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ORIGINAL ARTICLE

Heterogeneous dysregulation of microRNAs across the autism spectrum

Kawther Abu-Elneel · Tsunglin Liu · Francesca S. Gazzaniga · Yuhei Nishimura · Dennis P. Wall · Daniel H. Geschwind · Kaiqin Lao · Kenneth S. Kosik

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Abstract microRNAs (miRNAs) are ~21 nt transcripts capable of regulating the expression of many mRNAs and are abundant in the brain. miRNAs have a role in several complex diseases including cancer as well as some neurological diseases such as Tourette's syndrome and Fragile x syndrome. As a genetically complex disease, dysregulation of miRNA expression might be a feature of autism spectrum disorders (ASDs). Using multiplex quantitative polymerase chain reaction (PCR), we compared the expression of 466 human miRNAs from postmortem cerebellar cortex tissue of individuals with ASD ($n=13$) and a control set of non-autistic cerebellar samples ($n=13$). While most miRNAs levels showed little variation across all samples suggesting that autism does not induce global dysfunction of miRNA expression, some miRNAs among the autistic samples were expressed at significantly different levels compared to the mean control value. Twenty-eight miRNAs were expressed at significantly different levels compared to the non-autism control set in at least one of the autism samples. To validate the finding, we reversed the analysis and compared each non-autism control to a single mean value for each miRNA across all autism cases. In this analysis, the number of dysregulated miRNAs fell from 28 to 9 miRNAs. Among the predicted targets of dysregulated miRNAs are genes that are known genetic causes of autism such *Neurexin* and *SHANK3*. This study finds that altered miRNA expression levels are observed in postmortem cerebellar cortex from autism patients, a finding which suggests that dysregulation of miRNAs may contribute to autism spectrum phenotype.

Keywords Autism spectrum disorder · miRNA · Postmortem tissue · Cerebellum

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Kawther Abu-Elneel and Tsunglin Liu contributed equally.

K. Abu-Elneel · T. Liu · F. S. Gazzaniga · K. S. Kosik (✉)
Neuroscience Research Institute,
and Department of Molecular Cellular and Developmental Biology,
University of California Santa Barbara,
Santa Barbara, CA 93106, USA
e-mail: kosik@lifesci.ucsb.edu

Y. Nishimura · D. H. Geschwind
Program in Neurogenetics and Neurobehavioral Genetics,
Department of Neurology, David Geffen School of Medicine,
University of California at Los Angeles,
Los Angeles, CA 90095-1769, USA

D. P. Wall
The Center for Biomedical Informatics
& Department of Systems Biology, Harvard Medical School,
Boston, MA 02115, USA

K. Lao
Applied Biosystems,
850 Lincoln Centre Dr.,
Foster City, CA 94404, USA

Present address:
F. S. Gazzaniga
Biomedical Sciences, University of California San Francisco,
San Francisco, CA 94143, USA

Background

Autism describes a broad spectrum of clinical phenotypes and complex genetics estimated to occur in 1 in 150 children [1]. A few rare mutations, usually in genes which encode synaptic proteins, can give rise to the phenotype [2–7]. Many common variants, often in noncoding regions of the genome, bear a statistical association with the disease.

Linkage analyses have found evidence for autism on 20 different chromosomes with 1p, 2q, 5q, 7q, 15q, 16p, 17q, 19p, 22q, and Xq most frequently implicated [8–11]. Cytogenetic abnormalities at many different loci on all chromosomes have been detected in >5% of affected children [12], and de novo copy number variation (CNV) has been associated with autism spectrum disorders (ASDs) [13, 14, 10]. Most de novo copy number variations were smaller than microscopic resolution and affected highly heterogeneous genomic regions. Many of the identified variants are indeed inherited and may provide important information on inherited forms of autism. Inherited duplications involving the chromosomal region 15q11–15q13 are among the most common cytogenetic abnormalities in the ASDs, accounting for 1–2% of cases [15]. The phenotype of ASD is associated with the developmental disorder *Rett syndrome* [16]. Several single gene disorders are associated with an increased risk of ASD. The most prevalent single gene disorders in ASD are tuberous sclerosis (TSC) [17–19], fragile X syndrome (FRAXA) [20–24], and *Rett syndrome* [16]. More rare, single gene disorders are phenylketonuria (PK), Smith–Lemli–Opitz syndrome (SLO) [25, 26], and neurofibromatosis (reviewed in [27]).

