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Research Report

Gene and miRNA expression profiles in autism spectrum disorders

Mohammad M. Ghahramani Seno[a,b], Pingzhao Hu[a], Fuad G. Gwadry[a], Dalila Pinto[a], Christian R. Marshall[a], Guillermo Casallo[a], Stephen W. Scherer[a,c,d,*]

[a]The Centre for Applied Genomics and Program in Genetics and Genomic Biology, The Hospital for Sick Children, Toronto, Ontario M5G 1L7, Canada
[b]Department of Basic Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
[c]Department of Molecular Genetics, University of Toronto, Toronto, Ontario M5S 1A1, Canada
[d]McLaughlin Centre, University of Toronto, Toronto, Ontario M5S 1A1, Canada

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ABSTRACT

Accumulating data indicate that there is significant genetic heterogeneity underlying the etiology in individuals diagnosed with autism spectrum disorder (ASD). Some rare and highly-penetrant gene variants and copy number variation (CNV) regions including *NLGN3*, *NLGN4*, *NRXN1*, *SHANK2*, *SHANK3*, *PTCHD1*, 1q21.1, maternally-inherited duplication of 15q11–q13, 16p11.2, amongst others, have been identified to be involved in ASD. Genome-wide association studies have identified other apparently low risk loci and in some other cases, ASD arises as a co-morbid phenotype with other medical genetic conditions (e.g. fragile X). The progress studying the genetics of ASD has largely been accomplished using genomic analyses of germline-derived DNA. Here, we used gene and miRNA expression profiling using cell-line derived total RNA to evaluate possible transcripts and networks of molecules involved in ASD. Our analysis identified several novel dysregulated genes and miRNAs in ASD compared with controls, including *HEY1*, *SOX9*, miR-486 and miR-181b. All of these are involved in nervous system development and function and some others, for example, are involved in NOTCH signaling networks (e.g. *HEY1*). Further, we found significant enrichment in molecules associated with neurological disorders such as Rett syndrome and those associated with nervous system development and function including long-term potentiation. Our data will provide a valuable resource for discovery purposes and for comparison to other gene expression-based, genome-wide DNA studies and other functional data.

1. Introduction

Autistic disorder is the prototypic form of a group of conditions, the autism spectrum disorders (ASDs), which share common characteristics including impairments in reciprocal social interaction and communication, and the presence of restricted and repetitive interests and behaviors. Individuals with an ASD vary greatly in language ability and

* *Corresponding author*. The Centre for Applied Genomics, The Hospital for Sick Children, 14th Floor, Toronto Medical Discovery Tower/MaRS Discovery District, 101 College St., Toronto, Ontario, M5G 1L7, Canada. Fax: +1 416 813 8319.
E-mail address: stephen.scherer@sickkids.ca (S.W. Scherer).

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cognitive development, and may also show associated medical comorbidities. For example, cognitive development ranges from above average to intellectual disability (Chakrabarti and Fombonne, 2005).

Twin and family studies indicate a predominantly genetic basis for ASD susceptibility and provide support for considering these disorders as a clinical spectrum. Concordance of monozygotic twins for autistic disorder is ~60%, but rises to ~90% when less severe cognitive and social deficits are considered (Bailey et al., 1995). Additionally, subclinical autistic traits are sometimes observed in first-degree relatives (Hurley et al., 2007) and, to a lesser extent, in the general population (Constantino and Todd, 2005). ASD is more often observed in males compared to females with an approximate 4:1 difference.

The architecture of ASD genetic risk is complex (Veenstra-Vanderweele et al., 2004). Some 5–15% of individuals with an ASD have an identifiable genetic etiology corresponding to known rare single-gene disorders (e.g., fragile X syndrome, tuberous sclerosis 1 (*TSC1*), tuberous sclerosis 2 (*TSC2*), methyl CpG binding protein 2 (*MECP2*) in Rett syndrome and phosphatase and tensin homolog (*PTEN*)) and chromosomal rearrangements (e.g., maternal duplication of 15q11–q13) (Abrahams and Geschwind, 2008). Mutations have been identified in synaptic genes, including neuroligin 3 (*NLGN3*) and neuroligin 4 (*NLGN4*) (Jamain et al., 2003), as well as SH3 and multiple ankyrin repeat domains 2 (*SHANK2*) (Berkel et al., 2010; Pinto et al., 2010), SH3 and multiple ankyrin repeat domains 3 (*SHANK3*) (Durand et al., 2007; Moessner et al., 2007) and *PTCHD1* (Marshall et al., 2008; Pinto et al., 2010; Noor et al., 2010), further supporting a role for highly-penetrant rare variants in ASDs. Genome-wide microarray studies have revealed copy number variation (CNV) or CNV regions (CNVRs) as risk factors (Abrahams and Geschwind, 2008; Cook and Scherer, 2008). *De novo* CNVs have been reported in 5–10% of idiopathic ASD cases (Marshall et al., 2008; Pinto et al., 2010; Sebat et al., 2007; Szatmari et al., 2007), and some rare inherited CNVs are reported at higher frequencies in cases than controls (Marshall et al., 2008; Pinto et al., 2010; Sebat et al., 2007). CNV examples include ~550 kb hemizygous deletions and duplications of 16p11.2 (Kumar et al., 2008; Marshall et al., 2008; Weiss et al., 2008), deletions at neurexin 1 (*NRXN1*) (Szatmari et al., 2007), and some rare homozygous deletions in consanguineous families (Morrow et al., 2008).

Genome-wide association (GWA) studies using common single nucleotide polymorphisms (SNPs) have highlighted two different ASD risk loci: 5p14.1 (near cadherin 9 (*CDH9*)/cadherin 10 (*CDH10*)) (Wang et al., 2009) and 5p15.2 (between semaphorin-5A (*SEMA5A*) and taste receptor, type 2, member 1 (*TAS2R1*)) (Weiss et al., 2009). More recent GWA studies from the Autism Genome Project Consortium identified 20p12.1 (at MACRO domain containing (*MACROD2*)) as its most significant finding (Anney et al., 2010). However, none of these studies replicate each other and the associated odds ratios are small, suggesting common genomic variation will account for only a proportion of heritability in ASD and that additional studies need to be conducted with different experimental designs.

