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Genomic and genetic aspects of autism spectrum disorder

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ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with a strong genetic component. The past decade has witnessed tremendous progress in the genetic studies of ASD. In this article, we review the accumulating literatures on the monogenic forms of ASD and chromosomal abnormalities associated with ASD, the genome-wide linkage and association studies, the copy number variation (CNV) and the next generation sequencing (NGS) studies. With more than hundreds of mutations being implicated, the convergent biological pathways are emerging and the genetic landscape of ASD becomes clearer. The genetic studies provide a solid basis for future translational study for better diagnoses, intervention and treatment of ASD.

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1. Introduction

Autism spectrum disorder (ASD) refers to a wide range of neurodevelopmental conditions characterized by social communication and interaction deficits, stereotyped and repetitive behaviors. According to estimates from U.S. Centers for Disease Control and Prevention (CDC) [1], ASD affects 1 in every 68 children and is considered to be one of the most prevalent childhood disorders.

ASDs are diagnosed solely based on behavioral observation. The diagnostic criteria for ASD have been revised periodically with the reflection of the advances in research and practice. In the previous version of Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) [2], ASD was conceptualized as a manifestation of triad symptoms: impairment in social interaction, impairment in social communication, restricted and repetitive behavior pattern, and included three specific subgroups: Autism, Asperger syndrome, and Pervasive developmental disorder not otherwise specified (PDD-NOS). In the newest version of DSM-5 released in 2013 [3], these subgroups were merged into a single umbrella term as "autism spectrum disorders", and the triad of impairments has been folded into two, with social communication and social interaction combined as a single diagnostic category. In addition to main symptoms, approximately 31% ASD individuals also present intellectual disability (ID) [1] and 20–25% have seizures [4]. Other common comorbidities in ASD include anxiety disorders [5], sleep disorders [6], gastrointestinal (GI) disorders [7] and abnormal

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responses to sensory stimuli [8]. The core symptoms of ASD usually appear before 3 years old and the prevalence of ASD is strongly male-biased with the average male to female ratio around 5:1 [1].

ASD has a strong genetic basis. In the twin studies, the concordance rate of broad ASD phenotype in monozygotic twins is 70–90%, but 0–30% in dizygotic twins [9,10]. Family studies indicate that the prevalence of ASD is about 25 times higher in siblings of the affected individuals than in the general population [11]. Despite being highly heritable, ASD is genetically complex and the underlying genetic architecture is still not well understood. Currently there are two theories to explain the genetic architecture of common complex diseases including ASD [12]. The first is the common variant – common disease (CVCD) hypothesis, which suggests that the genetic risk factor of a complex disorder like ASD, is attributed to many high-frequency (minor allele frequency > 1%) genetic variants, each conferring a modest risk (odds ratio < 1.5). These common risk variants collectively contribute to the disease. The second one is the rare variant – common disease (RVCD) hypothesis, suggesting that genetic risk can be mainly explained by rare mutations carrying significant risk. Thus, ASD can be regarded as a collection of many forms of a rare monogenic disorder, each with a different etiology. The RVCD hypothesis is favored by current studies, while the CVCD model cannot be rejected.

In this review, we provide an overview of the ASD genetics by the methodology type, in a chronological order and highlight some key findings. Finally we summarized the convergent pathway and provide our perspective on the issues that should be addressed in the future study.

2. Monogenic forms of ASD and chromosomal abnormalities

The identification of the monogenic causes of ASD and the cytogenetic abnormalities provided the initial insight into the genetic components of ASD [13]. Following theses clues, a wide range of studies was conducted and greatly increased the understanding of both genetic and biological mechanisms of ASD.

About 10% of ASD patients have co-occurring single gene disorders including Fragile X syndrome (FXS), tuberous sclerosis (TSC), Rett syndrome, PTEN hamartoma tumor syndrome (PHTS) and others. FXS is caused by expansions of the trinucleotide CGG repeat in the FMR1 gene, an RNA-binding gene that plays a pivotal role in synaptic plasticity by regulating the mRNA transport and translation in the brain [14]. The loss-of-function mutations of TSC1 and TSC2 genes are responsible for TSC [15,16]. Both genes function as inhibitors in the mammalian target of rapamycin pathway (mTOR) – a key pathway that controls the local translation at the synapse. Rett syndrome, which is caused by mutations in methyl-CpG-binding protein 2 (MeCP2), was found in about 1% ASD individuals [17]. The MeCP2 protein has multi-functions and is involved in regulation of transcription; both activation and repression in neurons. The phosphatase and tension homolog (PTEN) gene mutations lead to cancer, seizures and ASD with macrocephaly [18,19]. PTEN negatively regulates the PI3K/AKT pathway and indirectly repress mTOR pathway. Collectively, these data implicated that the dysfunction of transcription/translation process in neurons and PI3K/AKT/mTOR pathways contribute to the pathophysiology of ASD.

