Abstract

The heterogeneity of symptoms associated with autism spectrum disorders (ASDs) has presented a significant challenge to genetic analyses. Even when associations with genetic variants have been identified, it has been difficult to associate them with a specific trait or characteristic of autism. Here, we report that quantitative trait analyses of ASD symptoms combined with case-control association analyses using distinct ASD subphenotypes identified on the basis of symptom profiles result in the identification of highly significant associations with 18 novel single nucleotide polymorphisms (SNPs). The symptom categories included deficits in language usage, non-verbal communication, social development, and play skills, as well as insistence on sameness or ritualistic behaviors. Ten of the trait-associated SNPs, or quantitative trait loci (QTL), were associated with more than one subtype, providing partial replication of the identified QTL. Notably, none of the novel SNPs is located within an exonic region, suggesting that these hereditary components of ASDs are more likely related to gene regulatory processes (or gene expression) than to structural or functional changes in gene products. Seven of the QTL reside within intergenic chromosomal regions associated with rare copy number variants that have been previously reported in autistic samples. Pathway analyses of the genes associated with the QTL identified in this study implicate neurological functions and disorders associated with autism pathophysiology. This study underscores the advantage of incorporating both quantitative traits as well as subphenotypes into large-scale genome-wide analyses of complex disorders.

Introduction

Autism spectrum disorders (ASDs) represent a group of neurodevelopmental disorders that are characterized by impaired reciprocal social interactions, delayed or aberrant communication, and stereotyped, repetitive behaviors, often with restricted interests [1,2]. With a concordance rate as high as 90% based on twin studies [3], ASDs are among the most heritable of neuropsychiatric conditions. Although specific genes have been found to be causal for several syndromes that are sometimes associated with autistic symptoms (for example, fragile X [4], Retts [5,6], and tuberous sclerosis [7,8]), there are no unequivocal genetic markers for non-syndromic idiopathic autism. Thus, a considerable amount of effort has been devoted to identifying genetic mutations or variants that associate with these perplexing and often devastating, life-long disorders. Because of the relatively high prevalence of ASDs in the general population (~1:110), genome-wide association (GWA) analyses have been used recently to search for common variants that may associate with increased susceptibility to this set of disorders [9–12]. However, despite case-control studies that have now exceeded many thousands of subjects and more than 500,000 SNPs, only a few significantly associated SNPs have been identified. In addition, replication of these SNPs in independent studies has not been successful. The inability to replicate findings from GWA analyses may be in part due to the genetic heterogeneity of the ASD population, thus giving rise to increased “noise” in the data. This genetic heterogeneity is likely responsible for the well-noted phenotypic and symptomatic heterogeneity among individuals with autism.

In a recent paper, we demonstrated that the ASD population can be divided into at least 4 phenotypic subgroups on the basis of cluster analyses of 123 severity scores taken from each individual’s diagnostic assessment using the Autism Diagnostic Interview-Revised [13]. The resulting subgroups included one with severe language impairment, another with mild severity across all items, a third of intermediate severity, and a fourth of moderate severity with a higher frequency of savant skills. We further demonstrated by gene expression profiling of lymphoblastoid cell lines from 3 of these subgroups (excluding the intermediate) and nonautistic controls that cells from each of these subgroups exhibited differentially expressed genes relative to those of the controls, but also were distinguishable from each other in terms of unique, subtype-specific differentially expressed genes [14]. These studies thus support the concept that different subgroups of autistic individuals may exhibit subtype-dependent biological differences due to genetic variation. We therefore hypothesized that genetic association analyses of such ASD subtypes with SNPs that are identified and filtered according to their association with...
quantitative traits relevant to ASDs should reveal more significant SNPs with increased statistical power. Here, we report the identification of 10 novel and highly significant SNPs that are associated with at least one of 4 different subtypes of ASDs, based on the combination of quantitative trait association analyses and subtype-dependent genetic association analyses using trait-associated SNPs, with 10 of these SNPs replicated in a second ASD subtype representing an independent case cohort.

**Results**

The overarching goal of these studies was to identify single nucleotide polymorphisms (SNPs) that are associated with both autistic traits and clinical subtypes of autism that are manifested by separate case cohorts. To accomplish this, we combined quantitative trait analysis and subphenotype association analyses using the wealth of genome-wide association (GWA) data published by Wang et al. in 2009 [9].

**Quantitative Trait Association Analyses**

The flowchart in Fig. 1 describes the experimental design and analyses that were used to derive the combined set of 18 novel and statistically significant SNPs that associate with 4 separate subtypes of ASDs. Raw item scores from the ADI-R score sheets of 2939 ASD cases were summed for spoken language skills, non-verbal communication, play skills, social development, and insistence on sameness/rituals, as described previously [13]. The specific items used to obtain the total score per “trait” category for each individual are shown in Table S1 and the profiles of total scores for each category are shown for the 2939 individuals in Fig. S1.

We then conducted quantitative trait association analyses using the distribution of scores in each of the categories to identify sets of SNPs that associate with symptomatic severity of each of the behaviors listed in Table S1. A nominal p-value $\leq 10^{-5}$ was used in this “discovery phase” designed to screen candidate SNPs that are potentially of functional significance with respect to the ASD traits. These sets of symptom-associated SNPs (or quantitative trait loci, QTL) are shown in Table S2.