Considering the established literature suggesting significant genetic heterogeneity in ASD, we propose two possible models that may help unify often disparate and complex data: 1) general cellular network deficits: dysregulation of gene expression in various genes/loci converging on the same or overlapping networks; and/or 2) general aggregation effects: combinatorial effects of different deleterious genetic variations cross a buffering threshold and result in expression of ASD. In support of these ideas, bioinformatics-based strategies have identified some positional ASD candidate genes that interact (Yonan et al., 2003). Furthermore, recent CNV analyses in ASD demonstrate enrichment for a subset of rare autism specific CNV deletions in cases over controls, and these variants often affect genes involved in signaling networks such as GTPase/Ras and kinase signaling networks (Pinto et al., 2010).

Global expression profiling provides the opportunity to evaluate possible transcriptomic deviations in ASD at both gene and gene-network levels. So far, only a handful of studies have investigated the ASD transcriptome in post-mortem brain samples (Abu-Elneel et al., 2008; Garbett et al., 2008; Purcell et al., 2001); and other studies have used RNAs from lymphoblastoid cell lines or blood (Baron et al., 2006; Gregg et al., 2008; Hu et al., 2006, 2009a,b; Nishimura et al., 2007; Sarachana et al., 2010; Talebizadeh et al., 2008) demonstrating their usefulness for this purpose.

Lymphoblastoid cell lines are not the best proxy for neural tissue, which is presumed to be the ideal material for study in ASD. However, lymphoblasts provide access to larger sample sizes and unlimited source of RNA. Moreover, for technical reasons lymphoblasts can be better controlled for various experimental confounds that may influence the fidelity of measuring gene expression data.

Choosing the most appropriate reference control samples for global gene expression studies in conditions such as ASD is always a challenge. While unaffected siblings of a test group seem appropriate for controls, some seemingly unaffected siblings may be just subclinical or have a broader autism phenotype (Hurley et al., 2007). Other sources of variation such as using individuals with different ancestry, and/or using cases ascertained in different centers may also confound the results.

We performed gene and miRNA expression profiling on 18 families using carefully selected cases and controls (siblings) where each sample was: 1) known to be of European ancestry based on genotype analysis; and 2) was extensively phenotyped in our center to confirm their ASD diagnosis, or lack thereof. Moreover, to account for the strongest genetic component involved and hence to increase the likelihood of capturing the ASD specific genetic variation(s) and decrease the level of heterogeneity in cases only stringently diagnosed ASD cases were included. For data analysis we took an approach to accommodate genetic heterogeneity associated with the disease. Our results identify numerous candidate genes and transcriptional units available for further interrogation in ASD.

2. Results

2.1. *Experimental design and data quality control*

Fig. 1 summarizes the analyses procedure followed. From our autism sample depository, we carefully selected 18 families

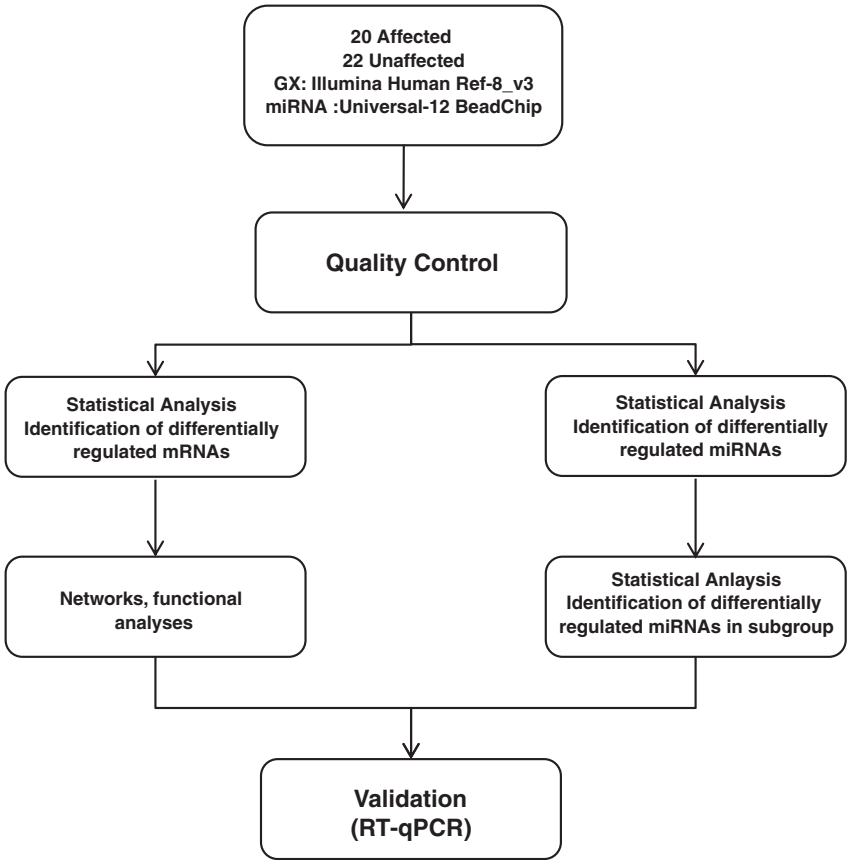

Fig. 1 – Flow chart summarizing the analysis procedure. Gene and miRNA expression profiling was performed on 20 individuals affected with ASD and their 22 unaffected siblings using Illumina Gene and miRNA expression arrays. Data quality was evaluated in BeadStudio (Illumina) and also by principal component analysis (PCA) and cluster analysis. Generalized estimating equations (GEE) were performed to identify statistically significant changes between affected and unaffected groups. Further, network analysis was performed in IPA. Differentially regulated genes and miRNA were validated using RT-qPCR.

with 20 individuals (13 males and 7 females) affected with severe autism and used their 22 unaffected siblings (19 males and 3 females) as reference control to conduct global gene and miRNA expression profiling. Autism Diagnostic Observation Schedule (ADOS) was applied in our center to ascertain the condition status in both affected and unaffected individuals; and further, all affected individuals were determined to be of European ancestry by genotype analysis. Additionally, none of the affected individuals who had high-resolution genotyping single nucleotide polymorphism (SNP) array data available had detectable CNVs overlapping known autism susceptibility genes (Marshall et al., 2008; Pinto et al., 2010). Lymphoblastoid cell lines from these individuals were grown under controlled conditions and high quality total RNA was extracted and assayed using Illumina gene and miRNA expression arrays (see Experimental procedures section for details). The expression signals were extracted from arrays and after quality control in BeadStudio (Illumina), the data was collected for further analysis.

