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Oligogenic heterozygosity in individuals with high-functioning autism spectrum disorders

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Autism spectrum disorders (ASDs) are a heterogeneous group of neuro-developmental disorders. While significant progress has been made in the identification of genes and copy number variants associated with syndromic autism, little is known to date about the etiology of idiopathic non-syndromic autism. Sanger sequencing of 21 known autism susceptibility genes in 339 individuals with high-functioning, idiopathic ASD revealed *de novo* mutations in at least one of these genes in 6 of 339 probands (1.8%). Additionally, multiple events of oligogenic heterozygosity were seen, affecting 23 of 339 probands (6.8%). Screening of a control population for novel coding variants in *CACNA1C*, *CDKL5*, *HOXA1*, *SHANK3*, *TSC1*, *TSC2* and *UBE3A* by the same sequencing technology revealed that controls were carriers of oligogenic heterozygous events at significantly ($P < 0.01$) lower rate, suggesting oligogenic heterozygosity as a new potential mechanism in the pathogenesis of ASDs.

INTRODUCTION

Autism spectrum disorders (ASDs) are a heterogeneous group of neuro-developmental disorders that are characterized by impaired social interaction and communication, and by restricted and repetitive behaviors. The autistic disorder (AD), Asperger syndrome (AS) and pervasive developmental disorder not otherwise specified (PDD-NOS) are recognized as three subgroups of the ASDs by the current version of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). The estimated prevalence of the ASD is 1/91 among 3–17 years old and 1/110 among 8 years old children (1,2).

ASDs are highly heritable, as evidenced by twin and family studies suggesting the heritability of autism to be >90%. Autism affects predominantly males, with an overall male-to-female ratio of 4:1. The male predominance is much more pronounced in high-functioning autism and AS, and may be as high as 14:1 within these subgroups (3). Recent advances in the field of autism genetics have led to the identification of several autism susceptibility genes and the appreciation of both *de novo* and inherited copy number variants (CNVs) in the etiology of ASDs (4,5).

In contrast to studies of CNV, genetic linkage and genome-wide association studies have been slower to identify susceptibility genes contributing to the heritability of autism, and many association analyses have had inadequate power. It is recognized that each genetic susceptibility locus identified to date accounts for only a small fraction of ASD cases (typically <1%). While significant progress has been made in the identification of genes and CNVs associated with syndromic autism (i.e. ASD as part of an underlying genetic syndrome as well as ASD associated with congenital malformations and facial

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Table 1. Novel, coding non-synonymous variants detected by Sanger sequencing of 21 autism susceptibility genes in 339 probands with the ASD