Cytogenetic abnormalities are found in 2% of ASD individuals by using technology like G banding karyotyping. The chromosomal abnormalities include 5p15, 15q11-q13, 17p11, 22q11.2 and others [20]. Of which, the 15q11-q13 locus is the most frequent cause leading to ASD, which may account for close to 1% of ASD cases. Depending on the mutation type and inherent pattern, this locus gives rise to Prader–Willi Syndrome (PWS), Angelman Syndrome (AS), and ASD [21]. A mouse model for 15q11-q13 duplication was generated by a chromosome-engineering technique based on Cre-loxP system and the mice carrying paternally inherited duplication display abnormal autistic-like behaviors such as reduced social interactions and stereotypical behavior [22]. The detailed analysis revealed that the abnormalities of serotonin (5-HT) signaling might be involved in the pathogenesis of ASD [23] and 5-HT pathway might be a promising therapeutic target for ASD.

3. Linkage and candidate gene studies

Linkage analysis is a traditional approach to locate the causal or susceptibility gene by examining the chromosomal regions that co-segregated with the trait among affected pedigrees. It is particularly powerful for Mendelian diseases. Linkage studies were successfully applied to identify MECP2 and FMR1 as causal genes for Rett syndrome and Fragile X syndrome respectively [17,24]. However, linkage analysis bears several limitations: first it has less power for complex disorder of which the inheritance pattern is not clear and the effect of susceptibility gene is weak; second it requires extended or multiplex families – which is not always feasible; third the chromosomal regions detected are usually broad (10 cM or more) and contain many genes, which requires further fine-mapping to narrow down the candidate region.

A number of genome-wide linkage studies have been performed since the first study by International Molecular Genetic Study of Autism Consortium (IMGSAC) in 1998 [25–27]. However, these studies met with limited success: suggestive linkage signals were found in almost all chromosomes and there is little consistency among the results. The most replicated linkage loci include chromosome 2q, 7q and 17q.

In all implicated linkage regions, chromosome 7q is the most well supported locus and has been implicated in multiple genome-wide scans and meta-analyses [28,29]. Several ASD candidate genes including *RELN*, *FOXP2*, *WNT2* and *CADPS2* are located in this region. *RELN* encodes an extracellular glycoprotein that has critical roles in the neuronal migration of cortical layers and synaptic remodeling. Despite several studies failed to find association [30,31], positive association have been found between polymorphisms in the 5′ UTR and intron of *RELN* and ASD [32,33], and meta-analysis support the association [34]. *FOXP2* is a gene important for language development and its mutations have been identified in individuals with speech and language impairment [35]. Despite the biological plausibility, there is little genetic evidence supporting the involvement of *FOXP2* in autism [36,37]. *WNT2* belongs to the large WNT gene family and is expressed particularly during the development of the central nervous system. *CADPS2* encodes a calcium binding protein and the knockout mouse has abnormal behaviors which are similar to human autistic phenotypes [38].

The candidate gene approach, which is based on known or suspected biological functions of a given gene, has also been widely applied and a number of candidate genes for ASD have been suggested. The identification of *NLGN3* and *NLGN4* mutations in ASD siblings is the first important evidence which links the synapse to ASD [39]. This study initiated a wave to identify rare mutations in synaptic genes and it also gave rise to a general concept of "synaptopathy" that suggests synaptic plasticity is an important etiology not only for ASD, but also for neuropsychiatry disorders in general such as schizophrenia and Alzheimer disorder [40–42]. Other well-established ASD genes include *SHANK3* [43], *CNTN4* [44], *CNTNAP2* [45], *NRXN1* [46], and *OXTR* [47,48].

4. Genome-wide association study (GWAS)

GWAS is a powerful data driven approach to identify, without prior knowledge, common variants with low penetrance. By

utilizing hundreds of thousands of single-nucleotide polymorphism (SNP) markers in the human genome, GWAS permits an unbiased and comprehensive scan for susceptibility genes and have more statistical power than linkage study.

The first GWAS was reported by Wang et al. [49]. By examining 780 ASD families in the discovery stage, an independent cohort of 1204 ASD cases and 6491 healthy controls in the replication stage, the authors identified 6 SNPs surpass the genome-wide significance level on chromosome 5p14.1 in combined analyses. The top associated SNP (rs4307059, $P = 3.4 \times 10^{-8}$) was located in a 2.2 Mb intergenic region between *CDH9* and *CDH10* – which encode type II classical cell-cell adhesion molecular cadherin-9 and cadeherin-10. Although rs4307059 was not associated with the expression level of either CDH9 or CDH10, a follow-up study found that a functional 3.9 kb noncoding RNA is actively transcribed from the top association region [50]. This noncoding RNA corresponds to moesin pseudogene 1 (*MSNP1*) and shares 94% sequence identity with the mRNA of *MSN*, and was termed as *MSNP1AS* (moesin psedogene 1, antisense). MSNP1AS significantly decrease the MSN expression level when overexpressed in vitro. Furthermore the expression of MSNP1AS is correlated with the genotype of rs4307059, where the ASD risk allele homozygote carriers have a 22-fold increase of expression compared with non-risk allele carriers, indicating the ASD associated SNPs may confer the risk by altering the protein level of moesin in certain neurodevelopment periods.

