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Review

# Next Generation Sequencing in Autism Spectrum Disorder

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**Abstract:**

Autism spectrum disorder is a clinically heterogeneous condition, characterized by social deficits, language impairment, repetitive behaviors, and restricted interest. Autism displays significant genetic heterogeneity. In the past one and a half decades, next generation sequencing has enabled identification of many variants that predispose to autism. These discoveries have improved understanding of the disease etiology of autism spectrum disorder. In this review article, we will address how development of next generation sequencing has helped answer the following questions: 1. What are the modes of transmission/inheritance of autism? 2. What is the nature of genetic risk factors that contribute to autism? 3. Why is there a higher prevalence of autism in males than females?

**Keywords**

Next generation sequencing, autism spectrum disorder, genetic variants, chromosome conformation, male bias, machine learning

## 1. Introduction

The first cases of autism spectrum disorder (ASD) were reported by Leo Kanner in 1943, characterized by social disconnection and impaired language skills beginning from early childhood. In subsequent years, more individuals with autism were identified, with the number of males being about four times that of females [1]. In the late 1970s, twin studies estimated the heritability of autism spectrum disorder to be greater than 80% [2]. From these early encounters, several questions arose that have puzzled us for decades: 1. What are the modes of transmission/inheritance of autism? 2. What is the nature of genetic risk factors that contribute to autism? 3. Why is there a male bias in autism?

During early years, genetic linkage analysis and cytogenetic tools were leveraged to identify genes involved in the pathophysiology of autism. A few autism risk loci were initially identified, namely regions 2q, 7q31-q33, 15q11-q13, 16p, 19p, and Xp [3-5]. While they struggled with inconsistent genetic association findings [6-9] and low prevalence of mutations in autism individuals [10], scientists quickly recognized the huge genetic heterogeneity of autism [11]. The power of genetic loci detection at the time was limited by both the capacity to manipulate large sample sizes and resolution of detection tools. With the advent of next generation sequencing in the 2000s, we witnessed a blossom of gene discovery, achieved unprecedented resolution to the level of the single nucleotide, and revolutionized our understanding of the etiology of autism. In this review article, we will present a timeline for the identification of autism risk loci (Figure 1), discuss how next generation sequencing helped us to approach the previously raised questions, and present challenges and future directions. Since many review articles have summarized earlier findings, we will mainly focus on findings from the past five years.

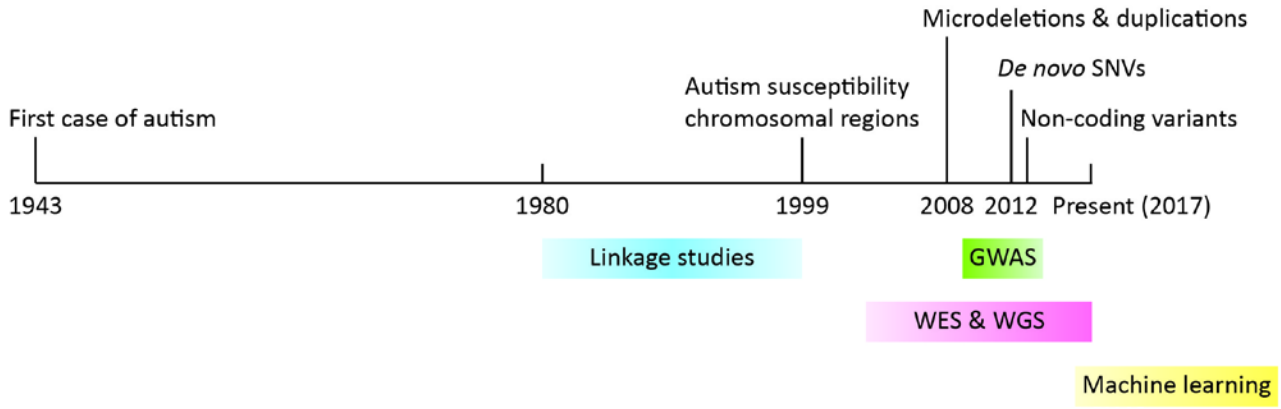


**Figure 1** Timeline of important findings and methods used in the discovery of autism risk loci. Years are denoted on the black line. Findings are denoted at the top while methods are indicated at the bottom.