**ASD subtype-dependent genetic association analyses with trait-associated SNPs**

Next, we performed cluster analyses as described by Hu and Steinberg [13] to divide the autistic cases into 4 non-overlapping phenotypic subgroups according to symptomatic severity profiles derived from 123 items on the ADI-R assessments. We postulated that this subtyping procedure, which reduces the behavioral/symptomatic heterogeneity among the cases within each subgroup, is likely to also restrict the genetic heterogeneity within each subgroup. The results of K-means cluster analyses (K = 4) of the ADIR data from the 2939 individuals (which included 1867 genotyped cases from the GWA study) is shown in Fig. S2A. An unsupervised principal components analysis (PCA) was used to demonstrate that the 4 subgroups of individuals with ASDs from the K-means cluster analyses results in effective separation of cases according to similarity of symptomatic profile as defined by the 123 item scores from the ADI-R (Fig. S2B). The resulting phenotypic subgroups were then used in genetic association analyses with each set of QTL derived from the five quantitative trait association analyses, where the cases were either divided into
the 4 ASD subtypes or used as a combined case group against the 2438 nonautistic controls. These analyses produced 5 sets of QTL that were associated with specific subtypes of ASDs (Tables 1, 2, 3, 4, 5). It is interesting to note that SNPs associated with certain genes (such as HTR4, BACH2, and TINAG among Language QTL, and OR5B3, OR5B17, and ERBB4 among Nonverbal QTL) are replicated in more than one ASD subtype. Finally, significant SNPs with Bonferroni-adjusted p-values \( \leq 0.05 \) from each of the 4 separate subtype-dependent association analyses with QTL were combined into a single set containing 18 unique SNPs, and case-control association analyses were performed for which the cases were either combined into one group or divided according to subphenotype.

Partial replication of SNPs between subtypes of ASDs

Table 6 shows the SNPs associated with each subtype of ASDs that resulted from the final association analyses using the combined highly significant QTL and 4 non-overlapping subgroups of ASD cases. Eighteen of the SNPs have p-values < 0.05 in at least one subtype even after using the stringent Bonferroni correction for multiple comparisons. Note that 10 of the SNPs, including rs317985, rs7785107, rs11671930, rs7950390, rs12266939, rs3861787, rs7725785, rs1827924, rs1231339, and rs757099, are associated with more than one subtype. Two of the replicated SNPs (rs317985 and rs7785107) are significant in two subtypes after Bonferroni adjustment (p < 0.05), while the remaining 8 exhibit lower levels of significance (nominal p-values from 0.0037–0.051 or FDR_BH adjusted p-values of 0.0087–0.088) in the second (or third) subtype. Association of these QTL with more than one subtype of ASD serves as a replication for these 10 SNPs. While the replication of SNPs in different subtypes may be interpreted as evidence for those SNPs being highly penetrant for ASDs, the subtype-dependent differences in minor allele frequency (MAF) and odds ratios (OR) associated with the shared SNPs suggest that the subtypes are genetically heterogeneous.

![Figure 2](image-url) summarizes the extent of SNP overlap among the 4 ASD subtypes (case cohorts) and clearly demonstrates that the odds ratios are distinctly different for different subtypes that share the same SNP. All of the QTL associated with specific genes are present in non-exonic (promoter, intronic, or intergenic) regions. Interestingly, all but one of the SNPs residing within intergenic regions can be associated by band position to rare copy number variants (CNV) that have been recently identified for ASDs [15], thus providing further support for the probable relevance of these SNPs to ASDs. These CNVs are noted in Table 6.

### Table 1. Language QTL associated with ASD subtypes.

| SNP             | SNP position | Band       | Location   | Gene       | UNADJ P | FDR_BH* | BONFb | Subtype                  |
|-----------------|--------------|------------|------------|------------|---------|---------|------|--------------------------|
| rs2277049       | chr5:147883281 | 5q33.1     | Intron     | HTR4       | 0.0002  | 0.0015  | 0.0036 | Language-impaired         |
| rs757099        | chr9:22757567 | 9p21.2     | CNV*       | BACH2      | 0.0003  | 0.0015  | 0.0055 |                        |
| rs7785107       | chr7:10247968 | 7p21.3     | CNV*       | BACH2      | 0.0003  | 0.0015  | 0.0059 |                        |
| rs77225785      | chr5:147882896 | 5q33.1     | Intron (boundary) | HTR4 | 0.0004  | 0.0015  | 0.0062 |                        |
| rs22875281      | chr5:53141022 | 5p13.3     | Intron (boundary) | CDH6 | 0.0007  | 0.0019  | 0.0111 |                        |
| rs1231339       | chr9:227530863 | 9p21.2     | CNV*       | BACH2      | 0.0007  | 0.0019  | 0.0116 |                        |
| rs128055        | chr22:47722427 | 22q13.32   | CNV*       | BACH2      | 0.0015  | 0.0036  | 0.0252 |                        |
| rs758138        | chr12:1895485 | 12p13.33   | Intron     | CACNA2D4   | 0.0032  | 0.0067  | 0.0538 |                        |
| rs17830215      | chr8:1602159 | 8p23.3     | Promoter   | KBTBD11    | 0.0043  | 0.0082  | 0.0739 |                        |
| rs10183984      | chr21:67181162 | 2q24.3    | CNV*       | BACH2      | 0.0060  | 0.0101  | 0.1013 |                        |
| rs11969265      | chr6:90739765 | 6q15       | Intron     | BACH2      | 0.0080  | 0.0124  | 0.1359 |                        |
| rs9474831       | chr6:54361040 | 6p12.1     | Intron     | TINAG      | 0.0099  | 0.0141  | 0.1688 |                        |
| rs17828521      | chr6:54328202 | 6p12.1     | Intron     | TINAG      | 0.0116  | 0.0145  | 0.1977 |                        |
| rs10886416      | chr6:90755541 | 6q15       | Intron     | BACH2      | 0.0120  | 0.0145  | 0.2036 |                        |
| rs6454792       | chr6:90743794 | 6q15       | Intron     | BACH2      | 0.0349  | 0.0395  | 0.5927 |                        |
| rs12893752      | chr14:25220350 | 14q12     | CNV*       | BACH2      | 0.0918  | 0.0975  | 1.0000 |                        |
| rs7785107       | chr7:10247968 | 7p21.3     | CNV*       | BACH2      | 0.0016  | 0.0265  | 0.0265 | Intermediate            |
| rs12047655      | chr1:14070302 | 1p36.21    | CNV*       | BACH2      | 0.0005  | 0.0093  | 0.0093 | Mild                     |
| rs12893752      | chr14:25220350 | 14q12     | CNV*       | BACH2      | 0.0068  | 0.0576  | 0.1151 |                        |
| rs6454792       | chr6:90743794 | 6q15       | Intron     | BACH2      | 0.0137  | 0.0747  | 0.2330 |                        |
| rs1231339       | chr9:227530863 | 9p21.2    | CNV*       | TINAG      | 0.0215  | 0.0747  | 0.3662 |                        |
| rs9474831       | chr6:54361040 | 6p12.1     | Intron     | TINAG      | 0.0220  | 0.0747  | 0.3736 |                        |
| rs10183984      | chr21:67181162 | 2q24.3    | CNV*       | TINAG      | 0.0356  | 0.0928  | 0.6059 |                        |
| rs17828521      | chr6:54328202 | 6p12.1     | Intron     | TINAG      | 0.0382  | 0.0928  | 0.6495 |                        |
| rs757099        | chr9:22757567 | 9p21.2     | CNV*       | BACH2      | 0.0441  | 0.0938  | 0.7503 |                        |
| rs77225785      | chr5:147882896 | 5q33.1     | Intron (boundary) | HTR4 | 0.0508  | 0.0960  | 0.8639 |                        |