Several postmortem studies have highlighted areas of anatomic abnormality in the autistic brain. Consistently observed findings occur in the limbic system, cerebellum, and the related inferior olive. In the limbic system, the hippocampus, amygdala, and entorhinal cortex have been shown to have small cell size and increased cell packing density at all ages, suggesting a pattern consistent with developmental curtailment (reviewed in [28]). Systematic studies indicate that anatomic abnormalities in autistic brains include reduced numbers of Purkinje cells in the cerebellum primarily in the posterior inferior regions of the cerebellar hemispheres [28–34]. A different pattern of change has been noted in the vertical limb of the diagonal band of Broca, cerebellar nuclei, and inferior olive with plentiful and abnormally enlarged neurons in the brains of young autistic subjects; whereas in adult autistic brains, small, pale neurons are reduced in number. A recent study presented data indicating elevated levels of oxidative stress markers in the cerebellum of autism cases [35].

microRNAs (miRNAs) represent an attractive class of genes as putative contributors to the ASD phenotype. miRNAs are abundant in the brain, play a large role in many facets of brain development, are involved in synaptic plasticity, and control multiple mRNA targets by finely regulating the levels of their encoded proteins (reviewed in [36]). Thus, the functions attributed to miRNAs overlap with the alterations found in the autism brain including growth abnormalities [37] and delays and disorganization in neuronal maturation [38]. Disruptions in the control over the multiple mRNAs targeted by each miRNA could lead to the quantitative phenotypes observed in ASD that depend on genetic background and stochastic factors. Furthermore, multiple miRNAs can target the same mRNA leading to convergent phenotypes arising from various miRNA loci. Using multiplex reverse transcriptase-polymerase chain reaction (RT-PCR), we profiled human miRNAs in autism and control brains. When miRNAs in individual autism cases were compared to an averaged sample of 13 control brains, 28 miRNAs deviated from the mean and ~70% of the autism cases had at least one miRNA that deviated from the mean.

Results

Differential expression of miRNAs in certain autistic samples

Methods of profiling all known human miRNAs make it possible to screen their expression levels in autism brains. We measured the expression level of 466 human miRNAs (ESM—Table 1) from postmortem cerebellar tissue taken from 13 autism cases and 13 control individuals (ESM—Table 2) using multiplex real-time PCR. From the 466 miRNAs tested, only 377 were detected and used for further analysis (ESM—Table 1). When Ct>28, the reproducibility of the real-time PCR measurements diminishes, and therefore, we selected 227 miRNAs in which the Ct values in all the control samples were Ct<28 (see "Material and Methods" section). The samples were normalized and statistically analyzed as described in the "Material and Methods" section. A Ct of 28 represents a stringent cutoff and is highly reliable for calculating the normalization constants (ESM—Figure 1 and [39]). Across all samples, most miRNA levels showed little variation, which increased the confidence in the accuracy of the measurements and reduced the chances of recovering false-positive candidates. However, some miRNAs among the autism samples were expressed at significantly different levels compared to the mean control value. A significantly different Ct value was defined to have a *P* value less than [0.05/(13*227)], based upon multiple hypotheses testing of 227 miRNAs in 13 autism samples (see "Material and Methods" section for details). We found 28 miRNAs expressed significantly differently from the non-autism control set in at least one of the autism samples (Fig. 1; Table 1). A few of the dysregulated miRNAs appeared in multiple autistic cases accounting for 35 appearances in total (Fig. 1; Table 1). Interestingly, one miRNA, hsa-miR-93, was upregulated in one case of autism and downregulated in another. In total, 28 miRNAs were dysregulated in the output table. The 28 dysregulated miRNAs were distributed

