Autism: genetics
Autismo: genética

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Abstract

Autism is a strongly genetic disorder, with an estimated heritability of greater than 90%. A combination of phenotypic heterogeneity and the likely involvement of multiple interacting loci have hampered efforts at gene discovery. As a consequence, the genetic etiology of the spectrum of autism related disorders remains largely unknown. Over the past several years, the convergence of rapidly advancing genomic technologies, the completion of the human genome project, and increasingly successful collaborative efforts to increase the number of patients available for study have led to the first solid clues to the biological origins of these disorders. This paper will review the literature to date summarizing the results of linkage, cytogenetic, and candidate gene studies with a focus on recent progress. In addition, promising avenues for future research are considered.

Keywords: *Autistic disorder; Genetics; Linkage (Genetics); Cytogenetics; Association*

Resumo

O autismo é um transtorno fortemente genético, com uma herdabilidade estimada de mais de 90%. Uma combinação de heterogeneidade fenotípica e o provável envolvimento de múltiplos loci *que interagem entre si dificultam os esforços de descobertas de genes. Conseqüentemente, a etiologia genética dos transtornos relacionados ao autismo permanece, em grande parte, desconhecida. Nos últimos anos, a convergência entre tecnologias genômicas em rápido avanço, a finalização do projeto genoma humano e os crescentes e exitosos esforços em colaboração para aumentar o número de pacientes disponíveis para estudo conduziram às primeiras pistas sólidas sobre as origens biológicas desses transtornos. Este artigo revisará a literatura até nossos dias, resumindo os resultados de estudos de ligação genética, citogenéticos e de genes candidatos com um foco no progresso recente. Além disso, são consideradas as vias promissoras para pesquisas futuras.*

Descritores: *Transtorno autístico; Genética; Ligação (Genética); Citogenética; Associação*

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Introduction

Among psychiatric disorders, autism and autism spectrum disorders (ASDs) have the strongest evidence for a genetic basis, yet the search for specific genes contributing to these often devastating developmental syndromes has proven extraordinarily difficult. Recently, advances in genomic technologies, the completion of the sequencing of the human genome, the increased availability of large collections of genetic samples from affected individuals, and a renewed commitment to autism genetics research on the part of both governmental agencies and private foundations has coalesced to result in dramatic progress. Already, the first reproducible evidence implicating specific chromosomal regions and genes in autism spectrum disorders has been presented. Over the next several years, there is little doubt that multiple risk alleles for autism spectrum disorders will be identified and confirmed and that significant progress will be made toward understanding how these genetic abnormalities may lead to pervasive developmental disabilities.

Autism is a genetic disorder

It has long been appreciated that genes play a central role in the pathophysiology of autism and related conditions. Though these calculations have been made in the absence of known disease-causing genes, the data nonetheless is quite convincing. Overall, the heritability, which is the proportion of phenotypic variance attributable to genetic causes, is thought to be approximately of 90%.[1]

An important line of evidence in this regard is derived from a comparison of the degree to which the diagnosis of autism is shared by monozygotic (MZ) versus dizygotic (DZ) twins. As MZ twins are genetically identical and DZ twins share the same amount of DNA as any sibling pair, the finding of a higher rate of concordance (sharing the diagnosis) among MZ pairs would suggest that genes make an important contribution to the etiology of a disorder. In the case of ASDs, the observed concordance rates for strictly diagnosed autism are 60% for MZ twins versus 0% for DZ twins.[1] This latter figure would be expected to approach the sibling recurrence rate if a larger group was sampled. For broader spectrum diagnoses, the concordance rates are 92% versus 10%,[1] which are highly divergent and suggest a strong genetic component of risk.

In a similar vein, one can divine a rough estimate of genetic contribution by determining the risk of having the disorder if one has an affected relative and comparing this to the risk found in the general population. This quantity is known as λ or, more specifically, as λ_s if siblings are the point of comparison. The current best estimate of the recurrence rate when a child has a sibling with autism is approximately 2.2%.[2] When this is compared to the prevalence of autism in the general population, which has increased over the last 40 years from approximately 4 in 10,000 to between 10 and 13 per 10,000, or 0.13%,[3] the resulting λ_s is 20, strongly supporting a genetic contribution.