## 2. From Linkage Disequilibrium Analysis to Genome Wide Association Studies

In the 1980s and 1990s, linkage studies were used to tackle the mode of inheritance in autism, using familial cases of autism, and with most results coming out consistent with an autosomal recessive inheritance model [12]. Human leukocyte antigen (HLA) genes and a few neuronal genes were tested as candidates. The genes tested included the HLA loci [13], fragile-X syndrome gene

*FMR1* [14], serotonin transporter gene *HTT* [7, 9], GABA receptor subunit gene *GABRB3* [9, 15], among others. However, association of these genes with ASD was either insignificant or inconsistent among different studies. Genome-wide screens with genetic markers, typically using a collection of 50-100 autism families, identified several autism susceptibility regions [3, 4, 16, 17], scattered across different chromosomes. Direct sequencing of genes that had been reported in autism probands in follow-up studies using larger cohorts usually led to conclusions that mutations in these genes were uncommon to autism [10]. No study was able to narrow the candidate regions to single genes, except for those known to cause syndromic autism. In the late 1990s, a consensus was reached that a fairly large number of genes may be involved in ASD, and that each individual gene may have a small effect [11].

With the implementation of next generation sequencing and accomplishment of the human genome project, scientists gained access to a larger number of tightly-spaced genetic markers, or single nucleotide polymorphisms (SNPs). The hope was to identify common variants underlying common disease, such as ASD, through regional or genome-wide association studies (GWAS) with collection of 1000-3000 patients and an unaffected control group. A handful of loci were reported as plausible risk loci for ASD [18-21], however, only SNPs near *CDH10* and *CDH9* and SNPs at 1p13.2 region were reproduced using independent cohorts [22-24]. Other studies found no association signal that met statistical significance [25-27]. Stratifying the samples by sex or subphenotypes helped to identify new candidate loci [28, 29], albeit this strategy met the same bottleneck of reproducibility. These findings highlighted the genetic heterogeneity of autism, and implicated that ASD may be largely attributed to rare variants.

## 3. Impact of Single Nucleotide Variants in Genes

With advancement of next generation sequencing technology, we were eventually capable of reading the genome at single nucleotide resolution. Studying families with shared ancestry greatly enhanced identification of risk loci with a recessive mode of inheritance [30-32], which included *UBE3B, CLTCL1, NCKAP5L, ZNF18, PCDH10, DIA1, NHE9, AMT, PEX7, SYNE1*, and others.

In 2012, three articles published back-to-back in Nature, highlighted the impact of *de novo* single nucleotide variants (SNVs) [33-35] on autism spectrum disorder. They performed whole exome sequencing (WES) on 500-1000 individuals respectively, and reported a significantly increased rate of gene-disrupting or loss-of-function *de novo* SNVs in subjects with ASD. The prevalence of these *de novo* changes was positively correlated with paternal age. Several autism risk genes with dominant effects were identified by these and other groups, including *SCN1A, SCN2A, CHD2, CHD8, KATNAL2, NTNG1, GRIN2B, LAMC3, DYRK1A, DAT1, SHANK1, SHANK3, SYNGAP1, TRIP12, PAX5, TCF20* [33-41]. Many of these genes are involved in FMRP-associated pathways or in the β-catenin/chromatin-remodeling protein network [34, 42]. Taken together, *de novo* SNVs may account for around 5-20% of ASD cases [43].

SFARI database is an involving database for genes implicated in autism susceptibility. We carefully curated all the SFARI genes associated with autism for their modes of inheritance, and found 432 dominant genes, 48 recessive ones, and 44 X-linked, with 357 genes showing insufficient evidence for a disease-driving effect (Figure 2, Supplementary Table 1). Beyond what was mentioned above, somatic mutations [44, 45], human-specific regions and developmental programs [46, 47], and increased burden of deleterious mutations in essential genes [48] have also

been implicated in ASD. Clinically and functionally validated autism risk genes have been reviewed in the following reference [49].