We used principal component analysis (PCA) and cluster analysis to qualitatively evaluate the expression data. PCA performed on normalized signals from 5000 randomly selected probes did not detect any major non-specific source of variation, but as expected showed relatedness (defined by sib-pairs) to be generally grouping the samples (Supplemental Fig. 1). Moreover, PCA showed there was not any gender effect in the data.

For selecting lists of differentially expressed genes, we decided to take heterogeneity associated with ASD into account. Therefore, we first generated a main list of differentially expressed genes between the affected and unaffected group using the generalized estimating equations (GEE) method (see Experimental procedures for details). The expression ratios (i.e. fold changes (FC)) between sib-pairs were then calculated for all genes present in this list. Due to the presence of more than two sibs in some families, this resulted in twenty four FCs. Finally, lists of differentially expressed genes were made by including all genes changing significantly, as determined by the *P* values reported by GEE, that also showed certain levels of FC (1.5-fold for the main list and 1.2-fold for network analysis) in at least 50% of all twenty four comparisons.

The expression ratios (between affected and unaffected siblings) calculated using the differentially expressed gene list used for network analysis (Supplemental Table 1; see section below and Experimental procedures section for details) was used to perform cluster analysis. Cluster analysis was performed using average-linkage hierarchical cluster analysis

with a correlation metric and displayed in the form of a clustered image map (CIM) using CIMminer (http://discover.nci.nih.gov/). CIM clearly clustered siblings together, which is another indication of soundness of the data (Fig. 2). CIM also determined two subpopulations, one with a highly similar gene expression pattern.

2.2. *Differentially regulated genes in ASD*

Table 1 lists the differentially regulated genes in the affected group compared to the unaffected group, as determined by GEE analysis, that also showed at least 1.5-fold change in at least twelve out of twenty four sib-pair comparisons. Several potentially interesting functional candidate genes for ASD are identified. Rho GTPase activating protein 24 (*ARHGAP24*) demonstrates the highest association (adjusted $P=0.01$) and it was found reproducibly differentially regulated in this set of experiments, as well as in one of our previous pilot gene expression study in eight different sib-pairs (data not shown). Notably, a recent study on CNV in autism detected overrepresentation of rare autism specific CNVs harboring genes involved in GTPase/Ras signaling (Pinto et al., 2010). *ARHGAP24* is not well studied; however it

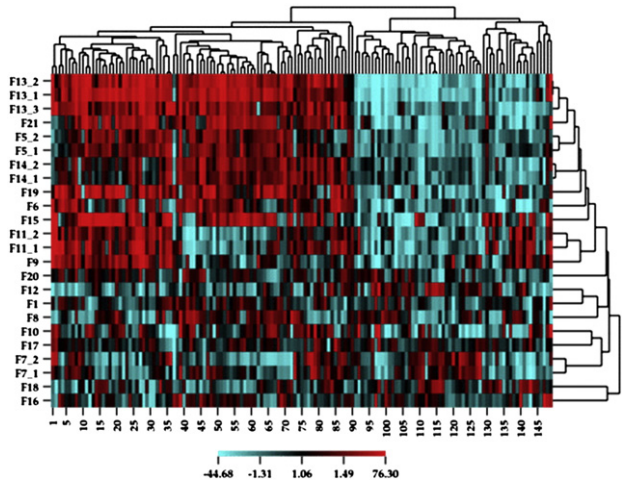

Fig. 2 – Clustered Image Map (CIM) of genes differentially regulated by autism. Ratio (FC) values (affected sib vs unaffected sib) from genes (149 genes) differentially regulated with FC 1.2 or greater (in either direction) and adjusted P values equal to or less than 0.15, changing in 12 or a greater number of comparisons out of 24 comparisons was used to generate CIM (see Experimental procedures for details). Both expression patterns in individuals and genes are clustered. While this analysis identified two subpopulations, one (red area of map) with a highly similar expression pattern, it rules out the presence of any non-specific variation in the data by tightly clustering ratios from family members. The color and intensity indicate direction and level of change. Blue spectrum colors indicate down-regulated expression; red spectrum colors, up-regulated expression. F: stands for family; and the number following "F" (e.g. F13) shows the family number. Identical numbering indicates member of the same family. Note: only families with more than two participating siblings have more than one ratio.

interacts with Filamin A (*FLNA*) (a chromosome X genes) and missense mutation of *FLNA* in 3 sibs has been associated with severe neurological disorder including verbal language deficit and seizures (Masruha et al., 2006). Over-expression of interferon induced transmembrane protein 3 (*IFITM3*) has reproducibly been reported in brain samples from schizophrenia (Arion et al., 2007; Saetre et al., 2007) and autism cases (Garbett et al., 2008). *IFITM3* is significantly up-regulated (adjusted $P=0.015$) in our study. Of note, a SNP at chromosome 1p22.2 (Guanylate binding protein (*GBP*) region) showed some level of association with autism in our GWAS study (Anney et al., 2010), though it did not hold as strong after multiple test correction was applied. *GBP4* in our experiment is significantly up-regulated in ASD (adjusted $P=0.034$).

HEY1 is another gene found to have a different expression profile in our ASD cases compared to controls (adjusted $P=0.0165$). *HEY1* is a member of basic helix–loop–helix (bHLH) family and is involved in NOTCH signaling which has several interesting regulatory functions during CNS development (Imayoshi et al., 2010; Lasky and Wu, 2005; Sakamoto et al., 2003). *HEY1* is also a negative regulator of dopamine transporter 1 (*DAT1*) (Fuke et al., 2005) and variations in DAT proteins levels have been associated with neurological disorders such as attention-deficit hyperactivity disorder (ADHD) and Tourette's syndrome (Krause et al., 2003; Muller-Vahl et al., 2000). Furthermore, *HEY1* positively regulates *p53* expression (Huang et al., 2004) and *p53* has been tested as a schizophrenia susceptibility gene (Allen et al., 2008). Notably, basic helix–loop–helix domain containing, class B5 (*BHLHB5*), another member of the bHLH family is also in our list of differentially regulated genes (Table 1).

Besides a small number of risk genes residing on the X-chromosome (Noor et al., 2010), the 4:1 ratio in male to female prevalence of ASD has not been well explained to date. We detect overexpressed SRY (sex-determining region Y)-box 9 (*SOX9*; chromosome 17) (adjusted $P=0.0347$) (Table 1), which might represent an interesting finding in this context as *SOX9* is involved in the development of the male phenotype (Barrionuevo and Scherer, 2010; Vilain and McCabe, 1998).

