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MicroRNAs as Biomarkers for Psychiatric Disorders With a Focus on Autism Spectrum Disorder: Current Progress in Genetic Association Studies, Expression Profiling, and Translational Research

Yubin Hu, Erik A. Ehli, and Dorret I. Boomsma

MicroRNAs (miRNAs) are a group of small noncoding RNA molecules, 18–25 nucleotides in length, which can negatively regulate gene expression at the post-transcriptional level by binding to messenger RNAs. About half of all identified miRNAs in humans are expressed in the brain and display regulatory functions important for many biological processes related to the development of the central nervous system (CNS). Disruptions in miRNA biogenesis and miRNA-target interaction have been related to CNS diseases, including psychiatric disorders. In this review, we focus on the role of miRNAs in autism spectrum disorder (ASD) and summarize recent findings about ASD-associated genetic variants in miRNA genes, in miRNA biogenesis genes, and miRNA targets. We discuss deregulation of miRNA expression in ASD and functional validation of ASD-related miRNAs in animal models. Including miRNAs in studies of ASD will contribute to our understanding of its etiology and pathogenesis and facilitate the discrimination between different disease subgroups. ***Autism Res*** *2017, 10: 1184–1203*. © 2017 International Society for Autism Research, Wiley Periodicals, Inc.

Keywords: microRNA; miRNA; psychiatric disorders; autism spectrum disorder; biomarkers; genetic variation; expression profiling; animal studies

Introduction

MicroRNAs (miRNAs) consist of a group of small noncoding RNA molecules (ncRNAs), 18–25 nucleotides long, which can suppress gene expression at the post-transcriptional level by binding to messenger RNAs (mRNAs). miRNAs are involved in almost all biological processes, regulating the expression of most protein-coding genes in animals and plants. The first miRNA was discovered in the nematode *Caenorhabditis elegans* (*C. elegans*) in 1993, marking the beginning of a journey to explore regulatory functions of noncoding RNAs in development [Almeida, Reis, & Calin, 2011; Lee, Feinbaum, & Ambros, 1993]. miRNAs have been found in nearly all animal species as well as in plants although the miRNA repertoire in animals and plants operate via distinct systems and are believed to have emerged independently [Berezikov, 2011; Guerra-Assunção & Enright, 2012]. In animals, miRNA transcripts are processed by enzymes encoded by Drosha and Dicer genes to produce mature miRNAs, which recognize the complementary sequences on targeted mRNAs and result in a down-regulation of gene expression.

At present, more than 2,500 mature miRNAs have been identified in humans [miRBase, Kozomara & Griffiths-Jones, 2014; URL: http://www.mirbase.org] and a nomenclature system for miRNAs has been developed [Issler & Chen, 2015]. miRNAs are estimated to regulate the expression of more than 60% of all protein-coding genes in humans [Stoicea et al., 2016]. The biogenesis and molecular action of miRNAs have been well established (Fig. 1). A primary miRNA transcript (pri-miRNA) with secondary hairpin structures is either directly transcribed from a miRNA gene or is processed from introns of another gene (host gene). Then, the 3′- and 5′- end of the pri-miRNA is cleaved by the Drosha/DGCR8 enzyme complex (Drosha is also known as ribonuclease 3 and DGCR8 stands for DiGeorge syndrome Critical Regions 8), generating a precursor miRNA (pre-miRNA). Next, the pre-miRNA is transported from the nucleus to cytoplasm and its hairpin structure is cleaved by the Dicer enzyme to form a miRNA duplex about 22 nucleotides long. After cleavage, only one strand of the miRNA duplex (mature miRNA) is integrated into the RNA-induced silencing complex (RISC). A 6–8 nucleotide long sequence in the 5′- end of the mature miRNA,

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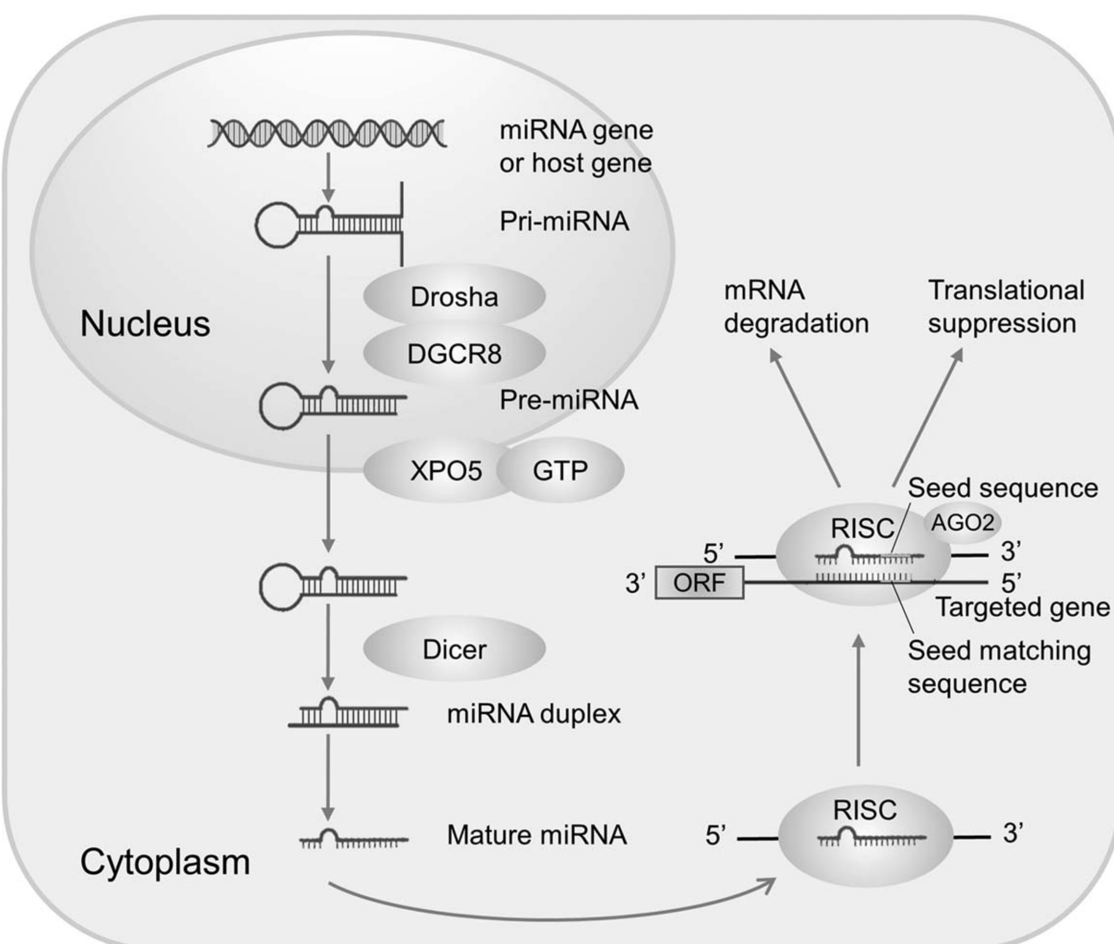

Figure 1. Biogenesis and molecular action of miRNAs. Primary miRNA transcripts (pri-miRNAs) are generated from miRNA genes or from the introns of other genes (host genes). The 3′- and 5′- end of pri-miRNAs are then cleaved by Drosha/DGCR8 enzyme complex (Drosha is also known as ribonuclease 3 and DGCR8 stands for DiGeorge syndrome Critical Regions 8), yielding precursor miRNAs (pre-miRNAs). Next, premiRNAs are transported from nucleus to cytoplasm by a complex composed of exportin 5 (XPO5) and RASrelated nuclear protein (RAN)-GTP. The hairpin structure of pre-miRNAs are then cleaved by Dicer enzyme, forming miRNA duplexes about 22 nucleotides long. Only one stand of the miRNA duplex (mature miRNA) is subsequently loaded into the RNA-induced silencing complex (RISC). A 6–8 nucleotide long sequence in the 5′- end of mature miRNA, which is called the seed sequence, complementarily binds to a sequence called the seed matching sequence that is located in the three prime untranslated region (3′-UTR) of the targeted gene and guides the RISC complex to suppress the translation of the targeted mRNA or induce the degradation of the targeted mRNA. AGO2, Argonaute 2; ORF, open reading frame.

called the seed sequence recognizes its complementary sequence in the mRNA of the targeted gene. The complementary sequence, called the seed match sequence is in most cases located in the three prime untranslated region (3′-UTR) of the targeted mRNA, a region that immediately follows the last codon (the terminate codon) in the mRNA. The recognition between the seed sequence and the seed match sequence guides the RISC complex to suppress subsequent translation of the mRNA or to promote degradation of the mRNA. Through base-pairing between the seed sequence and the seed match sequence, a single miRNA can regulate the expression of multiple genes which have a common seed match sequence. Similarly, the expression of a gene may be regulated by multiple miRNAs which have a common seed sequence. Genetic variants that disrupt such recognition could have an impact on an extensive genetic network involving multiple pathways. A detailed description of the miRNA biogenesis and molecular action is available in a review by Issler and Chen [2015].

With the development of miRNA microarrays and high-throughput sequencing techniques, variation in miRNA-target interaction is examined both within species and across species. One of the most important discoveries is that variation in miRNA-target interaction contributes to phenotypic variation, including variation in disease [Clop et al., 2006; Georges et al., 2006]. Studies during the early years in the 21st century mainly

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focused on the role of miRNAs in cancer. Deregulation of miRNAs was reported to be responsible for abnormal expression of a number of oncogenes and tumor suppressors in multiple cancers [Almeida et al., 2011; Saumet & Lecellier, 2015]. Mutations in miRNA genes and miRNA target sites as well as miRNA biogenesis genes (hereinafter referred to as miRNA-related genes) were identified for some cardiovascular diseases and autoimmune diseases, too [Dorn, Matkovich, Eschenbacher, & Zhang, 2012; Pauley, Cha, & Chan, 2009; Rao et al., 2009].