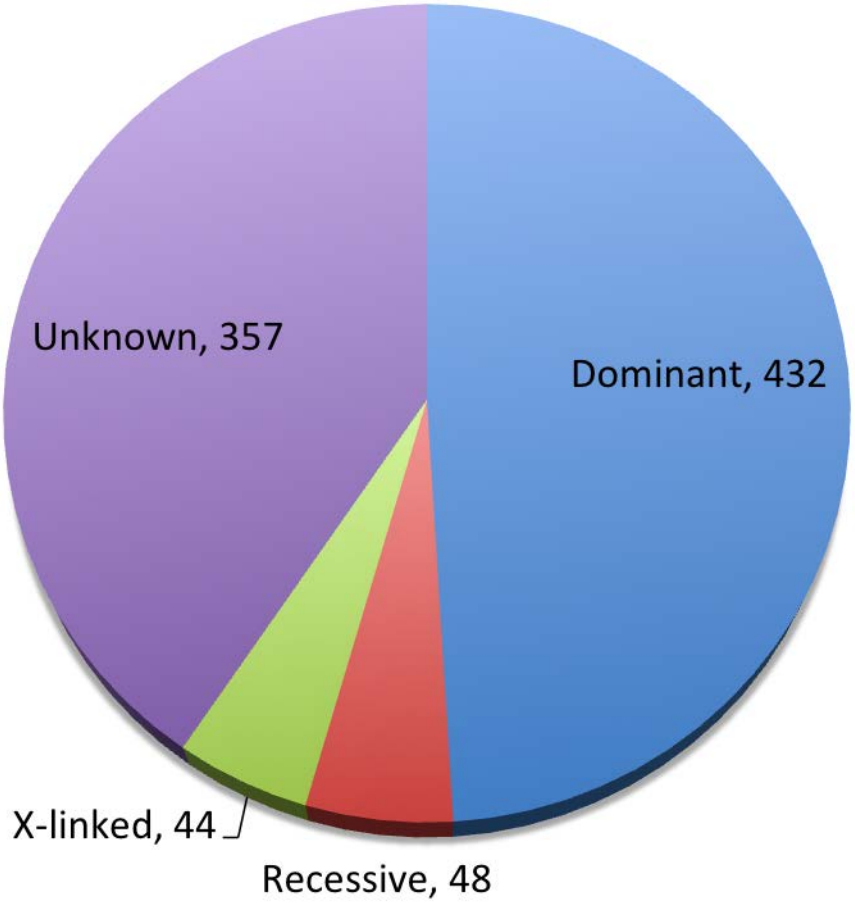


**Figure 2** Pie chart of inheritance patterns of SFARI genes.

**4. New Cytogenetic Findings**

Cytogenetic analysis represents another important means of uncovering the genetic cause of ASD. Next generation sequencing and the human genome project provided a reference genome for implementation of comparative genomic hybridization microarray (aCGH), which has a much higher resolution than traditional techniques. Such improvements led to identification of many inherited copy number variations (CNVs) that are associated with autism, including 1p34.2-p34.3, 2q23.1, 3q29, 7q11.23, 15q13.3, 16p11.2, 16q24.3, and 17q12-13 deletions or duplications [50]. Similarly, *de novo* CNVs present substantial risk for ASD, explaining approximately 2-7% of idiopathic autism cases [51-56]. Exonic CNVs and whole gene deletions or duplications that were present only in affected individuals overlapped with known ASD candidate genes and identified previously unreported loci [57, 58]. Notably, in the majority of autism families that harbor inherited or *de novo* CNVs, not all affected individuals carry the mutation, indicating that there are other risk factors that contribute to the disease manifestation [55].

Balanced chromosomal abnormalities (BCA) are powerful in pinpointing specific genes as well, in that the breakpoints usually disrupt expression of one to two genes. Next generation sequencing largely improved the resolution of breakpoint localization, and several potential candidate genes were implicated by *de novo* translocations and inversions, such as *TRIP8* [59],

*REEP3* [59], *NRXN1* [60], *CSMD3* [61], and *RAB11FIP5* [62]. Association of these genes with ASD will be better supported by additional evidence of variants within the genes and functional studies.

Compared with aCGH, whole genome sequencing (WGS) provides more detailed information about chromosomal rearrangements, albeit it has proven quite challenging to obtain copy number information. Recent progress involves identification of novel autism risk loci [63] and discovery of complex structural variations, in which different types of chromosomal rearrangements were generated at a single loci [64]. Application of *de novo* assembly of sequenced genomes uncovered previously undetectable mutations [65]. However the "gap" between read lengths of NGS (100 bp) and resolution of aCGH (5 kb) needs to be covered and will likely reveal many more variants.

To have a better idea of which autism risk loci were subject to copy number variation versus single nucleotide change, we curated all SFARI genes for evidence of SNV, CNV, and BCA. Our results show a substantial overlap between genes affected by SNV and CNV (Figure 3, Supplementary Table 1). In the future, it will be interesting to identify SNVs in genes that were initially associated with in CNVs alone.