Importantly, while the available twin and family data clearly implicate genetic mechanisms in the etiology of these disorders, the patterns of transmission observed do not correspond to Mendelian expectations. In short, in most cases, there does not appear to be a direct and simple correspondence between having a single genetic abnormality and having autism. Indeed, the data support the notion that in the vast majority of individuals, multiple loci interact to lead to manifestations of the syndrome. While it is widely accepted that there is not a single autism gene, the number of contributing genetic regions, or loci, is difficult to predict. It has been estimated that on the order of 15 genes may be involved.[4] However, this could turn out to be a significant underestimation of the total number that may lead to or increase the risk for developing an autism phenotype. This genetic complexity appears to be the rule rather than the exception for most common medical conditions. Gene discovery in autism may represent an even greater challenge than in other conditions, such as hypertension or diabetes, however, due to the vagaries of diagnosis, particularly at the boundaries of the syndrome, and the current absence of any biomarker that may reliably distinguish an affected from an unaffected individual.

In the face of these obstacles, researchers have relied on three approaches to identify disease genes in ASDs: linkage analysis, cytogenetic analysis, and candidate gene studies. Each is described below along with a selective review of findings to date.

Linkage analysis

Given the current uncertainty regarding specific genetic or cellular mechanisms underlying autism, many researchers have attempted "positional cloning" through genome-wide linkage analyses. At heart, linkage studies simply evaluate the transmission of a chromosomal segment from one generation to another generation within families, and seek to link the presence of this DNA interval with the presence of the phenotype of interest. Given the presumption that in most cases a genetic contribution to autism is unlikely to be transmitted in a simple Mendelian fashion (i.e., it is not likely to be simply dominant, recessive, or X-linked) many investigators have opted for "non-parametric" approaches to linkage that do not rely on first hypothesizing a precise mode of inheritance. In affected sib-pair studies, this is accomplished by evaluating whether autistic siblings share any region of the genome more frequently than would be expected by chance.

Several genome-wide scans have been performed for ASD and evidence in favor of linkage has been reported for the majority of the chromosomes. However, in most cases, this evidence has not reached statistical significance. For linkage studies, the most common statistic presented is the LOD (logarithm of the odds) score representing the logarithm of the likelihood ratio of observing the given data under a model of linkage compared to a model of free recombination (or no linkage). Using the most widely accepted criteria for evaluating linkage studies, a LOD score of 3.6 in a sib-pair analysis suggests that there is a 5% probability of seeing this result by chance in a single genome-wide study and is evidence for significant linkage.[5] To A LOD score of 2.2 is taken as "suggestive" evidence for linkage and a LOD score of 5.4 is considered highly significant linkage. Translating these thresholds into a more

tangible form: one would expect to see a suggestive peak (2.2) by chance once per each genome scan, or a significant peak (3.6) by chance at a minimum once per every 20 published scans (this is likely an underestimate as there is a bias toward publication of positive data.

To date, there have been more than a dozen genome-wide scans published in the autism literature. Despite increasing sample sizes and considerable methodological sophistication, there has been a vexing absence of direct agreement among studies. In fact, until very recently there have been no reported instances in which significant evidence for linkage was identified in two different studies at precisely the same genetic marker or among overlapping markers in the genome. This likely reflects in part the phenotypic and genetic heterogeneity discussed earlier. Investigators have attempted to address these difficulties by increasing further their sample sizes through international collaborative efforts, focusing on replicating specific genetic intervals from the individual scans, combining results from more than one study and reevaluating the data, and attempting to identify more homogenous subgroups of patients that may provide more power for genetic mapping.

At present, despite conflicting results, promising findings and interesting patterns have nonetheless emerged from these genome-wide studies. For instance multiple investigators have identified regions on chromosomes 2 and 7 showing suggestive or significant linkage to autism. Three groups have reported evidence implicating chromosome 2, findings that are strengthened when samples are stratified. In a study of 152 affected sib-pairs (ASPs), a maximum multipoint LOD score (MMLS) of 3.74 was calculated at 2q31.1. When the subset of ASPs that met "strict" diagnostic criteria (n = 127) was analyzed, the MMLS increased to 4.80. Of note, "strict" criteria included 84 ASPs in which one sib met criteria for a PDD other than autism.[6] An independent genome-wide analysis identified suggestive linkage within the same chromosomal band,[7] and a third more focused study also identified suggestive linkage when the patients were stratified based on certain language characteristics (discussed in more detail below).[8]

Chromosome 7q is the region most frequently implicated in genome-wide studies. In one instance a LOD score of 3.55 was reported for the band 7q32.1-34.[9] Despite this finding and four genome-wide analyses providing additional evidence for linkage on the long arm of this chromosome, results have been difficult to interpret.[10] No study has replicated any other study in precisely the same chromosomal region. In addition, in the largest genome-wide study published to date (345 multiplex families), essentially no evidence for linkage was identified over the entire region, with a maximum LOD score of 1.3 identified telomeric to the locus noted above.[11]