*p-value adjusted using Benjamini-Hochberg False Discovery Rate method; bBonferroni-adjusted p-value. CNV located in Chromosomal band as reported by Pinto et al. [15]. doi:10.1371/journal.pone.0019067.t001
Effect of subtyping of ASDs on association of previously identified SNPs within Chr5p14.1

Because none of the SNPs identified in this re-analysis overlapped with those of the previously published genome-wide association study [9], we examined the association of the 6 SNPs that were reported in the published study with our subphenotypes. Table 7 shows that only the “Moderate” ASD subtype (363 cases) is associated with two of the SNPs, with Bonferroni-adjusted p-values of 0.035 and 0.053. Interestingly, these 2 SNPs have the least significant combined p-values in the published study. The remaining 4 SNPs were suggestively significant with FDR_BH-
adjusted p-values of 0.074 in this subtype. The combined cases (1867 individuals in all) as well as the other 3 ASD subtypes show no association with any of the 6 SNPs even though there are more cases in each of these groups than in the Moderate group. This finding further illustrates the value of analyzing subphenotypes of ASDs in genome-wide association analyses in order to reduce the genetic heterogeneity of the population studied.

Pathway analyses of SNP-containing genes

To obtain a better understanding of how the novel SNPs identified in this study potentially relate to the biology of autism,
pathway analysis was conducted to develop a better sense of the relationships among the SNP-associated genes and their impact on higher level functions and diseases. Fig. 3 shows a gene network constructed using Pathway Studio 7 which includes seven of the 9 genes associated with SNPs found within gene promoters or introns. Of the 7 genes, HTR4 and GCH1 are “hubs” showing the highest “connectivity” with other components within the network. The specific relationships between these two genes and other network components are more clearly illustrated in Figures S3 and S4. It is noted that many of the cellular and higher level processes in this network, such as neurogenesis, axonogenesis, steroid metabolism, cell proliferation, long-term synaptic potentiation, learning and memory are relevant to identified deficits in ASDs.

Discussion

We have previously shown that the autistic population can be divided into subgroups of affected individuals according to symptomatic profile through cluster analyses of severity scores from the ADI-R assessment for each individual with an ASD [13]. Three of the 4 resulting subgroups were shown to exhibit distinct, though partially overlapping, gene expression profiles, each relative to a group of nonautistic controls, implying that both unique and shared genes are associated with the respective phenotypes [14]. We therefore applied the same rationale and methods in subtyping individuals with ASDs for this re-analysis of previously published genome-wide association data [9]. We first employed quantitative trait association analyses to the >500,000 SNPs tested in order to prioritize SNPs that might correlate with a behavioral or symptomatic “trait” relevant to ASDs. These quantitative traits for each individual with an ASD were derived from the sums of severity scores from the ADI-R items that described severity of language impairment, deficits in nonverbal communication, impaired play skills, insistence on sameness/rituals, and delayed social development. The specific items used to establish each quantitative trait (listed in Table S1) were shown by Hu and Steinberg to exhibit differential severity among the several subtypes of ASDs [13]. In this “discovery” stage of the experiment, quantitative trait association analyses across the 5 selected traits produced a filtered set of 167 unique SNPs from the original 513,312 SNPs (Table S2). Subsequent association analyses of these QTL with both combined and subtyped individuals with ASDs revealed 18 novel SNPs which were found to be highly significant (Bonferroni-adjusted p-value<0.05) in at least one subtype of autism (Table 6). Interestingly, many of the language QTL from Table S2 are strongly associated with the severely language-impaired ASD subtype. Of the 18 novel SNPs, 10 SNPs were replicated, albeit at a more modest significance level, in at least one of the other subtypes. Two are replicated with Bonferroni-significant p-values while the other 8 are suggestively significant (FDR-BH adjusted p-value<0.09). While it may be argued that the standard statistical methods used here to correct for multiple testing are not stringent enough, there is still no clear consensus on the significance level that is appropriate for a candidate gene (or SNP) study such as this, where the candidate SNPs are determined by prior quantitative trait association analyses. More significantly, different minor allele frequencies and odds ratios are observed for the SNPs that are associated with more than one subtype (Table 6 and Fig. 2), thus reflecting the genetic heterogeneity among the ASD phenotypes, which is teased apart by the subtyping procedures employed here. It is noteworthy that no significant SNPs were identified when all 1867 individuals with ASDs were analyzed against 2438 nonautistic controls, thus underscoring the importance of phenotypic subtyping to unearth SNPs associations with ASDs.