Gene name	Chr	Coordinate	Allele	Mutation type	RefSeqID	Ref AA	Var AA	Number affected patients	Number affected controls
ARX	chrX	24941366	T/G	Missense	NM_139058	Thr	Pro	1	0
CACNA1C	chr12	2437094	C/T	Missense	NM_000719	Arg	Cys	1	0
CACNA1C	chr12	2465684	T/C	Missense	NM_000719	Ile	Thr	1	2
CACNA1C	chr12	2484330	G/T	Missense	NM_000719	Gly	Val	0	1
CACNA1C	chr12	2529447	G/A	Missense	NM_000719	Gly	Arg	1	0
CACNA1C	chr12	2564900	G/A	Missense	NM_000719	Gly	Arg	1	0
CACNA1C	chr12	2564912	C/T	Missense	NM_000719	Pro	Ser	4	4
CACNA1C	chr12	2576670	T/G	Missense	NM_000719	Phe	Cys	1	0
CACNA1C	chr12	2590088	G/A	Missense	NM_000719	Val	Ile	0	1
CACNA1C	chr12	2656634	G/A	Missense	NM_000719	Ala	Thr	1	0
CACNA1C	chr12	2658877	G/A	Missense	NM_000719	Gly	Ser	2	0
CACNA1C	chr12	2658928	G/A	Missense	NM_000719	Ala	Thr	1	0
CACNA1C	chr12	2659126	G/A	Missense	NM_000719	Gly	Ser	0	1
CACNA1C	chr12	2659162	G/A	Missense	NM_000719	Gly	Arg	2	0
CACNA1C	chr12	2662090	T/C	Missense	NM_000719	Leu	Pro	1	0
CACNA1C	chr12	2665254	C/T	Missense	NM_000719	Arg	Cys	6	0
CACNA1C	chr12	2667958	G/C	Missense	NM_000719	Ala	Pro	0	1
CACNA1C	chr12	2668046	G/A	Missense	NM_000719	Ser	Asn	0	1
CACNA1C	chr12	2668129	G/A	Missense	NM_000719	Val	Ile	1	0
CACNA1C	chr12	2668205	C/G	Missense	NM_000719	Ala	Gly	0	1
CDKL5	chrX	18526627	A/G	Missense	NM_001037343	His	Arg	1	0
CDKL5	chrX	18581529	G/A	Missense	NM_001037343	Val	Ile	0	1
EML1	chr14	99445547	A/G	Missense	NM_004434	Asn	Ser	1	0
EML1	chr14	99450317	G/A	Missense	NM_004434	Asp	Asn	1	0
FOXP2	chr7	114081280	C/A	Missense	NM_148899	Pro	Thr	1	0
FOXP2	chr7	114091661	A/G	Missense	NM_148899	Asn	Ser	1	0
FOXP2	chr7	114117153	A/C	Missense	NM_148899	His	Pro	2	0
FOXP2	chr7	114117169	A/T	Missense	NM_148899	Glu	Asp	1	0
GRID2	chr4	94563072	C/T	Missense	NM_001510	Pro	Leu	1	0
GRID2	chr4	94766467	G/A	Missense	NM_001510	Val	Ile	2	0
HOXA1	chr7	27101672	C/A	Missense	NM_005522	Ala	Ser	0	1
HOXA1	chr7	27101822	T/C	Missense	NM_153620	Thr	Ala	0	1
HOXA1	chr7	27101874	G/C	Missense	NM_153620	Ile	Met	2	0
MAPK3	chr16	30035564	G/A	Missense	NM_002746	Pro	Ser	1	0
MAPK3	chr16	30040668	T/G	Missense	NM_002746	Thr	Pro	1	0
MECP2	chrX	152949868	G/C	Missense	NM_004992	Ala	Gly	1	0
PTEN	chr10	89680805	A/G	Missense	NM_000314	Thr	Ala	1	0
PTEN	chr10	89701980	C/G	Missense	NM_000314	Phe	Leu	1	0
SHANK3	chr22	49490328	G/A	Missense	NM_001080420	Arg	His	0	1
SHANK3	chr22	49506750	G/A	Missense	NM_001080420	Gly	Asp	1	0
SHANK3	chr22	49506884	A/T	Missense	NM_001080420	Ser	Cys	2	0
SHANK3	chr22	49507020	G/A	Missense	NM_001080420	Arg	Lys	1	0
SHANK3	chr22	49507097	G/A	Missense	NM_001080420	Ala	Thr	1	0
SHANK3	chr22	49507125	T/G	Missense	NM_001080420	Val	Gly	0	1
SHANK3	chr22	49507260	C/T	Missense	NM_001080420	Thr	Met	0	1
SHANK3	chr22	49507359	C/T	Missense	NM_001080420	Pro	Lys	0	1
SHANK3	chr22	49507406	G/A	Missense	NM_001080420	Val	Met	0	1
SHANK3	chr22	49516073	C/T	Missense	NM_001080420	Pro	Ser	1	0
SHANK3	chr22	49516107	G/T	Missense	NM_001080420	Ser	Ile	0	1
SHANK3	chr22	49516125	C/T	Missense	NM_001080420	Ala	Val	2	0
SHANK3	chr22	49516230	C/T	Missense	NM_001080420	Thr	Ile	1	0
SHANK3	chr22	49516308	G/A	Missense	NM_001080420	Arg	His	1	0
SHANK3	chr22	49516325	C/T	Missense	NM_001080420	Pro	Ser	1	0
SHANK3	chr22	49516329	C/T	Missense	NM_001080420	Ser	Lys	1	0
SHANK3	chr22	49516346	G/A	Missense	NM_001080420	Arg	Thr	1	0
SHANK3	chr22	49516365	G/A	Missense	NM_001080420	Gly	Asp	3	0
SHANK3	chr22	49516370	C/A	Missense	NM_001080420	Pro	Thr	1	1
SHANK3	chr22	49516374	G/A	Missense	NM_001080420	Gly	Asp	0	1
TSC1	chr9	134761739	T/G	Missense	NM_000368	Met	Leu	1	0
TSC1	chr9	134761835	C/T	Missense	NM_000368	Gly	Ser	1	2
TSC1	chr9	134767919	T/C	Missense	NM_000368	Asn	Ser	2	0
TSC1	chr9	134768873	G/A	Missense	NM_000368	His	Tyr	2	3
TSC1	chr9	134771026	T/C	Missense	NM_000368	Lys	Arg	7	1
TSC1	chr9	134771108	C/T	Missense	NM_000368	Gly	Ser	0	1
TSC1	chr9	134772035	G/A	Missense	NM_000368	Pro	Ser	1	0
TSC1	chr9	134775834	G/A	Missense	NM_000368	Ser	Leu	2	0

