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Genome-wide association study identifies *ITGB3* as a QTL for whole blood serotonin

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Serotonin has been implicated in common disorders involving the central nervous, gastrointestinal, cardiovascular, and pulmonary systems. We describe the first genome-wide screen to identify quantitative trait loci (QTLs) influencing whole blood serotonin in 567 members of a single large pedigree, using a novel association-based mapping approach. We identified an association between the *β*3 integrin (*ITGB3*) Leu33Pro polymorphism on 17q21 and whole blood serotonin levels (*P*-value $= 9.8 \times 10^{-5}$). This variant explained the evidence for linkage in this region when included as a covariate in the linkage analysis (change in LOD from 1.87 to 0.16), indicating that *ITGB3* may be an important serotonin QTL.

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is typically considered for its role as a brain neurotransmitter; however, it also serves diverse functions in the periphery as a neuromodulator, neurohormone, and vasoactive amine. Peripheral serotonin is produced in the enterochromaffin cells of the intestine and then taken up and stored in platelets, which later release serotonin to modulate vascular constriction and platelet aggregation.[1] Disruptions in the peripheral serotonin system have been implicated in a wide range of clinical phenotypes, from cardiac or vascular disorders such as arrhythmias,[2] coronary artery disease,[3] and migraine headache[4] to pulmonary diseases such as asthma[5] and pulmonary

hypertension.[6] Further, whole blood serotonin has been studied as a peripheral marker of brain disorders including autism,[7] bipolar disorder,[8] Alzheimer's disease,[9] and mental retardation[10] and of behavioral traits such as aggressi-on[11] and IQ.[12]

In a previous study on the heritability of 10 quantitative traits in the Hutterites, a founder population, whole blood serotonin levels had the highest heritability.[13] The narrow heritability, which includes only the additive genetic variance, was 0.52, similar to other quantitative traits such as body mass index (BMI) and high-density lipoprotein-cholesterol (HDL-c) levels. However, the broad heritability, which includes all genetic variance, was 0.99. These studies suggested not only that whole blood serotonin levels are highly heritable in the Hutterites, but also that at least one locus in the genome influences serotonin levels in a nonadditive manner (ie, dominant, recessive, or by interaction with other genetic or environmental factors).

Our strategy for identifying serotonin loci begins with a genome-wide analysis of serotonin levels in the Hutterites using recently developed homozygosity linkage and

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association mapping methods.[14] These methods allow us to utilize the inbreeding present in the Hutterites and detect QTLs with recessive main effects. Alone, each method only identifies broad candidate QTL regions. As we hope to identify specific genes that affect serotonin levels, we choose to implement a joint linkage and association analysis. Using joint analysis, we can assess the degree to which a linkage peak identified in the initial genome scan is the result of a particular QTL under that peak. Identifying association to a locus that simultaneously eliminates the linkage peak is a likely indicator of a true QTL.[15–18]

We initiated this study to identify the loci that influence whole blood serotonin levels in this founder population. Identifying such loci may not only increase our understanding of the intestinal and platelet serotonin systems, but may also suggest novel susceptibility loci or genetic modifiers for cardiovascular, pulmonary or brain disorders.

Materials and methods
Subjects
The Hutterites of South Dakota, the subjects of our studies, are descendants of only 64 ancestors who lived in the early 1700s to the early 1800s in Europe.[19] The usefulness of this population for genetic studies has been described previously.[20,21] A 1623-person pedigree was constructed to include all known ancestors of the 806 individuals in our studies.[22] The mean age of the participants was 28.7 years (SD 17.0 years; range 6–89 years). The mean inbreeding coefficient of the individuals in this sample is 0.034 (SD 0.015), slightly greater than that of $1\frac{1}{2}$ cousins.

Our sampling strategy was population-based,[20,23] and enthusiasm for our studies was high, with >95% of colony members participating. As a result, there are no known ascertainment biases that could influence our results. This study was approved by The University of Chicago and University of S Dakota Institutional Review Boards.

