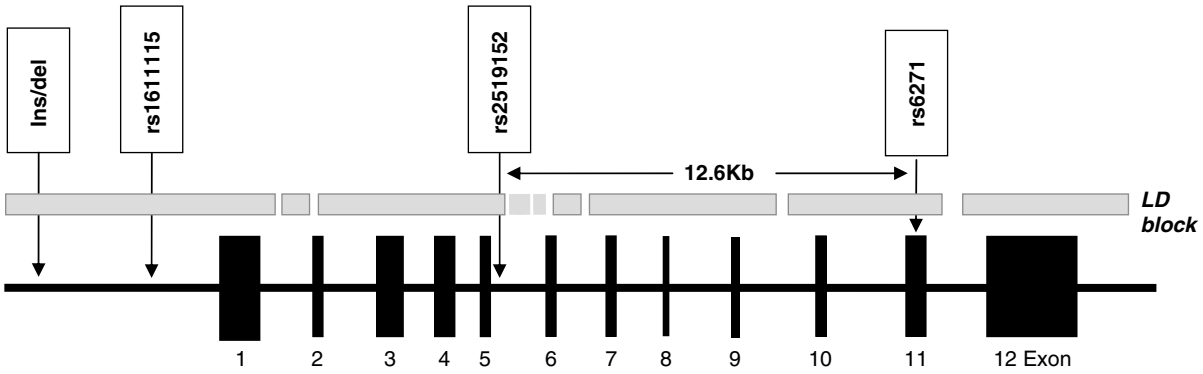

Figure 1 Schematic representation of the DBH gene located on human chromosome 9q34. ins/del, insertion/deletion of 19 bp. The LD structure in Yoruban population from HapMap database, as analyzed by the SNP Browser, is also shown as gray blocks.

Table 1 Association of individual diallelic polymorphisms at *DBH* to plasma DβH activity

Genotype	*Ins/del*	*rs1611115*	*rs2519152*	*rs6271*[a]
1	5.65 ± 2.24 (ins/ins = 28)	1.55 ± 1.23 (TT = 8)	5.47 ± 1.83 (CC = 17)	—
2	4.45 ± 1.90 (ins/del = 64)	3.71 ± 1.44 (CT = 28)	5.33 ± 1.87 (CT = 41)	7.22 ± 1.80 (CT = 6)
3	3.81 ± 1.76 (del/del = 17)	5.36 ± 1.91 (CC = 73)	3.85 ± 2.05 (TT = 51)	4.54 ± 2.00 (CC = 103)
ANOVA	$F(2, 106) = 5.52$	$F(2, 106) = 22.19$	$F(2, 106) = 8.32$	$F(1, 107) = 10.50$
	$P = 0.007$	$P = 8.9 \times 10^{-9}$	$P = 0.0004$	$P = 0.002$
Variance explained (R^2)[b]	0.065	0.287	0.116	0.082

Abbreviation: ANOVA, analysis of variance.
[a]There were no subjects with TT genotype at this locus.
[b]After adjustment for age and sex.

these polymorphisms on the trait. Before analysis, we transformed pDβH to follow an approximate normal distribution using a square-root transformation.[11,24] Square-root pDβH was therefore the dependent variable, and genotypes at the *DBH* SNPs the independent variables. For the stepwise regression procedures, we tested the incremental contribution of rs2519152, rs6271 and ins/del over and above rs1611115 in explaining variance in square-root pDβH. We explicitly tested the contributions of the SNPs in the following order: rs1611115, rs6271, and rs2519152 and ins/del, because our previous work demonstrated the independent contribution of rs1611115 and rs6271 to variance in DβH activity in samples from EAs.[11,13]

For haplotype analysis, we used a variation of linear regression[25] implemented in the WHAP program[26] to estimate haplotype frequencies and to assess the effects of specific SNP-based haplotypes on the square root of pDβH. Proper inference required the use of an EM algorithm to accommodate the haplotype ambiguity in the genotype data. We established empirical significance of these haplotype effects using permutation procedures.