Continued

Table 1. *Continued*

Gene name	Chr	Coordinate	Allele	Mutation type	RefSeqID	Ref AA	Var AA	Number affected patients	Number affected controls
TSC1	chr9	134776272	G/T	Missense	NM_000368	Thr	Asn	3	1
TSC1	chr9	134776684	G/A	Missense	NM_000368	Arg	Trp	1	0
TSC1	chr9	134776689	G/A	Missense	NM_000368	Ser	Leu	0	1
TSC1	chr9	134790812	A/C	Missense	NM_000368	Leu	Val	0	1
TSC2	chr16	2038634	G/A	Missense	NM_021056	Ser	Asn	0	1
TSC2	chr16	2040411	A/G	Missense	NM_021056	Met	Val	1	7
TSC2	chr16	2040453	A/G	Missense	NM_021056	Ile	Val	0	0
TSC2	chr16	2043393	A/T	Missense	NM_021056	Glu	Val	0	1
TSC2	chr16	2043407	G/A	Missense	NM_021056	Ala	Thr	0	1
TSC2	chr16	2052990	G/A	Missense	NM_021056	Ala	Thr	2	1
TSC2	chr16	2054427	A/C	Missense	NM_021056	Lys	Gln	1	0
TSC2	chr16	2055530	C/T	Missense	NM_021056	Arg	Cys	1	0
TSC2	chr16	2060557	A/G	Missense	NM_021056	Ile	Val	1	0
TSC2	chr16	2061577	T/G	Missense	NM_021056	Asp	Glu	0	1
TSC2	chr16	2061871	G/A	Missense	NM_021056	Ala	Thr	2	0
TSC2	chr16	2066142	C/G	Missense	NM_021056	Phe	Leu	1	1
TSC2	chr16	2067712	G/C	Missense	NM_000548	Glu	Gln	1	2
TSC2	chr16	2069567	C/T	Missense	NM_021056	Pro	Leu	1	0
TSC2	chr16	2070191	C/T	Missense	NM_021056	Ala	Val	1	0
TSC2	chr16	2074318	C/T	Missense	NM_021056	Ser	Leu	0	1
TSC2	chr16	2074330	G/A	Missense	NM_021056	Arg	Gln	1	0
TSC2	chr16	2074497	G/A	Missense	NM_021056	Gly	Arg	1	0
TSC2	chr16	2078075	C/A	Missense	NM_021056	Ser	Arg	1	0
UBE3A	chr15	23166916	T/A	Missense	NM_000462	Ser	Cys	0	6
UBE3A	chr15	23167822	C/T	Missense	NM_000462	Ala	Thr	10	13
UBE3A	chr15	23167903	T/C	Missense	NM_000462	Thr	Ala	1	0
UBE3A	chr15	23171810	T/G	Missense	NM_000462	Lys	Gln	0	9

Coordinates based on genome build hg18. Chr, chromosome; AA, amino acid.

dysmorphism), little is known to date about the etiology of idiopathic autism (ASD of unknown etiology, with no evident organic cause or underlying dysmorphisms). For the latter, a genetic model in which several genes interact with one another to produce the autism phenotype has been suggested (6). Using family history studies and twin studies of autism, Pickles *et al*. (7) rejected single-locus and heterogeneity models for the inheritance of autism in favor of a multi-locus model involving anything from 2 to 10 loci, with three interacting loci being most plausible. However, to date, there are no data to support or refute this model. In this study, we set out to evaluate whether sequence variations in genes known to cause syndromic autism contribute to the etiology of high-functioning, non-syndromic autism.

RESULTS

We sequenced a total of 21 genes (*ARX*, *ATRX*, *CACNA1C*, *CDKL5*, *EML1*, *FMR1*, *FOXP2*, *GRID2*, *HOXA1*, *KCTD13*, *MAPK3*, *MECP2*, *NLGN3*, *NLGN4X*, *PTEN*, *RS1*, *SHANK3*, *SLC25A12, TSC1*, *TSC2* and *UBE3A*) known to cause syndromic autism and other cognitive disorders (8–24), in 339 probands with high-functioning ASDs from the Simons Simplex Collection. Sequencing was performed by the traditional Sanger method, and coding non-synonymous variants and coding insertions or deletions (indels) were confirmed by a second, independent sequencing method (454 pyrosequencing).

A total of 818 coding non-synonymous events were detected at 92 sites, and 51 coding indels (11 sites) were identified. Excluding all variants annotated in dbSNP131 and the 1000 Genomes Project (data release pilot 2) resulted in a data set of 105 novel coding non-synonymous variants (66 sites) and 47 coding indels (8 sites) (Tables 1 and 2). Of note, no nonsense mutations were detected in any of the 21 genes among the 339 probands tested, and only one frame-shifting indel (*HOXA1*) that was inherited from a non-affected parent was identified.

We were able to follow up on 115 variants of interest (coding non-synonymous and coding indels), for which sufficient DNA from both parents was available. The analysis indicated that whereas the vast majority of events (108/115) were inherited from an unaffected parent, we did detect seven novel coding non-synonymous variants (in six patients) that were *de novo* events (Table 3). These *de novo* variants included three different small indels and three different missense mutations. All but one (a 3 bp deletion in *HOXA1*, present in two probands) were seen in single patients. One patient carried two *de novo* variants in the *HOXA1* gene, the aforementioned small deletion, and a missense mutation in a moderately conserved amino acid (p.I61M). One patient was found to carry a *de novo* 9 bp deletion in *TSC2*, which was out of frame, therefore deleting four amino acids and inserting an arginine in a highly conserved domain of the protein. Of note, this particular patient did not have a history of tuberous sclerosis, or a positive family history of tuberous sclerosis. The Simons Simplex Collection database does not contain information

Table 2. Novel, coding Indels detected by Sanger sequencing of 21 autism susceptibility genes in 339 probands with the ASD

Gene name	Chr	Coordinate	Mutation type	Number of basepairs	RefSeqID	Reference allele	Variant allele	Number affected patients	Number affected controls
HOXA1	chr7	27101863	Deletion	3	NM_153620	TGG	–	34	20
HOXA1	chr7	27101863	Insertion	3	NM_153620	–	TGG	1	0
HOXA1	chr7	27101863	Insertion	6	NM_153620	–	TGGTGG	1	0
HOXA1	chr7	27101863	Deletion	6	NM_153620	TGGTGG	–	0	1
TSC1	chr9	134761828	Insertion	3	NM_000368	–	CTG	0	1
UBE3A	chr15	23167844	Deletion	6	NM_130839	CTTTTC	–	1	0
HOXA1	chr7	27101857	Deletion	9	NM_153620	TGGTGGTGG	–	1	0
TSC2	chr16	2072468	Deletion	9	NM_021056	GCTGCCAAG	–	1	0
HOXA1	chr7	27100623	Frame shift deletion	1	NM_005522	C	–	1	0
FOXP2	chr7	114058841	Insertion	3	NM_148899	–	AGC	4	2
PTEN	chr10	89680779	Exon boundary deletion	5	NM_000314	TTAGT	–	1	0

Coordinates based on genome build hg18.

