Published in final edited form as:

Am J Med Genet B Neuropsychiatr Genet. 2010 March 5; 0(2): 477–483. doi:10.1002/ajmg.b.31003.

Association and gene-gene interaction of SLC6A4 and ITGB3 in autism

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Abstract

Autism is a heritable neurodevelopmental disorder with substantial genetic heterogeneity. Studies point to possible links between autism and two serotonin related genes: *SLC6A4* and *ITGB3* with a sex-specific genetic effect and interaction between the genes. Despite positive findings, inconsistent results have complicated interpretation. This study seeks to validate and clarify previous findings in an independent dataset taking into account sex, family-history (FH) and gene-gene effects. Family-based association analysis was performed within each gene. Gene-gene interactions were tested using extended multifactor dimensionality reduction (EMDR) and MDR-phenomics using sex of affecteds and FH as covariates. No significant associations with individual SNPs were found in the datasets stratified by sex, but associations did emerge when we stratified by family history. While not significant in the overall dataset, nominally significant association was identified at RS2066713 ($p=0.006$) within *SLC6A4* in FH– (family-history negative) families, at RS2066713 ($p=0.038$) in FH+ (family-history positive) families but with the opposite risk allele as in the FH– families. For *ITGB3*, nominally significant association was identified at RS3809865 overall ($p=0.040$) and within FH+ families ($p=0.031$). However, none of the association survived the multiple testing corrections. MDR-phenomics confirmed gene-gene effects using sex of affecteds ($p=0.023$) and family history ($p=0.014$, survived the multiple testing corrections) as covariates. Our results indicate the extensive heterogeneity within these two genes among families. The potential interaction between *SLC6A4* and *ITGB3*, particularly family history as a promising indicator of genetic architecture further illustrates the importance of covariates as markers of heterogeneity into genetic analyses.

Keywords

Association; interaction; SLC6A4; ITGB3

INTRODUCTION

Autistic disorder [OMIM 209850 (Online Mendelian Inheritance of Man, <http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=209850>)] (ASD) is a neurodevelopmental disorder characterized by three areas of abnormality: impairment in social interaction, impairment in communication, and restricted and repetitive patterns of interest or behavior. With improved surveillance and a broadening of the diagnostic criteria, the most recent prevalence studies suggest that ASD may affect as many as 1 of 150 children in the US and

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is at least three to four times more frequent in males vs. females. (<http://www.cdc.gov/od/oc/media/pressrel/2007/r070208.htm>).

Epidemiological studies have shown that both genetic and environmental factors are involved in this disorder. Twin and family studies indicate a higher concordance for monozygotic (MZ) than dizygotic (DZ) twins (1–4) and a sibling recurrence risk that is 50–100 times than the population risk (5) suggesting a major role for genetic factors in the etiology of autism. To date, no single major gene for autism has been identified conclusively through linkage or association studies, although quite a few genomic regions have been proposed to harbor autism genes (6–12). All of these observations are consistent with autism being a complex genetic disorder; thus heterogeneity and gene-gene interaction must be taken into account while searching for autism susceptibility genes (11, 13–15).

The serotonin system has been implicated in autism through clinically related investigation and genetic epidemiological studies. A clinical study indicated that nearly one third of autistic subjects have platelet hyperserotonemia (16). Another study provided direct support showing the treatment with selective serotonin reuptake inhibitors (SSRIs) was effective at reducing autistic traits such as rituals and aggression (17–19). The human serotonin transporter gene (*SLC6A4*) has been studied extensively in human genetic studies of autism. The gene possesses several polymorphic loci affecting its expression or function (Ile425Val, Gly56Ala, intron 2 VNTR and the 5'-*HTT*-linked polymorphic region [*5HTTLPR*]) (20, 21). This last polymorphism is commonly subdivided into *S* (short, lesser expressing) and *L* (long, greater expressing) alleles based on the presence of a 43 bp indel (22–25). It is the most widely tested polymorphism within *SLC6A4* with respect to its association with autism (26–30). Among the significant association studies, however, the findings are almost equally divided between positive association of the short allele and positive association of the long allele. Despite tremendous effort to use various subphenotypes and strict recruitment criteria, the findings for association studies remain inconsistent. A recent meta-analysis examining the association of *5HTTLPR* with autism failed to find a significant association and indicated significant genetic heterogeneity by ethnicity (31). Some studies have also shown that the inconsistent findings were possibly confounded by another polymorphism RS25531 within *5HTTLPR* (32).