By comparison, the original genome-wide study which was based on the combined analysis of 2503 cases and more than 7000

Table 4. Insistence on sameness QTL associated with ASD subtypes.

| SNP        | SNP Position | Band | Location | Gene | UNADJ | FDR_BH* | BONFb | Subtype |
|------------|--------------|------|----------|------|-------|--------|-------|---------|
| rs1827924  | chr:228377979| 2q3.3| Promoter | CCL20| 0.0002| 0.0053| 0.0053| Moderate|
| rs17738966 | chr:145437169| 14q22.2| Downstream | GCH1 | 0.0005| 0.0071| 0.0171| “      |
| rs7950390  | chr:14587928 | 11p15.4| Promoter | TRIM68| 0.0007| 0.0071| 0.0212| “      |
| rs3817871  | chr:14604346 | 11p15.4| CNV*    |      | 0.0012| 0.0078| 0.0385| “      |
| rs371985   | chr:56773558 | 5q13.1| CNV     |      | 0.0012| 0.0078| 0.0392| “      |
| rs3890467  | chr:7479914  | 3p26.1| Intron  | GRM7 | 0.0022| 0.0119| 0.0712| “      |
| rs3890468  | chr:7477700  | 3p26.1| Intron  | GRM7 | 0.0033| 0.0131| 0.1069| “      |
| rs1309622  | chr:7465129  | 3p26.1| Intron  | GRM7 | 0.0034| 0.0131| 0.1102| “      |
| rs6782718  | chr:7462776  | 3p26.1| Intron  | GRM7 | 0.0037| 0.0131| 0.1182| “      |
| rs9568011  | chr:134766081| 13q14.2| -       |      | 0.0047| 0.0152| 0.1518| “      |
| rs164187   | chr:16061526 | 1q23.3| Promoter | C1orf111| 0.0070| 0.0205| 0.2251| “      |
| rs10781238 | chr:76384432 | 9q21.13| Intron  | RORB | 0.0101| 0.0240| 0.3221| “      |
| rs9634811  | chr:134762176| 13q14.2| -       |      | 0.0103| 0.0240| 0.3303| “      |
| rs2469183  | chr:1584992924| 15q25.3| CNV     |      | 0.0105| 0.0240| 0.3363| “      |
| rs311785   | chr:56773558 | 5q13.1| CNV     |      | 0.0023| 0.0584| 0.0727| Mild   |
| rs1827924  | chr:228377979| 2q3.3| Promoter | CCL20| 0.0037| 0.0584| 0.1168| “      |
| rs2574852  | chr:173003467| 17q25.3| Intron  | SEPT9| 0.0057| 0.0609| 0.1826| “      |

* p-value adjusted using Benjamini-Hochberg False Discovery Rate method;
Bonferroni-adjusted p-value.
*CNV located in Chromosomal band as reported by Pinto et al. [15].
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controls across 2 independent datasets in the “discovery” phase, identified 1 SNP that reached genome-wide significance and 5 additional SNPs of nominal significance in one intergenic region on chr5p14.1 that was located between 2 cadherin genes [9]. In this study, we replicated the association of 2 of the SNPs but only in the “Moderate” subtype of ASDs which consisted of only 363 cases (Table 7). Neither the other 3 subtypes (with 387–639 cases per subgroup) nor the combined cases group (consisting of 1867 cases) showed any association with these 6 SNPs. Furthermore, while none of the 6 SNPs identified in the published study correlated with expression level of either cadherin (CDH9 or CDH10) in the cortical brain of 93 genotyped human subjects, we note that 2 of the SNP-associated genes identified in the current study, HTR4 and CCL25, were found to be differentially expressed (FDR, 5%) in lymphoblastoid cell lines from individuals with ASDs in a previous study by our laboratory [14]. This overlap of differentially expressed genes with those associated with at least some of the novel SNPs lends support to the potential functional relevance of these SNPs. Aside from the SNPs located within the promoter or intron regions of genes, seven of the other eight significant SNPs (Table 6) are located in intergenic regions that are linked by band position to rare copy number variants (CNVs) that

Table 5. Social development QTL associated with ASD subtypes.