Table 3. *De novo* mutations detected by Sanger sequencing of 21 autism susceptibility genes in 339 probands with the ASD

Patient(s)	Gene	Chrom	Coordinate	Mutation type	Reference allele	Variant allele	Reference amino acid	Variant amino acid	Conservation
11 598	*FOXP2*	7	114058841	Insertion	–	AGC	–	Gln	8/8 species
11 446	*FOXP2*	7	114117153	Missense	A	C	His	Pro	11/11 species
11 030	*HOXA1*	7	27101863	Deletion	TGG	–	H	–	6/9 species
11 452	*HOXA1*	7	27101863	Deletion	TGG	–	H	–	6/9 species
11 452	*HOXA1*	7	27101874	Missense	G	C	Ile	Met	5/7 species
11 532	*PTEN*	10	89680805	Missense	A	G	Thr	Ala	8/10 species
11 549	*TSC2*	16	2072468	Deletion	GCTGCCAAG	–	Ser/Cys/Gln/Gly	Arg	10/11 species

Based on genome build hg18. Conservation based on USCS Genome Browser.

about brain imaging studies; however, it is documented that this patient has a history of seizures. Another patient carried a *de novo* missense mutation in *PTEN*, altering a moderately conserved threonine to an alanine (p.T78A). The patient has no known history or documented features of *PTEN* harmatoma tumor syndrome and his head circumference was at the 25th percentile. Lastly, two patients carried *de novo* mutations in the *FOXP2* gene. One had a missense variant of an amino acid that is conserved throughout species (p.H603P) and another patient had a 3 bp insertion, adding a glutamine in yet another highly conserved domain of the protein (Fig. 1). For all *de novo* mutations, unaffected siblings were tested, in order to rule out the remote possibility of germline mosaicism. None of the respective siblings carried the mutation identified in the probands.

Aside from *de novo* mutations, we found an interesting pattern of inheritance to the inherited events. Notably, 23/339 probands (6.8%) were found to carry two or more novel coding non-synonymous variants or coding indels in the 21 genes analyzed, representing cases of oligogenic heterozygosity (Table 4). Follow up on these oligogenic variants in the respective unaffected parents and siblings revealed that only four of these combinations were present in one of the parents, while 15 represented oligogenic combinations unique to the affected proband. Two additional combinations could fall into the 'unique' category, as they involve novel variants in the maternal allele of the *UBE3A* gene. However, grandparental samples were not available to further test the inheritance of the *UBE3A* allele. For two oliogenic events, the inheritance pattern could not be established, given failure of amplification in at least one of the two parents. Studying the unaffected siblings of 23 probands with oligogenic events, only 2 siblings were carriers of the same oligogenic combination, while 15 did not carry the respective combination. Amplification failed in two siblings and four probands did not have a sibling enrolled in the study.

Eighteen of the 23 oligogenic events clustered among 7 genes (*CACNA1C*, *CDKL5*, *HOXA1*, *SHANK3*, *TSC1*, *TSC2* and *UBE3A*). We performed Sanger sequencing of the entire coding regions of these 7 genes in a total of 376 controls, the same methodology that was used in the autistic probands. Control individuals had undergone psychiatric screening by questionnaire. Individuals with known psychiatric disorder or phenotypes consistent with obsessive-compulsive behaviors were excluded from our study. While a total of 99 coding non-synonymous variants and coding indels were identified among controls in the 7 genes analyzed, only 6 control individuals were carriers of oligogenic heterozygous events of these genes. The incidence of oligogenic heterozygous variants in two or more of the seven genes is significantly different between probands (18/339, i.e. 5.31%) and controls (6/376, i.e. 1.59%), as evidenced by Fisher's exact test ($P < 0.01$) (Table 5).

Retrospective analysis of the clinical phenotypes of probands affected with oligogenic compound heterozygosity