ITGB3, another serotonin related gene on chromosome 17q, which encodes glycoprotein IIIa (GPIIIa), the beta subunit of the platelet membrane adhesive protein receptor complex GPIIb/IIIa, has been reported as a quantitative trait locus for whole blood serotonin levels in a founder population and associated with autism susceptibility in a multiplex sample with a gender effect (33, 34). The most significant association with autism was found with a functional variant, Leu33Pro (rs5918) (29) although the most significant associations with serotonin levels were with noncoding variation (34). Recently, linkage analysis has suggested an improved LOD score in male-only autistic affected subjects in the region harboring *SLC6A4* and *ITGB3* (35, 36). Association studies have also shown that these two genes are more strongly associated with serotonin levels in males (33, 37). More recently published studies also found significant *SLC6A4* and *ITGB3* interactions for both autism risk and serotonin levels (38–40).

Tremendous behavioral and clinical heterogeneity in autism is an obstacle in searching for susceptibility genes in genetic studies. Genetic studies in autism have shown increased linkage and association signals through the use of more specific types of families such as families with only male affected individuals, extended families and more accurate identification of participants. Family history of autism has been proposed as another potential index of genetic heterogeneity (41,42). A recent *de novo* copy number mutation study provided evidence suggesting a genetic distinction between sporadic and multiplex

autistic families with more copy number variants found in sporadic patients than multiplex families (43).

The current study uses the same dataset as Mei et al (40), however, that paper was method-oriented and focused primarily on a description of the novel MDR-P method. In this paper we expand the previous analysis, which considered only sex as a covariate and conducted only MDR-type analyses. Here we focus on the relevance to autism by considering the newly reported findings of both sex- and family history-specific genetic differences as possibly important variables to reduce genetic heterogeneity of genetic effects in *SLC6A4* and *ITGB3*, and conduct additional stratified analyses to help elucidate patterns of association. Our primary hypothesis is that families that are similar in terms of gender of affecteds and history of autism have similar genetic architecture, and genetic effects will be seen when these covariates are taken into account. What is unique about our study is that we applied new statistical methods which also address genetic heterogeneity captured by variables such as sex of affected individuals and family history. Our study illustrates that using such methods can reveal associations and interactions not seen in overall analysis and can show important genetic differences between groups.

MATERIAL AND METHODS

Patient ascertainment

Analysis was performed on 290 Caucasian (non Hispanic) families, of which 122 have a positive family history (FH+) defined as having at least two individuals with autism in the extended pedigree. By contrast, negative family history [FH−] is defined as the absence of any other individual with autism spectrum disorders in the extended pedigree. In addition, 208 of the 290 families are male only (families with only male affecteds). These families were recruited by the Collaborative Autism Team (CAT) from Miami Institute of Human Genomics and the WS Hall Psychiatric Institute. All families were ascertained on the basis of an individual with autism diagnosis using DSM-IV criteria (44) and supported by the Autism Diagnostic Interview-Revised (ADI-R) (45). Detailed diagnostic evaluations of the patients have been previously described (46).

SNP Selection and Genotyping

Blood was obtained from patients and other family members under IRB-approved procedures. DNA was extracted from whole blood using standard protocols (47).