As also highlighted by using Ingenuity Pathway Analysis (IPA) tool (Ingenuity Systems, Redwood City, CA) (see below), there are many other neural active genes such as dysbindin domain-containing protein 1 (*DBNDD1*) and nerve growth factor receptor-associated protein 1 (*NGFRAP1*) in our list of dysregulated genes, which can potentially contribute to ASD development. We tested the expression levels of six of the genes (*ARHGAP24*, *HEY1*, *bHLHB5*, *SOX9*, *DBNDD1* and *NGFRAP1*) mentioned above across 10 individuals by performing RT-qPCR on the same RNA samples used to generate array data. RT-qPCR corroborated the array data (Figs. 3a to f).

2.3. *Differentially regulated miRNAs in ASD*

Table 2 lists the differentially expressed miRNAs which are changing with FC 1.5 or greater (in either direction) in at least twelve of twenty four sib-pair comparisons. The top-scoring miRNA is miR-199b-5p (adjusted $P=0.018$). Though, this miRNA could not be accurately quantified by RT-qPCR due to its low abundance in lymphoblastoid cell lines, the good detection quality of this miRNA on expression arrays as demonstrated by low detection *P* values across all samples

Table 1 – Differentially regulated genes in autism.

Probe ID	Entrez ID	Symbol	Raw P value	Adj. P value	FC>1.5	FC<1.5	Average FC
ILMN_1801833	83478	ARHGAP24	2.60E-05	1.09E-02	12	3	1.47
ILMN_1805750	10410	IFITM3	6.48E-05	1.50E-02	15	3	1.60
ILMN_1788203	23462	HEY1	8.55E-05	1.65E-02	13	1	1.80
ILMN_1805466	6662	SOX9	5.48E-04	3.47E-02	13	2	1.61
ILMN_1771385	115361	GBP4	5.54E-04	3.49E-02	15	3	1.97
ILMN_2374352	79007	DBNDD1	1.19E-03	4.55E-02	12	2	1.48
ILMN_1789196	7169	TPM2	1.55E-03	5.17E-02	13	2	1.36
ILMN_1800078	4005	LMO2	1.58E-03	5.19E-02	2	12	−1.38
ILMN_1757604	7169	TPM2	2.23E-03	5.98E-02	13	3	1.36
ILMN_1715169	3123	HLA-DRB1	2.54E-03	6.18E-02	2	14	−1.32
ILMN_1733690	9465	AKAP7	2.83E-03	6.47E-02	14	4	1.96
ILMN_2358540	5937	RBMS1	5.00E-03	8.55E-02	12	5	1.24
ILMN_1719696	5337	PLD1	6.12E-03	9.22E-02	12	3	1.50
ILMN_2368530	9235	IL32	8.03E-03	1.02E-01	3	12	−1.46
ILMN_1685045	1117	CHI3L2	8.19E-03	1.02E-01	3	12	−1.35
ILMN_1802780	283316	M160	8.27E-03	1.03E-01	12	3	2.32
ILMN_1663793	116449	MIST	8.66E-03	1.05E-01	12	4	1.37
ILMN_1733998	10170	DHRS9	8.82E-03	1.06E-01	4	15	−1.55
ILMN_2370091	27018	NGFRAP1	1.23E-02	1.20E-01	6	14	−1.41
ILMN_1666444	648293	LOC648293	1.66E-02	1.34E-01	12	6	1.22
ILMN_1808238	348093	RBPMS2	1.68E-02	1.34E-01	12	3	1.35
ILMN_1804569	2620	GAS2	2.83E-02	1.69E-01	13	3	1.29
ILMN_1757387	7345	UCHL1	3.38E-02	1.81E-01	4	12	−1.58
ILMN_1756071	4240	MFGE8	3.55E-02	1.85E-01	12	4	1.44
ILMN_1699585	27319	BHLHB5	3.86E-02	1.90E-01	12	5	1.34
ILMN_1810962	5796	PTPRK	4.27E-02	1.97E-01	12	3	1.46
ILMN_1707137	400566	LOC400566	4.77E-02	2.07E-01	3	12	−1.47
ILMN_2213136	51176	LEF1	6.60E-02	2.37E-01	13	4	1.36
ILMN_1698318	56891	LGALS14	6.63E-02	2.38E-01	4	12	−1.46
ILMN_1659017	119710	LOC119710	7.02E-02	2.42E-01	13	5	1.43
ILMN_2384241	7048	TGFBR2	8.76E-02	2.63E-01	12	4	1.25
ILMN_2083066	91353	LOC91353	1.23E-01	3.00E-01	7	12	1.10
ILMN_2375184	404744	AAA1	2.48E-01	3.96E-01	12	7	1.11

Genes changing with fold changes (FC) of 1.5 or greater (in either direction) in at least 12 out of 24 sib-pair comparisons are displayed in this table. FC>1.5: shows the number of sib-pairs in which the expression is up-regulated by 1.5 fold or greater. FC<1.5: shows the number of sib-pairs in which the expression is down-regulated by 1.5 fold or greater. Average FC: average fold change between affected and unaffected groups. Table sorted by ascending adjusted *P* values.

(detection $P=0$ in 40 out 42 samples) may indicate that the array data is valid for this miRNA. This miRNA has two experimentally validated targets as well as several potential targets with many interesting neural active functions. Hairy and enhancer of split 1 (*HES1*) is one of the confirmed targets of miR-199b-5p (Garzia et al., 2009). *HES1* is involved in NOTCH signaling network and as mentioned earlier this network has an important role in CNS development (Imayoshi et al., 2010; Lasky and Wu, 2005; Sakamoto et al., 2003).

Protein phosphatase type 2A inhibitor (*SET*) is another validated target of miR-199b-5p (Chao et al., 2010). SET is a specific and non-competitive inhibitor of PP2A, which is involved in a variety of cellular activities including cell growth and differentiation (Li et al., 1996). PP2A is major phosphatase in the brain (Goedert et al., 1995) and is functionally well characterized to have a huge impact on CNS development.