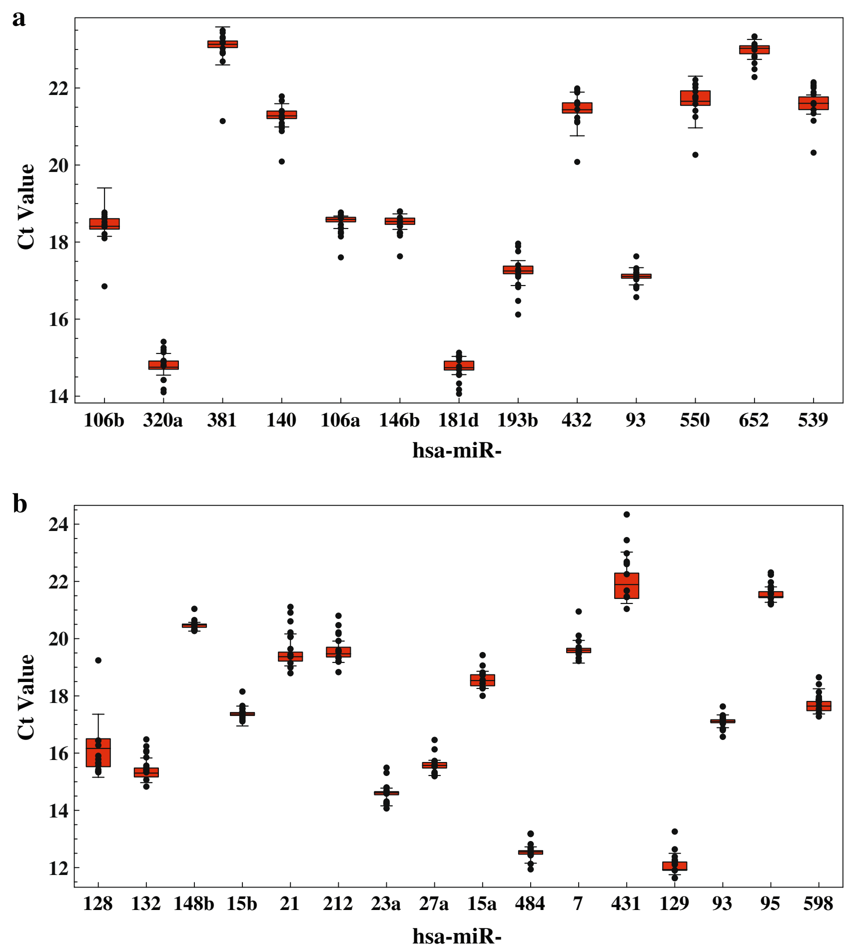

Fig. 1 Box plot of miRNAs dysregulated in autism. **a** Distribution of C_ts (SEM) in 13 autism samples and 13 non-autism controls for 13 miRNAs with a C_t in at least one autistic sample that fell below the significance range of the controls. The *red boxes* represent the distribution of C_ts in the non-autism control samples, and the *black dots* are the C_ts of the 13 autism samples. **b** Similar to (**a**), but for 16 miRNAs with a C_t value in at least one autism sample that fell above significance range of the controls

over nine cases of autism (Table 2). One autism case (B-1664) had relatively more miRNAs which differed in their expression compared to the other autism cases (Table 2).

When the comparison was reversed such that each non-autism control was compared by the z-test to a single mean value for each miRNA among all autism cases, nine miRNAs were outputted compared to the 28 miRNAs found in the above analysis (ESM—Table 1). The greater number of dysregulated miRNAs among the cases of ASD cannot be explained by age differences among the samples because grouping the cases according to age showed no significant differences in the expression levels of miRNAs.

Although these 28 miRNAs were notable in a subset of autism samples, the majority of the autism samples did not statistically differ from the non-autism controls by the z test. However, a Wilcoxon Rank Sum test was not statistically different between the two groups (ESM—Table 3) after correction for multiple hypotheses testing.