Results

Hardy–Weinberg Equilibrium (HWE) was evaluated for each SNP using the website based analysis from the website of Human Genetics, Munich (ihg.gsf.de). No genotypic proportions deviated significantly from HWE (all $P > 0.047$).

pDβH associates with genotypes at three different *DBH* SNPs

The association of each individual polymorphism to square-root plasma DβH activity (mean ± SD; numbers of subjects with each genotype given in parentheses) is shown in Table 1. Because some of the data for rs161115 were reported earlier,[11] we also examined the association of rs161115 to pDβH just in the 55 new samples. Consistent with the results from analysis of the combined group ($N = 109$), and with the original data reported by Zabetian *et al*.[11] There was a significant effect of genotype ($F = 7.035$, $P = 0.002$) in the new sample: Subjects with CC had the highest square-root plasma DβH activity ($N = 36$, 4.56 ± 1.65), followed by subjects with CT ($N = 14$, 3.96 ± 1.61) and TT ($N = 5$, 1.35 ± 0.60). Comparisons of mean square-root pDβH within genotype group from the original and new samples showed no significant differences (all $P > 0.2$). To maximize statistical power, the remaining analyses were therefore performed in the combined sample. As seen in Table 1, each polymorphism was by itself significantly associated with square-root pDβH, with the proportions of variance explained (as indexed by R^2) ranging from 0.065 to 0.29. Consistent with earlier

findings,[12] rs1611115 explained the greatest proportion of the variance.

Linkage disequilibrium analyses and haplotypes

We used the program Haploview[23] to calculate the LD measures D' and r^2 for all possible combinations of the SNPs. The results are shown in Figure 2. There was weak LD between rs1611115 and ins/del measured by D' (0.54), but no LD was observed among other polymorphisms; there was no substantial LD between any two polymorphisms as measured by r^2 (all $r^2 < 0.008$).

Simultaneous analysis of the contributions of all 4 SNPs

We performed a stepwise linear regression by using square root of pDβH as the dependent variable, with age and sex as covariates. Genotypes at rs1611115, rs2519152, rs6271 and ins/del, respectively, were then introduced into the model (coded in additive model) in that order, as suggested by previous results in EAs.[11,13,14] As shown in Table 2, three out of the four polymorphisms remained in the models, with rs1611115 explaining the largest proportion of variance (28.7%), and rs2519152 and rs6271 independently accounting for additional variance in pDβH (7.5 and 2.4%, respectively). The maximum value of the collinearity diagnostic parameter, variance inflation factor (VIF) was 1.095 in model 3, suggesting there was minimal collinearity among the three polymorphisms that entered the model.

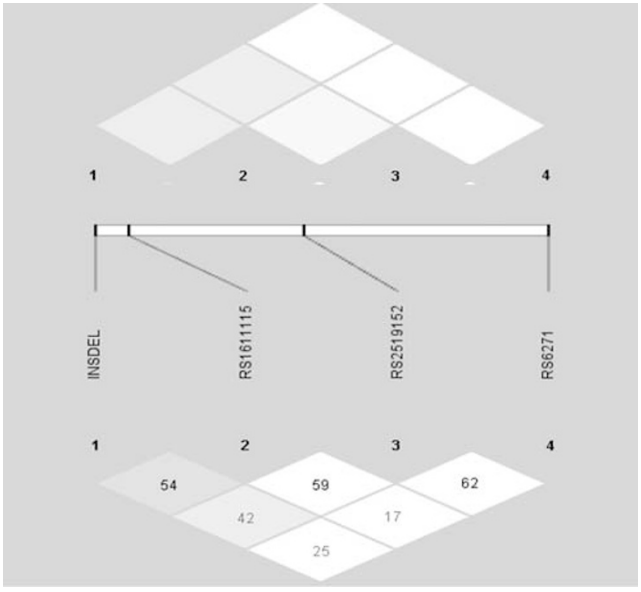

Figure 2 Pairwise LD among five polymorphisms in AA subjects measured by r^2 (above) and D' (below). There was weak LD between ins/del and rs1611115 measured by D', but no LD observed among other polymorphisms; there was no LD between any two polymorphisms observed by r^2.

Three-SNP haplotype analyses

We used the program WHAP[26] to calculate the haplotype frequencies of the three SNPs (rs1611115, rs2519152 and rs6271) because ins/del did not have an independent effect on pDβH. As shown in Table 3, the most common haplotype is C-C-C (~47%), followed by C-T-C (~31%) and T-C-C (~18%). Those three haplotypes account for ~94% of the observed haplotypes and only one of them carries the low DβH activity associated T allele at rs1611115. Table 3 also shows that the haplotypes C-T-C and T-C-C, were significantly associated with pDβH ($P = 0.0025$ and 0.0036, respectively).

