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Functional Impact of Global Rare Copy Number Variation in Autism Spectrum Disorder

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Abstract

The autism spectrum disorders (ASDs) are a group of conditions characterized by impairments in reciprocal social interaction and communication, and the presence of restricted and repetitive behaviors[1]. Individuals with an ASD vary greatly in cognitive development, which can range from above average to intellectual disability (ID)[2]. While ASDs are known to be highly heritable (~90%)[3], the underlying genetic determinants are still largely unknown. Here, we analyzed the genome-wide characteristics of rare (<1% frequency) copy number variation (CNV) in ASD using dense genotyping arrays. When comparing 996 ASD individuals of European ancestry to 1,287 matched controls, cases were found to carry a higher global burden of rare, genic CNVs (1.19 fold,

P= 0.012), especially so for loci previously implicated in either ASD and/or intellectual disability (1.69 fold, *P*= 3.4×10^{-4}). Among the CNVs, there were numerous *de novo* and inherited events, sometimes in combination in a given family, implicating many novel ASD genes like *SHANK2, SYNGAP1, DLGAP2* and the X-linked *DDX53-PTCHD1* locus. We also discovered an enrichment of CNVs disrupting functional gene-sets involved in cellular proliferation, projection and motility, and GTPase/Ras signaling. Our results reveal many new genetic and functional targets in ASD that may lead to final connected pathways.

Twin and family studies indicate a predominantly genetic basis for ASD susceptibility and provide support for considering these disorders as a clinical spectrum. Some 5–15% of individuals with an ASD have an identifiable genetic aetiology corresponding to known rare single-gene disorders (e.g., fragile X syndrome) and chromosomal rearrangements (e.g., maternal duplication of 15q11-q13). Rare mutations have been identified in synaptic genes, including *NLGN3*, *NLGN4X*[4] *and SHANK3*[5], and microarray studies have revealed copy number variation (CNV) as risk factors[6]. CNV examples include *de novo* events observed in 5–10% of ASD cases[7–9], *de novo* or inherited hemizygous deletions and duplications of 16p11.2[9–11] and *NRXN1*[7], and exceptionally rare homozygous deletions in consanguineous families[12]. Genome-wide association studies using single nucleotide polymorphisms (SNPs) have highlighted two potential ASD risk loci at 5p14.1[13] and 5p15.2[14], but these data suggest common variation will account for only a small proportion of the heritability in ASD.

To further delineate the contribution of rare genomic variants to autism we genotyped 1,275 ASD cases and their parents using the Illumina Infinium 1M-single SNP-microarray (Fig. 1). A set of 1,981 controls used for comparison studies was genotyped on the same platform[15] and both data sets were subjected to the same quality control (QC) procedures. Ultimately, we analyzed 996 ASD cases (876 trios) and 1,287 controls of European ancestry (EA) to minimize confounds due to population differences (Supplementary Fig. 1–2 and Supplementary Table 1)[16].

Two CNV prediction algorithms (QuantiSNP[17] and iPattern (unpublished)) and additional extensive QC were used to establish a stringent dataset of non-redundant CNVs called by both algorithms in an individual (Fig. 1, Supplementary Tables 1–3 and Supplementary Fig. 3). This stringent dataset of 5,478 rare CNVs in 996 cases and 1,287 controls of EA (Supplementary Table 4) had the following characteristics: (i) CNV present at <1% frequency in the total sample (cases and controls), (ii)≥CNV 30 kb in size (because >95% of these could be confirmed) and (iii) all CNVs further verified using combined evidence from the PennCNV algorithm[18] and child-parent intensity fold-changes, genotype proportions (to verify deletions) and visual inspection (for chromosome-X).