Gene ontology analysis on the predicted targets of the 28 miRNAs with dysregulated expression uncovered overrepresented GO terms among those with the headings neuron or nervous system related (ESM—Table 4). The term "central nervous system development" was over-represented in 13 out of the 28 miRNAs.

miRNAs dysregulated in autism are not associated with autism markers

Genome wide-scanning studies were collected to compile a list of genetic regions in linkage with ASD ([10, 27, 40], ESM-Table 5, see "Material and Methods" section). We tested whether the loci of 474 mature human miRNA loci ([41] Release 9.2) lie within a statistically significant physical distance of genetic markers for ASD. The significance of the distance between a marker and the closest miRNA is quantified by the probability to obtain a smaller or equal distance when we randomly position the marker across all the chromosomes. The null distribution represents the equally favorable probability for a marker to appear anywhere on the chromosome before performing a genomic screen.

Seven peak markers were close to a miRNA below a significance level $p=0.05$ (Table 3). The binomial probability

Table 1 miRNAs dysregulated in autism

miRNA	Chr	Lower C_t	Higher C_t
hsa-miR-484	16	0	B-5173, B-6677, B-5000
hsa-miR-21	17	0	B-6677, B-5144
hsa-miR-212	17	0	UMB-4721, B-6677
hsa-miR-23a	19	0	B-1664, UMB-1349
hsa-miR-598	8	0	B-1664
hsa-miR-95	4	0	UMB-1349
hsa-miR-129	11, 7	0	B-6677
hsa-miR-431	14	0	B-1573
hsa-miR-7	9, 15, 19	0	B-5666
hsa-miR-15a	13	0	B-5000
hsa-miR-27a	19	0	B-1664
hsa-miR-15b	3	0	B-1664
hsa-miR-148b	12	0	B-1664
hsa-miR-132	17	0	B-6677
hsa-miR-128	2, 3	0	B-1664
hsa-miR-93	7	B-1664	B-5144
hsa-miR-106a	X	B-6401, B-5000	0
hsa-miR-539	14	B-1664	0
hsa-miR-652	X	B-5000	0
hsa-miR-550	7	B-6401	0
hsa-miR-432	14	B-1664	0
hsa-miR-193b	16	B-5173	0
hsa-miR-181d	19	UMB-1349	0
hsa-miR-146b	10	B-1664	0
hsa-miR-140	16	B-1664	0
hsa-miR-381	14	B-1664	0
hsa-miR-320a	8	B-1664	0
hsa-miR-106b	7	UMB-1349	0

miRNAs from autistic samples with at least one C_t value outside the 0.17% tail (either lower or higher) of the control set C_t distribution (explained in the main text). The counts are the number of autism samples with a C_t value that significantly differed from the distribution of the non-autistic control set

to find at least 7 cases in 27 random trials with a probability of success 0.05 is ~0.0003, indicating that these peak markers do not distribute randomly across the whole genome. After setting the correction for multiple comparisons at a false discovery rate (FDR) of 5%, the peak markers D11S1314, D17S1294, D17S1800, and D17S2180, closest to hsa-miR-139, hsa-miR-423, hsa-miR-365-2, and hsa-miR-10a, respectively, remained significant. A similar result was observed by assuming that the miRNAs could show up in any position while the marker loci were fixed. These results were consistent with the findings obtained by fixing the miRNA loci (ESM—Table 6). The four miRNAs identified were not dysregulated in any of our autistic samples. Because linkage defines rare or common variation as opposed to association, the failure to find dysregulated miRNAs close to linkage signals neither confirms nor denies the rare variant hypothesis.