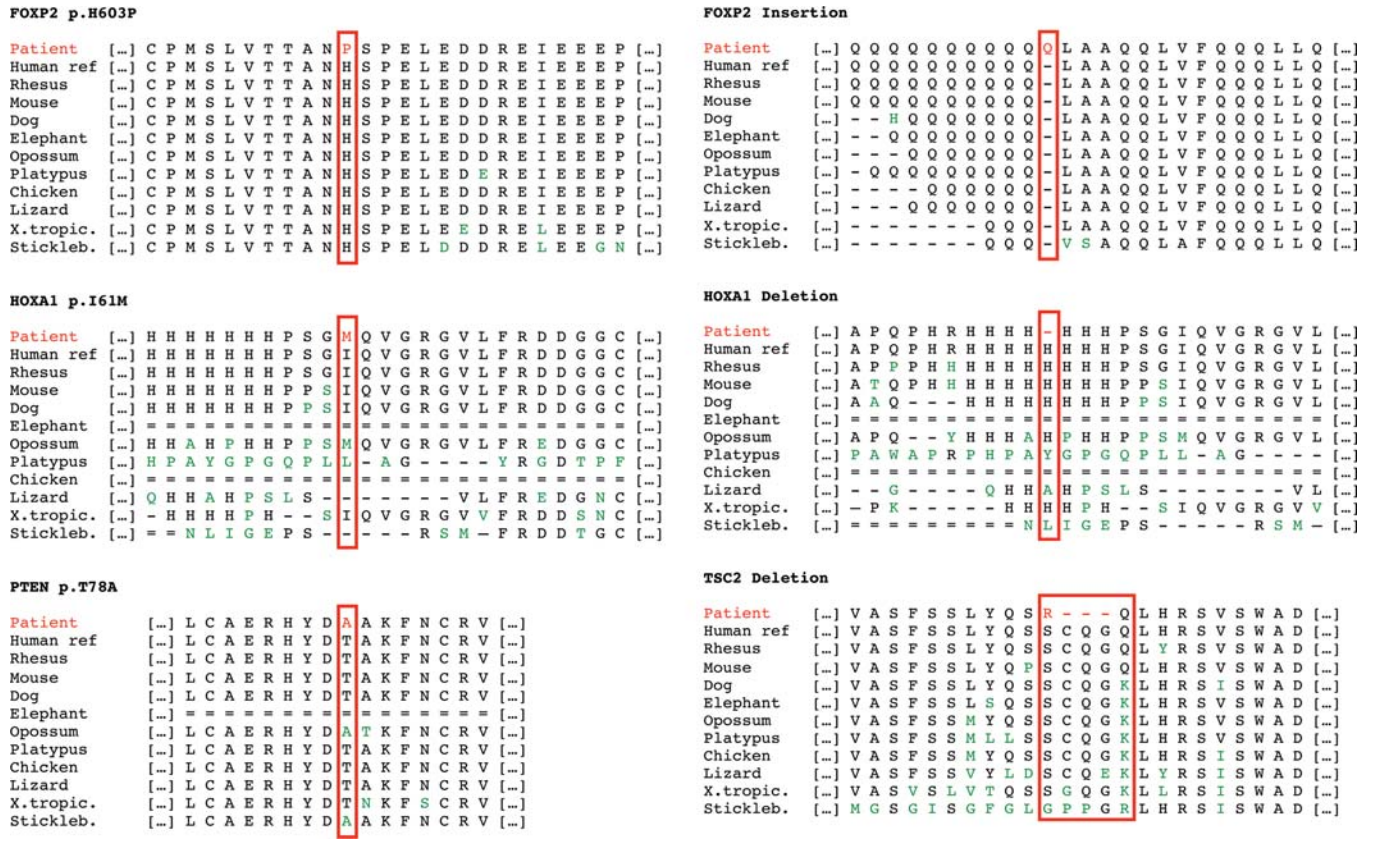

Figure 1. *De novo* mutations and evolutionary conservation. Six *de novo* mutations detected by Sanger sequencing of 21 autism susceptibility genes in 339 probands from the Simons Simplex Collection. Reference amino acids are displayed in black, variant amino acids in the patients in red color. Amino acids from different species which differ from the human sequence are displayed in green. Single line, no amino acids in the aligned species. Double line, aligned species has no alignable amino acids in the respective region. Information based on UCSC Genome Browser human genome build 18.

revealed that these individuals indeed represent a group of high-functioning autism with a total average IQ of 93.05 (SD = 22.75) (Table 6).

DISCUSSION

This study set out to identify the relationship of the genetics of syndromic and non-syndromic autism. The fact that only 6/339 probands (1.8%) carry a *de novo* novel, coding non-synonymous variant or coding indel in the 21 genes examined is consistent with their clinical presentation, as the patients selected represented cases of idiopathic autism rather than syndromic autism (which would be the expected phenotype caused by loss-of-function mutations in most of the genes tested). While this suggests that the individual mutations causing syndromic versus non-syndromic autism may be separate from each other, the actual number of *de novo* missense mutations in these genes is surprisingly high. It has been estimated that on average, a newborn carries 0.86 *de novo* amino acid altering mutations (25). Given this rate, our study of 21 genes in 339 probands should have revealed <1 (0.27) *de novo* missense mutations among these genes. The fact that the actual number of *de novo* mutations is much higher (22-fold increase for all tested genes) suggests that while severe loss-of-function mutations of given genes may cause syndromic autism, milder mutations of the same genes may be associated with non-syndromic autism. However, the comparison of *de novo* mutation rates between our own cohort and the per generation estimate cited above is limited by the fact that they rely on different detection methods and statistical analyses.

The two *de novo* mutations identified in *TSC2* and *PTEN* are clearly in genes known to cause syndromic autism. The other four *de novo* variants were identified in *HOXA1* and *FOXP2*, genes that are yet to be confirmed to be involved in autism or that show phenotypes on the autism spectrum. While a missense variant of *HOXA1* was reported in association with autism (15), most subsequent studies had failed to replicate an association of the gene to autistic phenotypes (26–31). As part of this study, we identified a *de novo* missense mutation of *HOXA1* in one patient, and a small *de novo* 3 bp deletion in a polyhistidine tract of the protein in another. The latter was seen at relatively high frequency (36 of 339 probands and 10 of 376 controls) and likely represents a common variant.