Nonetheless, chromosome 7q remains an area of intense interest for a number of reasons: First, as noted, multiple suggestive linkage signals have been reported in this interval and, while they are dispersed across a large area, it is not uncommon for linkage peaks in complex genetic disorders to be wide and shift between studies.[12] A second source of interest has been the identification a number of chromosomal rearrangements involving this interval in patients with ASD (Table 1). Finally, numerous brain-expressed transcripts map to the long arm of chromosome 7 and have known functions that could plausibly be involved in the pathophysiology of ASDs. These include *FOXP2* (forkhead box P2) at 7q31.1, which is mutated in a severe speech and language disorder,[13]

Table 1 – Linkage studies in autism[50-62]

Chromosome	Marker	LOD score (ref)
1p13.2	D1S1675	2.63 (50)
1p13.2	D1S1675	2.15 (4)
1q21.3	D1S498	2.32 (50)
1q22	D1S2721	2.88 (51)
1q23.3	D1S484	3.58 (51)
1q42.2	D1S1656	3.06 (52)
2q31.1	D2S2188	4.80 (6)
2q31.1	D2S335	3.32 (7)
2q33.1	D2S116	2.86 (8)
3p24.1	D3S2432	3.32 (51)
3p25.3	D3S3691	2.22 (15)
3q22.1	D3S3045-D3S1763	3.10 (53)
3q26.32	D3S3715, D3S3037	4.81 (50)
4q23	D4S1647	2.87 (52)
4q27	D4S3250	2.73 (52)
4q32.3	D4S2368	2.82 (51)
5p13.1	D5S2494	2.55 (54)
5p13.1	D5S2494	2.54 (11)
6q14.3	D6S1270	2.61 (52)
6q16.3	D6S283	2.23 (55)
7q21.2	D7S1813	2.2 (56)
7q21.2	D7S1813	2.17 (57)
7q22.1	D7S477	3.55 (6)
7q32.1-34	D7S530-D7S684	3.55 (9)
7q33	D7S640	2.01 (58)
7q34-36.2	D7S1824-D7S3058	2.98 (59)
7q36.1	D7S483	3.7 (60)
9p22.2	D9S157	3.11 (6)
9q34.3	D9S1826	3.59 (6)
11p11.2-13	D11S1392-D11S1993	2.24 (11)
13q12.3	D13S217-D13S1229	2.3 (56)
13q22.1	D13S800	3.0 (56)
13q22.1	D13S800	2.54 (57)
13q32.1-32.3	D13S793-1271	2.86 (51)
15q12	GABRB3	4.71 (14)
15q21.2	CYP19	2.21 (6)
16p13.13	D16S3102	2.93 (6)
16p13.2	D16S407	2.22 (6)
17p11.2	D17S1298-D17S1299	2.22 (53)
17q11.2	D17S1800-D17S1294	8.0 (39)
17q11.2	D17S1294-D17S798	4.3 (16)
17q11.2	D17S1294	2.85 (15)
17q11.2	D17S1800	2.83 (11)
17q11.2	HTTINT2	2.34 (6)
17q21.2	D17S1299	2.26 (15)
17q21.32	D17S2180	4.1 (17)
17q24.3	D17S1290-D17S1301	2.84 (53)
19p13.11	D19S930	2.77 (15)
19p13.12	D19S714	2.53 (54)
19p13.12	D19S714	2.31 (52)
21q21.1	D21S1437	3.4 (60)
Xq21.33	DXS6789	2.54 (61)
Xq25	DXS1047	2.67 (54)
Xq28	F8C	2.1 (62)

Abbreviation: LOD (logarithm of the odds)
Results from genome-wide (unshaded) and focused (shaded) linkage analyses are given for intervals generating LOD scores ≥ 2. Three groups report several additional loci with LOD ≥ 2; however, only loci for which markers were published are included in the table.[15,53,59]

and *EN2* (engrailed 2*),* discussed at length below, which has yielded strong evidence for association with autism in recent studies.