Discussion

Among a sampling of autism cases, miRNA expression levels in cerebellum postmortem tissues often deviated from a set of normal values compiled from a group of non-autistic controls matched for sex and postmortem interval. When age was studied as an independent variable, there was no detectable relationship to miRNA expression. We identified 28 miRNAs that were significantly dysregulated in the autistic cases compared to 9 miRNAs in the control

Table 2 miRNAs dysregulated in autism

Autism sample	No. of miRNAs
UMB-1445	0
UMB-4231	0
UMB-4721	1
B-1664	13
UMB-1349	4
B-6337	0
B-5569	0
B-5173	3
B-6677	5
B-5666	1
B-6401	2
B-5144	2
B-5000	4

Number of miRNAs for which their expression significantly differed from the non-autistic control set among the 13 autism samples

Table 3 Probability for 27 distinct autism peak markers to fall closer to a miRNA

Number	Author (year)	Chromosome	Marker	P_whole_genome
1	Buxbaum (2001)	2	D2S326	0.39292
2	Ylisaukko-Oja (2004)	3	D3S2432	0.36235
3	Alarcon (2005)	3	D3S1671	0.70923
4	Schellenberg (2006)	3	D3S1292	0.69703
5	Coon (2005)	3	rs1402229	0.60437
6	Schellenberg (2006)	4	D4S1572	0.53327
7	Yonan (2003)	4	D4S2460	0.52635
8	Liu/Yonan (2001/ 2003)	5	D5S2494	0.40512
9	Philippe (1999)	6	D6S283	0.46274
10	IMGSAC (2001)	7	D7S477	0.11600
11	Schellenberg (2006)	7	D7S530*	0.01865
12	Alarcon (2002)	7	D7S1826*	0.03427
13	Schellenberg (2006)	9	D9S164	0.34638
14	Yonan (2003)	11	D11S4102	0.54042
15	Szatmari (2007)	11	rs2421826	0.55865
16	Schellenberg (2006)	11	D11S1314**	0.00032
17	Ylisaukko-Oja (2004)	13	D13S159	0.10781
18	Lamb (2005)	15	D15S1011*	0.04111
19	Szatmari (2007)	15	Rs1433452	0.18145
20	McCauley/Stone (2005/2004)	17	D17S1294**	0.00317
21	Yonan (2003)	17	D17S1800**	0.00223
22	Cantor (2005)	17	D17S2180**	0.00109
23	Alarcon (2005)	17	D17S949	0.24199
24	Liu (2001)	19	D19S714	0.12035
25	McCauley (2005)	19	D19S930	0.25285
26	Molloy (2005)	21	D21S1432	0.06959
27	Liu (2001)	X	DXS1047	0.42460

Seven markers (with *, **) had a significance $p<0.05$, while four markers (**) survived the correction for multiple comparisons by FDR set at 5%. The list of literature is a modification of summary of publications in review [27] and articles ([10], [40])

cases. Among the autism cases, one sample had a disproportionate number of miRNAs dysregulated. While this case has a matched control in which the miRNA values do not deviate significantly from the norm, our conclusion that miRNAs are more frequently dysregulated among cases of ASD does not depend on this sample. The majority of the autism samples did not statistically differ from the non-autism controls. Thus, while miRNAs as a gene class tend to be dysregulated among individual cases of autism, no specific miRNA is consistently dysregulated across the entire autism sample set. This is not surprising, given the heterogeneity of ASD, and the fact that, so far, no one etiology explains more than 1% of cases in large scale studies. Because autism cannot be explained by one or even just a few genetic abnormalities and more likely will involve well over a hundred gene loci, it is possible that some of the convergent features of the phenotype may arise from dysregulation of miRNAs. We see no evidence that all miRNAs as a class of genes are dysregulated, but various individual miRNAs appear to be frequently dysregulated. Similar conclusions regarding miRNAs were drawn in a study of prefrontal cortex samples taken from schizophrenia patients [42]. As one traces pathways from underlying genetic errors to complex neurobehavioral phenotypes, a host of secondarily dysregulated genes, including miRNAs, are likely to surface. This study draws attention to a candidate set of such miRNAs in ASD.

In this study, we profiled miRNAs from cerebellum, a tissue in which anatomical abnormalities have frequently been reported [28–31, 33, 34]. Additional studies are needed to validate these findings in other brain regions.

Although many miRNAs are dysregulated, none were in statistically significant proximity to autism marker gene loci. Interestingly, four miRNAs hsa-miR-139, hsa-miR-423, hsa-miR-365-2, and hsa-miR-10a were not dysregulated in their expression levels, but did lie in statistical proximity to marker gene loci. These miRNAs deserve further analysis of their mature sequences, their precursor sequences, and their regulatory regions for possible contributory mutations to the ASD phenotype.