When we used the differentially expressed genes with FC 1.2 or greater (in either direction) and adjusted *P* values equal to or less than 0.15, changing in twelve or a greater number of comparisons out of twenty-four total comparisons for cluster analysis (Supplemental Table 1), a subgroup of samples showed a very similar pattern of expression (Fig. 2). In order to determine whether there is a relationship between miRNAs and gene expression in this subcluster, we reanalysed the miRNA expression data from the individuals forming this cluster. Table 3 shows the miRNAs differentially regulated in this subgroup. miR-486 is the top hit (adjusted $P=1.55\times10^{-8}$) in this analysis. In a pilot study of gene and miRNA expression in autism we previously performed on 8 different sib-pairs selected with a similar approach we had also identified this miRNA differentially regulated with the lowest *P* value (data not shown). Interestingly, it has recently been shown that this miRNA regulates Akt (a kinase) signaling network by targeting *PTEN* (Small et al., 2010). We also demonstrated that this miRNA targets splicing factor, arginine/serine-rich 3 *SFRS3* (Fig. 4). *SFRS3* gene is differentially regulated in males and females during memory formation in mice (Antunes-Martins et al., 2007). miR-181c is the second top miRNA in this analysis. miR-181c shares potential targets with miR-181b whose dysregulation has already been

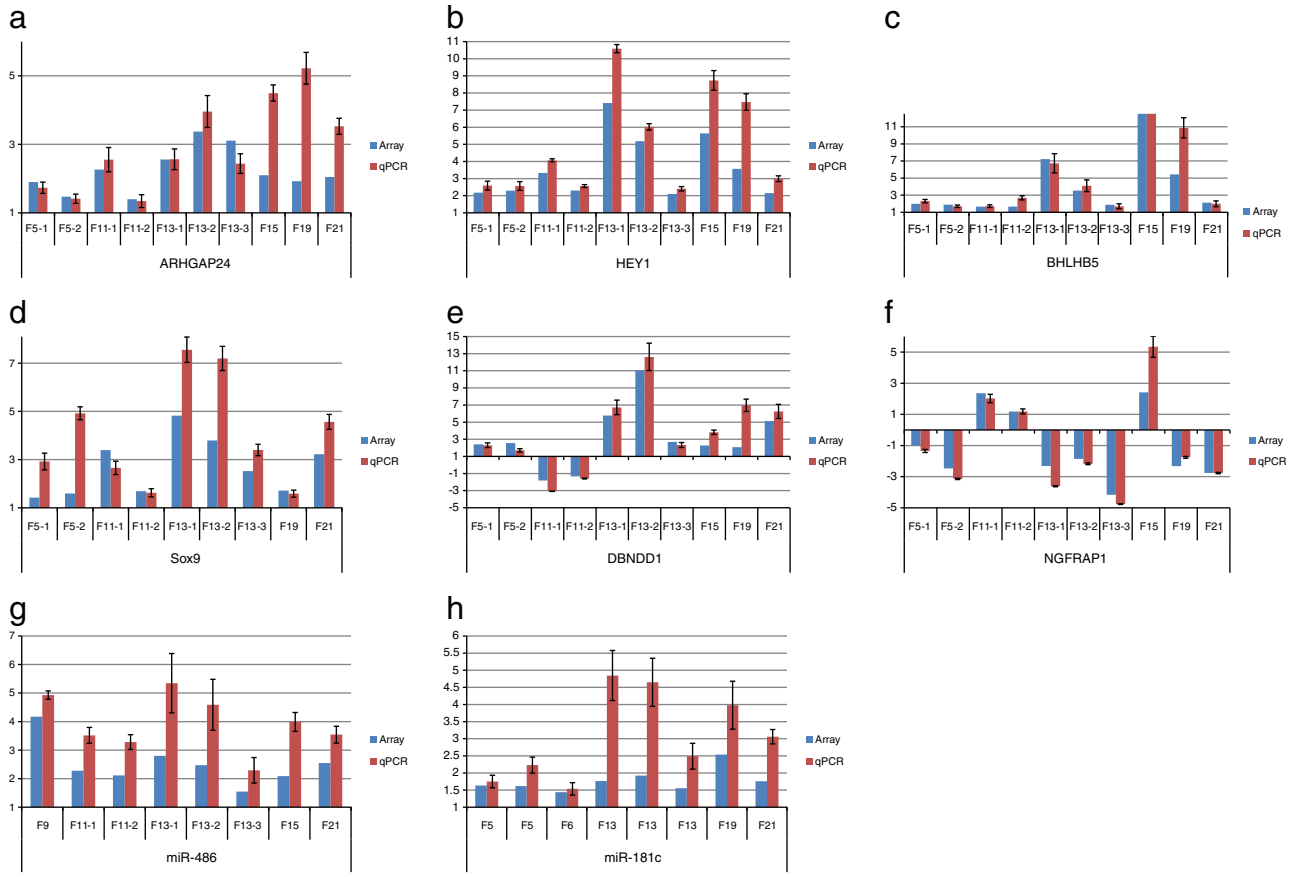

Fig. 3 – RT-qPCR validation of expression array data. Gene expression: expression levels of six genes relative to their reference control siblings were evaluated in six families using relative standard curve method of RT-qPCR. miRNA: expression levels of miR-486 and miR-181c relative to their reference control siblings were evaluated in six families using delta–delta CT method of RT-qPCR. The relative expression levels compared to the corresponding unaffected sibling based on qPCR and array data are reported. RT-qPCR confirmed variations observed based on microarray data. Also as demonstrated by array data, affected siblings show similar patterns of variations. F: stands for family.

associated with schizophrenia (Beveridge et al., 2008). It is notable that miR-181b is also present in this list of differentially regulated miRNAs (Table 3).

We confirmed the expression levels of miR-486 and miR-181c in the same RNA samples used for gene and miRNA expression profiling using RT-qPCR (Figs. 3g and h).

Table 2 – Differentially regulated miRNAs in autism.

Probe ID	miRNA ID	Raw *P* value	Adj. *P* value	FC>1.5	FC<1.5	Average FC
ILMN_3167259	hsa-miR-199b-5p	2.51E-05	1.80E-02	13	1	1.81
ILMN_3168671	hsa-miR-548o	6.67E-04	4.21E-02	15	4	1.84
ILMN_3167406	hsa-miR-577	2.82E-03	6.99E-02	12	4	1.51
ILMN_3168698	hsa-miR-486-3p	1.06E-02	1.15E-01	12	5	1.59
ILMN_3168749	hsa-miR-455-3p	1.61E-02	1.33E-01	13	5	1.66
ILMN_3167344	hsa-miR-338-3p	1.73E-02	1.36E-01	12	5	1.21
ILMN_3167976	hsa-miR-199a-5p	2.29E-02	1.52E-01	12	8	1.96
ILMN_3167524	hsa-miR-650	3.24E-02	1.81E-01	12	8	1.13
ILMN_3167240	hsa-miR-486-5p	3.29E-02	1.81E-01	12	6	1.42
ILMN_3168389	hsa-miR-125b	5.85E-02	2.15E-01	13	5	1.37
ILMN_3167552	hsa-miR-10a	1.53E-01	3.23E-01	12	4	1.18
ILMN_3167628	hsa-miR-196a	4.07E-01	4.61E-01	12	9	1.36

miRNAs changing with fold changes (FC) of 1.5 or greater (in either direction) in at least 12 out of 24 sib-pair comparisons are displayed in this table. FC>1.5: shows the number of sib-pairs in which the expression is up-regulated by 1.5 fold or greater. FC<1.5: shows the number of sib-pairs in which the expression is down-regulated by 1.5 fold or greater. Average FC: average fold change between affected and unaffected groups. Table sorted by ascending adjusted *P* values.