| SNP    | SNP Position | Band   | Location | Gene     | UNADJ P | FDR_BH* | BONFb | Subtype |
|--------|--------------|--------|----------|----------|---------|---------|-------|---------|
| rs1226938 | chr10:3852940 | 10p15.1 | CNV*     |          | 0.0001  | 0.0023  | 0.0023 | Mild    |
| rs10519124 | chr2:67819501 | 2p14   | -        |          | 0.0002  | 0.0040  | 0.0081 |         |
| rs2297172  | chr9:71563166 | 9q21.11 | Intron   | PTAR1    | 0.0006  | 0.0082  | 0.0245 |         |
| rs2255313  | chr12:102773924 | 12q23.3 | CNV     |          | 0.0014  | 0.0150  | 0.0613 |         |
| rs6698676  | chr1:84528583  | 1p31.1  | Promoter | SAMDJ3   | 0.0017  | 0.0150  | 0.0760 |         |
| rs2519866  | chr17:27859883 | 17q11.2 | Intron   | MYOID    | 0.0020  | 0.0150  | 0.0900 |         |
| rs10305860 | chr4:148625337 | 4q31.23 | Intron   | EDNRA    | 0.0028  | 0.0176  | 0.1229 |         |
| rs13205238 | chr6:413597    | 6p25.3  | CNV     |          | 0.0033  | 0.0182  | 0.1456 |         |
| rs4873815  | chr8:144796206 | 8q24.3  | Promoter | ZNF623   | 0.0042  | 0.0182  | 0.1836 |         |
| rs4832481  | chr2:16986218  | 2p24.3  | CNV     |          | 0.0045  | 0.0182  | 0.1959 |         |
| rs30746    | chr5:135366157 | 5q31.1  | CNV     |          | 0.0046  | 0.0182  | 0.2006 |         |
| rs10997162 | chr10:67906707 | 10q21.3 | Intron   | CTNNA3   | 0.0054  | 0.0196  | 0.2357 |         |
| rs38009282 | chr12:110192180 | 12q24.11 | Intron | CULT2    | 0.0061  | 0.0205  | 0.2664 |         |
| rs11215772 | chr9:133418328 | 9q34.13 | CNV     |          | 0.0069  | 0.0218  | 0.3049 |         |
| rs10874468 | chr2:96959718  | 2q11.2  | CNV     |          | 0.0099  | 0.0277  | 0.4361 |         |
| rs4809918  | chr20:50734607 | 20q13.2 | -       |          | 0.0106  | 0.0277  | 0.4642 |         |
| rs12962411 | chr18:27645521 | 18q12.1 | CNV     |          | 0.0107  | 0.0277  | 0.4712 |         |
| rs4959923  | chr6:6142773   | 6p25.3  | CNV     |          | 0.0129  | 0.0316  | 0.5679 |         |
| rs721087   | chr2:4942340    | 2p25.2  | CNV     |          | 0.0166  | 0.0385  | 0.7312 |         |
| rs9479482  | chr6:150399705 | 6q25.1  | -       |          | 0.0180  | 0.0397  | 0.7937 |         |
| rs1078819  | chr1:150161717 | 1q21.3  | -       |          | 0.0198  | 0.0414  | 0.8690 |         |
| rs4646421  | chr15:72803245 | 15q24.1 | Intron  | CYP1A1   | 0.0251  | 0.0502  | 1.0000 |         |
| rs11138895 | chr9:82693023  | 9q21.31 | CNV     |          | 0.0265  | 0.0507  | 1.0000 |         |
| rs11138885 | chr9:82676684  | 9q21.31 | -       |          | 0.0300  | 0.0550  | 1.0000 |         |
| rs4905110  | chr14:93342083 | 14q32.13 | -       |          | 0.0329  | 0.0570  | 1.0000 |         |
| rs2627468  | chr8:3812607   | 8p23.2  | Intron  | CSMD1    | 0.0349  | 0.0570  | 1.0000 |         |
| rs17192980 | chr5:7030768   | 5p15.31 | CNV     |          | 0.0350  | 0.0570  | 1.0000 |         |
| rs10886048 | chr10:118928872 | 10q25.3 | -       |          | 0.0426  | 0.0660  | 1.0000 |         |
| rs4811895  | chr20:55639775 | 20q13.31 | -      |          | 0.0435  | 0.0660  | 1.0000 |         |
| rs13384439 | chr2:187500106 | 2q32.1  | CNV     |          | 0.0462  | 0.0678  | 1.0000 |         |
| rs4416176  | chr2:26536150  | 2p23.3  | Intron  | OTOF     | 0.0507  | 0.0708  | 1.0000 |         |
| rs11627027 | chr14:93440170 | 14q32.13 | -       |          | 0.0529  | 0.0708  | 1.0000 |         |
| rs12183587 | chr6:150396301 | 6q25.1  | -       |          | 0.0531  | 0.0708  | 1.0000 |         |
| rs2151206  | chr9:14844072  | 9p22.3  | Intron  | FREM1    | 0.0562  | 0.0727  | 1.0000 |         |
| rs6022029  | chr20:50708572 | 20q13.2 | CNV     |          | 0.0606  | 0.0762  | 1.0000 |         |
| rs1996893  | chr9:14880268  | 9p22.3  | Intron  | FREM1    | 0.0726  | 0.0887  | 1.0000 |         |

*p-value adjusted using Benjamini-Hochberg False Discovery Rate method;
Bonferroni-adjusted p-value.
*CNV located in Chromosomal band as reported by Pinto et al. [15].
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Table 6. Highly significant SNPs associated with ASD subtypes.