We assessed the impact of rare CNV in cases compared to controls using three primary measures of CNV burden: the number of CNVs per individual, the estimated CNV size, and the number of genes affected by CNVs (Table 1). No significant difference was found in the former two measures (Supplementary Tables 4a and 5), even after controlling for fine-level ancestry differences by pair-matching cases and controls (Supplementary Information)[16]. In contrast, we discovered a significant increase in the number of genes intersected by rare CNV in cases when focusing on gene-containing segments (1.19-fold increase, empirical *P*= 0.012). This ASD association with genic CNV was stronger for deletions (1.26-fold increase, empirical *P*= 8.0×10^{-3}). These differences remained after we further controlled for potential case-control differences that could be present due to biological differences or technical biases. Restricting our analysis to autosomal CNVs (ie. after removing CNVs located on chromosome X) resulted in a consistent enriched gene count in ASD cases

compared to controls. Single-occurrence CNV deletions had increased rates in ASD over controls, suggesting some could be pathogenic.

We then examined parent-child transmission and confirmed that 5.7% (50/876) of ASD cases had at least one *de novo* CNV with >0.6% carrying two or more *de novo* events (Supplementary Tables 4a, 6 and 7). The *de novo* CNV rate in our simplex and multiplex families was 5.6% (22/393) and 5.5% (19/348), respectively, in contrast with previous studies showing a higher rate in simplex families[8,9]. A total of 226 validated *de novo* (7) and inherited (219) CNVs not observed in controls and affecting single genes were found (Supplementary Table 8).

Numerous novel candidate ASD loci such as *SHANK2*, *SYNGAP1*, and *DLGAP2*, were identified based on the observation that *de novo* CNV affects these genes in cases and not controls (Supplementary Table 6). The relatedness of *SHANK2* to the causal ASD gene *SHANK3*[5], involvement of *SYNGAP1* in ID[19], and interaction of DLGAP family proteins with SHANK proteins[20] further support their role in ASDs. Maternally-inherited X-linked deletions at *DDX53/PTCHD1* (7 cases) implicated this locus in ASD. We tested an additional 3,677 EA controls (Fig. 1) and again found no CNV at these genes, and *DDX53/PTCHD1* emerged as a significant ASD risk factor ($P= 3.1\times10^{-3}$ with the initial 1,287 controls; $P= 3.6\times10^{-6}$ with combined controls; Supplementary Fig. 4).

Association studies of individual rare CNV often have insufficient power to discriminate benign from disease-causing variants. Here, we assessed whether genes and CNVs previously associated with ASD and/or ID were enriched in cases compared with controls, in order to help identify pathogenic events. We defined three gene-lists based on evidence from previous studies of their involvement in ASDs (Supplementary Table 9): (i) 'ASD implicated' list consisting of 36 disease genes and 10 loci strongly implicated in ASD and identified in subjects with ASD or ASD and ID; (ii) 'ID' consisting of 110 disease genes and 17 loci implicated in ID but not yet in ASD; and, (iii) 'ASD candidates' including 103 genes from previous studies of common and rare variants.

We observed a higher proportion of cases with rare CNVs overlapping 'ASD implicated' disease genes compared to controls (4.3% versus 2.3%, Fisher exact test $P= 5.4\times10^{-3}$; Fig. 2a), corresponding to a significant enrichment for genes in this set (OR= 1.8; 95% CI 1.3–2.6, empirical $P= 2.6\times10^{-3}$; Fig. 2b, see also Supplementary Information). This effect was stronger for duplications, which may also disrupt genes (OR= 2.3; 95% CI 1.4–3.8, empirical $P= 9.4\times10^{-4}$). Enrichment was also found for rare CNVs overlapping ID genes, more notably for deletions (OR= 2.1; 95% CI 1.1–4.2, empirical $P= 5.3\times10^{-2}$). In contrast, there was no evidence of enrichment among case-CNVs compared to control-CNVs for genes in the 'ASD candidates' set (empirical P >0.3). When the two disease gene-sets 'ASD implicated' and 'ID' were combined, we observed 7.6% of cases with rare CNVs preferentially affecting ASD/ID genes compared to 4.5% in controls (Fisher exact test $P= 1.2\times10^{-3}$, Fig. 2a). The observed enrichments did not change when potential case-control genome-wide differences for CNV rate and size were considered.