Measurement of whole blood serotonin
The blood for measurement of whole blood 5-HT was drawn from 567 Hutterite subjects over 5 years old, after getting informed consent. The samples were immediately transported to the laboratory and stored frozen at −70°C until the time of batch assay. Previous work suggests that diet and/or fasting are unlikely to influence whole blood serotonin levels,[24] particularly when diet is as uniform as it is in the Hutterites. While time of sampling may affect whole blood serotonin levels,[25] nearly all of the blood sampling was done between 0900 h and noon. Similarly, an influence of seasonal variation on whole blood serotonin levels has been described,[25] but samples for these studies were collected during two consecutive winters. Further, the high heritability of whole blood serotonin in this population[13] suggests that diet, diurnal variation, and seasonal

variation are unlikely to be confounding factors in this study. Assays in platelet-poor plasma revealed that more than 99% of whole blood serotonin is contained in the platelet,[26] and whole blood serotonin correlates highly with measures of serotonin per unit number of platelets and serotonin per unit mass of platelet protein.[27]

Whole-blood 5-HT was analyzed by high-pressure liquid chromatography (HPLC) with fluorometric detection.[28] Intra-assay and inter-assay coefficients of variance were 0.8 and 3.6%, respectively. Assays were performed blind to genotypes, family relationships, and all demographic and medical information. The mean whole blood serotonin level in this sample was 191 ng/ml (SD 79). Data were ln-transformed to establish a normal distribution.

Genotyping
A genome screen using 658 autosomal microsatellite markers (Marshfield screening sets 9 and 51) was completed by the Mammalian Genotyping Service of the National Heart, Lung, and Blood Institute, yielding a ∼5-cM map (http://research.marshfieldclinic.org/genetics/). In addition, 226 microsatellite markers and 239 intragenic SNPs or in/dels related to asthma and cardiovascular diseases (eg see Newman *et al*[29]) were genotyped in this sample. None of the markers were selected because they were functional or positional 'candidates' for serotonin levels. Genotyping was performed blind to all phenotypic information. Distances for framework markers are based on the Marshfield map (http://research.marshfieldclinic.org/genetics/); all other markers were placed using the physical map (http://genome.ucsc.edu/) and estimations of recombination within the Hutterite pedigree by CRI-MAP (http://biobase.dk/Embnetut/Crimap/). The final map had an average intermarker distance of 3.2 cM.

Statistical analysis
Genome scans were performed using HBD linkage and association methods, and significance was assessed using a permutation-based method. The linkage method tests for correlations between regions inherited homozygous by descent (HBD) and trait value, while the association method, allele-specific HBD (ASHBD) tests for correlations between specific alleles at markers inherited HBD and trait value. These methods are the only multipoint methods available capable of analyzing the Hutterite pedigree unbroken and are described in detail elsewhere.[14] For both methods, we assess empirical locus-specific and genome-wide significance using a Monte Carlo permutation test. This test keeps the genotypes fixed while permuting trait values. This has the advantage of assessing significance conditional on characteristics of the genotype data (eg informativeness, heterozygosity, linkage disequilibrium, etc), while preserving the covariance structure in the phenotype data. Details of how this is accomplished can be found in Abney *et al*.[14] In addition to the empirical

P-values, the locus-specific *P*-values were Bonferroni corrected to adjust for multiple tests when several alleles were present at a marker locus. The Bonferroni-corrected *P*-values, which were very close to the locus-specific permutation-based *P*-values, are reported here. In addition, for each of the *P*-values, we report the equivalent one degree of freedom LOD score. Suggestive significance was met if the *P*-value at a locus was less than the expected minimum *P*-value under the null hypothesis.

In the covariate analyses, the probability of HBD for a specific allele at a marker is used as a covariate in the linkage analysis. Thus, we can determine the residual linkage at a locus after removing effects of the association. This approach has been the focus of recently developed methods aimed at identifying the specific genes and polymorphisms that produce an observed linkage peak (eg Cardon and Abecasis,[15] Fulker *et al*,[16] Soria *et al*,[17] and Sun *et al*[18]).