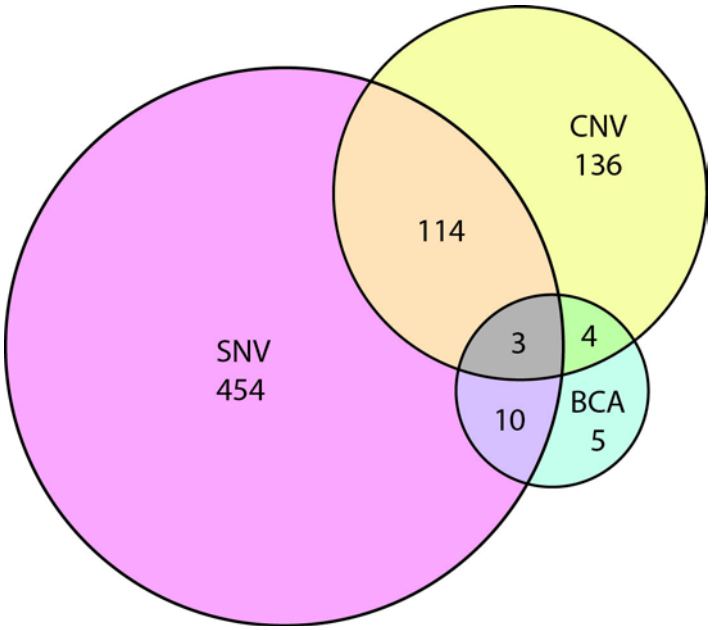


**Figure 3** Venn diagram showing the overlap of autism risk genes with evidence of copy number variation (CNV), single nucleotide variation (SNV), or balanced chromosomal abnormality (BCA).

**5. Non-coding Variants and Chromosome Conformation**

CNVs and SNVs that affect specific genes have been established as risk factors for autism spectrum disorder, which in total account for approximately 20% of ASD individuals [66]. Little effort had been invested into the vast intergenic regions of the genome until 2013, when rare inherited CNVs affecting only intergenic regions were reported to affect 3% of ASD cases [66]. Subsequently whole genome sequencing on ASD families, in which no CNV or SNV had been implicated, showed that probands had a significant enrichment of *de novo* loss-of-function mutations in DNase1 hypersensitive sites, which are putative regulatory regions [67]. MicroRNAs, long non-coding RNAs, and regions close to splicing sites have all been implicated in association with ASD through WGS and RNA profiling [68-71]. Dysregulation of the epigenome and

chromosome conformation may complement DNA mutations in the pathogenesis of autism [72-74], studies of which were enabled by ChIP-seq (Chromatin immunoprecipitation sequencing) and other techniques.

## 6. Rethinking Common Variants

Notably, some of the regulatory regions are enriched for genetic variants from GWAS studies [75]. Furthermore, 3D chromatin interaction map in the developing human brain showed that some GWAS loci regulate putative neuropsychiatric disease gene expression [74]. These findings urge us to rethink about common variants and their contribution to ASD and other neuropsychiatric disorders. In fact, some studies found that combinatorial effects of hypomorphic or common variants account for a major part of ASD heritability, yet rare variation contributes to variance in liability [76, 77].

Years of experience have taught us that such common variations scatter across the entire genome, with an impressive total number and a small individual impact. Machine learning emerged as a potential means of detecting the pattern of variation, dissecting genetic components and subphenotypes, as well as improving clinical diagnosis. Pioneering studies have applied machine learning to develop an observation-based diagnostic classifier of autism [78], and to predict new candidate genes based on features of the known ones [79]. We foresee unprecedented findings from machine learning of patient and unaffected individuals at a multi-dimensional level.

## 7. Possible Explanations for Male Bias

Autism has been consistently reported to affect more males than females, with the commonly referenced male-to-female ratio being 4:1. This cannot be explained simply by X-linked autism, due to the fact that there are more autosomal genes associated with autism than genes on the sex chromosome, and the prevalence of the X-linked genes alone does not account for the substantial difference between male and female cases. A female protective model has been proposed and supported by higher mutational load of *de novo* CNVs, *de novo* SNVs, and inherited variants in affected females compared to males [1, 49, 80]. Sex chromosomal genes, other sex related risk loci, hormone factors, and innate sex differences in neurodevelopmental and immune systems that may contribute to the sex bias of ASD have been identified [1, 81, 82]. While they cannot fully explain the sex-skewed prevalence of autism, one of the most convincing explanations emerged from an RNA-seq study, which discovered that genes highly expressed in males were significantly enriched for those that were upregulated in postmortem ASD brains [83]. Further investigations into naturally occurring sexual dimorphism and gene-environment interactions will help elucidate the mechanism of male bias in autism.