Our global analyses of these putative pathogenic loci use somewhat subjective boundaries for CNV overlap. Manual inspection of the data yields more accurate results. After eliminating CNVs that are less likely to have an aetiological role (heterozygous CNVs that disrupt autosomal recessive loci, events outside the critical region of overlap of genomic disorders, X-linked genes in females inherited from non-ASD fathers, duplications inherited from non-ASD parents, and intronic CNVs in *NRXN1*), 25 CNVs remained in the ASD group, compared to only four in the controls ($P= 3.6\times10^{-6}$; Supplementary Table 10). Moreover, the latter four CNVs were all duplications at 1q21.1, 16p11.2 or 22q11.2, loci

known to exhibit incomplete penetrance and variable expressivity[6]. The population attributable risk provided by the combination of all ASD-CNVs that overlap ASDs and/or ID genes is estimated to be 3.3% (Supplementary Table 11). We also identified rare *de novo* chromosomal abnormalities and large CNVs likely to be aetiologic (Supplementary Table 10).

We then tested for functional enrichment of gene-sets among those genes affected by CNVs to identify biological processes involved in ASD (Fig. 3). Here, the term gene-set refers to groups of genes that share a common function or operate in the same pathway. Such a functional enrichment mapping approach can combine single-gene effects into meaningful groups[21].

We compiled comprehensive collections of gene-sets (Supplementary Table 12) and used the Fisher's exact test to assess which gene-sets were more frequently affected by rare CNV events in ASD cases compared to controls. An estimate of the false-discovery rate (FDR) at each gene-set was obtained by random permutation of case and control labels (Supplementary Information). To visualize enriched gene-sets, overlap scores were used to graphically organize these sets into a functional enrichment map (or network) using Cytoscape[22]. We identified the 'seed' genes for the network at an FDR q-value of 5% and further relaxed the thresholds to 12.5% to better capture the network topology[23].

Using these criteria only deletions were found significantly enriched in gene-sets in cases over controls (Supplementary Fig. 5), consistent with the global burden results (Table 1). Specifically, 76 gene-sets affected by deletions (2.18% of sets tested) were found enriched and used to construct a functional map (Figure 3a, Supplementary Fig. 6–7). We tested for possible bias, including measures of CNV size and number for cases versus controls per gene-set, as well as genome proximity, but no differences were found that might explain the observed enrichments (Supplementary Fig. 8–9).

We identified enrichments in gene-sets known to be involved in ASDs and also discovered new candidate ASD pathways (Fig. 3a, Supplementary Table 13). For example, gene-sets involved in cell and neuronal development and function (including projection, motility, and proliferation) previously reported in ASD-associated phenotypes, were identified[24]. Novel observations included gene-sets involved in GTPase/Ras signaling, with component Rho GTPases known to be involved in regulating dendrite and spine plasticity and associated with ID. We also found a tentative link to sets in the kinase activity/regulation functional group where only minorities of these sets meet a stringent 5% FDR q-value threshold (Supplementary Fig. 10).

We further assessed the relationship of our functional enrichment map with known ASD/ID genes (Fig. 3b, Supplementary Fig. 11) and found genes enriched in sets linked to microtubule cytoskeleton, glycosylation and CNS development/adhesion[25]. The two groups of genes found enriched in deletions (Fig. 3a) also displayed connectivity to the ASD/ID disease gene-sets, either directly or through intermediates (Fig 3b, Supplementary Fig. 12). Although ASD genes appear to be enriched in different subsets of genes compared to ID-only genes, we cannot discount the possibility that this is the result of selection bias, and we expect that more ID genes may yet be linked to ASD.