An independent GWAS was conducted by Weiss et al. [51]. Despite that a large proportion of the sample in the screening stage overlap with that of the previous GWAS by Wang et al., the association of 5p14.1 was not replicated in this study. A single SNP rs10513025 in 5p15 was identified associated with ASD with the genome wide significance ($P = 2.1 \times 10^{-7}$). This SNP lies between Taste Receptor Type 2 Member 1 (*TAS2R1*) and semaphorin 5A (*SEMA5A*). *SEMA5A* has been implicated in the axonal guidance and is likely to be a susceptibility gene for ASD. The expression of SEMA5A was found significantly lower in the whole blood as well as occipital lobe cortex in autistic patients compared with healthy controls. In addition, this study also detected several suggestive association signals at loci including *JARID2* and *JMJD2C*, which are plausible ASD candidate genes.

Autism Genome Project consortium further reported two GWAS. In the first stage of this study [52], a genome-wide significant association of *MACROD2* was detected by including 1558 ASD families. However an independent replication study using 1170 ASD cases and 35,307 controls could not provided any support for this association [53]. Furthermore in the second stage, no SNP was found to be associated with ASD at the genome-wide significance level, even after an increase of the sample size to 2705 ASD trios [54]. In addition, the previously reported association at *MACROD2* was not retained with additional samples.

Xia et al. reported an ASD GWAS from the Chinese population [55]. Three SNPs were found with genome-wide significance and gene expression analyses suggest *TRIM33* and *NRAS-CSDE1* might be novel candidate genes for ASD. TRIM33 is an E3 ubiquitin ligase and is functionally intriguing, as ubiquitin pathway has been implicated in ASD, Angelman Syndrome and X-linked mental retardation.

In addition, we also performed a GWAS using Japanese and Chinese ASD individuals. Nominal associations were found in or near ASD candidate genes including *CNTN4, JARID2* (Liu et al., unpublished data). In the future, meta-analysis combined or mega-analysis that combines multiple datasets is necessary to increase the power and may shed light on the genetic architecture of ASD.

5. Copy number variation (CNV)

CNV is loosely defined as duplication or deletion of a section of DNA with a length usually from 1 kb to 5 Mb. CNVs are widespread in the human genome and account for a substantial proportion of genetic variation and accumulating data highly suggested a critical role of CNV in the etiology of neuropsychiatric disorders, including ASD, schizophrenia and cancer. CNV studies, to some extent, are inspired by the early cytogenetic studies and could be regarded as the continuation of such studies but with a much higher resolution. Since a typical size of cytogenetically visible regions is around 5–10 Mb, this low resolution greatly limits the ability to identify small-size chromosomal abnormalities including micro-duplication or micro-deletion. With the advancement of technologies including array-comparative genomic hybridization (aCGH) and microarrays, the resolution was highly improved and CNV studies became feasible.

In an early CNV study using 10K SNP array, the deletion encompassing *NRXN1* was identified as a risk factor for ASD [25]. In addition to *NRXN1*, 1q21, 17q12 and 22q11.21 were also implicated. Sebat et al. conducted an important study which focused on *de novo* CNVs by using aCGH [56]. They observed that rare *de novo* CNVs were present in 10% of ASD cases from simplex families, 3% in multiplex families, but only 1% in the general population. This indicates that *de novo* CNVs may account for a significant proportion of the risk factors especially for sporadic ASDs. The high *de novo* CNV rate in ASDs was further confirmed by other studies, which found *de novo* rates from 5.8% to 8.4% in sporadic ASDs [57–59]. Marshall et al reported another CNV study on 427 ASD families and they found a similar *de novo* rate (7.1% and 2.0% for simplex and multiplex families respectively) [57]. Furthermore they highlighted the recurrent CNV loci at 16p11.2 together with postsynaptic density genes (*SHANK3-NLGN4-NRXN1*), synapse complex genes (*DPP6-DPP10-PCDH9*) and others. The association of 16p11.2 was replicated by another study [60] and a meta-analysis estimated that 16p11.2 may account for approximately 0.76% of ASD cases [61]. The mouse model for 16p11.2 has also been generated [62].

By using case-control design, Hakonarson and colleagues found the neuronal cell-adhesion molecules and ubiquitin pathway were enriched with CNVs in ASD individuals [63]. In a similar approach, Pinto et al. found that ASD cases have a 1.19-fold higher global burden of rare, gene-containing CNVs. Several genes including *SHANK2*, *SYNGAP1* and *DLGAP2* were only found in *de novo* CNV from ASD cases, and were suggested as novel ASD candidate genes [64]. Furthermore by examining maternal inherited CNVs, X-linked deletions at *DDX53–PTCHD1* locus was identified to be significantly associated with ASD. Pathway analysis implicated gene sets that involved in (1) cell and neuronal development and function and (2) GTPase/Ras signaling may play a role in the pathogenesis of ASD.