Table 3 – Differentially regulated miRNAs in clustering families.

Probe ID	miRNA ID	Raw P value	Adj. P value	Average FC
ILMN_3167240	hsa-miR-486-5p	2.36E-11	1.55E-08	2.1
ILMN_3168280	hsa-miR-181c	4.33E-11	1.55E-08	1.4
ILMN_3168698	hsa-miR-486-3p	7.12E-11	1.70E-08	2.4
ILMN_3167127	hsa-miR-181a	1.11E-10	2.00E-08	1.2
ILMN_3167158	hsa-miR-30a*	2.48E-09	3.56E-07	1.6
ILMN_3168257	hsa-miR-181b	1.13E-08	1.35E-06	1.3
ILMN_3168361	hsa-miR-181a*	8.85E-08	9.05E-06	1.3
ILMN_3168671	hsa-miR-548o	9.54E-07	8.54E-05	2.4
ILMN_3167552	hsa-miR-10a	1.67E-06	1.20E-04	1.7
ILMN_3168643	hsa-miR-10a*	3.19E-06	1.84E-04	1.7
ILMN_3167259	hsa-miR-199b-5p	1.27E-05	5.68E-04	2.1
ILMN_3168102	hsa-miR-502-3p, hsa-miR-500*	5.69E-05	2.04E-03	1.2

miRNAs changing with average fold changes (FC) of 1.2 or greater (in either direction) between affected and unaffected groups and adjusted *P* values < 0.002 are displayed. Table sorted by ascending adjusted *P* values. Note: all displayed miRNAs are up-regulated.

2.4. *Networks and functions dysregulated in ASD*

In order to systematically evaluate the genes differentially expressed between ASD cases and controls we used Ingenuity Pathway Analysis (IPA). As mentioned earlier in order to accommodate the heterogeneity associated with ASD, genes differentially regulated in twelve or more comparisons (of twenty four total sib-pair comparisons) with FC 1.2 or greater (in either direction) and adjusted *P* values equal to or less than 0.15 (Supplemental Table 1) were used for this purpose. By taking this approach we avoided losing informative genes when building networks. Indeed, this approach was informative as IPA results showed the best dysregulated network to be associated with nervous system development and neurological disorders (Supplemental Table 2). Notably, this network is nucleated by several kinases from ERK1/2 group and other mitogen-activated protein kinases (MAPK) (Supplemental Fig. 2) implying some roles for kinases in the development of autism.

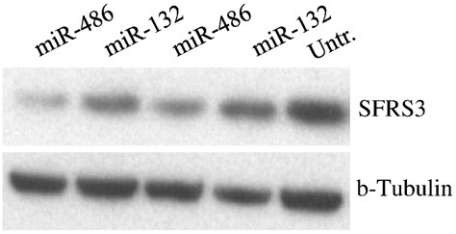

Fig. 4 – Western blot showing miR-486 down-regulates SFRS3 expression. The figure shows down-regulation of endogenous SFRS3 in HeLa cells upon transfecting the cells with miR-486 specific Pre-miRs (Ambion). Transfection of cells with miR-132 Pre-miRs does not result in this effect. Lysates were prepared from two separate transfected cultures. Untr.: Lysates from cells not transfected. Similar results were obtained by using 293T cells (data not shown) b-tubulin was used for loading control.

IPA allocated the best enrichment score (based on Fisher exact test) to molecules associated with neurological disorder under diseases and disorders category (Table 4; See Supplemental Table 3 for a full list of associated diseases and disorders) and to those associated with nervous system development and functions under physiolological system development and function (Supplemental Table 4). Interestingly, molecules associated with Rett syndrome demonstrate the most significant association (raw $P=1.52\times10^{-4}$; adjusted $P=4.55\times10^{-2}$) under neurological disorder category as have the molecules associated with long-term potentiation (raw $P=4.42\times10^{-6}$; adjusted $P=4.88\times10^{-3}$) under nervous system development and function category (Table 5; see Supplemental Tables 5 and 6 for the full lists).

3. Discussion

We considered etiologic heterogeneity in ASD and examined genes differentially regulated between discordant ASD siblings including performing a network analysis, and identified numerous new molecules as candidates for involvement in ASD susceptibility. One of the limitations of our approach was that lymphoblastoid cell lines were used. However, irrespective that this might not be the most appropriate RNA source for a disorder that is generally considered to be one of early development and brain-specific, our approach identified many genes, miRNAs and networks of molecules associated with nervous system and neurological disorder development (Tables 1, 2, 3, 4 and 5; and Supplemental Tables 2, 5 and 6). Of note, molecules associated with Rett syndrome and long-term potentiation were identified to have the best enrichment scores under their relevant categories (Table 5).

The top network revealed by IPA having some function in nervous system development (Supplemental Table 2) is nucleated by kinases such as ERK1/2 group, AKT and other mitogen-activated protein kinases (MAPK) (Supplemental Fig. 2). Interestingly, our pilot gene and miRNA expression profile study on 8 discordant sib-pairs also showed a similar pattern (data not shown). This implies a systematic dysregulation in kinases in the development of ASD. Moreover, the role of kinases in signal transduction and translating environmental signals into cellular behavior (responses) makes them more appealing with regard to autism development.

In addition to the presence of several kinases such as A kinase (PRKA) anchor protein 7 (*AKAP7*) and p38 mitogen-activated protein kinase (*p38MAPK*) amongst differentially regulated genes, differentially regulated miRNAs could also contribute to this dysregulation by targeting factors regulating kinases. miRNA-486 was reproducibly detected to be dysregulated in autism in this and our previous pilot gene and miRNA expression profiling studies on autism. It has recently been shown that miR-486 regulates v-akt murine thymoma viral oncogene homolog 1 (AKT1) activity by targeting *PTEN* (Small et al., 2010). Notably, there are also indications for the association of *PTEN* mutations and autism (McBride et al., 2010).