From a list of 26 genes that are associated with autism from published data [43, 44]), TargetScan [45] predicted miRNA target sites for the majority of the genes targeted by the miRNAs recovered from our data analysis (ESM—Table 7). Genes such as *MECP2*, *DISC1*, and *CNTNAP2* were targeted by the majority of the miRNAs we identified. Among the dysregulated miRNAs are some miRNAs which are predicted to target genes which are known genetic causes of autism. These include *NRXN1* which is potentially targeted by the miRNAs, hsa-miR-129, hsa-miR-

181d, hsa-miR-381, hsa-miR-128, hsa-miR-23a, hsa-miR-27a, hsa-miR-539, hsa-miR-328, and hsa-miR-218, and *SHANK3* which is potentially targeted by the dysregulated miRNAs hsa-miR-15a, hsa-miR-484, hsa-miR-7, hsa-miR-128, hsa-miR-15b, hsa-miR-328, and hsa-miR-27a [46].

De novo CNVs are associated with autism [10, 13, 14]. A few of the dysregulated miRNAs were significantly associated with genomic CNVs or common SNPs. However, hsa-miR-484 is found in a CNV [47] and hsa-miR-7-1 [48]. The SNP rs12884005 is found in hsa-miR-431 and rs895819 and rs11671784 are in hsa-miR-27a. Further analysis is needed to elucidate the cascade of molecular events leading to ASD through miRNA dysregulation.

Conclusion

Dissecting the basis for genetic diseases requires picking up the molecular trail that ultimately is likely to reveal the phenotype. Underlying gene mutations initiate or predispose to a phenotype, but may not be the most proximate cause of the phenotype. Rather, a cascade of downstream molecular events leads is likely to lead to the ASD phenotype, and based on the work presented here, miRNA dysregulation is likely to be among those events.

Materials and methods

Postmortem tissue

This study was approved by the Institutional Review Board of the University of California Santa Barbara. The request for postmortem human brain tissue was reviewed and approved by the Autism Tissue Program [49]. Tissues were obtained from the Harvard Brain Tissue Resource Center [50] and the Brain and Tissue Bank of Maryland [51]. Tissue consisted of frozen blocks (300–500 mg/block) from the cerebellum cortex. Cases number B-1664 and B-4272 were a gift from Dr. Margaret Bauman and were obtained as 10-µm frozen slices on slides. The tissues were group matched for age, gender, PMI, and hemisphere (ESM—Table 2).

RNA purification from normal and autistic tissue samples

Postmortem tissues were transferred from −70°C to a prechilled slide sitting on dry ice and finely sliced using a surgical blade. The cut tissue was grinded to powder, and RNA was isolated using a mirVana™ miRNA Isolation Kit (Ambion) following the manufacturer's instructions. For cases B-1664 and B-4272, tissue was scrubbed from slide and cut fine and processed for RNA purification. RNA was quantified using Nanodrop. RNA was intact in all the tissues.