As noted, in an effort to increase the genetic homogeneity of affected individuals, several groups have stratified samples using a variety of phenotypic measures. Two groups have done so using the criteria of phrase speech delay (PSD) past 36

months of age. One study of 95 families reported a maximal multipoint nonparametric linkage score (NPL) of 2.39 at 2q31.3. When a subset of 49 families meeting a "narrow" diagnosis of autism and having PSD was analyzed, the LOD score increased to 3.32.[7] A second group focused on the long arm of chromosome 2 and found an MMLS of 1.12 at 2q33 studying 99 families. This subsequently increased to 2.86 in a subset of 45 families with PSD.[8]

These examples suggest that there may be considerable value to sub-grouping samples and identifying so called endophenotypes that is measureable heritable traits that are present along the pathway from gene to syndrome. Intuitively, this is an attractive notion: if autism is not a single entity, but a collection of overlapping phenotypes resulting from the combined action of multiple risk alleles, it seems logical that an approach which parses the clinical presentation into biologically relevant components might be more powerful than one that relies on standard diagnostic criteria. Of course, any time one engages in multiple comparisons, it comes at the cost of an increased risk for false positive results. Consequently, one must be cautious interpreting an initially negative linkage result that improves after multiple subsequent analyses unless these have been taken into account in establishing an appropriate statistical threshold.

It is interesting to note that few of the genome-wide scans provide evidence for linkage on chromosome 15q11-13, the most frequent site of chromosomal abnormalities (other than the Fragile X site) detected in ASD. One group used a novel statistical method known as ordered subset analysis (OSA) and identified evidence for linkage with the phenotype "insistence on sameness." The approach increased the LOD score for the 15q11-13 region, at the *GABRB3* locus, from 1.45 to 4.71 under a dominant model of inheritance.[14] *GABRB3*, which encodes the receptor for GABA, the chief inhibitory neurotransmitter in the brain, has been studied as a candidate gene for autism (see below) with inconsistent results.

Perhaps the most exciting recent findings have focused attention on chromosome 17q. A study of 345 multiplex families from the Autism Genetic Resource Exchange (AGRE) database yielded its highest LOD score, 2.83, for 17q11.[11] Similarly, a study of 158 multiplex families produced its highest LOD score, 2.9, for 17q11.2.[15] Given the male predominance in autism (male: female ratio = 4:1), it has been hypothesized that stratifying samples by sex may uncover loci that predispose boys to this disorder. 257 AGRE families were subdivided into male-only and female-containing affected groups. Linkage analysis yielded a LOD score of 3.2 at 17q11 in the total data set, which increased to 4.3 in the male-only families.[16] Of note, these results were replicated at the same markers in an independent sample of 91 families with a LOD score of 4.1 at 17q11-21 in the male-only affected group.[17] This finding represented the first formal replication of an autism-linkage finding presented in the literature. One attractive candidate gene in the interval is *SLC6A4*, which encodes the serotonin transporter, involved in the reuptake of serotonin from the synapse. Platelet hyperserotonemia is found in a third of individuals with autism,[18] representing one of the oldest and most reliable findings in biological psychiatry. In a similar vein, the gene *ITGB3* (integrin beta-3) mapping to 17q21 is a neuronally expressed cell adhesion molecule that has been identified as a quantitative trait locus (QTL) for male serotonin levels,[19] suggesting that it, too, should be considered as a strong candidate.

Typically, larger sample sizes have more power to detect disease genes than smaller samples. This is not necessarily true in a complex genetic disorder such as autism, because increasing sample size may only serve to dilute the presence of each of several disease genes. This problem has been addressed by using the posterior probability of linkage (PPL), a statistical method designed to analyze heterogeneous data sets. The 345 AGRE families were subdivided into six classes according to diagnosis of autism or another PDD and the presence of phrase speech delay past 36 months. The linkage statistic is calculated and updated as the subsets are analyzed sequentially, allowing for heterogeneity within and across subsets. The largest probability of linkage, 55% (> 2% favors linkage), was calculated for 1q23-24, a region that was not detected by the original study with these families. For comparison, when the subsets were pooled, treating the families as one homogeneous group as in the original study, the probability decreased to 1.7% for this locus. A 15% probability of linkage was also calculated for 17q11.[20]

Certainly, the paucity of highly significant findings and the difficulty in replicating linkage at individual markers have been a disappointment to those seeking to identify autism risk alleles. Given the accumulating number of genome-wide investigations, one would expect multiple regions to yield LOD scores between 2 and 3.6 simply by chance. On the other hand, recent studies have begun to point repeatedly to a relatively small number of chromosomal intervals and at least one formal replication has already been confirmed. These findings coupled with the increasing availability of autism samples and genome-wide datasets, the decreasing costs of high-resolution genotyping and the promising results from other approaches to gene discovery, discussed below, are all reason for considerable optimism.