The investigation of the role of miRNAs in normal and abnormal development of the central nervous system (CNS) revealed that more than half of all identified miRNAs were expressed in the brain and are involved in several biological processes in CNS development, including cell proliferation, cell differentiation, synaptogenesis, synaptic plasticity, and apoptosis [Alural, Genc, & Haggarty, 2017; Reschke & Henshall, 2015] and mutations in miRNA-related genes and abnormal expression of miRNAs were found to be associated with several neurodegenerative diseases [Nelson, Wang, & Rajeev, 2008], including Alzheimer's Disease and Parkinson's Disease.

Recognizing miRNAs as a "master regulator" of gene expression has led to the aim to understand the role of miRNAs in human complex disease, including psychiatric disorders. Genome-wide association studies (GWASs) have shown that a number of common genetic variants contribute, albeit to a small extent, to the genetic risk for psychiatric disorders like schizophrenia [Ripke et al., 2014], bipolar disorder [Hou et al., 2016], or symptoms of depression [Okbay et al., 2016]. However, biological functions of many genetic variants remain largely unknown. Studies tapping into the link between genetic variation and miRNAs have identified genetic variants, including single nucleotide polymorphisms (SNPs) [Hauberg, Roussos, Grove, Børglum, & Mattheisen, 2016] and copy number variations (CNVs)]Warnica et al., 2015], that are related to miRNAs and their targets. Abnormal expression of miRNAs has been reported in various tissues from patients with psychiatric disorders. Results regarding miRNA-related genetic variants and abnormal expression of miRNAs have recently been reviewed for schizophrenia, bipolar disorder, and major depressive disorder [Alural et al., 2017; Hommers, Domschke, & Deckert, 2015] and, in spite of limitations such as population heterogeneity for genetic association studies and tissue specificity for expression profiling studies, point to several consistent findings (e.g. hsa-miR-137 for schizophrenia). These studies, although in their infancy, suggest miRNAs have an important role in the etiology and pathogenesis of psychiatric disorders.

miRNAs are present in diverse biological fluids, including cerebrospinal fluid (CSF), blood, saliva, and urine. Therefore, miRNAs could be potential noninvasive biomarkers for the diagnosis, prognosis, and treatment responses for psychiatric disorders and may have applications in precision medicine in the future [Alural et al., 2017; Santulli, 2015; Stoicea et al., 2016]. Due to the fact that miRNAs are small in size, traditional RNA isolation procedures can be inefficient in the recovery of these molecules; special care should be considered prior to RNA extraction to enhance capture.

Here, we focus on the role of miRNAs in autism spectrum disorder (ASD) and summarize recent findings from three perspectives: (1) genetic variants related to miRNAs in ASD; (2) deregulation of miRNA expression in ASD; (3) functional studies of relevant miRNAs in animal models. For the first part, we begin with an overview about the prevalence and location of common and rare variants that are in miRNA genes, miRNA biogenesis genes, and miRNA targets, followed by an examination of whether these variants are captured with common techniques used in population genetics, including SNP arrays, exome arrays, whole exome sequencing (WES), and whole genome sequencing (WGS). Then, we present studies which primarily use bioinformatics tools to identify an overlap between ASD-associated genetic variants and miRNA-related variants. For the second part, we review discoveries from expression profiling of miRNAs in ASD patient derived tissues such as post-mortem brains, lymphoblastoid cell lines, blood, CSF, and stem cells. For the third part, we try to evaluate biological relevance of miRNA-related variants or deregulated miRNAs based on studies in animal models. Last, we discuss strengths and limitations of these studies and evaluate the potential of miRNAs as novel biomarkers for psychiatric disorders.

Primary literature for this review was collected in two stages. During the first stage, we aimed to have a broad picture about miRNA-related genetic variation and we searched all English reviews between 2011 and 2016 in Web of Science™ Core Collection and MEDLINE® databases using the following search criteria, yielding 145 results (June, 2016): "(miRNA or microRNA or noncoding RNA) AND (genomic variation or genetic variation) AND human." From this pool, we selected seven reviews about genetic variants related to miRNAs, four reviews about current techniques to detect genetic variants, and two reviews about miRNA-related variation for psychiatric disorders. During the second stage, we focused on ASD and searched all English publications between 2006 and 2016 in Web of Science™ Core Collection and MEDLINE® databases using the following search criteria, yielding 125 results (June, 2016): "(miRNA or microRNA or noncoding RNA) AND (expression or variation) AND

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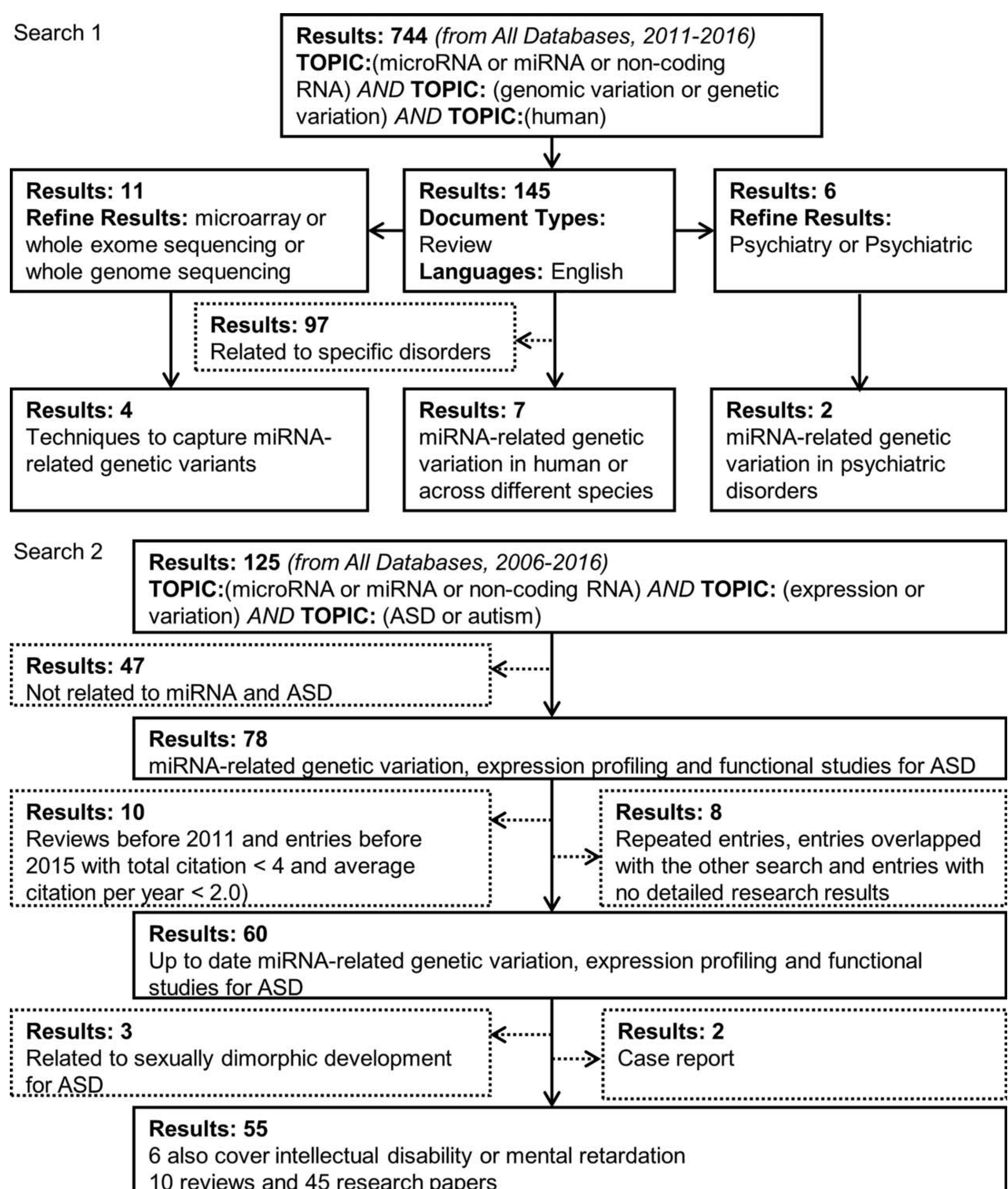

Figure 2. Procedures to select primary literatures using Web of Science™ service. Solid boxes represent inclusion while dashed boxes represent exclusion. The number of relevant publications and searching criteria are described inside each box. Topic indicates searching for terms in the title, abstract and keywords of a publication.

(ASD or autism)." From this pool, we selected another 55 research and review articles covering miRNA-related genetic variants for ASD, expression profiling of miRNAs in ASD patient-derived tissues, and functional studies in animal models. Figure 2 shows the literature mining process in detail. In total, 68 publications (23 reviews and 45 research articles) were included for primary review.

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Genetic Variants Related to miRNAs in ASD

More than 60,000 genes have been annotated across the human genome, containing 19,815 protein-coding genes, 15,941 long (>200 bp) noncoding RNA genes, 9,882 small (<200 bp) noncoding RNA genes and 14,505 pseudogenes (GENCODE V24, URL: http://www.gencodegenes.org/releases/current.html). About 40% of all annotated genes are noncoding RNA genes, including approximately 2,000 validated miRNA genes [Bhartiya & Scaria, noncoding RNAs: Structure, function and regulation. Genomics, 107, 59–68. [PMC][10.1016/j.ygeno.2016.01.005] [26790601]" 2016; Hrdlickova, de Almeida, Borek, & Withoff, 2014]. Compared to other genic or inter-genic regions, genetic variants are much less abundant within miRNA genes [Li & Zhang, 2013]. However, genetic variants including single nucleotide polymorphisms (SNPs) and copy number variants (CNVs) in miRNA genes have been found and associated with various diseases like cancer and psychiatric disorders.