## 8. Overlap with Other Neuropsychiatric Disorders

Individuals with ASD show high rates of comorbidity with other neuropsychiatric disorders, including intellectual disability (ID, 45%), attention deficit hyperactivity disorder (ADHD, 28-44%), clinical depression (12-70%), epilepsy (8-30%), schizophrenia (SCZ), and bipolar disorder [84]. A number of CNVs and specific genes are associated with multiple neuropsychiatric conditions [85,

86], suggesting genetic correlation between these different neuropsychiatric disorders. Identification of specific risk loci allowed us to further investigate what genetic factors may be shared or distinct. Studies demonstrate that few genes are specific to ASD. Risk loci of autism and intellectual disability have extensive overlap [87], and schizophrenia risk genes overlap with genes implicated in autism and ID [88]. On the other hand, ADHD may be genetically different from autism [89, 90].

## 9. Clinical Diagnosis

Diagnosis of autism spectrum disorder can be difficult, given the variation in clinical manifestations and the absence of medical tests or strong, measurable biomarkers. Currently, individuals with autism are diagnosed based on the DSM-5 criteria (American Psychiatric Association, 2013). With mounting knowledge of autism susceptibility loci, next generation sequencing has great potential to facilitate clinical diagnosis of ASD, to refine clinical features of related syndromes, and may suggest specific interventions to modify phenotype, the power of which has been demonstrated by a few groups [91, 92]. However, autism is and will continue to be a clinical diagnosis. No single genetic test will make a diagnosis of autism, in part because of incomplete penetrance, pleiotropy, and variable expressivity.

## 10. Challenges and Future Directions

With an overwhelming, accelerating number of new variants being identified in individuals with autism, one of the challenges is to prevent false-positive reports of causality. A guideline has been put forward to regulate the process from study design to both gene-level and variant-level assessments of evidence [93]. During variant interpretation, it is always helpful to refer to the allele frequency in the ExAC database, which contains exomes from approximately 60,000 individuals, who had no clinical manifestations and various ethnic backgrounds (exac.broadinstitute.org). There also exist databases that can help determine whether a specific gene/variant is linked to ASD, for example the SFARI gene (https://sfari.org/resources/sfari-gene), the Geisinger Developmental Brain Disorder Genes database (http://geisingeradmi.org/care-innovation/studies/dbd-genes/), and ClinGen (https://www.clinicalgenome.org).

Pipelines for analysis of variants in gene-coding regions have been maturing in the past decade. However, non-coding variants were commonly filtered out, with a lack of appreciation of their potential consequence. Development in computational algorithms and functional assays for these non-coding regions promise to open new avenues of understanding the genetics of autism.

## 11. Conclusions

We started this review by asking the following questions: 1. What are the modes of transmission/inheritance of autism? 2. What is the nature of genetic risk factors that contribute to autism? 3. Why is there a male bias in autism? Obviously, there are no easy answers to these questions. Copy number variations, X-linked inheritance, autosomal recessive or dominant inheritance, *de novo* mutations, and common variants, all play a role in autism susceptibility. There is tremendous genetic heterogeneity in autism with several hundred genes contributing to the overall prevalence. Many of the well-established genes and proteins are involved in synaptic

and chromatin remodeling pathways. Sexual dimorphism of gene expression contributes to the female protective effect of autism. Other innate differences in neurodevelopmental and immune systems are waiting to be uncovered. With more large-scale sequencing projects, unprecedented advances in single-cell sequencing, chromosome conformation capture, and human *in vitro* models, we foresee a better understanding of the etiology of autism at multiple levels.

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**Author Contributions**

Jiani Yin and Dr. Christian Schaaf conceived the outline of the manuscript. Jiani Yin and David Oleoson wrote the manuscript. Dr. Christian Schaaf supervised the work.

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**Competing Interests**

The authors have declared that no competing interests exist.

**Additional Materials**

The following additional materials are uploaded at the page of this paper.
1. Supplementary Table 1

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