Cytogenetic analysis

It has been appreciated for some time that children with developmental delay and/or autism carry chromosomal abnormalities at a greater frequency than the typically developing population. For example, a recent review showed that 78 of 1826 karyotypes (4.3%) in children with autism were abnormal. Even when those with Fragile X were excluded, 54 (3.0%) were abnormal.[21] Abnormalities have been found on every chromosome, and there is overlap at only a few intervals. These findings support the notion that no single genetic variant or chromosomal rearrangement is likely to account for a significant proportion of patients with autism. Nonetheless, the study of chromosomal abnormalities may be of significance for both clinical and research purposes.

From a clinical standpoint, chromosomal analysis (and other genetic tests) in patients with ASD may point to a known syndrome, such as Fragile X or Angelman syndrome, or the presence of a translocation or other chromosomal

rearrangement that may require genetic counseling. Certainly the finding of syndromic features or non-specific dysmorphology on exam would suggest that standard cytogenetics should be performed. As discussed further below, the high relative incidence of Fragile X mutations in patients diagnosed with an ASD suggests that testing for this syndrome should be routine.

As genomic technologies advance, the question arises as to whether additional, higher resolution chromosomal studies are warranted clinically. For instance, molecular studies of subtelomeric rearrangements show an increased prevalence among patients with mental retardation, with an average rate of 4.6% across various studies.[22] The findings in patients with ASDs have been more equivocal. One study that examined 10 children with autism identified a single 2q37 deletion.[23] However, another search for subtelomeric abnormalities found none among 50 children with ASD.[24] A third investigation, and the largest to date, also found no rearrangements among 71 patients.[25] At present the studies have been too small to determine definitively whether molecular cytogenetic studies of this kind should be a routine part of an initial ASD work-up. However, given the findings to date in patients with mental retardation, it is clear that a child presenting with clear dysmorphology or significant delay should be studied in this fashion.

From a research standpoint, chromosomal abnormalities offer an avenue for the rapid identification of candidate regions for gene discovery. This is particularly the case for balanced translocations and chromosomal inversions in which two discrete "breakpoints" interrupt the normal chromosomal architecture. Likewise, small deletions may point to chromosomal intervals that warrant further study. The value of these types of findings has been demonstrated repeatedly with respect to developmental disorders. Initially, the gene for Angelman syndrome was identified as the result of a rare translocation that was found disrupted the gene *UBE3A*.[26] Of course, the gene for Fragile X was first localized as the result of the telltale cytogenetic findings.

More recently, chromosomal abnormalities have led to the identification of the *NLGN* (neuroligin) family of genes as strong candidates for involvement in developmental delay and autism. Based on the initial observation that three of eight girls with deletions at Xp22.3 had autistic features,[27] one research group elected to screen 158 individuals with ASD for mutations in genes within this interval.[28] A frameshift mutation in *NLGN 4* was identified in two affected brothers, one with autism and one with Asperger disorder, as well as their unaffected mother. This substitution was predicted to lead to significant truncation of the resulting protein, which is involved in synaptogenesis. The finding represented the first identification of a clearly functional mutation that segregated in patients with autism without other physical findings. *NLGN3* was also screened in this sample, and a point mutation at a highly conserved amino acid was found in a second family that included two affected brothers (one with autism and one with Asperger disorder) and their unaffected mother.[28] Shortly after the publication of these findings, a second frameshift mutation in *NLGN4* was identified by an independent research group that conducted a linkage analysis on 13 affected members of one large pedigree: two with autism, one with PDD-NOS, and the rest with mental retardation.[29] These results represent an independent replication of the initial findings and suggest a single mutation in this gene may lead either to developmental delay alone and/or autism spectrum phenotypes.

While these results, particularly with respect to *NLGN4*, are quite exciting and provide an avenue for investigation of the molecular consequences of an autism-related mutation, the frequency with which *NLGNs* may contribute to idiopathic autism appears to be low, as would be expected based on the initial findings. No coding mutations in *NLGN3* and *NLGN4* were discovered in a total of 292 patients in two studies.[30-31] Four missense mutations in *NLGN4* were found among 148 patients,[32] though the mutations did not clearly segregate with ASD when family members of the four cases were screened. In addition, the presence of a homologue to *NLGN4* on the Y chromosome has led some to question whether the loss of one copy, observed in the patients described above, actually results in the absence of this gene product in males.

The most frequent site of chromosomal abnormalities found in autistic patients without syndromic features involves the region 15q11-13. The presence of DNA segmental duplications makes this region vulnerable to rearrangements. Deletion of the maternally inherited copy leads to Angelman syndrome while deletion of the paternally inherited copy leads to Prader-Willi syndrome, due to imprinted genes that are expressed from only one or the other chromosomes. Duplication of the maternal, but not the paternal, chromosomal segment in this region have been reported numerous times in ASDs.[33] Several candidate genes map to this interval, but clear mutations have not yet been identified and no association of common alleles in this region has been conclusively demonstrated.