Prenatal stress has been associated with increased risk of several neuropsychiatric disorders including schizophrenia and autism (O'Donnell et al., 2009). It has been demonstrated

Table 4 – Top diseases and disorders categories (identified by IPA) associated with differentially expressed genes in autism.

Category	P value	Adj. P value	Molecules
Neurological disease	1.52E-04–1.82E-02	4.55E-02–8.51E-02	DPYSL2, DPYD, MBP, CD96, MSX1, HLA-DRB1, CETP, CXCL10, IL1R2, RHOB, MT2A, ZHX2, RAB38, RIMS3, DLL1, TJP2, CSRP2, GRK5, KCNK12, CHI3L2, CLNK, SERINC2, FLOT2, NGFRAP1, RARRES3, DUSP4, TNIK, BANK1, SERPINB1, GBP1, SPATA13, AKAP7, APP, NRGN, SOX9, ENPP2, SORBS3, CD24, PRNP, PSAT1, MYOM2, DST, UPP1, F13A1, LARGE, CLCF1, HNF1B, TMEM2, CBS, HERC6, FCER1G, RBMS1, PHACTR2, GLDC, KCNMB1.
Genetic disorder	1.52E-04–1.84E-02	4.55E-02–8.56E-02	PSAT1, DLL1, TJP2, DST, MBP, CD96, MSX1, TPM2, F13A1, GRK5, APP, CLEC2B, CETP, UBD, HNF1B, SOX9, RHOB, FCER1G, NGFRAP1, DUSP4, ENPP2, FXYD2, PRNP.
Skeletal and muscular disorders	1.76E-04–1.52E-02	4.55E-02–8.51E-02	DPYSL2, CD163L1, BANK1, SERPINB1, DPYD, GBP1, MBP, LMO2, SPATA13, CD96, MSX1, HLA-DRB1, IKZF1, MAPK13, APP, NRGN, CLEC2B, CXCL10, IL1R2, SOX9, LCK, MT2A, ZHX2, ENPP2, AIF1, FAM101B, CD24, PRNP, PSAT1, MTSS1, MYOM2, CSRP2, DST, TPM2, F13A1, GRK5, LARGE, PLD1, CLNK, TRIM69, TMEM2, FCER1G, RARRES3, RBMS1, PHACTR2, KCNMB1, TNIK.

Differentially regulated genes from Supplemental Table 1 were used by IPA for this analysis. Only terms with the lowest Benjamini–Hochberg adjusted P values < 0.05 are displayed. Molecules: The differentially regulated genes associated with each category.

that prenatal stress in rats decreases the levels of active forms of Jun N-terminal kinases (i.e. p-JNK1 and p-JNK2) in the hippocampus and p38-MAPK [i.e. p-p38] in the frontal cortex and most of these changes are reversed by antidepressant drugs (Budziszewska et al., 2010). Since p-JNK and p-p38 inhibit glucocorticoid receptor (GR) function, their lower levels may enhance glucocorticoid action observed in prenatal stress (Budziszewska et al., 2010; Mueller and Bale, 2008).

Of note, males seem to be more susceptible to the adverse effects of prenatal stress (Mueller and Bale, 2007). Higher prevalence of ASD in males and also increased male brain susceptibility to adverse effects of prenatal stress may indicate that the male brain is more prone to subtle alterations in proper neurogenesis. We detected up-regulation of *SOX9* in autistic cases in our study (Table 1). *SOX9* which has been shown to regulate and also be regulated by several kinases such as ERK1/2, MAPK and PI3K/AKT (Dupasquier et al., 2009; Murakami et al., 2000; Tew and Hardingham, 2006; Yagi et al., 2005) is involved in the development of the male phenotype (Barrionuevo and Scherer, 2010; Kojima et al., 2008; Vilain and McCabe, 1998), and therefore can potentially contribute to the observed increased risk of autism in males by turning the brain more vulnerable.

HEY1, whose transcript was detected to be up-regulated in autism in our study, has been shown to act as an androgen receptor corepressor (Belandia et al., 2005). However, it has also been demonstrated that a Leu94Met polymorphism at this locus can reverse this function and turn HEY1 to an androgen receptor activator (Villaronga et al., 2010). Interestingly, this polymorphism also abolishes HEY1-mediated activation of p53. This HEY1 dual action may propose HEY1 as another risk factor which may increase male brain susceptibility to autism when dysregulated. In line with this, we showed that miR-486 targets *SFRS3* (Fig. 4) and there are indications for male specific regulation of SFRS3 expression during memory formation (Antunes-Martins et al., 2007). Hence, miRNA-486 is another factor, which could potentially explain the involvement of kinases and gender-biased pattern in ASD.

It has been shown that HEY1 inhibits the expression of dopamine transporter 1 (DAT1) (Fuke et al., 2005) and changes in DAT proteins levels have been associated with some neurological disorders including ADHD (Krause et al., 2003). Notably, 30–80% of ASD cases meet the criteria for ADHD diagnosis (Rommelse et al., 2010).

NOTCH signaling has a major role in nervous system development and function, and there have been some indications for its involvement in schizophrenia (Imayoshi et al., 2010; Lasky and Wu, 2005). It is notable to observe dysregulation in expression levels of genes (e.g. *HEY1* and

Table 5 – Top disease and system development and function categories (Benjamini–Hochberg adjusted P values < 0.05) associated with genes differentially regulated in autism.

Disease/disorder	Disorder	P value	Adj. P value	Molecules
Neurological disease	Rett syndrome	1.52E-04	4.55E-02	DUSP4, ENPP2, FCER1G, GRK5, MBP, NGFRAP1, RHOB.

System development/function	Function	P value	Adj. P value	Molecules
Nervous system development and function	Long-term potentiation of mice	4.42E-06	4.88E-03	APP, CBS, NRGN, PRNP.
Nervous system development and function	Synaptic transmission of muscle	8.26E-05	3.12E-02	APP, CD24.

Differentially regulated genes from Supplemental Table 1 were used by IPA for this analysis (see Supplemental Tables 5 and 6 for the full lists). Molecules: The differentially regulated genes associated with each category.

bHLHB5) and a miRNA (miR-199b-5p) targeting molecules (*HES1*) involved in this network in autism.