Multiplex real-time PCR

The expression profile of the human 466-plex was measured as described previously [52]. Briefly, reverse transcriptase reactions were performed in total volume of 5 µl containing 1× cDNA Archiving Kit buffer (Applied Biosystems), 10 U MMLV reverse transcriptase, 5 mM of deoxyribonucleotide triphosphate (dNTP) mixture, 0.26 U RNase inhibitor (Applied Biosystems), 5 nM each reverse primer, 3.4 mM $MgCl_2$, and 20 ng of purified total RNA from cerebellum cortex. Reactions were cycled 30× at 20°C for 30 s, 42°C for 30 s, and 50°C for 1 s. The enzyme was subsequently inactivated at 85°C for 5 min. Then, the cDNAs were preamplified using a common universal reverse primer (UR) and forward primers (FPs) containing 3′ sequences that correspond to the 5′ end of different miRNAs and 5′ sequences zip-coded to each RNA to increase the T_m of annealing above 65°C. The amplification was performed in 25-µl volume reaction containing 1× Universal Master Mix with no UNG (Applied Biosystems), 51 nM of each FP, 5 µM of UR, 6.25 u AmpliTaq Gold polymerase (Applied Biosystems), 2 mM of dNTPs mixture, 1 mM MgCl2, and 5 µl of the initial reverse transcriptase amplification. Reactions were cycled 1× at 95°C for 10 min, 55°C for 2 min, and 18× at 95°C for 1 s and 65°C for 1 min. For the real-time PCR, 25 µl of the amplified products was diluted 8× with 175 µl H_2O and 0.1 µl was used for each of the 466 singleplex real-time PCR reactions performed in 10 µl containing 1× Universal Master Mix with no UNG (Applied Biosystems), 0.5 µM FP, 0.2 µM TaqMan® probe, 0.5 µM UR. The miRNA specificity for each singleplex reaction was provided by the miRNA-specific sequences of the FP (ESM—Table 1) and TaqMan® probes. Real-time PCR was performed in a Biosystems 7500HT 96-well plate Sequence Detection System using 40 cycles of 95°C for 15 s and 60°C for 1 min. The threshold cycle (C_t) is determined as the fractional cycle number at which the fluorescence passes the fixed threshold. The experiment of using the 466-plex was repeated once. All the miRNAs with C_t values deviating from the general pattern of expression across the control and autism cases were repeated to eliminate experimental error. In addition, a partial repeat with a 240-plex replicated those miRNAs represented on that array.

Statistical analysis of multiplex real-time PCR data

We sought to identify any miRNA among the autism samples whose level differed significantly from the mean value among the non-autism control samples. With the expression levels of 466 miRNAs (ESM—Table 1), we first excluded 60 miRNAs which were undetectable [C_t=40 in all samples) and 29 miRNAs which displayed a noisy background $\left(C_{t_{NTC}} < \max\left(C_{t_{samples}}\right)\right)$. When the miRNA

expression levels are low, usually the C_t values show larger fluctuations. Therefore, among the remaining 377 miRNAs, we focused on a group containing 227 miRNAs in which Ct values in all the control samples were C_t<28. The cutoff 28 was set by observing relatively large fluctuations above the cutoff in the scatter plot (ESM—Figure 1). All "undetermined" values were converted to a C_t=40 which is commonly done because, in the indeterminate range, we cannot discriminate between a miRNA that is present in vanishingly small amounts from one that is not present at all.

For the group of miRNAs with lower C_t values, and therefore less fluctuation, we performed a global normalization by adding a constant to the C_t of each miRNA for each sample so that the majority of miRNAs had similar levels across different samples. The constant was determined according to invariant set normalization [53]. Invariant set normalization is a standard option of "dChip', which is commonly used to process microarray data. This approach was adopted because the majority of the miRNAs were expressed "similarly" in both the non-autism controls and the autism cases. Rank ordering each miRNA according to its expression level for each sample resulted in similar rankings. Those miRNAs that fell outside this "invariant set" as well as those with Ct values greater than 28 were not used to obtain the normalization constant. All samples were normalized to a base sample, sample case no. UMB-4543, which had a median mean C_t value. The normalization constants for 26 samples are (UMB-1445: -0.79; UMB-1714–0.14; UMB-1793: -0.23: UMB-1796: -0.78: UMB-4231: 1.14: UMB-4721: 0.68; B-4272: 0.53; B1664:0.85: UMB-4543: 0: UMB-1349: -1.19: UMB-1500: -0.52: UMB-1080: -1.05: B-6337: 0.36: B-6207: 1.32: B-5569: 0.99: B-5173: -0.02: B-5251: 0.12: B-6677: 0.97: B-5666: 1.01: B-6401: -0.40: B-4756: -0.72: B-5144: -0.05: B-5333: 0.13, B-6756: -0.01: B-5873: -0.55: B-5000: 0.04; ESM-Table 1). After normalization, the fluctuation in C_ts across the 26 case samples for most miRNAs were relatively small compared with those before normalization.