Our findings provide strong support for the involvement of multiple rare genic CNVs, both genome-wide and at specific loci, in ASD. These findings, similar to those recently described in schizophrenia[26], suggest that at least some of these ASD-CNVs (and the genes that they affect) are under purifying selection[27]. Genes previously implicated in ASD by rare variant findings have pointed to functional themes in ASD pathophysiology[6,28]. Molecules such as NRXN1, NLGN3/4X and SHANK3, localized presynaptically or at the post-

synaptic density (PSD), highlight maturation and function of glutamatergic synapses. Our data reveal *SHANK2*, *SYNGAP1* and *DLGAP2* as new ASD loci, which also encode proteins in the PSD. We also found ID genes to be important in ASD[29]. Furthermore, our functional enrichment map identifies new groups such as GTPase/Ras, effectively expanding both the number and connectivity of modules that may be involved in ASD. The next steps will be to relate defects or patterns of alterations in these groups to ASD endophenotypes. The combined identification of higher-penetrance rare variants and new biological pathways, including those identified in this study, may broaden the targets amenable to genetic testing and therapeutic intervention.

Methods Summary

Cases were classified using the Autism Diagnostic Interview-Revised (ADI-R) and Autism Diagnostic Observation Schedule (ADOS) instruments and those with known karyotypic abnormalities or genetic disorders were excluded. Informed consent was obtained from all families and procedures had approval from institutional review boards. DNA was obtained from blood or buccal-swabs (73% of cases; 75% of controls) or cell-lines (22% of cases; 25% of controls) (in 5% of cases the DNA source was not identified). The 1,287 EA controls passing all QC-filters included 1,261 individuals recruited as controls for the study of addiction (SAGE)[15] and 26 HapMap samples (from Illumina). An additional 3,677 EA controls from three separate studies genotyped on other platforms were also used. Raw data from ASD family (Accession pending) and SAGE control (Accession: phs000092.v1.p1) genotyping are at NCBI dbGAP. CNVs were analysed using PLINK v1.07[30], R stats and custom scripts. Primary analyses were robust to potential systematic measurement differences between cases and controls; it was not possible to control for site but we controlled for the overall extent and number of CNVs for all burden comparisons, and obtained a consistent enriched gene count in ASD cases compared to controls.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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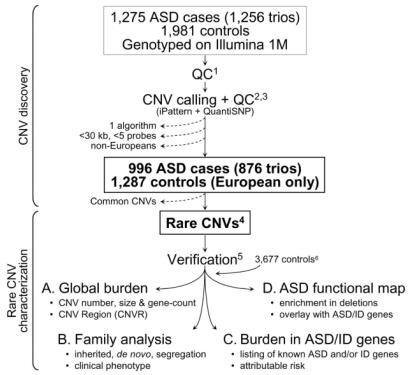

Figure 1. CNV discovery and characterization

Comprehensive procedures were used to identify the rare CNV dataset (boxed). Dashed arrows indicate CNVs not included in downstream analyses. [1] SNP and intensity quality control (QC) with ancestry estimation. [2] QC for CNV calls. [3] Pilot validation experiments using quantitative-PCR were used to evaluate the false discovery-rate. [4] Rare CNVs in samples of EA ancestry were defined as 30 kb in size and present in the total sample set at a frequency <1%. 70/996 (17%) of ASD cases were analyzed on different lower-resolution arrays in previous studies[9,10,28]. [5] All CNVs were computationally verified and at least 40% of case-CNVs were also experimentally validated by qPCR and/or independent Agilent or other SNP microarrays. [6]3,677 additional EA controls were used to test specific loci from the primary burden analyses. Additional details are in the Methods Summary and Supplementary Information

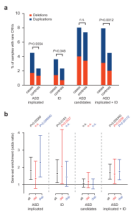

Figure 2. CNV burden in known ASD and/or ID genes

a, Proportion of samples with CNVs overlapping genes and loci known to be associated in ASD with or without ID or ID only, as well as published candidate genes and loci for ASD (Supplementary Table 9). To select for CNVs with maximal impact, they needed to intersect genes, and overlap the target loci by ≥50% of their length. Fisher's exact test *P*-values for significant differences ($P \leq 0.05$, one tailed) are shown. **b**, enrichment analysis for genes overlapped by rare CNVs in cases compared to controls for the three gene-sets in panel a, relative to the whole genome. Odds ratio (OR) and 95% confidence intervals are given for each gene set. Empirical *P*-values for gene-set enrichment are indicated above each OR. All *P*-values <0.1 are listed.