As noted, chromosome 7q abnormalities have also been found in a number of cases of ASD. The combination of linkage data, the presence of language-related loci, and the fact that multiple intriguing candidate genes map to this interval has attracted considerable interest from autism researchers. A maternally inherited balanced translocation between chromosomes 7 and 13, t(7;13)(q31.3;q21)mat, was found to disrupt the gene *RAY1,* a suppressor of tumorigenicity.[34] In a second case, a balanced translocation in two monozygotic twins concordant for autism was found to disrupt the novel transcript *AUTS2*[35] at 7q11.2, which is highly expressed in brain. However no mutations have been found in either gene in cytogenetically normal ASD patients.

In addition to *RAY1* and *AUTS2*, other rearrangements in individuals with ASD have been found to disrupt the following genes: *PAX3* (paired box gene 3) at 2q36.1, *MMP16* (matrix metalloproteinase 16) at 8q21.3, *NBEA* (neurobeachin) at 13q13.3, *GRPR* (gastrin-releasing peptide receptor) at Xp22.2, and *A2BP1* (ataxin 2-binding protein 1) at 16p13.2 (reviewed in [21]). Again, neither association of common alleles of these genes nor rare functional mutations have yet been reported in a larger population of affected individuals. Attention has also turned to children with the 22q11 deletion syndrome (DiGeorge syndrome, Velo-cardio-facial syndrome), some of whom have social skills deficits. One study reported that a third of 32 individuals with 22q11 deletion syndrome met criteria for

ASD.[36] A specific transcript in this interval contributing to these findings has not yet been identified.

Candidate genes

Studies of candidate genes are largely divided into two types, those that seek to determine if a common variant of a gene confers an incremental risk for the autism phenotype (candidate gene association studies) and those that seek to determine if rare, functional mutations might be present in a gene of major effect for autism or related conditions (mutation screening). Of late, investigators have been combining both analyses, particularly when studying candidate genes implicated by their location within a linkage interval, a region of disruption due to one or more chromosomal abnormalities, or being implicated in a related syndrome such as Rett disorder.

Over a hundred genes have been evaluated for association with the ASD with multiple positive results; however, replication has been the exception rather than the rule. As the use of association strategies, which are essentially the geneticist's version of a case-control study, have grown in popularity, the propensity for false positive, or at least non-replicable findings has been widely noted.[37] The reasons for this observation are the subject of debate and beyond the scope of this discussion. However, in light of the widespread difficulties not just with regard to autism but across multiple medical disorders, it is clear that true replication in an independent sample, implicating the same allele at the same locus, is the standard to which common-variant association studies should be held.

With regard to autism, a candidate gene with a particularly long and venerable history is *SLC6A4*. As noted, the transcript codes for the serotonin transporter, which mediates reuptake of serotonin from the synapse. Interest in this gene and its protein products derives from a plausible role for serotonin in the repetitive behaviors observed frequently in patients as well as the highly reliable finding of increased platelet serotonin among a substantial subset of autistic individuals.

Two variable number tandem repeat (VNTR) polymorphisms, one in the promoter (HTTLPR-s short and HTTLPR-l long alleles) and one in the second intron are known to alter expression of the transporter and presumably the level of serotonin in the synapse, and have been the subject of multiple studies.[38] The results have been contradictory. Some groups have reported excess transmission of the short allele, while others have reported excess transmission of the long allele or stronger association with single nucleotide polymorphisms (SNPs) in the region. Still others did not find any significant association of *SLC6A4* with ASD.

However, interest in this transcript has not waned, in part because the gene has recently evolved from being a biologically plausible candidate to one implicated by multiple positional cloning efforts. As noted, the evidence in favor of an autism gene on chromosome 17q has been quite strong, especially with respect to a sex-specific risk allele. In a linkage analysis involving 341 families, a LOD score of 5.8 was calculated at 17q11.2 under a recessive model of transmission. This increased to 8.0 for the 202 families that only had male patients and decreased to 0.06 for the 138 families containing female patients.[39] Only nominal evidence for association was found when two SNPs were evaluated, and the researchers concluded that these could not account for the linkage peak observed. As a consequence, they elected to mutation screen the promoter and coding exons in the 120 families with the highest family-specific LOD scores. Four sequence variants that changed highly conserved amino acids were identified. In each case, segregation data was inconclusive but generally supported a relationship between the allele and affected status. Moreover, additional analyses suggested that the coding variants were associated with increased severity of rigid-compulsive behaviors. These data suggest that multiple rare variants of *SLC6A4* may contribute to susceptibility to ASD.[39] However, intensive study of this interval in search of a common variant that might account for the replicated linkage signals continues.