After normalization, we tested the normality for the distributions of C_ts in the control samples for each of the 227 miRNAs. Using Kolmogorov–Smirnov statistics, none of the null hypotheses, i.e., the C_{t_s} follow a normal distribution, were rejected (data not shown) with a significance level 0.05. For each miRNA, we ran a *z*-test on each C_t value of the 13 autism samples, based on the fact that the normalized C_t values of the control approximated a normal distribution, and applied the Bonferroni correction for multiple hypotheses testing.

Autism spectrum-disorder associated markers

We compiled a list of chromosomal regions having the most statistical support for linkage to ASD based on the literature (ESM—Table 5) [10, 27, 40]. Regions were identified by considering a 1 lod drop around markers reaching a prespecified level of suggestive significance (P<0.005). Some exceptions were included because of their high linkage score (rs2421826 and rs1433452 have a Zir score 3.33 and 3.41, respectively; D17S1294 and D17S2180 have a multipoint LOD score 4.3 and 4.1, respectively). Two markers with a P<0.005 in the latest review were not selected (Chen and Lauristen [54, 55]) because no genetic marker information was given in Chen's study [54] and no figures to estimate the marker region was shown in Lauristen's study [55]. We extracted markers with a peak score and the one lod drop interval (peak score −1). This range of linkage was estimated in cM on the left and right of the peak loci from each study (ESM—Table 5) and converted into a physical distance based on Ensembl Genome Build 36. For studies in which the linkage peak was between markers or not explicitly related to the peak marker, we identified the closest marker from the Marshfield genetic map [56] based on proximity to the reported peak cM. The physical loci on chromosome for all the markers were obtained from the search engine Ensembl [57].

Proximity measures between miRNA loci and autism markers

The probability that a marker is within a certain distance to the closest miRNA on a chromosome was obtained by exploring all possible loci for the marker along the chromosome. Because both a marker and a miRNA were represented by a physical region, we defined the distance between a marker and a miRNA as the head to tail distance. To calculate the probability for the observed distances, we first constructed a histogram of distances between a set of markers randomized for start sites at any base along a chromosome and the closest miRNA. The exact probability was obtained from this distribution of distances. The whole genome result was the average of each single chromosome result weighted according to its length. We considered 22 autosomes and the X chromosome because no markers for the Y chromosome are in the literature. We also constructed a histogram of the number of miRNAs within a marker region and used these data to define a probability of coverage. A miRNA was defined as covered by a marker region when the two regions overlapped by at least one base.

Gene ontology analysis

To determine which GO terms were overrepresented among predicted miRNA targets, we downloaded the gene association file (gene association.goa_human, GOC validation

date: 12/08/2007) and ontology file ([58] gene ontology. obo, v1.0 format, date: 12/07/2007). We processed these files to obtain genes associated with each GO term (including genes associated with all the descending terms) in the ontology "Biological Process". Given a query list of genes, the overrepresentation of a GO term is determined by Fisher's exact P value, P_F, subjected to multiple hypotheses testing. We controlled the family-wise error rate by an adjusted P value, which was obtained by comparing 1,000 random queries. Thus, an adjusted P value is the percentage of 1,000 random queries for which the smallest P_F among all the GO terms is equal to or smaller than the P_F of that GO term. An overrepresented GO term was defined to have an adjusted P value<0.05 and has at least five associated genes.

The predicted conserved targets of the 453 miRNAs (13 probes for miRNAs were not in TargetScan) were downloaded from TargetScan website [45]. We obtained the overrepresented GO terms in the predicted targets for each miRNA (some predicted targets were not associated with any GO terms and were excluded from the query list). To determine whether the 28 miRNAs with dysregulated expression have common overrepresented GO terms, we calculated the percentage of the 28 miRNAs that have an overrepresented GO terms compared to the 453 miRNAs. In this way, we account for an overrepresented GO term that may be very common among all miRNAs. We selected those overrepresented GO terms for which the percentage among 28 miRNAs was at least twice the percentage among all 453 miRNAs and at least five of the 28 miRNAs had the overrepresented term.

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