| CHR | SNP   | BP     | A1* | F_A1* | F_U1* | A2* | CHISQ* | OR1 | Band      | Location | Gene  | UNADJ | FDR_BH | BONFp | Subtype                  |
|-----|-------|--------|-----|-------|-------|-----|--------|-----|-----------|----------|-------|-------|-------|-------|--------------------------|
| 5   | rs2277049 | 147883281 | 0.115 | 0.081 | 0.137 | 1.48 | 5q33.1 | Intron | HTR4 | 0.0002 | 0.0017 | 0.0041 |
| 9   | rs757099 | 25727567 | 0.391 | 0.447 | 1.293 | 0.79 | 9p21.2 | CNV* | 0.0003 | 0.0017 | 0.0061 |
| 7   | rs7785107 | 10247968 | 0.031 | 0.056 | 1.279 | 0.54 | 7p21.3 | CNV | 0.0003 | 0.0017 | 0.0066 |
| 5   | rs7725785 | 147882896 | 0.114 | 0.082 | 1.271 | 1.44 | 5p33.1 | Intron | HTR4 | 0.0004 | 0.0017 | 0.0069 |
| 5   | rs2285781 | 33140122 | 0.078 | 0.053 | 1.162 | 1.51 | 5p13.3 | Intron | CDH6 | 0.0007 | 0.0022 | 0.0124 |
| 9   | rs1231339 | 25730863 | 0.534 | 0.480 | 1.154 | 1.24 | 9p21.2 | CNV | 0.0007 | 0.0022 | 0.0129 |
| 22  | rs2180055 | 47722427 | 0.146 | 0.113 | 1.101 | 1.34 | 22q13.32 | CNV | 0.0015 | 0.0040 | 0.0281 |
| 19  | rs11671930 | 8023308 | 0.135 | 0.160 | 1.409 | 0.82 | 19p13.2 | Promoter | CCL25 | 0.0283 | 0.0598 | 0.5379 |
| 7   | rs7785107 | 10247968 | 0.083 | 0.056 | 1.011 | 1.52 | 7p21.3 | CNV | 0.0016 | 0.0296 | 0.0296 |
| 11  | rs7950390 | 4587928 | 0.107 | 0.080 | 1.751 | 1.38 | 11p15.4 | Promoter | TRIM68 | 0.0061 | 0.0370 | 0.1166 |
| 10  | rs12266938 | 3852940 | 0.236 | 0.198 | 1.714 | 1.25 | 10p15.1 | CNV | 0.0075 | 0.0370 | 0.1432 |
| 11  | rs3861787 | 4604346 | 0.102 | 0.076 | 0.800 | 0.38 | 11p15.4 | CNV | 0.0078 | 0.0370 | 0.1480 |
| 5   | rs1827924 | 228377979 | 0.256 | 0.326 | 1.424 | 0.71 | 2q36.3 | Promoter | CCL20 | 0.0002 | 0.0031 | 0.0031 |
| 14  | rs17738966 | 54371969 | 0.131 | 0.090 | 1.199 | 1.52 | 14q22.2 | Downstream | GCH1 | 0.0005 | 0.0042 | 0.0102 |
| 11  | rs7950390 | 4587928 | 0.144 | 0.080 | 1.159 | 0.53 | 11p15.4 | Promoter | TRIM68 | 0.0007 | 0.0042 | 0.0126 |
| 11  | rs3861787 | 4604346 | 0.043 | 0.076 | 1.495 | 0.54 | 11p15.4 | CNV | 0.0012 | 0.0047 | 0.0229 |
| 5   | rs317985 | 66773558 | 0.321 | 0.264 | 1.045 | 1.32 | 5q13.1 | CNV | 0.0012 | 0.0047 | 0.0233 |
| 5   | rs7725785 | 147882896 | 0.058 | 0.082 | 0.518 | 0.69 | 5q33.1 | Intron | HTR4 | 0.0230 | 0.0729 | 0.4372 |

| 10  | rs12266938 | 3852940 | 0.137 | 0.198 | 1.633 | 0.64 | 10p15.1 | CNV | 0.0001 | 0.0009 | 0.0010 |
| 16  | rs730168 | 73707776 | 0.173 | 0.234 | 1.434 | 0.68 | 16q23.1 | Intron | LDHD | 0.0002 | 0.0009 | 0.0029 |
| 2   | rs10519124 | 67819501 | 0.187 | 0.137 | 1.399 | 0.46 | 2p14 | CNV | 0.0002 | 0.0009 | 0.0035 |
| 6   | rs1284516 | 18879356 | 0.310 | 0.247 | 1.391 | 1.37 | 10p12.33 | Intron | NSUN6 | 0.0002 | 0.0009 | 0.0037 |
| 19  | rs11671930 | 8023308 | 0.212 | 0.160 | 1.321 | 1.42 | 19p13.2 | Promoter | CCL25 | 0.0003 | 0.0011 | 0.0053 |
| 9   | rs2297172 | 71563166 | 0.093 | 0.139 | 1.192 | 0.64 | 9q21.1 | Intron | PTAR1 | 0.0006 | 0.0018 | 0.0106 |
| 5   | rs317985 | 66773558 | 0.212 | 0.264 | 0.840 | 0.75 | 5q13.1 | CNV | 0.0002 | 0.0002 | 0.0002 |
| 2   | rs1827924 | 228377979 | 0.380 | 0.326 | 0.845 | 1.26 | 2q36.3 | Promoter | CCL20 | 0.0037 | 0.0087 | 0.0693 |
| 9   | rs1231339 | 25730863 | 0.436 | 0.480 | 0.820 | 0.83 | 9p21.2 | CNV | 0.0215 | 0.0455 | 0.4092 |
| 9   | rs757099 | 25727567 | 0.486 | 0.447 | 0.451 | 1.17 | 9p21.2 | CNV | 0.0441 | 0.0839 | 0.8386 |

*Minor allele;  
†Minor allele frequency-autistic cases;  
‖MAF-unaffected controls;  
*Major allele;  
*Chisquare value;  
Odds ratio with respect to major allele;  
P-value adjusted using Benjamini-Hochberg False Discovery Rate method;  
Bonferroni-adjusted p-value.  
CNV located in Chromosomal band as reported by Pinto et al. [15].  
doi:10.1371/journal.pone.0019067.t006

have been recently associated with autism [15]. The finding that all 18 of the novel SNPs are in non-exonic (promoter and noncoding) regions further suggests that gene regulatory processes likely leading to differential gene expression, rather than to alterations of gene products, may contribute to the hereditary component of ASDs. The presence of the identified QTL in ASD-related CNVs provides additional support for the potential relevance of these novel SNPs to ASDs. The current study thus illustrates the advantage of combining both quantitative traits and defined ASD phenotypes in analyzing genome-wide genetic data from individuals with this complex disorder.