Other neurotransmitter systems have been investigated in autism. Studies of the GABA-A receptor genes at 15q11-13 were described briefly above; no consistent association with sequence variants has been found. The same is true for the genes encoding the dopamine receptors D2, D3, and D5; tyrosine hydroxylase; and dopamine beta hydroxylase (reviewed in [40]). The glutamate receptor genes, *GRIK2* at 6q21[41] and *GRM8* at 7q31-33,[42] have been associated with autism in single studies and need further investigation. *SLC25A12*, which encodes the mitochondrial aspartate/glutamate carrier, has received interest since it is located at 2q31.1, a locus for which high LOD scores have been calculated by two genome-wide linkage studies. Association between two SNPs in the gene and ASD was reported in two independent samples.[43-44]

There is a long list of genes, many on chromosome 7, which were considered promising positional and/or functional candidates but have not been conclusively associated with ASD thus far. Some noteworthy ones include: *HOXA1* at 7p15.2 and *HOXB1* at 17q21.32 (homeobox) regulates hindbrain development; *DLX6* (distal-less homeobox) at 7q21.3 regulates forebrain development; RELN (reelin) at 7q22.1 is involved in neuronal migration; *FOXP2* (forkhead box P2) at 7q31.1 is involved in speech and language disorder; *NRCAM* at 7q31.1 is a neuronal cell adhesion molecule; *WNT2* (wingless-type MMTV integration site 2) at 7q31.2 is involved in CNS development and interacts with DVL1, of which knockout mice have diminished social interaction; *AVPR1A* (arginine vasopressin receptor 1A) at 12q14.2 influences affiliative behavior in transgenic mice; ADA (adenosine deaminase) at 20q13.12 is involved in mRNA editing.

Finally, and most recently, exciting results have been reported for *EN2* (engrailed 2) at 7q36.3.[45-46] *EN2* is a homeobox gene that regulates development of the cerebellum. It has attracted attention as a result of the fact that cerebellar abnormalities are among the most consistent findings from pathological and neuroimaging studies in ASD. Mice that express mutant *EN2* or lack the protein exhibit cerebellar pathology similar to postmortem findings in some ASD samples. Its chromosomal locus has also been a focus of attention based on linkage studies (reviewed in [47]). Therefore, *EN2* is both a functional and positional candidate gene.

Initially, four SNPs were analyzed, two in the single intron of *EN2* and one each in the flanking exons. Significant association was found between the two intronic SNPs and

ASD in 167 AGRE families.[45] A subsequent study analyzed the four SNPs and an additional 14, spanning the entire gene. Significant association with the two intronic SNPs was also detected in 222 different AGRE families and 129 NIMH families.[46] Therefore, the association was replicated in multiple, independent study populations. The total set of 518 families (2336 individuals) is one of the largest association studies done in ASD. The p value for the haplotype (allele) containing the two SNPs for the total set was 0.00000035, providing strong evidence that *EN2* is a susceptibility gene for ASD. Furthermore, given the high frequency of the haplotype in the sample (approximately 67%), the population attributable risk was calculated to be 40%, i.e., sequence variations in *EN2* may influence as many as 40% of cases of ASD.

The two intronic SNPs were predicted to lie within consensus binding sites for transcription factors. However, it is the non-associated alleles which abolish binding to the factors.[46] Therefore, the precise risk allele has yet to be determined, but this important study should direct more interest and energy into scrutinizing *EN2.*

Future directions

Whether describing linkage analysis, cytogenetic studies, or association strategies, the foregoing discussion highlights both the obstacles confronting autism genetics researchers as well as the dramatic progress of late. It has taken some time, but the field is poised to deliver on the promise of identifying multiple autism genes. In addition to the types of methodological approaches already noted above, such as the widespread attempt to identify useful endophenotypes, several recent additional developments have made a notable contribution to the recent achievements and will continue to drive progress in this area.

The availability of biomaterials: perhaps the single most important advance over the last decade has been the least technologically driven; it is the availability of DNA and cell lines from well-characterized patients. One only needs look at the number of papers that acknowledge the Autism Genetic Resource Exchange to get a sense of how much impact the widespread dissemination of high quality phenotypic data and biological samples has had on the field. This effort on the part of the private foundation Cure Autism Now to create a freely available, web accessible DNA and phenotype bank, has been joined by the National Institute of Mental Health in the United States with the result that talented researchers outside the field are able to readily test novel hypotheses on sample sets they would have been unlikely to access in the past. In addition, groups long-dedicated to autism genetics have been able to quickly increase their subject numbers or use these public samples for replication sets, both critical issues in the effort to find genes that contribute relatively small increments of risk. In a similar vein, researchers have banded together to form national and international consortia that are driving samples sizes up an order of magnitude. Coupled with the strong commitment to funding research in the US on the part of organizations like National Association for Autism Research (NAAR) and Autism Speaks in addition to the federal government, current optimism is well justified.