Results

Association analysis by allele-specific homozygosity-by-descent (ASHBD)

Using the multipoint ASHBD association method[14] for whole blood serotonin level, two loci reached the threshold for suggestive significance (see Materials and methods). Results with *P*-value <0.01 are shown in Table 1. The Leu33 allele at the β3 integrin (*ITGB3*) locus on 17q21 at 66 cM from P-terminus (p-ter) provided the strongest signal and was associated with lower levels of whole blood serotonin levels (*P*-value = 9.8×10^{-5}, genome-wide *P*-value = 0.21). Three other alleles at flanking loci between 57.6 and 66.3 cM on chromosome 17 showed some evidence for association, with *P*-values <0.01. *ITGB3* is about 17 cM distal to the serotonin transporter locus

(*SLC6A4*), which showed only a modest association with serotonin levels in this study (*P*-value = 0.015). A second locus, the vitamin D receptor (*VDR*) on chromosome 12q12–q14, also met the criteria for suggestive significance (*P*-value = 2.4×10^{-4}). However, the association of whole blood serotonin levels with *VDR* can be explained by a primary association of *VDR* with platelet count (data not shown). The association with *ITGB3* has no primary association with platelet count, and an association with serotonin levels remains after accounting for platelet number (data not shown).

Linkage analysis by locus-specific HBD

Using the HBD linkage method,[14] one locus reached the threshold for suggestive linkage (Figure 1). This signal was on chromosome 16p13-p12 at 35 cM from p-ter (LOD = 2.5, *P*-value = 0.0007). The peak included the locus *D16S764*, at which homozygosity for the 108 bp allele was also associated with higher serotonin levels by the ASHBD test (*P*-value = 0.008). The second largest linkage peak was on chromosome 17 at 70 cM, near the *ITGB3* locus (LOD = 1.87, *P*-value = 0.0033).

Covariate analysis by locus-specific HBD

To determine whether HBD for the Leu33 allele at the *ITGB3* locus accounted for the linkage signal on chromosome 17q, we repeated the HBD linkage analysis including probability of HBD for the Leu33 allele as a covariate. The variation in whole blood serotonin levels attributable to HBD for the Leu33 allele at the *ITGB3* locus is removed, and evidence for linkage with the remaining variation is evaluated. In the covariate analysis, the linkage peak in the *ITGB3* region on 17q was nearly completely eliminated (LOD = 0.16, *P*-value = 0.39 at 67 cM; Figure 2). Thus, HBD for the Leu33 allele, or other variants that are in linkage

Table 1 Results of ASHBD association test with whole blood serotonin level

Locus	Chr	cM	Allele	Frequency	Direction	P-value
D3S3054	3	215	386	0.25	−	0.003
D9S1838	9	164	169	0.13	+	0.007
VDR_1	**12**	**63**	**T**	**0.54**	**+**	**0.0002**
D12S398	12	68	140	0.28	+	0.002
ATA069	14	82	204	0.11	−	0.001
GATA153F11	15	49	221	0.44	+	0.008
D16S764	16	30	108	0.46	+	0.008
D16S266	16	94	100	0.47	−	0.002
D17S1539	17	58	400	0.19	−	0.007
D17S1299	17	62	196	0.55	−	0.0008
ITGB3_33	**17**	**66**	**L**	**0.80**	**−**	**0.0001**
D17S2180	17	67	122	0.23	−	0.001
D21S2052	21	25	137	0.24	−	0.003
IFNAR	21	31	4	0.50	−	0.002