Table 3 Haplotype analyses of square root of pDβH in AA subjectsΔ

Haplotype rs1611115-rs2519152-rs6271	*Frequencies*	*Association with pDβH (P)*
C-C-C	0.474	—
C-T-C	0.306	0.0025
T-C-C	0.176	0.0036
T-T-C	0.029	0.209
C-T-T	0.015	0.246

ΔData were analyzed by the program WHAP (URL: http://pngu.mgh.harvard.edu/~purcell/whap/). All *P*-values were based on 10 000 permutations

Table 2 Model Summary of linear regression using stepwise model

Model	*R*	R^2	*Adjusted* R^2	*SE of the estimate*	*Change statistics*					*VIF*
					R^2 *change*	*F change*	*d.f.1*	*d.f.2*	*Significant F change*	
1	0.541[a]	0.293	0.287	1.75859	0.293	44.373	1	107	1.2×10^{-9}	1.000
2	0.611[b]	0.374	0.362	1.66285	0.081	13.676	1	106	0.0003	1.001
3	0.635[c]	0.403	0.386	1.63095	0.029	5.187	1	105	0.025	1.012–1.095

Abbreviations: d.f., degrees of freedom; SE, standard error.
[a]Predictors: (constant), genotype at rs1611115.
[b]Predictors: (constant), genotype at rs1611115, rs6271.
[c]Predictors: (constant), genotype at rs1611115, rs6271 and rs2519152.
VIF, variance inflation factor.

Discussion

This study examined the relationship between DBH gene polymorphisms and pDβH in AAs more closely than our prior study,[11] in a larger set of samples. Our results, both in the combined group ($N=109$) and in the group of new samples ($N=55$), replicated the association between SNP rs1611115 and pDβH reported in the prior AA sample.[11] Results from analysis of additional polymorphisms showed apparently significant associations between pDβH and each of the three polymorphisms, including a 19-bp insertion/deletion and three SNPs. However, the failure of the former polymorphism to remain in the regression model after controlling for other genotypes strongly suggests the apparent difference in activity associated with this variant can be explained entirely by LD to rs1611115.

As in subjects of European origin,[11,13,14] rs1611115 explained the largest proportion of variance in pDβH, However, as we reported previously,[11] rs1611115 accounts for a lower proportion of the variance in pDβH than in subjects of European origin. Also in accordance with previous results from EAs,[14] rs2519152 and rs6271 appear to account for additional variance in pDβH independent of rs1611115. Finally, the data reported here are the first to examine the haplotype structure of *DBH* in an AA sample. It should be noted, however, that more detailed studies are necessary, as the variants examined do not tag all the common variation at *DBH* present in AAs.

Substantial differences in allele and haplotype frequencies often occur across populations of differing geographic origins.[27] Although such differences can complicate efforts to identify causal polymorphisms for specific phenotypes, consistency in the pattern of association between genotype and phenotype in different populations can validate genotype–phenotype associations. We found that both allele and haplotype frequencies of the variants examined here differ in AAs and those of European origin.[7,9,11–14] For example, the minor allele frequency (MAF) for C of rs2519152 in this sample was 34.8% (based on Table 1), whereas it was 42.8% in EAs as reported by Tang *et al*.[14] In addition, rs2519152 was in stronger LD with rs1611115 and rs6271 in EAs than in AA, especially as measured by r^2 ($D'=0.59$ and 0.62, respectively; $r^2=0.04$ and 0.019, respectively). That lower degree of LD is consistent with the *DBH* LD structure apparent in the data from Yorubans in the HapMap database (www.hapmap.org). Comparisons of allele and haplotype frequencies in other samples of AAs and EAs in our lab further support differences in allele and haplotype frequencies in the two populations: among 31 SNPs typed across *DBH* and a neighboring gene, *SARDH*, 13 showed significantly different allele frequencies in AA and EA (Y.-L. Tang, E.B. Binder and J.F. Cubells (2006), unpublished data), including rs6271 ($P=0.000029$). Despite these clear differences in allele and haplotype frequencies, comparison of the patterns of genotype–pDβH associations in the two populations reveal similar patterns. In addition, the *direction* of genotype–pDβH associations at rs1611115, the major contributor to the variance of pDβH, was identical in both populations, so that the same allele associated with higher or lower pDβH in both analyses.

In summary, we conclude that: (1) in AA and EA subjects, there are similar associations between *DBH* polymorphisms and pDβH; (2) two common haplotypes, C-T-C and T-C-C are significantly associated with pDβH in AA; (3) the association of ins/del with pDβH reflects its LD with rs1611115; but (4) the other two SNPs (rs6271 and rs2519152) appear to contribute small but independent effects on pDβH. Further work is necessary to determine whether the significantly associated SNPs studied here are functional, or in LD with other functional variants. Clearly, analyses of association between SNPs at *DBH* and clinical phenotypes requires analysis of multiple SNPs across the locus.

Acknowledgements

This work was supported by a NIDA INVEST fellowship (to YLT), K02 DA015766 and R01 DA12422 (to JFC), K08 NS044138 (to CPZ). The authors are grateful to Laura Heizman and Matthew Girgenti for their expert technical assistance, and to all the study participants for their donation of blood for analysis.

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