Advancing genomic technologies: a second contributor to the acceleration in high quality autism genetics research has been the development of high-throughput, low cost technologies especially in the area of genotyping. The mainstay of both linkage and association studies is the evaluation of known polymorphic DNA markers, known as genotyping. Recent advances in micro-array technologies (in which many thousands of spots of DNA can be arrayed on a single microscope slide) now allow researchers to query hundreds of thousands of SNPs in a single hybridization reaction and at comparatively low cost. This capacity allows small laboratories to conduct genome wide linkage analyses quickly, and has opened the door to a new type of analysis known as whole genome association.

As noted above, the common case-control association study has involved the specification of a hypothesis about a gene or set of genes that are believed to play a role in autism that are then tested by determining whether a DNA polymorphism in or near the gene(s) is more common in affected versus unaffected individuals. For reasons that a beyond the score of this review, to be successful, such studies must choose a marker which is quite close to whatever genetic change is actually leading to the disease or increased risk. With the recent development of micro-array based SNP genotyping platforms that carry 300-500,000 markers, there is more than sufficient coverage of the genome to look for association without having to *a priori* choose a single gene or set of genes. The power of this type of approach was recently demonstrated in several studies reporting the identification of a gene for age related macular degeneration.[48] There is tremendous excitement in the field about leveraging these methods to perform positional cloning in autism and related disorders.

Advances in cytogenetic technologies: in a similar vein, micro-array technology is transforming the identification of chromosomal deletions or duplications, a method now referred to as copy number analysis. There are several new techniques available to accomplish this, one in widespread use is known as array-based competitive genomic hybridization or aCGH. This method uses patient DNA and control DNA labeled with different colors via a fluorescent tag. Equal amounts of patient and control materials are hybridized to known regions of the human genome that are pre-arrayed on a slide. If patient and control have equal copy numbers at a given locus, the colors mix equally. If the patient has lost (deleted) a locus, only the control color is visualized. Conversely, if the patient has extra copies at a locus (duplication), the patient color predominates.

With currently available micro-arrays, this type of analysis can currently identify copy number changes smaller than 50,000 basepairs, which is approximately 100 times more sensitive than standard high-resolution cytogenetics, and the resolution is still increasing at a rapid pace. The technique has already been used to identify many more deletions and duplications in the human genome than were previously imagined.[49] However, as these technologies have been developed it has become clear that there is considerably more structural variation to the normal human genome than anyone suspected. Consequently it is not possible to draw a simple conclusion that for instance a small lost region of

one chromosome in a patient with autism is related to the appearance of symptoms, even the interval contains an interesting candidate gene. Indeed there have been many instances of copy number loses at important brain expressed genes that have been found commonly in apparently normal individuals.

Despite having to address these unexpected complexities, the ability to identify submicroscopic chromosomal changes holds tremendous promise. It is likely that in certain instances, formerly cryptic deletions or duplications will point to a gene of major effect in autism that is only relevant for a small number of patients, such as has been the case with *NLGN*. It also appears increasingly likely that copy number variations may contribute to disease risk in a more complex fashion, analogous to other types of genomic variation, such as SNPs, that sometimes subtly alter protein function. It will be both challenging and exciting to sort out these new possibilities.

Conclusions

After several decades of halting progress, the entire field of autism genetics is moving forward at a remarkable pace. Over just the past couple of years, a specific genetic mutation in *NLGN4* has been identified as being responsible for rare cases of mental retardation and/or pervasive developmental disabilities, *EN2* has emerged as a strong candidate for association with the autism phenotype, and a linkage region on chromosome 17q has been confirmed in independent samples using rigorous statistical criteria. These are just a handful of the exciting recent findings in the field that offer avenues for real progress. Of course, the identification of risk alleles or rare causative mutations is just one important step in unraveling the biology of ASDs, and effort that will require the combined contributions of a variety of fields including geneticists, clinical researchers, developmental neurobiologists and neuroimagers. Though the final goal of leveraging an understanding of pathophysiology to develop new treatments and to reveal strategies for prevention is still over the horizon, we have clearly now have started to take the necessary first steps in that direction.

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