In 2011, three studies were published by using the simplex autism families from the Simons Simplex Collection (SSC). These families consist of two healthy parents, a single affected individual and at least one normal sibling. By using different genotyping platform, Sanders et al. and Levy et al. both identified about 80 rare *de novo* CNVs, and two recurrent *de novo* CNVs at 16p11.2 (both duplication and deletion) and 7q11.23 (only duplication) were strongly associated with ASD [58,59]. It is interesting to note that the deletion of 7q11.23 has been linked to Williams syndrome, implying that the dosage of genes in this regions is important for the social behaviors. The *de novo* rates observed in the SSC samples are consistent with previous studies (7.9% in probands and 2.0% in unaffected siblings) and it has been found that the female probands carry more and larger *de novo* CNVs than male probands, suggesting there is a protective mechanism for female to buffer the harmful genetic mutations. Furthermore, Network analysis

Table 1
Frequent chromosomal abnormalities associated with ASD.

Locus	Comment
15q11.2-q13.1	The maternal deletion or mutation of *UBE3A* gene causes Angelman Syndrome. The paternal deletion leads to Prader–Willi Syndrome. And 15q11-q13 maternal duplication is the most frequent cause for ASD.
16p11.2	A typical 500 kb microduplication or microdeletion of 16p11.2 is associated with ASD and schizophrenia. The deletion is more penetrant than duplication. *KCDT13* gene has been implicated to be major contributor to the neuroanatomical phenotype.
2p16.3	Deletions which disrupted *NRXN1* gene were frequently reported in ASD cases.
1q21.1	The 1.35 Mb deletion is associated with development delay, intellectual disability, schizophrenia and the duplication is linked to intellectual disability and ASD. This region is gene rich and structurally complex. A paralog of the *HYDIN* gene in this locus might account for microcephaly or macrocephaly
7q11.23	Deletion of 7q11.23 is known to be responsible for Williams syndrome where as the duplication is associated with ASD and speech delay.
3q29	3q29 deletions are associated with ASD and schizophrenia. *FBXO45*, *PAK2* and *DLG1* might be causal genes in this region.
22q11.2	The 22q11.2 deletion is associated with DiGeorge syndrome, ASD, schizophrenia and ADHD. The symptom of the duplication is very heterogeneous but mostly leads to mental retardation, developmental delay and ASD.
17p11.2	Deletion of 17p11.2 results in Smith–Magenis syndrome. Duplications of this region leads to Potocki–Lupski syndrome. Both syndromes are associated with ASD.
17q12	Deletion of 17q12 has been reported to be associated with diabetes, renal cysts, epilepsy, intellectual disability and ASD.

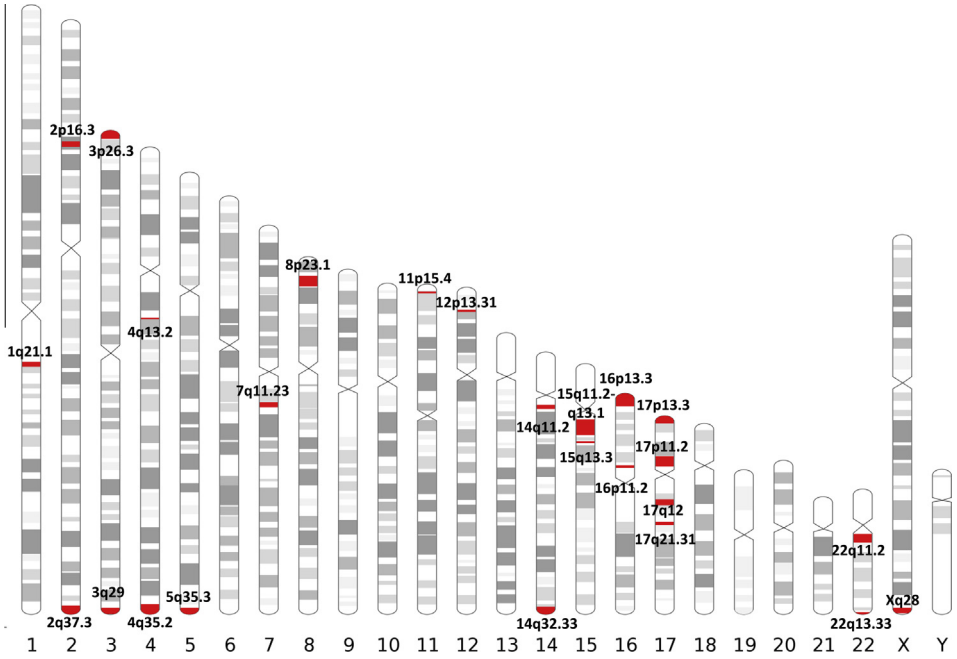

Fig. 1. Idiogram for most frequent chromosomal abnormalities associated with ASD A total of 25 loci were shown in this ideogram. These loci were represented as red bands. Note that many loci are located in telomere proximal regions.

highlighted genes set involved synaptogenesis, axon guidance and neuronal motility [65].

Finally, we summarized the most frequent CNVs in Table 1 and Fig. 1. With the development of technology, we expect more smaller-size CNV will be identified and characterized in future studies.