Significant efforts have been underway to understand the role of genetic variation in miRNA genes, miRNA biogenesis genes, and miRNA targets. Although most SNP arrays are designed to capture common genetic variation in the general population, techniques and analysis methods have been developed to examine genetic variants in miRNA genes utilizing the genotypes captured by these arrays. In order to detect rare or de novo variants, more sensitive approaches are required such as exome arrays or high-throughput next generation sequencing (NGS) techniques. If an array designed to detect common SNPs is utilized to measure genetic variation, imputation may be applied to increase the density of SNPs under investigation. Therefore, it is also important to know how these techniques enable discoveries of common and rare miRNA-related variants in order to link findings from genetic association studies of a specific disease with miRNAs.

In part one, we examine the relative abundance and functional consequences of different types of miRNA-related genetic variants. Then, we evaluate whether different techniques like SNP or exome arrays, NGS, and imputation used in these studies have covered or may potentially cover miRNA-related variants. Next, we focus on genetic association studies of ASD. Last, we present recent studies that are aimed at identifying the overlap between miRNA-related genetic variants and ASD-related variants.

Genetic Variants in miRNA Genes, miRNA Biogenesis Genes, and miRNA Targets

Although miRNA gene sequences are conserved both within-species as well as across species, genetic variants have been identified in different regions of miRNA genes. The density of SNPs decreases from the flanking region of miRNA genes to the pre-miRNA sequence to the mature miRNA sequence outside the seed sequence and finally to the seed sequence [Cammaerts, Strazisar, De Rijk, & Del Favero, 2015; García-López, Brieño-Enríquez, & del Mazo, 2013]. SNPs in human miRNA loci are continuously curated in several databases like miRvar [Bhartiya, Laddha, Mukhopadhyay, & Scaria, 2011; URL: http://genome.igib.res.in/mirlovd] and miRNA SNiPer [Zorc et al., 2012; URL: http://www.integratomics-time.com/miRNA-SNiPer]. In the miRvar database, 188 SNPs were mapped by June 2016 to 157 miRNA genes (miRvar, Current LOVD status). However, according to statistics based on the current version of miRNA SNiPer database (miRNA SNiPer 4.0), more than 1,500 miRNA genes contain a polymorphic pre-miRNA region and more than 400 human miRNAs contain a polymorphic seed region out of 1,881 miRNA genes [Zorc, Obsteter, Dovc, & Kunej, 2015]. The abundance of SNPs in miRNA loci is inconsistent across different databases due to various integration procedures of resources. Nonetheless, it is clear that SNPs are sparse in miRNA genes given the fact that there are about 84.7 million SNPs across the whole human genome (The 1000 Genomes Project Consortium, 2015). The functional relevance of SNPs close to or within miRNA genes is primarily predicted with bioinformatics approaches [Cammaerts et al., 2015]. These SNPs could influence biogenesis of miRNAs and/or miRNA-target interaction depending on the location of the SNP [Cammaerts et al., 2015; Issler & Chen, 2015]. First, SNPs in regulatory regions, including promoter regions of miRNA genes or host genes may change the abundance of pri-miRNAs. Second, SNPs in pri-miRNA or pre-miRNA sequences may affect the secondary structure of pri-miRNAs or pre-miRNAs, thus altering their binding affinity with Drosha/DGCR8 enzyme complex or Dicer enzyme, yielding different cleavage sites on these miRNA transcripts. Third, SNPs in the miRNA duplex could change strand preference during the loading of a single strand into the RISC complex [Sun et al., 2009]. Last, SNPs in the seed sequence of the mature miRNA, though not common, will result in a partial loss of the original targets or create new targets. For a limited number of SNPs that are associated with certain diseases (cancer in most cases), their functional impact on miRNA biogenesis and target recognition has been validated with experiments in vitro and in vivo [Bhartiya & Scaria, 2016; Cammaerts et al., 2015].

In addition to SNPs, CNVs involving miRNA loci (miRNA-CNVs) have also been investigated. Consistent with findings regarding the location of SNPs in the genetic architecture of miRNAs, a very limited number of miRNA loci are located in highly polymorphic CNV

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regions [Marcinkowska, Szymanski, Krzyzosiak, & Kozlowski, 2011]. However, miRNA-CNVs may have a greater impact on miRNA expression. Deletion or duplication of miRNA genes will alter the expression level of specific miRNAs [Issler & Chen, 2015]. Moreover, it is suggested that CNVs could affect long-range interaction between DNA segments, which may cause a wide-range deregulation of miRNA expression and target recognition [Persengiev, Kondova, & Bontrop, 2013].

Apart from genetic variants in miRNA genes, variants have also been identified in miRNA biogenesis genes. For example, SNP rs2073778 in the DGCR8 gene and SNP rs3742330 in the Dicer gene have been found to be associated with nonmuscle bladder cancer progression and the survival rate of T cell lymphoma patients respectively, although their functional relevance is unclear [Hrdlickova et al., 2014]. The 22q11.2 deletion contains the DGCR8 gene and was found to result in reduced levels of many miRNAs in a mouse model [Issler & Chen, 2015; Merico et al., 2015]. The burden of genetic variants in miRNA biogenesis genes, to our best knowledge, has not been systematically evaluated yet.

Genetic variants in miRNA loci (especially in the seed sequence) and in miRNA biogenesis genes are predicted to be under high selective pressure considering their potential deleterious effects [García-López et al., 2013; Li & Zhang, 2013]. Therefore, these variants are expected to be rare in populations. However, another important source of genetic variation that may change miRNA-target interaction is variation within the miRNA target (the protein coding mRNA transcript). In spite of evolutionary constraints on the seed match sequence in the 3′-UTR of miRNA targets, SNPs in miRNA targets are less rare than variants in the actual miRNAs. MirSNP is a database that annotates human SNPs in predicted miRNA target sites [Liu et al., 2012; URL: http://bioinfo.bjmu.edu.cn/mirsnp]. In total, 414,510 SNPs, including 32,822 SNPs with a minor allele frequency (MAF) larger than 0.01 have been collected so far spanning 17,569 genes. Another database, PolymiRTS, has included experimentally validated SNPs ($n = 24,879$) as well as 1,217 small insertions and deletions (INDELs) in miRNA target sites [Bhattacharya, Ziebarth, & Cui, 2014]. Genetic variation in miRNA target sites may disturb the recognition between miRNAs and miRNA targets and thus may contribute to genetic risk for complex disorders like psychiatric disorders [Geaghan & Cairns, 2015]. Figure 3 illustrates the influences on miRNA functionality of different genetic variants in miRNA genes, miRNA biogenesis genes, and miRNA target sites.

Detection of miRNA-Related Genetic Variants

Commercially designed SNP arrays used in GWASs are designed to capture common genetic variation in populations [Ombrello, Sikora, & Kastner, 2014]. Using SNP array data for association analysis could cover common SNPs in the 3′-UTR of miRNA targets, but not rare variants. As mentioned previously, many SNPs in miRNA target sites are rare ($MAF < 0.01$) and most genetic variants in miRNA genes and miRNA biogenesis genes are also rare. Exome arrays and next generation sequencing, like WGS and WES could help researchers to identify less-frequent and rare variants [Ombrello et al., 2014]. Since WGS is relatively expensive for large-scale association studies and requires more complex data management and analysis, exome arrays and WES are alternatives which capture variants in protein-coding regions, including miRNA biogenesis genes. miRNAs are produced from either their own genes or from introns of other genes and miRNA target sites are located in the 3′-UTR of miRNA targets, both of which are outside protein-coding regions and thus are not covered by exome arrays and WES. However, the two techniques described above (exome arrays and WES) could allow for the detection of rare variants in miRNA loci and miRNA target sites if combined with imputation. Imputation is a statistical method to infer ungenotyped variants. For rare variants, appropriate reference panels are necessary, for example, 1000 Genomes reference panel]Panoutsopoulou, Tachmazidou, & Zeggini, 2013; The 1000 Genomes Project Consortium, 2015] or the Haplotype Reference Consortium reference panel [McCarthy et al., 2016], both of which integrate WGS data to construct the reference panel. With more WGS data being used to build reference panels for the human genome, variants at a wider range of the MAF spectrum may be imputed more precisely [McCarthy et al., 2016]. As for identification of de novo variation, trio-sequencing of both parents and their offspring could be performed using WES or WGS [De Rubeis et al., 2014; Iossifov et al., 2014], although with WES only protein-coding regions are sequenced prohibiting assessment of the burden of de novo variants in miRNA genes and miRNA target sites.