Quantitative trait and phenotype incorporation into genetic analyses

There have been relatively few large-scale genetic studies on ASDs that incorporate quantitative traits or phenotypes into genome-wide analyses. The value of using phenotypic subsets of ASDs in genetic analyses was demonstrated in an early linkage
study by Nurmi et al. who found that subsetting the subjects (and families) according to presence or absence of savant skills increased the HLOD score of the D15S511 marker within the GABRB3 gene on chr15q11–q13 from 0.8 in the entire study population to 2.6 for the savant-skills-positive subset of individuals [16]. Later, Alarcon et al. used quantitative trait analysis of 2 language scores on the ADI-R combined with ordered subsets analysis for subject stratification to reveal a strong language QTL on 7q35, and observed additional linkage peaks on several other chromosomes without stratification [17]. Chen et al. used a combination of 7 ADI-R scores describing nonverbal communication and the same approach to uncover QTL for nonverbal communication [18]. Similarly, Duvall et al. used quantitative trait analysis based upon scores from the Social Responsiveness Scale to discover QTL for social endophenotypes with strong linkage to chromosomes 11 and 17 across all samples and additional linkages to chromosomes 4, 8, and 10 in “male-only” families [19]. More recently, Nijmeijer et al. identified loci overlapping ASDs and attention-deficit/hyperactivity disorder (ADHD) in a genome-wide study of children with ADHD and autistic traits (but not classical or atypical autism) by using total and subscale scores from the Social Communication Questionnaire (an autism diagnostic instrument) as well as by including scores related to severity of ADHD as covariates [20]. Along a similar vein, Ronald et al. examined both social and non-social autistic-like traits in the general population, quantified on the basis of DSM-IV social and non-social scales, to tease out 22 and 20 SNPs that associated with these traits, respectively [21]. However, only one SNP survived testing in an independent sample and its p-value was still only nominally significant in an all male sample. Recently, quantitative trait analysis was applied in a genome-wide linkage study of two repetitive ASD behavior phenotypes, “insistence on sameness” (IS) and “repetitive and stereotyped behavior” (RSMA) [22]. In this study, Cannon et al. demonstrated IS and RSMA were linked to 2 separate loci, each exhibiting respective HLOD scores of 3.42 and 3.93, suggesting that more narrowly-defined phenotypes improve linkage signals. When social behavior, as reflected by severity scores on the Social Responsiveness Scale was used as a quantitative trait, Coon et al. not only replicated a susceptibility locus identified previously in the Duvall et al. study [19], but also identified additional linkage peaks with HLOD scores ranging from 2.23–3.64 [23]. Interestingly, St. Pourcain et al. recently reported that one of the SNPs identified at Chr5p14 by GWA analyses [9] also associates by quantitative trait analyses with the social communication phenotype [24]. They
suggested that this SNP, rs4307059, may also associate with multiple subthreshold impairments across a variety of ASD-related functions.

In the present study, we observe that rs4307059 is suggestively associated only with the Moderate ASD subtype, with an unadjusted p-value of 0.074 (Table 7). Thus, the above-mentioned studies, in combination with ours, reinforce the concept that the incorporation of quantitative traits into genetic analyses greatly improves the ability to elicit significant genetic information on complex disorders such as ASDs. Furthermore, the genes associated with the QTL in Tables 1, 2, 3, 4, 5 and Table S2 may provide clues to the biology underlying the various autistic phenotypes across the whole spectrum of ASDs (severe to mild), based on severity profiles across a broad range of behaviors and symptoms characteristic of ASDs.
Biological relevance of SNP-containing genes

To examine the biological processes and pathways that might be impacted by the SNP-associated genes, we performed pathway analyses on the genes using Pathway Studio 7 software. Fig. 3 shows that 7 of the 9 SNP-containing genes are included in a gene network with many genes leading to "axonogenesis," a function which has been reported to be enriched among genes overlapping copy number variants in autistic patients [25]. HTR4 and GCH1 are network "hubs" connecting with many other genes, cellular processes and disorders (see Figs. S3 and S4 for specific connections). As illustrated in Fig. S3, HTR4 [5-hydroxytryptamine (serotonin) receptor 4] regulates neurogenesis, long-term synaptic potentiation and, in turn, learning and memory, as well as the release of neurotransmitters (dopamine, acetylcholine), peptide hormones (AVP, OXT, PRL, VIP) and steroid compounds (cortisol, corticosterone). Thus, any alteration in the expression or function of this gene can be expected to have wide-ranging consequences on processes known to be affected by ASDs. It is notable that one of the SNPs associated with HTR4, rs7725785, is associated with three ASD subtypes (Table 6). However, the odds ratio is 1.44 for the severe language-impaired subtype while it is 0.68 and 0.74 for the moderate and mild subtypes, respectively. Interestingly, a reduction in HTR4 expression was observed only for the language-impaired subtype of ASDs and not for the moderate (savant) or mild subtypes in our earlier gene expression study [14], suggesting that this SNP, which is located at an intron boundary within HTR4, may play a role in level of expression. Genetic variants in HTR4 have also been associated with schizophrenia [26], bipolar disorder [27] and attention deficit/hyperactivity disorder [28], further suggesting some functional overlap among these neurological disorders. More recently, a de novo translocation on chromosome 5 close to HTR4 has been identified in an autistic boy [29], lending additional support for the relevance of this gene in ASDs.