It has been shown that up-regulation of *HEY1* and also of *HES1* result in increased p53 activity (Huang et al., 2004). This is interesting because of an indication for the involvement of p53 in the development of schizophrenia (Allen et al., 2008). *HEY1* has also been shown to have a role in the maintenance of neural precursor cells in the brain (Sakamoto et al., 2003). In line with this, tight regulation of *HES1* is also important for controlling neural differentiation and maintenance of neural progenitor cells (Ishibashi et al., 1994; Kageyama et al., 2008).

In summary, our list of differentially regulated genes is enriched with molecules associated with nervous system development and function, and most of these molecules seem to build around core networks such as those involved in kinase and/or NOTCH signaling networks. Therefore, our results support the involvement of various genetic factors (heterogeneity) in the development of autism, while suggesting these different factors can be converging at, or diverging from central networks such as kinase signaling networks. Accordingly, focused studies on interrogating the role of kinases in ASD development especially by using neuronal post-mortem samples or induced pluripotent stem cells can be very helpful in this regard.

4. Experimental procedures

4.1. Lymphoblastoid cell-line culture and RNA extraction

Lymphoblastoid cell-line cultures were grown and maintained in RPMI 1640 (Wisent, Canada) complemented with 5 mML-Glutamine (Wisent), 100 U/ml penicillin (Wisent) and 100 μg/ml streptomycin (Wisent). The cells were split every 3 days. Forty hours before harvesting the cells for RNA extraction, cells were split 1:3 in 10 ml fresh complete media and 40 h later cells were transferred to room temperature (RT) and left for ~1 h to reach RT. The cells were then centrifuged at 200 g/10 min and the pellets were used for RNA extraction.

4.2. RNA isolation

Total RNA was extracted using mirVana miRNA isolation kit (Ambion) according to the manufacturer recommended protocol. The quality of RNAs was checked using Agilent Bioanalyzer (Santa Clara, USA). RNAs were stored at −80 °C until used.

4.3. Microarray hybridization

Illumina HumanRef-8_V3 gene expression arrays (San Diego, USA) were used for global gene expression studies. 250 ng total RNA was used to prepare amplified and labeled cRNAs using Illumina TotalPrep RNA amplification kit (Ambion). 1.5 μg cRNA was then hybridized to each microarray chip following manufacturer instructions. The signals were detected and gathered using BeadArray Reader (Illumina). The data were uploaded to BeadStudio software (Illumina) and after qualitative evaluation, the text file data were extracted and statistically analyzed as explained below.

Illumina Universal-12 BeadChips (San Diego, USA) were used for global miRNA expression studies. 400 ng total RNA was used to prepare labeled and amplified probes using Illumina miRNA kit according to the manufacturer's instruction. The labeled probes were then hybridized to arrays following the manufacturer's instructions. The signals were detected and gathered using BeadArray Reader (Illumina). The data were uploaded to Bead Studio software (Illumina) and after qualitative evaluation, the text file data were extracted and statistically analyzed as explained below.

4.4. RT-qPCR and primers

For gene expression, RNA samples were first treated with DNase I (Invitrogen, USA) as instructed by the manufacturer to remove possible DNA contamination. 1 μg total RNA was then reverse transcribed using the Supercript III reverse transcription kit (Invitrogen) following the manufacturer's instructions. The product was diluted 5-fold in dH_2O, and 1–2 μl per reaction was used for qPCR. qPCR was performed using SYBR Green (Stratagene) and the relative standard curve method. The *TMEM32* was used as an internal normalizer, because it showed the least variation (based on our microarray data) among all various samples. RT-qPCR primers (Supplemental Table 7) were designed using Primer 3 software (Rozen and Skaletsky, 2000). To test the primers an end point PCR was performed using SYBR Green mastermix (Stratagene) and melting curves of products were evaluated on a qPCR device for the possible presence of primer–dimers. The products were also run on agarose gel to evaluate the product sizes.

For miRNA expression assays, the TaqMan microRNA assay kits were purchased from Applied Biosystems (USA) and the general protocol by the manufacturer was followed.

4.5. Hela cell culture and Pre-miR transfection

Pre-miRs were purchased from Ambion (USA). Hela cells were cultured at 1.5×10^5/well in 12-well dishes in 1.5 ml complete DMEM medium containing 10% fetal bovine serum (FBS) and penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma). Twenty-four hours later cells were transfected with 100 nM Pre-miRs using siPORTAmine (Ambion) transfection reagent according to the manufacturer's instruction. 1–2 h prior transfection, media were replaced with 500 μl fresh DMEM containing 10% FBS without antibiotics. 7 h after transfection 1 ml DMEM containing 10% FBS was added to each well. 72 h after transfection cells were lysed for western blot experiment.

4.6. Antibodies and western blotting

Mouse monoclonal primary antibodies detecting SFRS3 and beta tubulin as well as HRP conjugated anti-mouse secondary antibody were purchased from Santa Cruz Biotechnology (USA).

To determine the total protein amount in samples, the BCA protein assay kit and protocol (Perbio Science, UK) was used. Twenty micrograms of each of the samples was run on Bis–Tris NuPAGE gels (Invitrogen) using MES-SDS running buffer (Invitrogen). The proteins were transferred to nitrocellulose membranes following general protocol for western blotting.

The proteins were detected using primary and secondary antibodies according to the manufacturer instructions.

4.7. Statistical analysis

Unless otherwise stated, all calculations were done using R (www.r-project.org). The raw data was pre-processed using lumi R\Bioconductor package (Du et al., 2008). Data were background corrected in BeadStudio (Illumina). The quantile normalization method (Bolstad et al., 2003) implemented in lumi R was used to normalize the data.

To reduce the number of comparisons and the chances of false positive findings, statistical comparisons were performed on selected probes based on an average signal intensity of ≥7.0 ($\log_2$ scale) instead of the total number of probes present on the arrays. This resulted in a total of 9593 of the initial 24526 (~40%), and 708 of the initial 1145 (~60%) probes for gene expression and miRNA arrays, respectively. Principal component analysis (PCA) was performed on the selected probe sets to identify outlier arrays.

Generalized Estimating Equations (GEE) (Hanley et al., 2003; Ziegler et al., 2000) was performed for each gene to identify statistically significant changes between affected and unaffected groups using family as a clustering variable and using an exchangeable correlation structure, which takes into account family dependence. Gender indicators were also added to the model to reduce gender effects. *P* values were corrected using Benjamini and Hochberg False Discovery Rate (FDR) method.

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