Based on Hapmap data using a cutoff of $r^2 = 0.8$, four independent linkage disequilibrium (LD) blocks were identified within *SLC6A4* (25549032–25586841 bp). We selected the extensively tested polymorphism 5HTTLPR and another three tagging SNPs (RS1042173; RS140700; RS2066713) to cover the haplotype block delineation determined by Hapmap data. Four SNPs in *ITGB3* (42686207–42745075 bp) including functional SNP RS5918 were selected to evenly cover the gene since there is little LD in the region (i.e., few large LD blocks). The functional polymorphism 5HTTLPR in the promoter of *SLC6A4* is a 43 base pair insertion/deletion polymorphism, which was detected using fluorescent primers on a 6% denaturing polyacrylamide gel. SNPs RS5918 and RS140700 were identified in the NCBI SNP database (www.ncbi.nlm.nih.gov/SNP/), and ordered as custom Taqman® SNP genotyping assays (ABI, Foster City, CA). The remainder of the SNPs were identified among Applied Biosystems (ABI, Foster City, CA) Taqman® SNP genotyping assays. All SNPs were genotyped using Taqman® allelic discrimination method, according to the manufacturer's recommendations. For quality control procedures, two CEPH standards were included on each 96-well plate, and samples from six individuals were duplicated across all plates as quality controls (QCs), with the laboratory technicians blinded

to their identities. Analysis required that identical QC samples within and across plates had matching genotypes, in order to identify errors in loading and reading, and thus minimize the error rate in genotypes assignment.

Statistical Methods

Hardy-Weinberg equilibrium was assessed using exact tests implemented in the Genetic Data Analysis program (48). Pairwise linkage disequilibrium (LD) measures (D' and r^2) between markers within each gene were calculated using the software package GOLD (49), and are presented in table 1. The pedigree disequilibrium test (PDT) (50,51) and the geno-PDT (52) were used to examine disease association in the overall sample and subsets of autism family data. Subset analysis was performed by classifying the families into two groups based on the presence or absence of female affected individuals (male only families) and also based on the presence or absence of a family history of autism (FH+, FH-). Bonferroni corrections were employed to correct for multiple testing within each stratum (PDT P-value/number of markers being tested) and across strata (corrected P-value/4) recognizing that this is likely to be conservative due to correlations between markers and overlapping families between strata.

The extended multifactor dimensionality reduction (EMDR) program was used (53) to test for potential high-order gene-gene interactions. This test is based on the MDR method, a data reduction approach (54, 55), and includes two steps: 1) best model identification for different locus combinations, and 2) permutation testing to obtain an empirical P-value including an adjustment for multiple testing. Given the sample size of 290 Caucasian families and even smaller sample of female families in this study, we tested only for up to a 2-way interaction (53). Empirical p-values were computed using a permutation test and assessed as statistically significant at p-value < 0.05. These p-values are adjusted for multiple testing across SNPs and SNP pairs in the permutation test.

MDR-Phenomics (MDR-P) was developed to test for high-order interaction with the consideration of genetic heterogeneity (40). It inherits the advantages from MDR that no assumption of genetic model is required and that high dimensional data can be analyzed efficiently and powerfully through data reduction. Further, it can improve the power by integration of relevant phenotypic covariates into analysis that can decrease the effect of heterogeneity. In this study, presence or absence of female affecteds and family-history of autism were used as covariates while testing for interaction between *SLC6A4* and *ITGB3*. P-values were computed using a permutation test and assessed as significant at p-value < 0.05. As above p-values are adjusted for multiple testing across SNPs and SNP pairs in permutation test.

RESULTS

Table 1 shows estimates of minor allele frequency and the LD pattern among SNPs within each gene in our dataset. Similar to the hapmap phase II CEU LD data, the four SNPs within either *SLC6A4* or *ITGB3* gene display only mild pair-wise LD between each other (r^2 = [0.025, 0.299]; r^2 = [0.014, 0.441] respectively) in affecteds suggesting they provide independent information in association analysis.