Genetic Variants Associated With ASD

Autism spectrum disorder (ASD) refers to a group of heterogeneous neurodevelopmental disorders characterized by social interaction deficits, communication problems, and repetitive behaviors. Except for some monogenic forms, most disorders within this spectrum have a complex genetic background [Fett-Conte, Bossolani-Martins, & Rosan, 2015]. As with other psychiatric disorders, GWASs of ASD using SNP arrays investigate the association between common genetic variation and predisposition for the disorder. Although no consistent GWAS hits for ASD have been reported so far, probably due to limited sample sizes, common genetic variation is estimated

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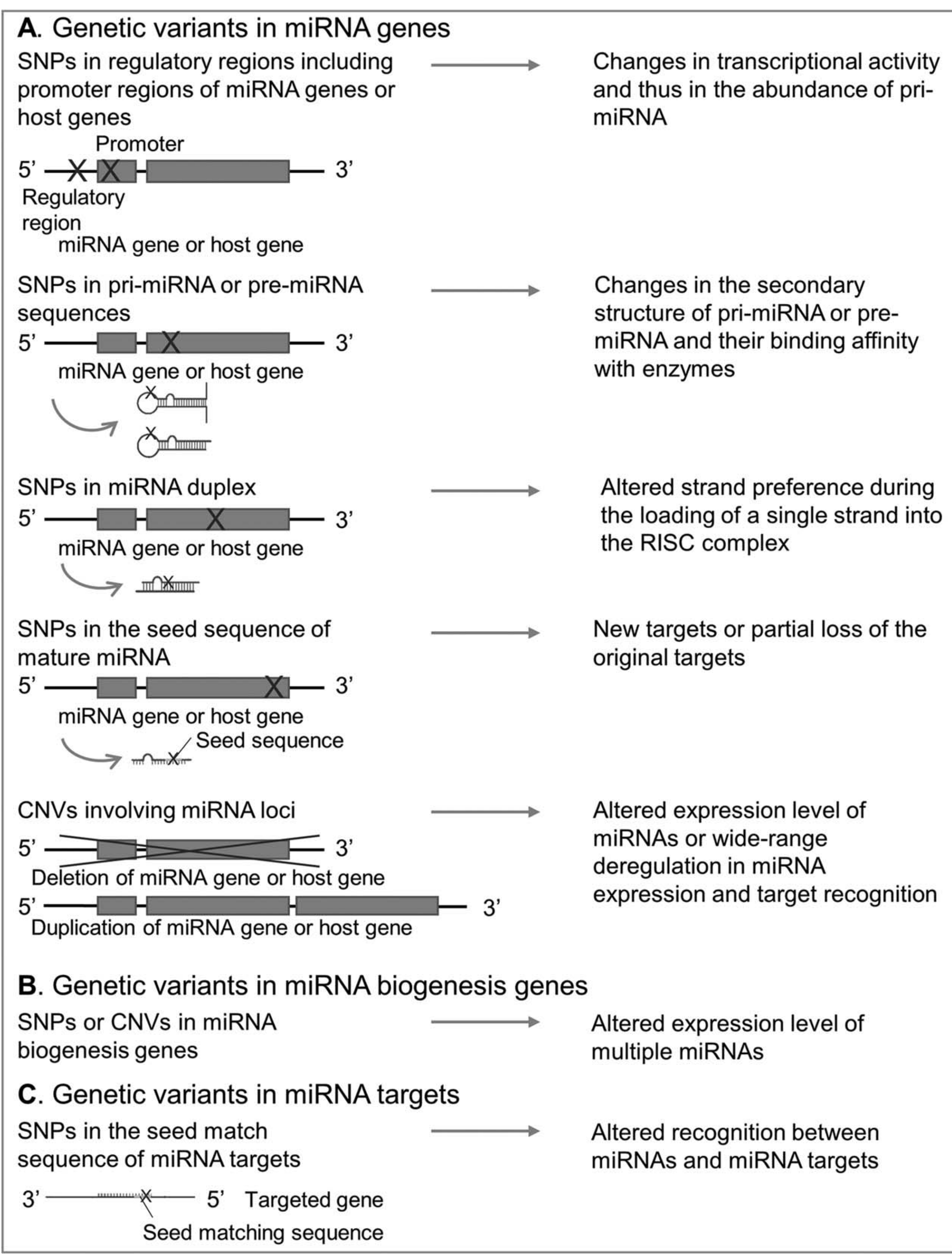

Figure 3. Influences on miRNA functionality of genetic variants in miRNA genes, miRNA biogenesis genes, and miRNA targets. (A) For genetic variants at miRNA loci, single nucleotide polymorphisms (SNPs) in regulatory regions including promoters may affect the transcriptional activity of miRNA genes or host genes, thus influencing the abundance of pri-miRNAs. SNPs in pri-miRNA or pre-miRNA sequences may change the secondary structure of pri-miRNAs or pre-miRNAs and their binding affinity to Drosha/DGCR8 enzyme complex and Dicer enzyme respectively. SNPs in miRNA duplexes may alter the strand preference during the loading of a single strand into the RISC complex. Besides, SNPs located in the seed sequence of mature miRNAs may yield new targets or result in partial loss of the original targets. Apart from SNPs, copy number variations (CNVs) involving miRNA loci, including deletion and duplication of miRNA genes or host genes, will change the expression level of miRNAs or cause a wide-range deregulation in miRNA expression and target recognition. (B) SNPs or CNVs in miRNA biogenesis genes may alter the expression level of multiple miRNAs. C. SNPs in the seed match sequence of miRNA targets may disturb the recognition between miRNAs and miRNA targets.

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to contribute to nearly half of the variation in the risk for ASD [Gaugler et al., 2014]. Recent studies using WES and WGS highlight an important role of rare and de novo variants in ASD. An excessive burden of single-nucleotide variants (SNVs), INDELs, CNVs, including de novo CNVs and de novo loss-of-function mutations (LOF mutations) have been reported in ASD cases [Krumm et al., 2015; Ronemus, Iossifov, Levy, & Wigler, 2014]. These variants are only present in a small fraction of the collected ASD cases (5–10%), but their individual effect size is relatively high especially for de novo variation compared to the effect size of a single SNP [De Rubeis & Buxbaum, 2015]. De novo variation occurs when a mutation happens during meiosis. Although it only accounts for about 3% of the variance in the risk for ASD, de novo variation could considerably change the ASD risk for a certain person [De Rubeis & Buxbaum, 2015]. One of the well-established CNVs that are associated with ASD is 22q11.2 microdeletion or microduplication. Approximately 80% of the 22q11.2 CNVs are de novo, suggesting a substantial contribution of de novo mutations to ASD occurrence [Hiroi et al., 2013].

Genetic association studies for ASD enable researchers to identify the role of miRNAs in ASD at a genetic level, but the genotyping or sequencing techniques used in these studies may not necessarily cover all of the miRNA-related variants. Since most miRNA-related variants are rare, imputation is required, but is difficult for rare variants. More trio-WGS data might capture ASD-associated de novo variants in miRNA genes and miRNA target sites.

Overlap Between miRNA-Related Genetic Variants and ASD-Related Genetic Variants

ASD-related genetic variants have been identified in miRNA genes, miRNA biogenesis genes, and miRNA target sites. Two studies systematically examined the presence of miRNA loci in ASD-associated CNVs with bioinformatics tools. Vaishnavi, Manikandan, Tiwary, and Munirajan [2013] reported about 11% of the well-validated ASD-associated CNVs harbor miRNAs and Marrale, Albanese, Calì, and Romano [2014] showed miRNA loci are over-represented in ASD-associated de novo CNVs on chromosome 1, 2, and 22. Eight affected miRNAs are identified in both studies (Chr1: hsa-mir-429, hsa-mir-200a, hsa-mir-200b; Chr2: hsa-mir-149; Chr22: hsa-mir-185, hsa-mir-1306, hsa-mir-1286, hsa-mir-649). These miRNAs have previously been linked to ASD [Marrale et al., 2014]. The most frequent rare CNVs in ASD subjects include a 15q11-q13 duplication and a 22q11.2 deletion [De Rubeis & Buxbaum, 2015; Fett-Conte et al., 2015], which disrupt a miRNA gene (*MIR211*) and an important miRNA biogenesis gene (*DGCR8*) respectively [Alural et al., 2017; Miller et al., 2009]. However, the 22q11.2 deletion is also associated with other disorders, including schizophrenia [Alural et al., 2017] and early childhood epilepsy [Reschke & Henshall, 2015], which is not surprising considering the central role of DGCR8 in the miRNA biogenesis. Actually, children with a 22q11.2 deletion often display autistic behaviors, which are suggested to be early signs for schizophrenia too [Fett-Conte et al., 2015].

More ASD-related variants are found in miRNA target sites. Vaishnavi, Manikandan, and Munirajan [2014] also implemented computational approaches to identify SNPs that disturb miRNA-target recognition in the 3′-UTR of ASD-associated genes. They reported nine SNPs in the seed match sequence that block the binding or change the binding affinity between miRNAs and miRNA target sites. Another 12 SNPs were discovered which create new miRNA target sites in the 3′-UTR of ASD-associated genes. The functional relevance of some of the genes that harbor these SNPs has been demonstrated previously in animal studies [Vaishnavi et al., 2014]. An example of a SNP that can create a new miRNA target site is the rs1045881 SNP which is located in the 3′-UTR of the Neurexin-1 gene (*NRXN1*). This SNP, with the C-allele generating a novel binding site for hsa-miR-1274a and hsa-miR-339-5p, is identified to be associated with sensorimotor performance in healthy subjects which is impaired in ASD patients [Voineskos et al., 2011]. In addition, X-linked SNPs in the gene family linked with fragile X syndrome were found to be associated with autistic traits. This association is possibly due to a disruption in the recognition between hsa-miR-181 and the corresponding seed match sequences in these genes [Stepniak et al., 2015].

No common ASD-associated variants have been found in miRNA genes and miRNA biogenesis genes so far. Even for variants in miRNA target sites, those SNPs identified in the study of Vaishnavi et al. [2014] are not common (a few SNPs with $MAF < 0.05$ and most others with $MAF < 0.005$). More interestingly, the MAF of some of these SNPs contrasts greatly across different populations [Vaishnavi et al., 2014]. It implies that the high selective pressure on miRNA-related variants may act in opposite directions for different populations.