6. Next generation sequencing (NGS) studies

The rapid advances of next-generation sequencing technology have led to the discovery of many rare and *de novo* point mutations in ASD in the past few years. O'Roak et al. reported the first whole-exome sequencing study on 20 ASD trios in 2011 [66]. A total of 11 missense mutations were identified. Four genes including *FOXP1*, *GRIN2B*, *SCN1A* and *LAMC3* were highlighted as potentially causative genes. However no recurrent mutation was found, which was likely due to the small sample size.

In the first half of 2012, four ASD exome studies were published by independent groups. Wigler and colleagues sequenced exomes of 343 ASD families from SSC [67]. Despite that the overall *de novo* mutation rate is not significantly different between affected and unaffected siblings, the gene-disrupting *de novo* mutations occur two times more frequent in ASD patients. Furthermore, the likely gene disruption mutations are enriched in FMRP-associated gene set, implicating a shared pathogenesis mechanism between Fragile X syndrome and ASD.

Sanders et al. reported the study with another 238 families of SSC [68]. Consistent with the previous study, they found the number of non-synonymous *de novo* mutations were significantly higher in ASD probands than in normal siblings. And the rate of *de novo* mutation is positively correlated with the paternal age. Furthermore, based on statistic modeling and simulation, the authors suggested that it is unlikely to observe two independent *de novo* mutations in the same gene by chance. They found recurrent mutations in three genes: *SCN2A*, *KATNAL2* and *CHD8*. The first

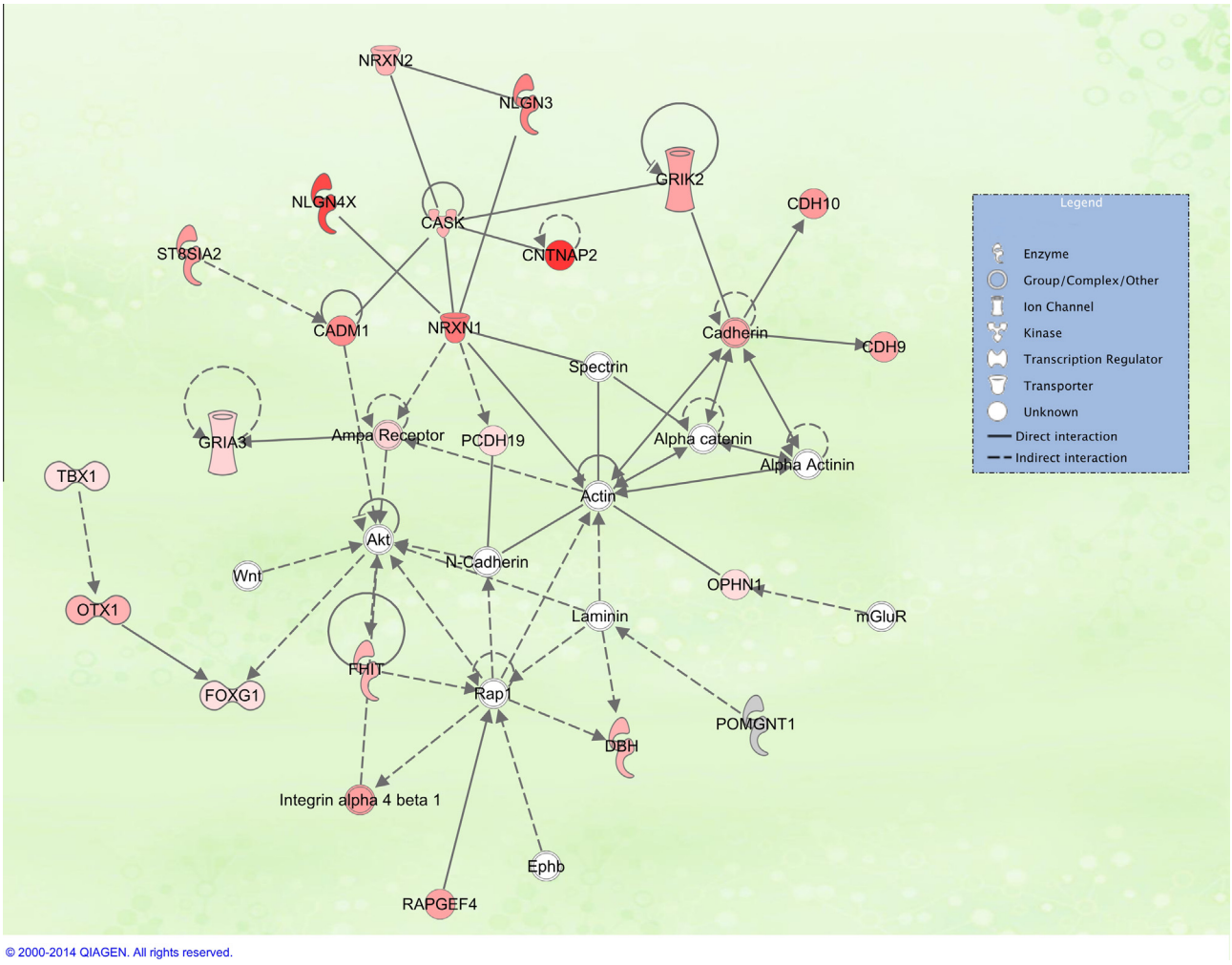

Fig. 2. Network 1 from Pathway analysis. Ingenuity pathway analysis (IPA) was performed to identify the biological networks underlying ASD. Genes or their products were shown as nodes. Lines between each node indicate biological relationships, such as transcriptional suppression or protein–protein interaction. The genes with colors are included in AutismKB core dataset. The gradation of the color is positively correlated with the overall evidence-based score of the gene as ASD related genes summarized in AutismKB. The shape represents the functional categories of the gene. The network 1 contains genes that carry functions in the regulation of behavior, cell-to-cell signaling and interaction, nervous system development.