The other hub gene, GCH1 [GTP cyclohydrolase I], is the rate-limiting enzyme in the de novo biosynthesis of tetrahydrobiopterin which is, in turn, required for the biosynthesis of folate, serotonin, dopamine, and catecholamines (Fig. S4), all of which are important for neural development and functions. It is interesting to note that elevated expression of GCH1 has been implicated in mood disorders [30], while genetic polymorphisms or mutations in GCH1 have been associated with pain sensitivity [31–33], and dystonia [34], which are often associated with ASDs. Although these genes are not likely to be causal for ASDs, genetic polymorphisms in them may be associated with some of the comorbid symptoms or pathobiology of ASDs.

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**Figure 3. Gene interaction network of genes associated with the intronic SNPs identified in Table 6.** Genes from Table 6 are shown with blue highlights while other genes in the network are shown in pink and small molecules are green. Processes are shown in yellow and disorders are shown in purple. The orange entities represent functional complexes. doi:10.1371/journal.pone.0019067.g003
To obtain the total score per category for development, and insistence on sameness/ritualistic behaviors. (ADI-R) score sheets of 2939 ASD cases were summed for spoken ADI-R data, and 513,312 SNPs on the Illumina HumanHap550 Bead Chip. The specific items used to obtain the total score per category for each individual, shown in Table S1, were noted in an earlier study [13] to exhibit average differences in severity among several subtypes of ASDs, described below. The sums of item scores within each of the 5 categories were used as quantitative traits for genetic association analyses using the genotype data reported by Wang et al. [9].

Subtyping of individuals with ASDs

Two thousand nine hundred and thirty nine (2939) individuals with ASDs were divided into phenotypic subgroups using clustering tools within the MeV software package [35], as previously described by Hu and Steinberg [13]. Briefly, subtyping of individuals with ASDs involved K-means cluster analysis (with K = 4) of scores from 123 items from the ADI-R score sheets of each individual which were adjusted as described to fall into a severity range of 0 (normal) to 3 (highest severity). A figure of merit analysis (not shown) indicated that the individuals with ASDs were optimally represented by 4 subgroups. A principal components analysis (PCA) was then used to verify that the 4 subgroups of individuals identified by the K-means cluster analyses were distinguishable by this unsupervised test. Fig. S2 shows the symptomatic profiles of the 4 ASD subtypes as well as their separation into discernible clusters by PCA. The subtypes are named “Language-impaired,” “Intermediate,” “Moderate,” and “Mild,” and contain 639, 478, 363, and 387 cases, respectively.

Quantitative trait association analyses

Using the score totals for the 5 categories of autistic symptoms exhibited by each of 1867 cases as quantitative traits, we utilized PLINK [36], a program to analyze whole genome association data, to perform quantitative trait association analyses with the genotype data reported by Wang et al. [9]. From the results of each of the 5 analyses (Table S2), we selected SNPs with unadjusted p-values < 10^-5, which prioritized SNPs filtered by association with quantitative traits relevant to ASDs. These filtered sets of SNPs were then used in case-control association analyses, as described below.

Case-control association tests

Using the 5 sets of QTL generated for each symptom category, association analyses using PLINK were performed with each set where the cases, in addition to being treated as a single group of 1867 cases vs. 2438 controls, were also divided into the 4 ASD subtypes that were determined by the ADI-R cluster analyses described above. It should be noted that each ASD subgroup represents an entirely separate cohort of cases. From each of the 5 sets of genetic association analyses with subtypes and QTL (Tables 1, 2, 3, 4, 5), we selected SNPs associated with each ASD subtype with a Bonferroni-adjusted p-value ≤ 0.05 and combined them (a total of 18 unique SNPs) for a second case-control association analysis using the combined and subtype ASD cases against controls. The results of this final analysis are shown in Table 6.

Pathway analysis

Pathway Studio 7 software (Ariadne Genomics, Inc.) was used to generate relational gene networks using the SNP-containing genes listed in Table 6.

Supporting Information

Figure S1 Quantitative trait profiles generated by summing the severity scores for ADI-R items for each trait listed in Table S1. (TIF)
Figure S2 Identification of ASD subtypes by cluster analyses. A) Symptomatic profiles of the 4 ASD subtypes that resulted from K-means cluster analyses of 123 ADI-R severity scores per individual. In this figure, each row represents an individual and each column represents an item on the ADI-R. Black represents a score of 0 which is considered “normal,” while the intensity of red indicates severity scores ranging from 1–3. Gray represents unavailable data. The wide band of intensely red items in the language-impaired subgroup corresponds to spoken language. The 12 columns at the extreme right in each block represent items corresponding to “Savant skills,” which appear to be present at a slightly higher frequency in the group labeled “Moderate.” This group had been labeled “Savant” in our previous study [13]. Note that each cluster contains an independent cohort of cases. B) Principal components analysis (PCA) of the individuals based on the 123 ADI-R severity scores. Each subgroup of individuals identified in (A) is assigned a color, which identifies individuals from that subgroup in the PCA. Red: Language-impaired; Green: Intermediate; Yellow: Moderate; Blue: Mild. Each point on the PCA represents an individual with an ASD whose position is defined by his/her scores for the 123 ADI-R items.

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