Deregulation of miRNA Expression in ASD

The identification of differentially expressed miRNAs in ASD individuals is undertaken by comparing the expression profile of miRNAs in patient-derived tissues and healthy tissues. Common techniques for miRNA expression profiling include microarrays, RNA sequencing (RNA-seq), and quantitative reverse transcription polymerase chain reaction (RT-qPCR). The different miRNA profiling techniques have been described and evaluated

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by Pritchard, Cheng, and Tewari [2012]. Early studies primarily used commercially designed miRNA microarrays to efficiently quantify the relative abundance of many miRNA species in parallel [Ghahramani Seno et al., 2011; Sarachana, Zhou, Chen, Manji, & Hu, 2010; Talebizadeh, Butler, & Theodoro, 2008]. With the advent of next generation sequencing, high-throughput techniques like RNA-seq began to be applied to miRNA expression profiling [Hicks, Ignacio, Gentile, & Middleton, 2016]. RNA-seq enables the characterization of miRNAs that differ by a single nucleotide or have varying lengths, which is an important advantage for some studies like those that focus on SNP-specific effects on miRNA expression, but it is still much more expensive than microarrays [Pritchard et al., 2012]. It is important to note that mRNAs and miRNAs cannot be sequenced together on these next generation platforms and must be assayed separately due to the drastic differences in size. Fortunately, in many instances the same total RNA preparation can be utilized to create the sequencing libraries for both the large (mRNAs and lncRNAs) and small RNAs (miRNAs) in separate workflows. Both microarrays and RNA-seq are unable to quantify the absolute abundance of miRNAs, so it is sound practice to utilize RT-qPCR to validate the results from microarrays or sequencing [Ghahramani Seno et al., 2011; Huang et al., 2015; Mor, Nardone, Sams, & Elliott, 2015; Sarachana et al., 2010; Talebizadeh et al., 2008; Vasu et al., 2014].

Possibly more important than the profiling techniques, is what kind of patient-derived tissue to use for the miRNA expression profiling. ASD is a neurodevelopmental disorder, but brain tissues from live individuals are obviously nearly impossible to acquire. An alternative is profiling of post-mortem tissues for ASD subjects and healthy controls [Abu-Elneel et al., 2008; Ander, Barger, Stamova, Sharp, & Schumann, 2015; Stamova, Ander, Barger, Sharp, & Schumann, 2015; Wu, Parikshak, Belgard, & Geschwind, 2016]. Other studies use more accessible tissues like peripheral blood or saliva [Hicks et al., 2016; Huang et al., 2015; Vasu et al., 2014]. Cell lines transformed from peripheral blood lymphocytes, like lymphoblastoid cell lines (LCLs), can be also used for miRNA expression profiling with an advantage of unlimited source of miRNAs and an easy control of confounding factors in experiments [Ghahramani Seno et al., 2011]. Although it has been demonstrated that miRNA expression patterns are similar in brain and in peripheral blood lymphocytes [Anitha & Thanseem, 2015], tissues that resemble neural stem cells may be a better proxy for the brain in early developmental stages. Induced pluripotent stem cells (iPSCs), are stem cells induced from patient tissues and iPSC technology has been used to model neurodevelopmental disorders [Brennand et al., 2011; Zhao et al., 2015]. Therefore, miRNA expression profiles in iPSC-derived neurons have been investigated to study deregulation of miRNAs for schizophrenia and may be applied to other neurodevelopmental disorders, including ASD in the future [Zhao et al., 2015]. Nguyen et al. [2016] utilized olfactory stem cells from living patients which are close to neural stem cells and identified miRNA signatures that are unique to ASD patients, suggesting olfactory stem cells as a relevant model for expression profiling studies of ASD.

Table I provides a list of deregulated miRNAs in ASD patients reported by more than one expression profiling study of ASD. In spite of technique and tissue specificity (**Supporting Information** Table S1), these miRNAs are consistently differentially expressed between ASD subjects and healthy controls and the deregulation of hsa-miR-146, hsa-miR-21, hsa-miR-181, hsa-miR-320, and hsa-miR-103 was validated by RT-qPCR. Interestingly, several mature miRNAs with closely related sequences, for example, hsa-miR-23a and hsa-miR-23b, hsa-miR-146a and hsa-miR-146b, are present in the list, suggesting that the targets of these closely related sequences may be overlapping and these shared targets could be of interest in terms of the pathogenesis of ASD.

However, only hsa-miR-7, hsa-miR-195, hsa-miR-211, and hsa-miR-484 are in ASD-associated CNV loci that are identified in the study by Vaishnavi et al. [2013]. None of these deregulated miRNAs are reported to be in ASD-associated de novo CNV loci [Marrale et al., 2014]. In addition to tissue specificity, population heterogeneity and small sample sizes were significant limitations in the expression profiling studies. It should be noted that miRNAs are continuously being identified in humans, so each individual expression profiling study described here only covers a part of all mature miRNAs except for some recent studies using RNA-seq. Therefore, it should not be surprising that the current findings are not consistent between studies that focus on genetic variation and those that focus on expression.

Functional Studies of ASD-Related miRNAs in Animal Models

Functional validation of miRNA-related genetic variants that are associated with ASD and the differentially expressed miRNAs in ASD could provide insights into the biological relevance of miRNAs and their targets. Animal models form an important way to link specific changes in miRNA biogenesis and molecular action to pathophysiological and behavioral abnormalities [Issler & Chen, 2015]. One approach is to manipulate specific miRNA genes, miRNA biogenesis genes, or target genes to generate animal models with knock-out, knowdown, or overexpression of the gene. The manipulation

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Table I. Summary of Deregulated miRNAs in Autism Spectrum Disorder Patients Reported by More Than One miRNA Expression Profiling Study

miRNA	#Study	Literature
hsa-miR-23	5	hsa-miR-23: Hicks et al. [2016] (↓) hsa-miR-23a: Wu et al. [2016] (↑); Sarachana et al. [2010] (↑); Talebizadeh et al. [2008] (↑); Abu-Elneel et al. [2008] (↓) hsa-miR-23b: Sarachana et al. [2010] (↑); Talebizadeh et al. [2008] (↑)
hsa-miR-146[a]	4	hsa-miR-146a: Nguyen et al. [2016] (↑); Mor et al. [2013] (↑); Talebizadeh et al. [2008] (↑) hsa-miR-146b: Talebizadeh et al. [2008] (↑); Abu-Elneel et al. [2008] (↑)
hsa-let-7	3	hsa-let-7a: Mor et al. [2013] (↓); Huang et al. [2015] (↓) hsa-let-7d: Huang et al. [2015] (↓) hsa-let-7f: Huang et al. [2015] (↓) hsa-let-7g: Wu et al. [2016] (↑)
hsa-miR-7	3	Hicks et al. [2016] (↑); Mor et al. [2013] (↑); Abu-Elneel et al. [2008] (↓)
hsa-miR-19	3	hsa-miR-19a: Mor et al. [2013] (↑) hsa-miR-19b: Mor et al. [2013] (↑); Huang et al. [2015] (↓); Vasu et al. 2014 (↑)
hsa-miR-21[a]	3	Wu et al. [2016] (↑); Mor et al. [2013] (↑); Abu-Elneel et al. [2008] (↓)
hsa-miR-27	3	hsa-miR-27a: Hicks et al. [2016] (↓); Vasu et al. [2014] (↑); Abu-Elneel et al. [2008] (↓)
hsa-miR-30	3	hsa-miR-30a: Ghahramani Seno et al. [2011] (↑) hsa-miR-30c: Sarachana et al. [2010] (↑) hsa-miR-30e: Hicks et al. [2016] (↓)
hsa-miR-92	3	hsa-miR-92a: Huang et al. [2015] (↓) hsa-miR-92a1: Talebizadeh et al. [2008] (↓) hsa-miR-92a2: Talebizadeh et al. [2008] (↓) hsa-miR-92b: Mor et al. [2013] (↓)
hsa-miR-106	3	hsa-miR-106a: Abu-Elneel et al. [2008] (↑) hsa-miR-106b: Vasu et al. [2014] (↑); Sarachana et al. [2010] (↑↓); Abu-Elneel et al. [2008] (↑)
hsa-miR-132	3	Sarachana et al. [2010] (↓); Talebizadeh et al. [2008] (↑); Abu-Elneel et al. [2008] (↓)
hsa-miR-148	3	hsa-miR-148a: Wu et al. [2016] (↑) hsa-miR-148b: Sarachana et al. [2010] (↓); Abu-Elneel et al. [2008] (↓)
hsa-miR-181[a]	3	hsa-miR-181a: Ghahramani Seno et al. [2011] (↑) hsa-miR-181b: Vasu et al. [2014] (↓); Ghahramani Seno et al. [2011] (↑) hsa-miR-181c: Ghahramani Seno et al. [2011] (↑) hsa-miR-181d: Abu-Elneel et al. [2008] (↑)
hsa-miR-195	3	Huang et al. [2015] (↓); Vasu et al. [2014] (↑); Sarachana et al. [2010] (↑)
hsa-miR-320[a]	3	hsa-miR-320: Talebizadeh et al. 2008 (↓) hsa-miR-320a: Vasu et al. 2014 (↓); Abu-Elneel et al. [2008] (↑)
hsa-miR-451	3	hsa-miR-451: Sarachana et al. [2010] (↑) hsa-miR-451a: Mor et al. [2013] (↑); Huang et al. [2015] (↓)
hsa-miR-10	2	hsa-miR-10a: Wu et al. [2016] (↑); Ghahramani Seno et al. [2011] (↑)
hsa-miR-15	2	hsa-miR-15a: Huang et al. [2015] (↓); Abu-Elneel et al. [2008] (↓) hsa-miR-15b: Huang et al. [2015] (↓); Abu-Elneel et al. [2008] (↓)
hsa-miR-20	2	hsa-miR-20a: Huang et al. [2015] (↓) hsa-miR-20b: Wu et al. [2016] (↑)
hsa-miR-34	2	hsa-miR-34a: Mor et al. [2013] (↓) hsa-miR-34b: Huang et al. [2015] (↑) hsa-miR-34c: Huang et al. [2015] (↑)
hsa-miR-93	2	Sarachana et al. [2010] (↑); Abu-Elneel et al. [2008] (↑↓)
hsa-miR-103[a]	2	hsa-miR-103: Sarachana et al. [2010] (↑) hsa-miR-103a: Huang et al. [2015] (↓)
hsa-miR-107	2	Wu et al. [2016] (↑); Sarachana et al. [2010] (↑)
hsa-miR-130	2	hsa-miR-130a: Vasu et al. [2014] (↑) hsa-miR-130b: Wu et al. [2016] (↑)
hsa-miR-140	2	Hicks et al. [2016] (↑); Abu-Elneel et al. [2008] (↑)
hsa-miR-155	2	Wu et al. [2016] (↑); Mor et al. [2013] (↑)
hsa-miR-191	2	Hicks et al. [2016] (↑); Sarachana et al. [2010] (↑)
hsa-miR-199	2	hsa-miR-199a: Ghahramani Seno et al. [2011] (↑) hsa-miR-199b: Ghahramani Seno et al. [2011] (↑); Sarachana et al. [2010] (↓)
hsa-miR-211	2	Mor et al. [2013] (↓); Sarachana et al. [2010] (↓)
hsa-miR-219	2	Mor et al. [2013] (↑); Sarachana et al. [2010] (↓)
hsa-miR-221	2	Wu et al. [2016] (↑); Nguyen et al. [2016] (↓)
hsa-miR-335	2	Wu et al. [2016] (↑); Hicks et al. [2016] (↑)
hsa-miR-363	2	Wu et al. [2016] (↑); Talebizadeh et al. [2008] (↓)