Single-locus association analysis with the PDT and sample size for each subgroup is shown in Table 2. Significant association was identified at RS2066713 [*SLC6A4*] with risk allele G over transmitted (p=0.006) in the FH- subset. There was also nominally significant evidence of association with autistic risk (p=0.038) in FH+, but the opposite allele 'A' was found to be over-transmitted to patients in this subset. Nominally significant association was found within the *ITGB3* gene at RS3809865 (p=0.040) in the overall dataset, but was seen only in

the FH+ ($p=0.031$) in the stratified analysis. Notably, no association survived Bonferroni correction ($p<0.05/8/4\approx0.0016$) across strata. Also, no association was found for the functional 5HTTLPR polymorphism in *SLC6A4* or RS5918 in *ITGB3*, either in the overall dataset or any subsets despite previous evidence of association in other studies.

Gene-gene analysis by EMDR yielded no significant results for one or two- locus analysis in the overall or in the male-only datasets. A 2-locus (RS2066713 and RS5918) joint effect was close to significant ($p=0.07$) in the FH+ subset and significant in FH- ($p=0.014$). This result was confirmed by MDR-P (Table 3) by using family-history as phenotypic covariate ($p=0.014$). EMDR identified another nominally significant 2-locus joint effect in female families ($p=0.028$, RS1042173 and RS3809865), which was again confirmed by MDR-P ($p=0.023$) (Table 3) using gender of affecteds as covariate. Among all these gene-gene tests, only the P-value from MDR-P using family-history as covariate survived the multiple testing correction. (Empirical p-value/the number of strata: $0.05/2=0.025$)

DISCUSSION

The purpose of this study was to explore sex and family-history as possibly important covariates for decreasing genetic heterogeneity that has lead to the inconsistent findings for *SLC6A4* and *ITGB3* in autism. Although previous studies reported gender-specific effects in *ITGB3* in multiplex families (34), we failed to confirm that sex was an important covariate in our single-locus analyses of either gene, overall or in just the multiplex families (data not shown). Stratification for family-history, however, did yield interesting findings with several markers being nominally significant and a significant joint effect detected with MDR-P. A single non-functional variant (RS2066713) in *SLC6A4* almost met our Bonferroni correction for significance. On closer inspection, the association signals present different patterns between FH+ vs. FH- groups. Specifically, we found that FH+ and FH- groups show nominal association with *SLC6A4* at RS2066713 in opposite directions (i.e. with the opposite risk allele). This association flip-flop phenomenon has been discussed thoroughly in our group (56, 57). This result could reflect allelic heterogeneity between familial and sporadic patients with different unobserved causative variants occurring on different marker haplotype backgrounds. If this is a true flip-flop, failure to account for family history could explain why other studies have shown different alleles to be associated at the 5HTTLPR locus. Various combinations of family types across studies may result in a different degree of phenotypic and genetic heterogeneity leading to inconsistent findings across studies. Of course, it could also be the result of chance alone, resulting from multiple tests across strata.

While the 5HTTLPR and L33P (RS5918) variants in *SLC6A4* and *ITGB3*, respectively, have been reported as associated functional variants, neither show association evidence in our dataset, overall or stratified by covariates. However, given that numerous studies have shown the association between *SLC6A4* with autism, our identification of association, which almost survived the Bonferroni correction (RS2066713: 25575791), provides additional support for the true association between *SLC6A4* with autism. One question is whether this association signal reflects the previously reported signals in *SLC6A4* [Ile425Val (25562500 bp), Gly56Ala (25572936 bp), intron 2 VNTR (25572535–25572734 bp) (20, 21)]. We did not genotype these markers in our dataset and none of them have been genotyped on hapmap dataset, however based on the physical location, these markers are in the second LD block, the same block as RS140700 (25567515 bp). This marker is in only moderate LD ($r^2=0.30$) with our peak marker RS2066713 and shows no significant association in our analyses. Thus, we hypothesize that this may represent a different association signal due to extensive allelic heterogeneity within this gene. To further validate our findings and more thoroughly compare with previously reported associations, we

analyzed the genotypes for the samples genotyped on our recently completed GWAS using the Illumina 1M chip. The GWAS included RS15908 [*ITGB3*] and also suitable proxies for Ile425Val, Gly56Ala and the intron 2 VNTR in *SLC6A4*. We were unable to detect significant interaction with RS15908 and 5HTTLPR that has been previously detected. Nor did we confirm associations with the proxies in *SLC6A4* in our dataset (data not shown).