gene has been implicated in epilepsy where the latter two have not been reported to be associated with ASD.

Neale et al. analyzed the exomes of 175 ASD trios [69]. The correlation between *de novo* mutation rate and paternal age was further confirmed in this study. Three genes were found in ASD probands with two *de novo* mutations: *BRCA2*, *FAT1* and *KCNMA1*. By using in silico protein–protein interaction analysis, the authors found significant enrichment of *de novo* mutations in 5 ASD candidate genes including *STXBP1*, *MEF2C*, *KIRREL3*, *RELN* and *TUBA1A*, and the average distance of non-synonymous variants found in ASD individuals was significant smaller than the comparator, indicating a proportion of the *de novo* events confer the risk of ASD.

In a fourth independent study, O'Roak added 189 new families to the previously reported 20 trios [70]. They found the *de novo* mutations are strongly paternal in origin, and a highly interconnected β-catenin/chromatin remodeling protein network was highlighted which containing 39% of a total of 120 severely damaging *de novo* mutations.

In a follow up study, targeted sequencing was performed on 44 candidate genes in 2446 ASD probands from the SSC [71]. A total of 27 *de novo* events were observed in 16 genes. Six genes including *CHD8*, *DYRK1A*, *GRIN2B*, *PTEN*, *TBL1XR1* and *TBR1* were found with recurrent *de novo* mutations. The results further strengthen the previous observation and the author concluded that these six recurrent genes might contribute to 1% of sporadic ASDs.

7. Convergent ASD pathways

With the accumulated data, several biological networks are emerging as potential key mechanisms for ASD. On the molecular level, these networks include: synaptic gene transcription and translation pathway (*FMR1*, *TSC1*, *TSC2*, *PTEN*, *NF1*, *CYF1P1*); neural cell adhesion molecules (Nerexin and Neuroligin families, *CNTN4*); ubiquitination pathway (*UBE3A*, *PARK2*, TRIM33); scaffolding protein in synapse (*SHANK2* and *SHANK3*); ion channel proteins (*CACNA1A*, *CACNA1H*, *SCN1A*, *SCN2A*); neuron transmitter reporter (*GRIN2A*, *GRIN2B*, *SLC6A3*); chromatin remodeling (*CHD8*, *BAF155*) and others. On a higher level, these networks seems to convergent to affect the development and functions of neurons by altering the neuron growth, excitation/inhibition (*E*/*I*) balance [72] and activity-dependent signaling [73].

Systems biology approach and network analysis have also been utilized to understand the convergence of ASD risk factors. By using temporal and spatial gene coexpression network analysis, which is based on nine high-confidence ASD genes identified from