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Table I. Continued

miRNA	#Study	Literature
hsa-miR-455	2	Ghahramani Seno et al. [2011] (↑); Sarachana et al. [2010] (↓)
hsa-miR-484	2	Wu et al. [2016] (↑); Abu-Elneel et al. [2008] (↓)
hsa-miR-494	2	Mor et al. [2013] (↑); Huang et al. [2015] (↑)
hsa-miR-663	2	hsa-miR-663: Talebizadeh et al. [2008] (↑) hsa-miR-663a: Vasu et al. [2014] (↓)
hsa-miR-940	2	Wu et al. [2016] (↑); Huang et al. [2015] (↓)

Note. Whether the miRNA is up-regulated (↑) or down-regulated (↓) in ASD cases for a specific study is indicated between parentheses. For miRNAs that are annotated with lower case letters (e.g., hsa-miR-23a and Hsa-miR-23b), their mature miRNA sequences are closely related and these mature miRNAs show similar structure. If mature miRNAs with identical sequences are generated from different precursor sequences and genomic loci, these miRNAs are annotated with an additional number (e.g., hsa-miR-92a1 and Hsa-miR-92a2) (Issler & Chen, 2015)

[a] Validated by quantitative reverse transcription polymerase chain reaction.

of genes can be systematic or limited to a specific region in order to study the site-specific effects the gene has on the phenotype. In addition, the manipulation can be induced at a particular developmental stage in order to investigate temporal effects. Alternatively, the expression level of miRNA-related genes in a region can be manipulated at the post-transcriptional level with tools like miRNA mimics, antagomirs (anti-miRNAs) or miRNA sponges [Issler & Chen, 2015].

Some studies discussed above incorporate functional validations in animal models or cell cultures derived from the animal models. For example, Nguyen et al. [2016] found that the overexpression of miR-146a in mice astrocytes results in an increased glutamate uptake capacity and a reduced growth of neurites, which supports the role of glial cells in neurodevelopmental disorders. Other studies have investigated the biological relevance of hsa-miR-132 and its targets during neurodevelopmental processes. Overexpression of miR-132 in murine models increases neurite outgrowth, spine density and yields more stable and mature spines, but it impairs the performance on novel object recognition tasks [Hansen, Sakamoto, Wayman, Impey, & Obrietan, 2010; Tognini, Putignano, Coatti, & Pizzorusso, 2011]. An optimal expression level of miR-132 is important for maintaining synaptic plasticity in the mouse visual cortex [Tognini et al., 2011]. In addition, it has been shown that miR-132 plays a role in modulating circadian clock, cocaine addiction, inflammation, and tumor proliferation [Anitha & Thanseem, 2015; Tognini & Pizzorusso, 2012].

A disturbed regulation of genes that are involved in circadian clock rhythms has been associated with social timing deficits in autism and sleeping disorders in ASD, implying a role of miR-132 in ASD [Sarachana et al., 2010]. The targets of miR-132 include the methyl CpG-binding protein 2 gene (*MECP2*), the phosphatase and tensin homolog gene (*PTEN*), the fragile X mental retardation 1 gene (*FMR1*), and the 5-hydroxytryptamine receptor 3A gene (*HTR3A*), all of which are related to ASD [Talebizadeh et al., 2008]. Several recent studies elaborated on the molecular regulatory network centered on methyl CpG-binding protein 2 (MeCP2) involving multiple miRNAs. MeCP2 is recognized as a transcriptional repressor by binding with methylated DNA [Cheng & Qiu, 2014]. Loss of function mutations of *MECP2* contribute to Rett syndrome while duplication of *MECP2* may result in a wide spectrum of disorders, including autism and intellectual disability (ID) [Cheng et al., 2014; Han et al., 2013]. Phosphorylated MeCP2 directly binds to DGCR8 protein and inhibits the formation of the Drosha/DGCR8 enzyme complex, thus regulating miRNA expression at the post-transcriptional level [Cheng & Qiu, 2014; Cheng et al., 2014].

Expression profiling of hippocampal tissues in *MECP2* knock-out mice demonstrated a marked increase in expression levels of nearly half of all mature miRNAs [Cheng et al., 2014] One of the miRNAs that are regulated by MeCP2 is hsa-miR-137 and the expression of MeCP2 is regulated by hsa-miR-483-5p and hsa-miR-132 [Cheng & Qiu, 2014; Han et al., 2013; Lyu, Yuan, Cheng, Qiu, & Zhou, 2016]. The two miRNAs, hsa-miR-137 and hsa-miR-132, were recently reported to mediate the reciprocal regulation between MeCP2 and PTEN proteins. Knock-down of *MECP2* results in an elevated level of miR-137 which in turn suppresses the expression of *PTEN* while knock-out of *PTEN* leads to an increased level of miR-132 which subsequently inhibits the expression of *MECP2* [Lyu et al., 2016]. Another study using an *MECP2* transgenic nonhuman primate, demonstrated that overexpression of *MECP2* leads to autism-like behaviors and specifically reduced the social interactions in these animals [Liu et al., 2016].

The forkhead box P2 gene (*FOXP2*), which expresses a transcription factor related to language development, is another validated target of hsa-miR-132. Expression of *FOXP2* in the mouse neocortex at an abnormal time during cortical development disrupts radial migration of neurons. Abnormal expression of *FOXP2* is partially

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repressed by endogenously expressed miR-132 and miR-9 in mice, attenuating the effects on neuronal radial migration [Clovis, Enard, Marinaro, Huttner, & Tonelli, 2012; Davis, Haas, & Pocock, 2015]. These studies collectively suggest that deregulation of hsa-miR-132 and its targets explain at least partially the pathogenesis of ASD. Another ASD-related miRNA, hsa-miR-7, was found to regulate the expression of the SH3 and multiple ankyrin repeat domains 3 gene (*SHANK3*) which encodes an important scaffolding protein at the post-synaptic site of glutamatergic synapses. Deregulation of this miRNA influences the dendritic spine density of mouse hippocampal neurons [Choi et al., 2015].

Functional consequences of the microdeletion or microduplication at 22q11.2, disrupting *DGCR8*, have also been studied in animal models. Monoallelic deletion of *DGCR8* in mice leads to down-regulation of many miRNAs in the prefrontal cortex. Moreover, it affects the structural and electrical properties of pyramidal neurons, which impairs the excitatory synaptic transmission in the prefrontal cortex [Schofield et al., 2011]. Microduplication of *DGCR8* may result in hyperactive miRNA biogenesis, but it has not been validated by functional studies in vivo [Xu, Hsu, Karayiorgou, & Gogos, 2012]. Another miRNA, hsa-miR-185, is also affected by the 22q11.2 microdeletion or microduplication [Alural et al., 2017; Xu et al., 2012; Zhao et al., 2015]. Putative targets of hsa-miR-185 are involved in important pathways for neurodevelopment like the brain-derived neurotrophic factor (BDNF) signaling pathway and these pathways are implicated in multiple psychiatric disorders [Alural et al., 2017; Sarachana et al., 2010].

Since our original review of literature as described in Figure 2, two more papers were published which discussed the functional relevance of Drosophila miR-980 and hsa-miR-21 in the context of ASD. Guven-Ozkan et al. demonstrated that miR-980 suppressed olfactory learning and memory formation in Drosophila through its regulations on the expression of Ataxin2 binding protein 2 (*A2bp2*), an ASD-related gene [Guven-Ozkan et al., 2016]. Drosophila miR-980 belongs to the miR-22 family in humans. It is possible that hsa-miR-22 also has a role in learning and memory functioning in humans and may be relevant for certain cognitive abnormalities in ASD. However, there is no evidence of associations between genetic variants in hsa-miR-22 and ASD or the deregulation of hsa-miR-22 expression in ASD cases, at least based on our review. One reason for negative findings could be that learning and memory functions in human are more advanced than Drosophila and depend less on odors. The link between the expression level of *A2bp2* and memory formation may be not as direct as that in Drosophila. The clarification of the role of hsa-miR-22 in ASD awaits more studies. The other miRNA, hsa-miR-21, was found to be up-regulated in prefrontal/frontal cortices [Mor et al., 2015; Wu et al., 2016] and down-regulated in cerebellar cortices of ASD patients [Abu-Elneel et al., 2008]. The up-regulation of hsa-miR-21 in prefrontal/frontal cortices may be responsible for the social behavioral deficits in ASD patients because one of the targets of hsa-miR-21 is the oxytocin receptor (*OXTR*), which is a major regulator for social behaviors [Mor et al., 2015]. The OXTR protein/mRNA ratio was demonstrated to be negatively correlated with the expression level of hsa-miR-21 in ASD cases although the expression level of oxytocin receptors was elevated [Mor et al., 2015], which suggests that hsa-miR-21 can prevent the translation of OXTR mRNAs rather than promoting the degradation of OXTR mRNAs. Moreover, hsa-miR-21 also targets *PTEN*, another ASD-associated gene. The PTEN/PI3K/AKT (PI3K stands for phosphatidylinositol-3 kinase and AKT refers to serine–threonine protein kinase) signaling pathway is important for multiple cellular processes, including cell growth, cell differentiation, and apoptosis. A disruption of this pathway has been observed in ASD patients [Minami, Murai, Nakanishi, Kitagishi, & Matsuda, 2015]. Another target of hsa-miR-21 is *DLGAP1* (Discs, large (Drosophila) homolog-associated protein 1), which expresses a scaffold protein that interacts with SHANK3, an ASD-associated gene [Wu et al., 2016]. However, these findings regarding hsa-miR-21 either come from bioinformatics analyses or in vitro assays. Animal studies with a direct manipulation on hsa-miR-21 would help to elucidate the effects of hsa-miR-21 deregulation on ASD-related functions, including social behaviors, learning, and memory.