All ASHBD results with *P*-value <0.01 are shown with the marker name, chromosome and cM location, associated allele, frequency of associated allele, direction of the association, and allele-specific Bonferroni-corrected *P*-value. A plus sign indicates association with increased serotonin levels, while a minus sign indicates association with decreased serotonin levels. Results in boldface type met criteria for suggestive significance.
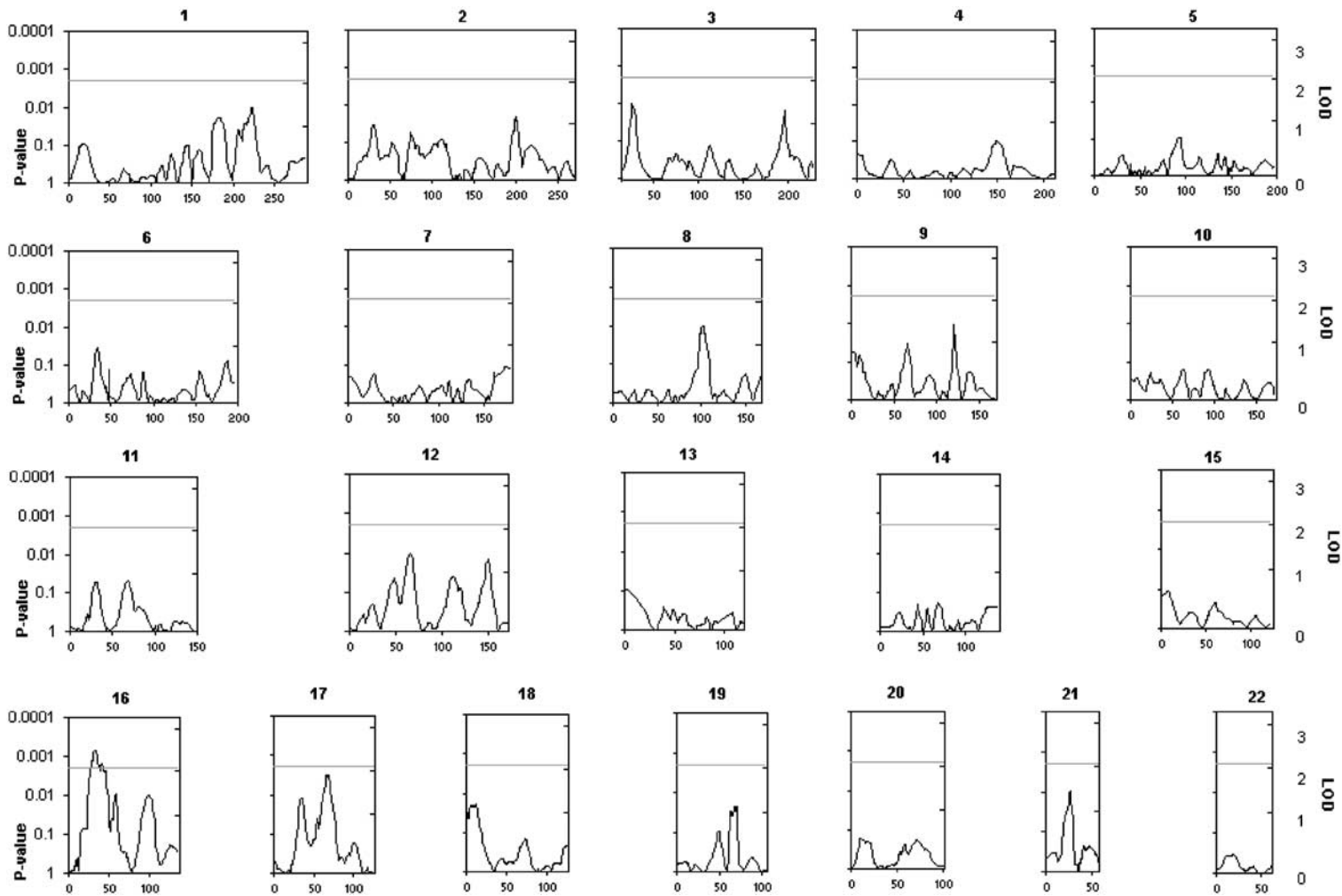
Figure 1 HBD linkage results. Each chromosome is shown p-ter to q-ter. Locus-specific *P*-values are shown on the left axis and LOD equivalents on the right axis (see Materials and methods). The gray line denotes the threshold for suggestive significance.
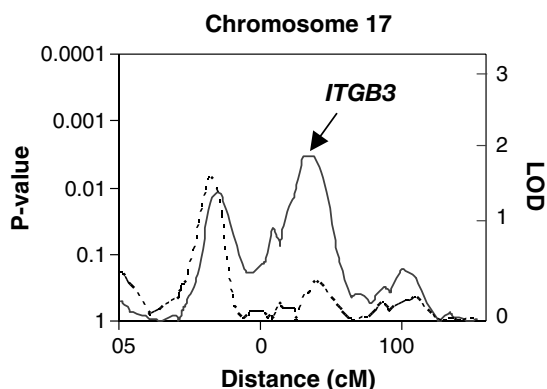
Figure 2 Covariate linkage analysis of whole blood serotonin for chromosome 17. The solid line is the original linkage signal (Figure 1) and the dashed line indicates the linkage results with *ITGB3* Leu33Pro as a covariate. Locus-specific *P*-values are shown on the left axis and LOD equivalents on the right axis.