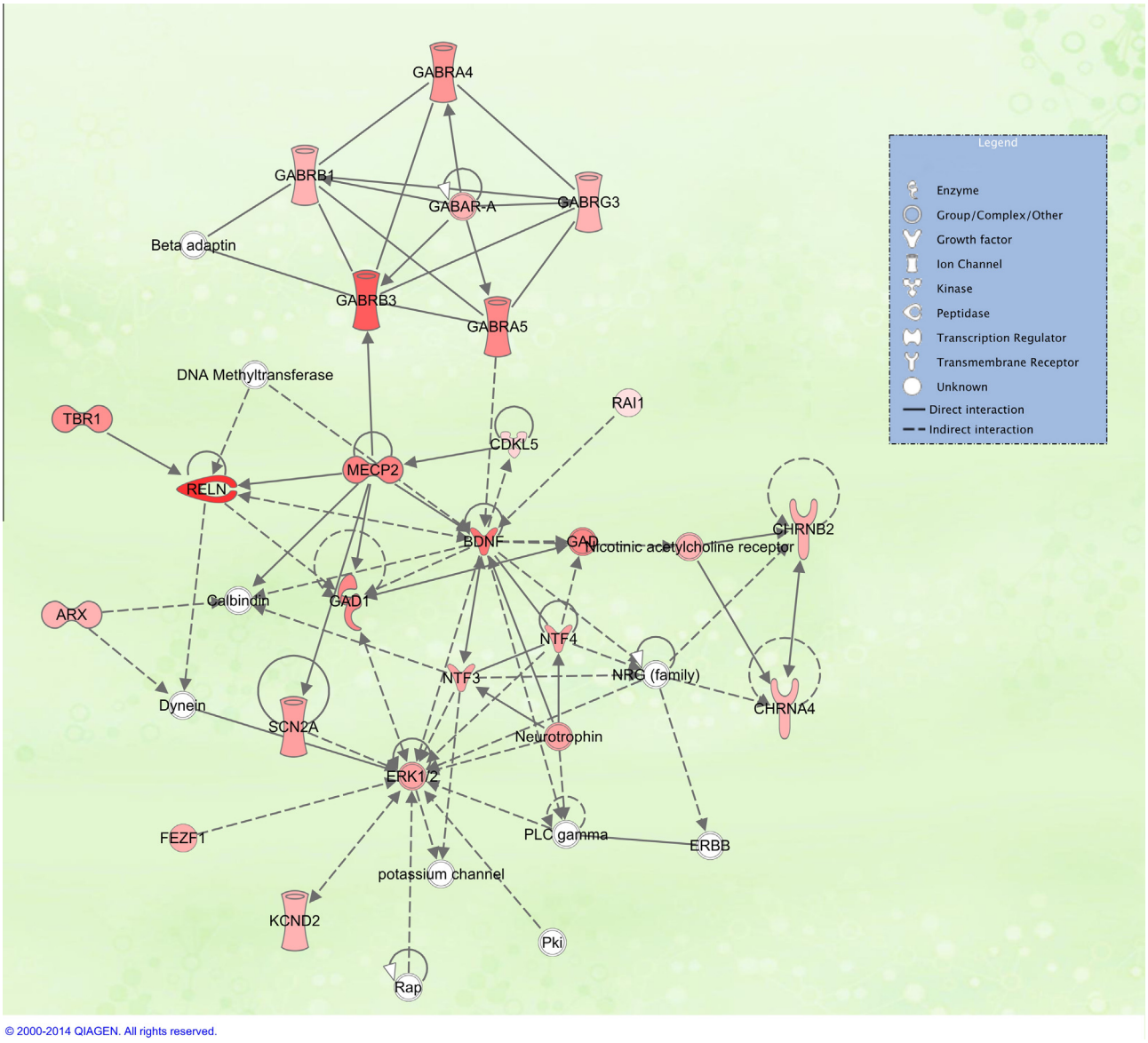

Fig. 3. Network 2 from Pathway analysis. The genes in network 2 are characterized as related with neurological diseases, psychological disorders, organismal injury and abnormalities. BDNF, MECP2, ERK1/2, and GABA receptors were identified as hubs of this network.

NGS studies, a single group of cortical neurons in layer 5/6 during midfetal development was emerged as significant risk factors to ASD [74]. In a similar methodology but based on a larger list of ASD candidate genes, Geschiwnd and colleagues identified two co-expression modules involved in early transcriptional regulation and synaptic development as key components for ASD [75]. The analysis also suggests ASD genes are more likely to influence superficial cortical layers and glutamatergic projection neurons.

Several ASD databases are now available with detailed annotation of genes and CNV associated with ASD. AutismKB is an evidence based, comprehensive knowledge base for known ASD candidate genes and CNV [76]. By using the curated list of high confidence core ASD genes (171 genes) from AutismDB, we performed the Ingenuity Pathways Analysis (IPA) to identify molecular networks connected with ASD. (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity). The top three networks were shown in Fig. 2–4. The first network is associated with behavior, cell-to-cell signaling and interaction, nervous system development and function. Several network hubs were suggested including AMPA receptor, AKT, actin and RAP1. Abnormal actin is likely to underlie the morphological deficit of neurons in some ASD cases. Recent study reported mutations in *SHANK3* affect the dendritic spine morphology via an actin-dependent mechanism [77]. *RAP1* has not been implicated as ASD candidate gene, but it was demonstrated as the key regulator for activity-dependent dendritic spine structural plasticity [78]. In the second top network, GABA receptors, ERK1/2, MECP2 and BDNF were highlighted. BDNF is the central hub in this network, given its prominent role in neuron growth and differentiation, early BDNF hyperactivity has been hypothesized to be linked with ASD and may account for phenotypes such as early brain outgrowth in ASDs [79]. In the third network, AP1, PTEN and RAS/MAPK signaling pathways were implicated. In addition, a highly conserved gene *YWHAE*, which plays a role in signal transduction and might responsible for Miller–Dieker syndrome [80], may warrant further study in its relation to ASD.