Our findings also suggest that the difficulty in replication of association studies could be attributed in part to failure to consider gene-gene or gene-environment interaction, both of which have been proposed as strong factors in autism (58, 59). The program MDR-P (40) allowed us to test for gene-gene effect while controlling for heterogeneity by integrating phenotypic covariates such as sex and family history in the analysis. The simulation results shown by Mei and colleagues (40), who also analyzed this dataset as an illustration of this method, suggested increased power for MDR-P as compared to traditional MDR-PDT and conditional logistic regression if the phenotypic covariate correctly captures the genetic heterogeneity. This is consistent with what we observed in this study where we detected significant joint effects with MDR-P not seen in EMDR analysis; though whether this can be attributed to true interaction or not is driven by marginal association needs to be further validated in independent datasets.

Our results provide some explanations for inconsistent association findings with *SLC6A4* and *ITGB3* in autism. First, functional variants (5HTTLPR and RS5918) are not likely the sole causative variants. Second, family history of autism may be an important index of allelic heterogeneity, which needs to be taken into account while estimating the genetic effect of these two genes. Third, powerful tools for testing gene-gene and gene-environment interaction are needed that can test gene effects in autistic families given genetic heterogeneity. In conclusion, our results show compelling evidence of associations with *SLC6A4* and autism, particularly in families with a negative family history. Evidence for the involvement of *ITGB3* and interactive effects is suggestive but weaker.

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Table 1

Linkage Disequilibrium in affected samples for markers within each gene.

	SLC6A4	Minor allele frequency	RS1042173	RS140700	RS2066713	5HTTLPR
1	RS1042173	0.42		1	0.776	0.298
2	RS140700	0.07	0.086		1	0.572
3	RS2066713	0.36	0.299	0.058		0.501
4	5HTTLPR	0.20	0.074	0.025	0.101	
	ITGB3		RS11657517	RS5918	RS5919	RS3809865
5	RS11657517	0.29		0.944	0.873	0.207
6	RS5918	0.14	0.441		1	0.788
7	RS5919	0.07	0.022	0.014		1
8	RS3809865	0.29	0.033	0.235	0.037	

Shaded cells show D' values, while plain cells show r^2 . Numbers on the left of the SNPs correlate with coding used in Table 3.

Table 2

Association results (p-value) for all markers.

marker	Overall (n=290) pdt_pvalue*	Fam -history+ (n=122) pdt_pvalue	Fam-history- (n=168) pdt_pvalue	Male_only (n=208) pdt_pvalue	Female (n=82) pdt_pvalue
SLC6A4					
RS1042173	0.196	0.755	0.021	0.302	0.437
RS140700	0.854	0.816	0.547	0.722	0.884
RS2066713	0.874	0.038	0.006	0.743	0.856
5HTTLPR	0.847	0.626	0.865	0.499	0.460
ITGB3					
RS11657517	0.852	1.000	0.787	0.454	0.149
RS5918	0.225	0.233	0.596	0.125	0.889
RS5919	0.558	0.873	0.493	0.777	0.532
RS3809865	0.040	0.031	0.455	0.062	0.361

* : p-values for pedigree disequilibrium test

Table 3

Gene-gene effects tested by MDR-phenomics

SLC6A4	ITGB3	p-value	covariate
RS2066713	RS5918	0.014	family history
RS1042173	RS3809865	0.023	gender