Appraisal of Different Approaches

The exploration of the role of miRNAs in ASD by bioinformatics analyses of genetic association results, by expression profiling in patient-derived tissues or by functional animal models leads to different results and insights. Bioinformatics analysis enables the integration of well-established resources from genetic association studies of ASD and genomic annotations of miRNAs and provides a systematic examination of the variants in miRNA genes, miRNA biogenesis genes, and miRNA targets that overlap with ASD-associated variants in a quick and cheap way. However, results from bioinformatics analyses depend on the analysis pipeline, algorithms, as well as the resources to be integrated. Since the pool of ASD-associated variants and miRNAs are both being continuously updated, some results may be outdated. These specificities make it difficult to compare and integrate results from different studies. In fact, there are very limited overlapping results between

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different studies [Issler & Chen, 2015]. The targets of a specific miRNA are often predicted with bioinformatics algorithms [Hommers et al., 2015]. Most expression profiling studies adopt this method to predict the targets of deregulated miRNAs and conduct pathway analysis or other follow-up analyses. As the integrated resources expand, the results will be more error-prone even with appropriate statistical control on the frequency of false-positive discoveries [Issler & Chen, 2015]. Therefore, results from bioinformatics analyses require experimental validations with in vitro and in vivo studies.

High-throughput technologies for expression profiling of miRNAs in patient-derived tissues allow for the quantification of multiple miRNAs in parallel. With the application of the next-generation sequencing technology, extensive profiling of genome-wide miRNAs becomes possible. A large number of deregulated miRNAs in ASD and other psychiatric disorders, including schizophrenia, bipolar disorder, and major depressive disorder have been reported by multiple studies [Alural et al., 2017; Geaghan & Cairns, 2015; Hommers et al., 2015]. More importantly, differentially expressed miRNAs in easily-accessible tissues like peripheral blood and saliva may be potential noninvasive biomarkers for a specific disorder. Nonetheless, consistent findings across studies are limited. Apart from technical issues and tissue specificity as well as population heterogeneity, complications may occur in several procedures, including sample collection, miRNA extraction, and data analysis, leading to inconsistent findings [Stoicea et al., 2016]. First, most miRNAs are stable against RNA degradation and have long half-lives, but the half-lives of some miRNAs are shorter than 1 hr [Agwa & Ma, 2013; Cammaerts et al., 2015]. For post-mortem tissues, these miRNAs with short half-lives will suffer from different degrees of degradation due to different delays from the decease of the donor till the collection of brain samples [Cammaerts et al., 2015]. Secondly, expression profiling of miRNAs also can be performed using total RNA or isolated small RNA (<200 bp) depending on protocols. The different kits that are available for sample preparation may influence the results [Stoicea et al., 2016]. Thirdly, high-throughput qPCR and RNA-seq require a stably expressed miRNA as a reference for data normalization to control for batch effects [Hommers et al., 2015]. Different choices of the reference miRNA will affect the profiling consistency across studies. Therefore, sample collection, miRNA extraction, and data analysis procedures should be standardized to minimize technical confounding factors across different studies. More important than these technical issues is the correlative rather than causative nature of the findings from expression profiling studies [Issler & Chen, 2015]. Any identified deregulated miRNA is not necessarily directly related to a specific disorder. Instead, the observed differential expression between cases and controls for the miRNA may be a consequence of other phenotypes that correlated with the disorder (e.g. ID vs. ASD) or even some irrelevant confounding factors (e.g., diets vs. ASD). In addition, high-throughput profiling methods are error-prone, especially when the sample sizes of most studies are very small. As with the results from studies using bioinformatics tools, differentially expressed miRNAs should be further validated with in vitro and in vivo studies.

Functional studies using animal models allow the study of effects of a specific miRNA in a strictly controlled condition and may possibly reveal the causative role of the miRNA in the pathogenesis of a specific disorder [Issler & Chen, 2015]. In addition, the behavioral consequences of specific manipulations on miRNA expression may be observed, which has advantages over in vitro assays. However, generating a mutant animal model is expensive and time-consuming. Therefore, only those miRNAs with multiple evidences from bioinformatics analyses and expression profiling studies will be subject to functional validation in animal models. In the case of ASD, very limited miRNAs or miRNA-related variants have been investigated in animal studies. The biological functions of most ASD-associated miRNAs remain unclear. An important caveat for studies using animal models is that the sequence of the miRNA under investigation may be not conserved across different species. There were two bursts of novel miRNAs during the speciation of the animal kingdom. One happened at the time when bilaterian species (animals with bilateral symmetry) appeared and the other happened at the time when vertebrates appeared [Berezikov, 2011]. Naturally, miRNA sequences are more conserved within vertebrate species than between invertebrate species and vertebrate species. Moreover, sequence conservation is prevalent across different primates for most miRNAs, including those enriched in the central nervous system [Chan & Kocerha, 2012]. Nonetheless, there are still many miRNAs with high sequence conservations across distantly related species [Mor & Shomron, 2013]. For example, some brain-enriched miRNAs, including miR-9, miR-34, miR-124, miR-128, miR-132, and miR-219 have identical mature sequences across humans, monkeys, mice, Drosophila, and *C. elegans* [Anitha & Thanseem, 2015; Chan & Kocerha, 2012]. Among these miRNAs, miR-9 and miR-124 possess identical pre-miRNA sequences in humans and mice [Chan & Kocerha, 2012]. As a consequence, the regulatory networks of these miRNAs probably resemble each other in the corresponding species. It is noteworthy that miR-132, for which the relevance for ASD has been suggested by multiple expression profiling studies as well as some animal studies, is among the miRNAs with

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identical mature sequences across a variety of species. Therefore, findings about miR-132 in animal studies are probably applicable to human situations. At present, mice and rats are the most frequently used animal models while several studies include nonhuman primates. The advantage of studies in monkeys is that their brain structures and biological functions resemble those in humans to a large degree [Chan & Kocerha, 2012], which supports translational studies for higher cognitive functions like learning and memory.

Discussion

A systematic understanding of miRNA-related genetic variants, for example, the relative abundance and functional relevance of the genetic variants located in different regions of miRNA genes, will help to select candidates for follow-up studies and contribute to the understanding of ASD. As more sequencing data are acquired, more miRNAs and their targets will be validated for the multiple miRNA databases. Such progress will facilitate discoveries of genetic variants in miRNA genes, miRNA biogenesis genes, and miRNA target sites and the association of these variants to ASD. The primary challenges lie in the development of efficient algorithms and their cross-validation.

Meta-analyses of genetic association studies for a specific disorder are increasingly successful, with more well-validated common variants being discovered as sample sizes increase and the methodology of meta-analysis improves [Ripke et al., 2014; The Psychiatric GWAS Consortium, 2009]. Thus, it is not surprising that psychiatric disorder studies focusing on the overlap between schizophrenia-associated genetic variants and miRNA-related variants indeed identified multiple schizophrenia-associated SNPs and CNVs in miRNA genes, miRNA target genes as well as miRNA biogenesis genes (including *DGCR8* and *Dicer*, among which the rs1625579 SNP in *MIR137* was consistently reported by several studies [Alural et al., 2017; Hommers et al., 2015]. The same roadmap could also be applied to ASD. However, recent studies for ASD also highlight a role of rare and especially de novo variation for the genetic architecture of ASD [De Rubeis & Buxbaum, 2015; Krumm et al., 2015; Ronemus et al., 2014]. Therefore, more trio whole-genome sequencing data are beneficial for an in-depth investigation of de novo genetic variation not only limited to coding regions for ASD.

Expression profiling studies for psychiatric disorders have reported many deregulated miRNAs in various tissues from patients. For example, up-regulated hsa-miR-181b is identified in several regions of post-mortem brain samples and peripheral blood samples from schizophrenia patients [Alural et al., 2017]. Although expression profiling studies in ASD-patient derived samples are much fewer than those for schizophrenia, several consistently deregulated miRNAs have been detected as described before. In particular, deregulated circulating miRNAs, those present in various body fluids like CSF, blood, saliva, and urine, may be of interest for the discovery of noninvasive biomarkers for psychiatric disorders [Kichukova, Popov, Ivanov, & Vachev, 2016]. Circulating miRNAs are relatively resistant to degradation because these miRNAs are pre-dominantly bound to stabilizing proteins or wrapped in microvesicles (exosomes) and apoptotic bodies [Anitha & Thanseem, 2015; Issler & Chen, 2015]. Circulating miRNAs in peripheral blood, in particular, may to a degree reflect the expression pattern of miRNAs in the brain because neurons and glial cells could secrete these nucleic acids into the periphery across the blood brain barrier (BBB). However, some peripheral tissues could also secrete encapsulated miRNAs or protein-bound miRNAs into peripheral blood, which makes peripheral blood a less ideal tissue for biomarker identification than CSF [Stoicea et al., 2016]. The correlation of the expression pattern of miRNAs in the brain and in tissues outside the brain should be checked in order to find noninvasive biomarkers that are more relevant for the etiology of psychiatric disorders. Besides, the expression pattern of miRNAs can be affected by a number of factors, including age, gender, circadian rhythms, medication, and some chronic factors like diet [Hommers et al., 2015; Hunsberger et al., 2013; Stoicea et al., 2016; Ziats & Rennert, 2014]. Researchers should take these potential confounding factors into consideration and perform stricter sample selection and/or adopt appropriate statistical analyses.