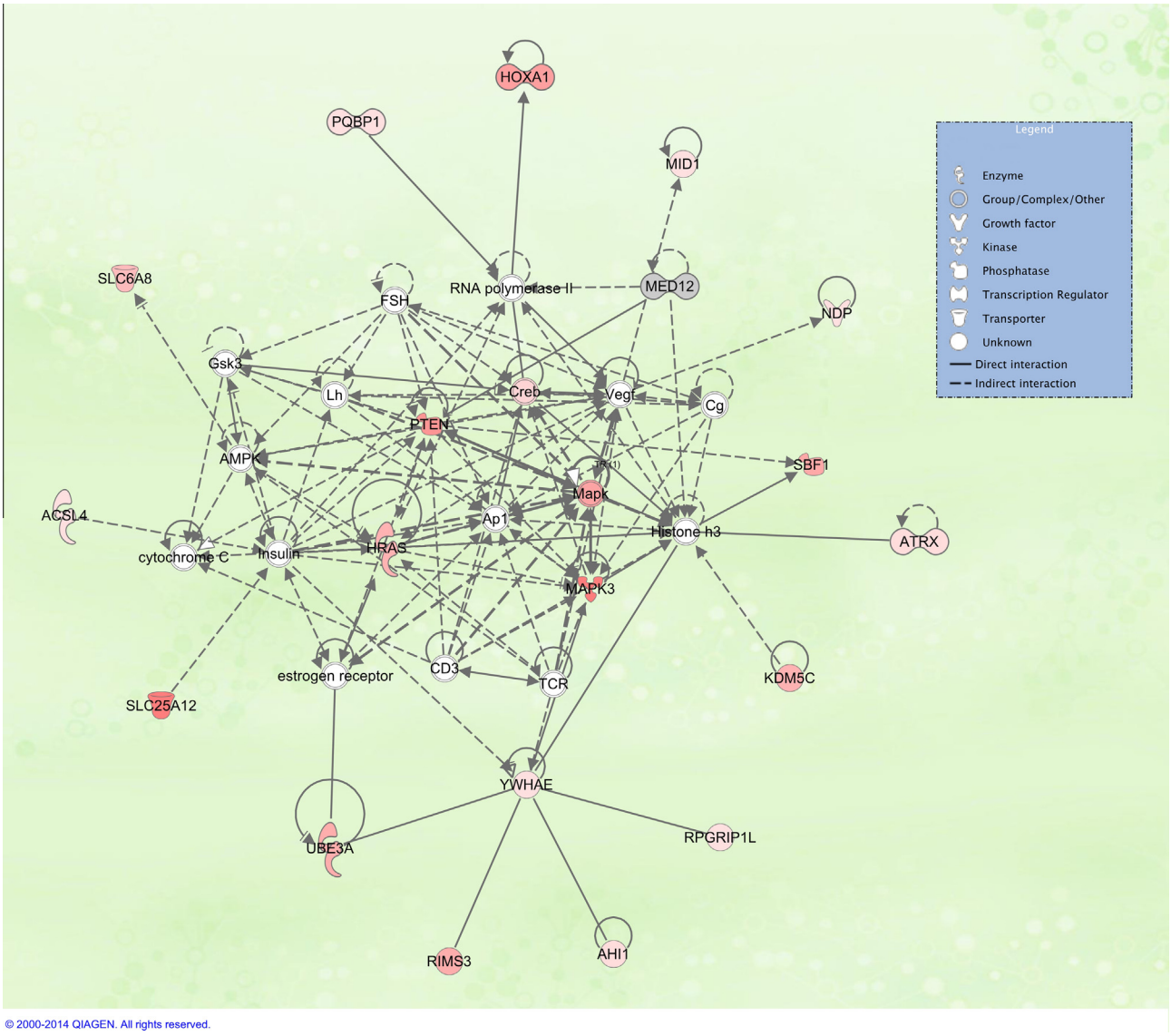

Fig. 4. Network 3 from Pathway analysis. The functions of the third network are associated with developmental disorder, hereditary disorder and neurological disease. The central hub in this network is AP-1 transcription factor, which closely connected by HRAS, PTEN, Creb and Mapk and MAPK3.

8. Future perspective

To date, hundreds of genetic variants have been implicated in ASD. Although it is estimated that only 1% of the ASD variance could be explained by common variants – a challenge to the CVCD hypothesis. However, this does not mean that the CVCD hypothesis must be rejected, given that the current sample size has limited power to detect common variants with a modest effect. Future studies with a larger sample size are necessary for the detection of common variants associated with ASD. The importance of rare *de novo* mutations in the etiology of ASD has been greatly appreciated, but *de novo* mutations may only account a limited proportion of the genetic risk factor especially in the non-idiopathic ASD cases. A recent study has demonstrated that a large proportion of missing heritability in complex diseases can be explained by genetic interactions from multiple loci [81]. We propose the gene-gene interaction may play significant roles in the etiology of ASD.

Furthermore male predominance in ASD should be addressed in the future studies. It is likely that the female are either under diagnosed or female are more resistant to the genetic perturbation. It is possible that the same ASD risk variant may have a high penetrance in male but a modest effect in female.

Finally the phenotypic and genetic heterogeneity of ASD poses a great challenge to the future study. Since the behavioral and physiological manifestations of ASD vary considerably, appropriate subgrouping by endophenotypes might help to elucidate the divergent mechanisms of ASD. In regard to the phenotypic heterogeneity, it is critical to examine the effect of the variant on the molecular and brain circular level. The emergence of the next generation genome-editing tool like TALEN and CRISPR permit a systematic investigation by creating isogenic mutated cell or animal model [82]. An integrated approach using multiple methodologies from different disciplines including genetics, neuroscience, molecular biology and others is needed to understand the etiology of ASD.

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