Mutations in the forkhead-domain gene *FOXP2* provided evidence that the gene is critical for human speech and language (14), but the number of autistic patients identified with *FOXP2* mutations has been very limited (32–35). In this study, we identified two patients with *de novo* mutations in *FOXP2*. While one of the two adds an additional glutamine to a polyglutamine tract of the protein, which may represent a

Table 4. Parental and sibling data for all variants participating in oligogenic heterozygous events in 23 ASD probands

Patient	Gene	Chrom	Coordinate	Mutation type	Patient status	Mother status	Father status	Designated sib status
11 445	*HOXA1*	7	27101863	Indel (3 bp del)	1	1	0	0
11 445	*TSC2*	16	2061871	Missense G/A	1	1	0	1
11 450	*CACNA1C*	12	2668129	Missense G/A	1	0	1	1
11 450	*SHANK3*	22	49516308	Missense G/A	1	–	0	–
11 450	*SHANK3*	22	49516346	Missense G/A	1	–	0	–
11 184	*HOXA1*	7	27101863	Indel (3 bp del)	1	1	1	1
11 184	*UBE3A*	15	23167822	Missense C/T	1	1	0	1
11 542	*HOXA1*	7	27101863	Indel (3 bp del)	1	0	1	0
11 542	*TSC1*	9	134775834	Missense G/A	1	0	1	–
11 370	*TSC1*	9	134771026	Missense T/C	1	1	0	1
11 370	*UBE3A*	15	23167822	Missense C/T	1	0	1	0
11 590	*CACNA1C*	12	2437094	Missense C/T	1	1	0	0
11 590	*TSC2*	16	2067712	Missense G/C	1	0	1	1
11 049	*CACNA1C*	12	2658877	Missense G/A	1	1	0	0
11 049	*CDKL5*	X	18526627	Missense A/G	2	1	0	0
11 444	*CACNA1C*	12	2564912	Missense C/T	1	1	0	1
11 444	*TSC1*	9	134776272	Missense G/T	1	0	1	0
11 256	*HOXA1*	7	27101863	Indel (3 bp del)	1	1	0	1
11 256	*TSC1*	9	134776272	Missense G/T	1	0	1	1
11 402	*HOXA1*	7	27101860	Indel (6 bp del)	1	1	0	0
11 402	*TSC2*	16	2052990	Missense G/A	1	–	–	0
11 540	*TSC1*	9	134776684	Missense G/A	1	0	1	0
11 540	*TSC2*	16	2070191	Missense C/T	1	0	1	1
11 540	*TSC2*	16	2078075	Missense C/A	1	1	0	1
11 028	*CACNA1C*	12	2665254	Missense C/T	1	1	0	0
11 028	*FOXP2*	7	114117153	Missense A/C	1	1	0	0
11 598	*FOXP2*	7	114058841	Indel (3 bp ins)	1	0	0	x
11 598	*GRID2*	4	94766467	Missense G/A	1	0	1	x
11 546	*TSC1*	9	134768873	Missense G/A	1	1	0	0
11 546	*UBE3A*	15	23167822	Missense C/T	1	0	1	0
11 468	*HOXA1*	7	27101863	Indel (3 bp del)	1	1	0	x
11 468	*UBE3A*	15	23167822	Missense C/T	1	1	0	x
11 376	*TSC1*	9	134771026	Missense T/C	1	1	0	1
11 376	*GRID2*	4	94766467	Missense G/A	1	0	1	0
11 202	*CACNA1C*	12	2665254	Missense C/T	1	1	0	x
11 202	*TSC1*	9	134771026	Missense T/C	1	2	0	x
11 202	*EML1*	14	99450317	Missense G/A	1	0	1	x
11 290	*SHANK3*	22	49516073	Missense C/T	2	–	–	–
11 290	*TSC1*	9	134771026	Missense T/C	1	0	1	1
11 685	*CACNA1C*	12	2658928	Missense G/A	1	0	1	0
11 685	*MECP2*	X	152949868	Missense G/C	2	1	0	0
11 714	*HOXA1*	7	27101863	Indel (3 bp del)	1	1	0	–
11 714	*CACNA1C*	12	2564900	Missense G/A	1	0	1	0
11 780	*CACNA1C*	12	2662090	Missense T/C	1	–	1	x
11 780	*GRID2*	4	94563072	Missense C/T	1	–	1	x
11 724	*UBE3A*	15	23167822	Missense C/T	1	1	0	0
11 724	*SHANK3*	22	49516370	Missense C/A	1	–	–	–
11 543	*UBE3A*	15	23167844	Indel (6 bp del)	1	0	1	0
11 543	*TSC2*	16	2054427	Missense A/C	1	1	0	1

'0', homozygous for reference allele; '1', heterozygous for variant allele; '2', homozygous or hemizygous for variant allele; '–', data not available; 'x', no sibiling. Coordinates based on genome build hg18.