disequilibrium (LD) with it, explained all of the evidence for linkage on chromosome 17q.

Discussion

This is the first genome-wide association or linkage study of the quantitative trait whole blood serotonin. Our study was conducted in a sample that was unselected with respect to any particular phenotype, allowing us to study normal levels of variation in this important signaling molecule. Further, the reduced environmental variation resulting from this population's communal lifestyle should enhance the relative influence of genetics on variation in a quantitative trait. This is likely reflected in the high heritability of whole blood serotonin in the Hutterite population, although this does not necessarily correspond to simple inheritance or even the presence of a major QTL.[20] However, the association with the Leu33Pro polymorphism in *ITGB3*, when inherited HBD, was strong ($P = 9.8 \times 10^{-5}$) and accounted for nearly all the evidence for linkage in the region, indicating that this polymorphism lies on a haplotype that acts as a recessive QTL for whole blood serotonin. In addition to the *ITGB3* locus, this study identified a linkage signal on chromosome 16p that coincides with linkage peaks for autism[30] and attention-deficit hyperactivity disorder.[31]

ITGB3 encodes a *β*-integrin that comprises part of the platelet-specific heterodimeric receptors for fibrinogen and the widely expressed heterodimeric receptors for vitronectin. Fibrinogen receptors regulate platelet aggregation, hemostasis, and thrombosis. In tissues other than platelets, integrin activation of kinases and small GTP-binding proteins through vitronectin receptors is critical for processes such as cell migration, proliferation, survival, differentiation, and synapse maturation.[32-34]

The *ITGB3* Leu33Pro polymorphism determines the platelet-specific antigen Pl^{A1}/Pl^{A2} (corresponding to Leu33/Pro33, respectively). The protein carrying the Pl^{A2} allele has been shown to have increased and more stable interactions with fibrinogen,[35] enhanced platelet aggregability and thrombin generation,[36-38] and enhanced cell migration.[39] Further, the Pl^{A2} allele has been associated with coronary thrombosis,[40,41] stroke,[42,43] and myocardial infarction.[44,45] Studies of *ITGB3* polymorphisms and whole blood serotonin in patients with cardiovascular diseases and controls will be required to evaluate whether altered serotonin levels mediate the association between Pl^{A2} and cardiovascular phenotypes. In fact, because previous association studies of *ITGB3* considered only the Leu33Pro polymorphism, it is possible that the associated Leu33 allele in some of these earlier studies, as well as in this study, is a marker for other variation in the gene.

In summary, we identified a novel association between a polymorphism in *ITGB3* and whole blood serotonin levels, using methods that harness the extensive linkage disequilibrium in this founder population. A direct connection between platelet integrin function and whole blood serotonin has not previously been noted, but our results suggest that integrin signaling may affect the uptake, storage, or release of serotonin in the platelet. Lastly, this study identifies the *ITGB3* gene as a tool for further genetic dissection of the serotonin signaling pathway and as a candidate for other common disorders that have been associated with the serotonin system.

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