In terms of the correlative nature of findings from case-control expression profiling studies, miRNA expression profiling of ASD-discordant monozygotic (MZ) twin pairs, where one twin is affected while the co-twin is not, could corroborate the causative nature of the correlation between a specific deregulated miRNA and ASD. Since MZ twin pairs have an identical genetic background and shared environment, including prenatal environment, the identified correlation is not due to confounding effects of genes or environmental factors and thus indicates causality [van Dongen, Slagboom, Draisma, Martin, & Boomsma, 2012; Vink et al., 2015]. The direction of causality, that is, whether the deregulation of the miRNA contributes to ASD or the other way around, could be investigated with Mendelian randomization (MR), an approach to infer the causality between X and Y by introducing genetic variants as an instrumental variable Z [Latvala & Ollikainen, 2016], or by performing functional studies in animal models.

Functional investigation of biomarker candidates will benefit from more efficient techniques to manipulate

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Table II. Summary of Autism Spectrum Disorder-Related miRNAs That Have Been Validated With More Than One Approach among Genetic Association Analyses, Expression Profiling, and Functional Research

miRNA	Genetic association studies	Expression profiling studies	Functional studies	Specificity to ASD
hsa-miR-132	—	Sarachana et al. [2010]; Talebizadeh et al. [2008]; Abu-Elneel et al. [2008]	Lyu et al. [2016]; Clovis et al. [2012]; Tognini et al. [2011]; Hansen et al. [2010]	Deregulations reported for SCZ, BP, and MDD
hsa-miR-7	Vaishnavi et al. [2013]	Hicks et al. [2016]; Mor et al. [2013]; Abu-Elneel et al. [2008]	Choi et al. [2015]	Deregulation reported for SCZ
hsa-miR-195	Vaishnavi et al. [2013]	Huang et al. [2015]; Vasu et al. [2014]; Sarachana et al. [2010]	—	Deregulation reported for SCZ
hsa-miR-146	—	Nguyen et al. [2016]; Mor et al. [2013]; Talebizadeh et al. [2008]; Abu-Elneel et al. [2008]	Nguyen et al. [2016]	Deregulation reported for MDD
hsa-miR-181	Stepniak et al. [2015]	Vasu et al. [2014]; Ghahramani Seno et al. [2011]; Abu-Elneel et al. [2008]	—	Deregulations reported for SCZ and BP
hsa-miR-211	Vaishnavi et al. [2013]	Mor et al. [2013]; Sarachana et al. [2010]	—	—
hsa-miR-484	Vaishnavi et al. [2013]	Wu et al. [2016]; Abu-Elneel et al. [2008]	—	—

Note. Whether the findings about these miRNAs are specific to ASD is also indicated.
—, no evidence; ASD, autism spectrum disorder; SCZ, schizophrenia; BP, bipolar disorder; MDD, major depressive disorder.

the expression of miRNAs in vivo [Barry, 2014]. It is possible that endogenous regulation of miRNA expression will counteract the expected effects so an understanding of this type of regulation is also important. Moreover, models generated from human tissues allow for the preservation of individual genetic backgrounds so functional validation in these models may be more relevant than that in animal models. Neurons derived from iPSCs are promising models, but expression signatures of these neurons are more similar to those in stem cells rather than neurons [Okita & Yamanaka, 2011]. Other models like cerebral organoids that mimic the three-dimensional structure of native brain tissues will also contribute to advances in this field [Lancaster et al., 2013; Ziats, Grosvenor, & Rennert, 2015].

Identifying novel miRNA biomarkers for ASD necessitates an integration of findings from these three fields (Table II). Some promising candidates are hsa-miR-132, hsa-miR-7, and hsa-miR-195. Deregulation of hsa-miR-132 is detected in LCLs and post-mortem cerebellar cortices from ASD patients and the biological functions of hsa-miR-132 and its targets have been validated by many animal studies. hsa-miR-7, which is located in an ASD-associated CNV locus, is up-regulated in the saliva and post-mortem anterior prefrontal cortices and its functional relevance has been validated using animal studies. Deregulation of hsa-miR-195 is found in peripheral blood, serum samples, and LCLs and it is also disrupted by an ASD-associated CNV locus.

The specificity of these miRNA biomarker candidates need to be addressed before they can be applied to create medical tests. A number of studies have investigated the phenotypic and genetic overlap between ASD and other neurodevelopmental disorders, including schizophrenia and ID [Cristino et al., 2014; De Rubeis & Buxbaum, 2015; Lee et al., 2013]. Lee et al. reported a low, but still statistically significantly genetic overlap estimated from genome-wide SNPs between ASD and schizophrenia [Lee et al., 2013]. The 22q11.2 microdeletion that involves *DGCR8* and *MIR185* is found in both ASD and schizophrenia [Hiroi et al., 2013; Merico et al., 2015; Zhao et al., 2015]. As for ASD and ID, both are associated with many genes on the X chromosome [De Rubeis & Buxbaum, 2015]. A burden of de novo CNVs in miRNA genes and miRNA targets has also been reported for ID [Qiao et al., 2013]. Disruptions of *SHANK3* located at 22q11.3 locus have been associated with ASD, ID, and schizophrenia [Choi et al., 2015]. These data suggest that in spite of the more diverse symptoms of neurodevelopmental disorders, there is a convergent molecular mechanism covering some important functional domains during brain development like synaptic transmission [Chen et al., 2014; Cristino et al., 2014; Millan, 2013]. The targets of some ASD-related miRNAs listed in Table II include important molecules for synaptic transmission (e.g., SHANK3, target of hsa-miR-7) and neuronal migration (FOXP2, target of hsa-miR-132). Therefore, it is not surprising to find that these miRNAs are also deregulated in

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other neuropsychiatric disorders (Table II). Despite these overlaps between different neurodevelopmental disorders, some ASD-related miRNAs may be specific for ASD, for example, hsa-miR-211 and hsa-miR-484. Wu et al. also reported that the targets of hsa-miR-21 showed enrichment for ASD-associated genes, minor enrichment for SCZ-associated genes and no enrichment for ID-associated genes [Wu et al., 2016]. Cristino et al. found that genetic variation in intergenic regions, including miRNA loci can explain the difference between these neurodevelopmental disorders [Cristino et al., 2014] and Nguyen et al. suggested that it is possible to discriminate ASD and ID with the expression signatures of several miRNAs [Nguyen et al., 2016]. Therefore, a combination of some miRNAs may be used as novel biomarkers for ASD or even as a signature to define subgroups of ASD.

The clinical presentation of ASD is heterogeneous, especially in terms of cognitive functioning. The genetic architecture differs between low-functioning and high-functioning subgroups. Robinson et al. found that the burden of de novo LOF mutations was higher in ASD patients with a low IQ, while common inherited genetic variants play a major role in those with a high IQ [Robinson et al., 2014]. Although the study by Robinson et al. using exome sequence data did not cover de novo LOF mutations in miRNA genes or miRNA target sites, it is highly possible that the genetic architecture in miRNA genes, miRNA biogenesis genes, and miRNA target sites for low-functioning ASD cases contrasts, even to a greater extent, with that for high-functioning ones, considering the fact that most miRNA-related variants are rare. The inherited common genetic variants which contribute more genetic risk to the high-functioning subgroup are unlikely to include variants in miRNA genes, miRNA biogenesis genes, miRNA target sites. Instead, if de novo LOF mutations, which are rare, happen in these miRNA-related genes (especially in miRNA biogenesis genes or miRNA genes that are involved in important neurodevelopmental processes like synaptic formation), it could have a deleterious effect on cognitive functioning, characterizing the low-functioning ASD groups. In this sense, de novo LOF mutations in miRNA-related genes may help explain the differences in cognitive functioning in ASD patients. An examination of genetic profiles at miRNA-related sites among low-functioning ASD subgroups may contribute to our understanding of cognitive development.

Concluding Remarks

Although in their infancy, studies of miRNAs have suggested a role in psychiatric disorders, including ASD. ASD-associated common, rare, and de novo variants have been identified in miRNA genes, miRNA biogenesis genes, and miRNA target sites. Deregulation of multiple miRNAs have been detected in a number of tissues derived from ASD patients. For some of these miRNA-related variants and deregulated miRNAs, their biological relevance has been validated with animal studies. Due to some technical and methodological limitations, only a few consistent findings are available for ASD. The role of miRNAs in ASD and other psychiatric disorders will be further elaborated as relevant approaches are being continuously improved and validated. Meanwhile, meta-analysis of studies that investigate miRNAs at multiple levels, including genetic variation, expression, and biological function will provide valuable information for miRNA research in the field of psychiatric disorders. Potential miRNA biomarkers may be identified and validated for the diagnosis and prognosis of psychiatric disorders or even can be used to distinguish different subgroups of a specific psychiatric disorder.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Suppl Table S1. Overview of studies from 2006 to 2016 that investigate differentially expressed miRNAs between ASD subjects and healthy controls

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