Table 5. Fisher's exact test analysis for oligogenic heterozygous events

Observed	Case	Control	Total
Oligogenic event	18	6	24
No oligogenic event	321	370	691
Total	339	376	715

Probands with high-functioning ASD and control individuals display significantly different frequencies of oligogenic heterozygous events in two or more of seven genes ($P = 0.00653$, Fisher's exact test).

benign variant, the other represents a missense mutation (p.H603P) in a protein domain that is highly conserved throughout species. The two patients identified to carry *de novo* mutations of *FOXP2* were both diagnosed with AD. Testing of their communication skills by the communication domain of the Vineland Adaptive Behavioral Scale II (VABS-II) revealed low scores in both individuals (74 in individual 11 598, and 77 in individual 11 446), suggesting moderate to significant impairment of communicative skills in both probands. These findings strengthen the role of *FOXP2* and its contributions to the ASDs.

Table 6. Clinical phenotypes and the genes involved in oligogenic heterozygous events among 23 probands with ASD

Patient ID	Sex	Age (years)	Diagnosis	IQ score (total)	First gene	Second gene
11 445	Male	8	AD	78	*HOXA1*	*TSC2*
11 450	Male	5	PDD-NOS	77	*CACNA1C*	*SHANK3*
11 184	Male	9	AD	93	*HOXA1*	*UBE3A*
11 542	Female	12	PDD-NOS	116	*HOXA1*	*TSC1*
11 370	Male	14	AD	96	*TSC1*	*UBE3A*
11 590	Male	10	Asperger	96	*CACNA1C*	*TSC2*
11 049	Male	7	PDD-NOS	137	*CACNA1C*	*CDKL5*
11 444	Female	16	Asperger	104	*CACNA1C*	*TSC1*
11 256	Male	11	AD	108	*HOXA1*	*TSC1*
11 402	Male	8	PDD-NOS	91	*HOXA1*	*TSC2*
11 540	Male	8	PDD-NOS	57	*TSC1*	*TSC2*
11 028	Male	9	AD	108	*CACNA1C*	*FOXP2*
11 598	Male	5	AD	71	*FOXP2*	*GRID2*
11 546	Male	11	Asperger	119	*TSC1*	*UBE3A*
11 468	Male	10	PDD-NOS	87	*HOXA1*	*UBE3A*
11 376	Male	7	AD	91	*TSC1*	*GRID2*
11 202	Male	11	PDD-NOS	82	*CACNA1C*	*TSC1*
11 290	Male	11	AD	131	*SHANK3*	*TSC1*
11 685	Male	5	AD	98	*CACNA1C*	*MECP2*
11 714	Male	6	Asperger	95	*CACNA1C*	*HOXA1*
11 724	Male	10	AD	55	*SHANK3*	*UBE3A*
11 543	Male	16	AD	57	*TSC2*	*UBE3A*
Average		9.5		93.05		
SD		3.19		22.75		

AD, autistic disorder; PDD-NOS, pervasive developmental disorder not otherwise specified.

As part of this study, 18 of 339 probands were found to be carriers of novel oligogenic heterozygous coding variants, even among the small number of genes analyzed. The occurrence of oligogenic heterozygous events is of particular interest, as it has been suggested before that autism could represent a complex genetic disorder that results from simultaneous genetic variations in multiple genes (4). Following the same concept, a two-hit model for CNVs has been proposed for severe developmental delay (36) and subsequently been discussed for epilepsy as well (37). For autism, Pinto *et al.* (38) reported the occasional combination of *de novo* and inherited CNVs within a given family. While this study of 21 genes provides limited insight in the actual complexity of autism genetics, the data show significant increase in oliogogenic heterozygous combinations of novel coding variants in genes such as *CACNA1C*, *CDKL5*, *HOXA1*, *SHANK3*, *TSC1*, *TSC2* and *UBE3A* among autistic probands compared with control individuals. Given the uncertain significance of the aforementioned 3 bp deletion in the polyhistidine tract of HOXA1, we re-analyzed our data set excluding this common variant. This would leave 14 oligogenic heterozygous events among 339 probands and 4 oligogenic heterozygous events among 376 controls, which is still highly significant by Fisher's exact test ($P = 0.01448$).

Studying the parents and unaffected siblings for the presence of oligogenic events revealed that the vast majority of these combinations are unique to the proband. However, the fact that four parents and two siblings carried the same combinations of oligogenic heterozygosity reveals that at least some of these events on their own are not sufficient to cause autism. One might speculate that the accumulation of several, if not many of such hypomorphic mutations causes a genetic load, which will ultimately cross a given threshold and lead to clinical manifestation of the ASD in the respective individuals (Fig. 2). Our study is limited by the small number of genes tested, and the full range of oligogenic heterozygous events contributing to the etiology of autism will only become evident once large scale, whole exome or whole genome data sets of sequences from autistic individuals are analyzed to evaluate for such combinatorial events. Also, while our study detected a significant difference in the incidence of oligogenic heterozygous variants between probands and controls for the aforementioned genes, it might be the case that controls have different heterozygous combinations with other genes that were not tested.

'Synergistic heterozygosity' has been described as a potential disease mechanism in some metabolic disorders, with the idea that concurrent partial defects in more than one pathway, or at multiple steps in one pathway may lead to disease, even though no complete deficiency in any one enzyme is present (39). In the field of autism genetics, several hypomorphic variants may accumulate either in a specific signaling pathway, or a subcellular compartment (such as the synapse) to exceed a threshold and result in phenotypic manifestation. This would be consistent with the data from clinical studies whereby children from families in which both parents manifest subthreshold autistic traits are more likely to show more severe impairment in reciprocal and social behavior (40).