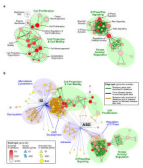

Figure 3. A functional map of ASD

Enrichment results were mapped into a network of gene-sets (nodes) related by mutual overlap (edges), where the color (red, blue, or yellow) indicates the class of gene-set. Node size is proportional to the total number of genes in each set and edge thickness represents the number of overlapping genes between sets. **a,** Gene-sets enriched for deletions are shown (red) with enrichment significance (FDR q-value) represented as a node color gradient. Groups of functionally related gene-sets are circled and labeled (groups, solid line; sub-groups, dashed line). **b,** An expanded enrichment map shows the relationship between gene-sets enriched in deletions (panel a) and sets of known ASD/ID genes. Node color hue represents the class of gene-set (i.e. enriched in deletions, red; known disease genes (ie. ASD and/or ID genes), blue; enriched only in disease genes, yellow). Edge color represents the overlap between gene-sets enriched in deletions (green), from disease genes to enriched sets (blue), and between sets enriched in deletions and in disease genes or between disease gene-sets only (orange). The major functional groups are highlighted by filled circles (enriched in deletions, green; enriched in ASD/ID, blue).

Table 1

Global burden of genic rare CNVs in cases vs. controls[1]

Type	Classification	Total CNVs (n)	*P*	Case/Ctrl. ratio	Baseline rate (Ctrl.)	P_{corr}
	None					
All	All	5,478	**0.012**	1.19	3.59	**0.003**
Deletions only	All	2,757	**0.008**	1.26	1.08	**0.006**
Duplications only	All	2,721	0.072	1.16	2.51	**0.015**
	CNV frequency					
All	2-6x	1,831	0.058	1.26	1.03	0.062
	1x	1,419	0.375	1.03	0.83	0.180
Deletions only	2-6x	1,094	**0.004**	1.57	0.43	**0.011**
	1x	880	**0.036**	1.26	0.30	**0.036**
Duplications only	2-6x	977	0.203	1.16	1.05	0.118
	1x	896	0.749	0.92	0.72	0.442
	CNV size					
All	30 – 500 kb	5,086	0.313	1.03	2.72	0.181
	≥500 kb	392	**0.005**	1.69	0.88	**0.005**
Deletions only	30 – 500 kb	2,645	**0.004**	1.24	0.85	**0.004**
	≥500 kb	112	0.209	1.32	0.23	0.143
Duplications only	30 – 500 kb	2,441	0.801	0.93	1.86	0.518
	≥500 kb	280	**0.007**	1.82	0.65	**0.012**

[1]Gene-count in cases vs. controls. Total burden for genes intersected by CNVs in ASD cases (n = 996) compared to controls (n = 1,287). Gene coordinates were defined by the RefSeq boundaries plus a 10 kb region on either side. All genomic analyses used NCBI Build 36. Genome-wide *P*-values were estimated in 100,000 permutations (one-sided) and additionally corrected (P_{corr}) for global case-control differences in CNV rate and size. Significant differences ($P \leq 0.05$) are bold. Analyses were further stratified according to CNV type ('Deletions only' and 'Duplications only') and frequency (single occurrences (1x) or CNVs observed two to six times in the total sample (2-6x)). Note that for the two lower-frequency groups, the 'Deletions only' and 'Duplications only' counts are not expected to sum to the 'All' count (see ref. [26] and Supplementary Information).