It is noteworthy that the average full-scale IQ of individuals with *de novo* mutations in some of the 21 autism susceptibility genes was 71.6 (SD = 19.2), whereas the average full-scale IQ of those with oligogenic heterozygous events without *de novo* mutations was 94.1 (SD = 22.2). While evidence is emerging that intellectual disabilities might be widely attributable to *de*

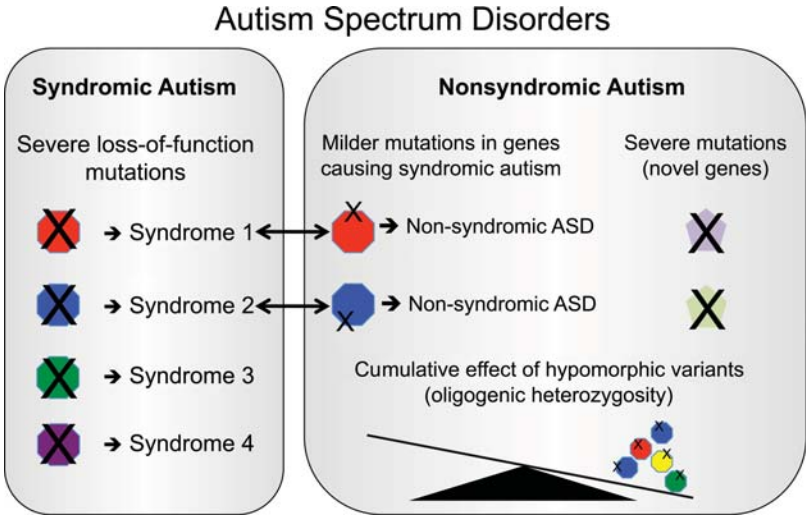

Figure 2. Proposed models of inheritance for ASDs. Left panel: syndromic autism is mostly caused by severe loss-of-function mutations of specific genes, with each gene causing a specific syndrome. Right panel: non-syndromic autism may be caused by milder mutations in genes that are known to cause syndromic autism or by mutations in novel genes, unrelated to syndromic autism. Oligogenic heterozygosity of hypomorphic variants in genes known to cause syndromic autism may have a cumulative effect, resulting in non-syndromic autism. Mutations may be point mutations or coding indels, as well as CNVs.

novo mutations (41), cases of the high-functioning ASD may rather be attributable to co-inheritance of subtle, yet functionally significant variants in respective genes.

In summary, our data uncovered *de novo* mutations in 1.8% of the ASD patients we studied and suggest that oligogenic heterozygosity of coding non-synonymous variants and coding indels may constitute a novel pathogenic mechanism or risk for ASDs. The data from this study provide a framework upon which to expand investigations into oligogenic events in larger data sets. A model of oligogenic heterozygosity may offer at least a partial explanation for why traditional linkage analysis and mapping approaches have been rather unsuccessful in identifying genetic variants predisposing to ASDs. Whole exome sequencing analyzed in the context of genes involved in pathways critical for neuronal development and function is likely to be a productive approach to unravel oligogenic and combinatorial events that might increase an individual's risk for ASDs.

MATERIALS AND METHODS

Subject recruitment

We obtained DNA samples (from lymphoblast cell lines) from probands and their family members through the Simons Simplex Collection (SSC), a resource of the Simons Foundation Autism Research Initiative (SFARI). The SSC represents a repository of clinical, neuropsychological, phenotypic and genetic data of >2000 families with simplex autism. This is a collection of cases of sporadic ('simplex') autism with unaffected parents and unaffected siblings. On average, probands in the SSC exhibit moderate-to-severe autistic symptoms with relatively little intellectual disability (42). Control DNAs were obtained from the NIMH through the Center for Collaborative Genetic Studies on Mental Disorders. Control individuals had undergone a comprehensive online psychiatric questionnaire.

Controls were ruled out if they

- Replied 1 (Yes) to both A8d and A8e (Depression);
- Replied 1 (Yes) to both B14 and B15 (Generalized Anxiety Disorder);
- Replied 3, 4 or 5 to G2a or replied 5 to G7a (Alcohol Dependence);
- Replied 1 to H3 AND 3, 4 or 5 to H3a (Drug Dependence);
- Replied 1 or −1 to any question of section I (Obsessive Compulsive Behavior).

Probands and controls were sex matched at a ratio of M:F = 6.8:1.

Sequencing

We have designed primers and amplified coding regions and intron/exon junctions of the 21 genes according to standard protocols. polymerase chain reaction (PCR) products were sequenced using traditional Sanger fluorescent di-deoxy methods on ABI 3730 capillary sequencers. Resulting sequences were analyzed and single nucleotide variants and Indels detected using SNPdetector software (43).

Validation

All coding non-synonymous variants and coding indels detected in Sanger sequencing were assayed with PCR-directed orthogonal sequencing validation. Targets were re-amplified, and resulting PCR reactions pooled and sequenced using 454 pyrosequencing. Resulting 454 reads were mapped to the human reference sequence using BLAT and CrossMatch alignment software. We required coverage

of >50 at the site and variant allele fraction >20% to validate a variant.

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Conflict of Interest statement. None declared.

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