ABSTRACT
Autism is a severe neurodevelopmental disorder with both genetic and epigenetic etiological elements. Currently it is unclear how many genes are associated with autism and how strong the evidence is. Micro-RNA gene expression profiling is considered a promising tool for discovery of autism-related genes and biological pathways because of the dynamic nature of the whole blood transcriptome. The objective of this study was to identify miRNA expression changes in children with autism compared to general population controls. In the present study, we examined miRNA gene expression changes applying custom-made LC Science miRNA expression profiling service, using pooled whole blood-derived total RNA samples in order to evaluate possible miRNA transcripts and networks of molecules associated with the disease. Here, we report molecular evidence for a differentially expressed miR486-3p which has shown brain-specific expression with possible roles in human neuronal differentiation. This study outlines altered miRNAs expression levels observed in peripheral whole blood from autism patients, a finding which suggests that dysregulation of miRNAs may contribute to autism phenotype. Potential and validated target genes for these microRNAs were shown, which include several autism susceptibility genes. The miRNA expression changes involved may help to define the etiology, genetics, and clinical phenotype, as well as the outcome in autism. Further molecular analysis on miRNA gene expression changes will give a more detailed picture about the miRNA associated mechanism in autism.

Materials and Methods
Participants
Thirty autistic patients were recruited at the Medical University Hospital of Plovdiv and an autistic center in Plovdiv. Diagnosis of autistic disorder (299.00 – DSM IV TR criteria) was assigned according to routine clinical interview and psychiatric examination. Written informed consent form approved by the Medical University of Plovdiv Ethics Committee was taken from the parents of the children. The control group included 25 healthy subjects matched by gender (p = 1.0) and age (p = 0.839). Demographic information about the autistic and control cohorts is given below (Table 1). The patients did not receive any medication before blood sampling and they had standard breakfast. Persons with other chronic medical illness were also excluded.

Blood collection and RNA isolation
An aliquot of whole blood (2.5 mL) for each subject (autistic and healthy controls) was collected directly into PAXgene blood RNA tubes (PreAnalytiX) and stored at room temperature for minimum 4 h and then frozen at ~20 °C, as this method shows the highest yield of RNA. After collection of all samples, total RNA was isolated using the PAXgene blood miRNA kit (PreAnalytiX), according to the manufacturer’s protocol. A260/A280 ratios revealed that all samples appeared to be of sufficient quality for microarray analysis (1.93–2.10). RNA quality and purity were analyzed by Epoch Micro-Volume Spectrophotometer System (BioTek), and RNA integrity was...
analyzed using agarose gel electrophoresis. RNA integrity of pooled samples (autistic and healthy controls) was checked by Agilent 2100 Bioanalyzer. Pooled samples were created by adding an equivalent amount of total RNA from each individual sample from autistic and healthy controls to a final concentration of 5 µg RNA samples.

**MicroRNA expression profiling (LC Science)**

The µParaflo™ miRNA microarray assay was performed using a service provider (LC Sciences, Houston, TX) with a proprietary microfluidic array based on the Sanger miRBase v18.0 database (http://www.sanger.ac.uk/Software/ Rfam/mirna), designed to detect 1898 unique mature human miRNA sequences. MiRNA microarray analysis was performed to determine differential expression in blood miRNAs between pooled samples of healthy individuals and pooled RNA samples derived from patients with autistic disorder. The assay required 5 µg of total RNA sample, which was size fractionated using a YM-100 Microcon centrifugal filter (Millipore, Billerica, MA) and the isolated small RNAs (300 nt) were 3’-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining; one tag was used for the two RNA samples in one sample experiments. Hybridization was performed overnight on a µParaflo microfluidic chip using a microcirculation pump (Atactic Technologies, Houston, TX). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to all 1898 target genes and therefore has the potential to modulate multiple pathways. In order to explore what targets and pathways may be regulated by these differentially expressed miRNAs, we used the miRWalk database as an important resource for the functional investigation of individual miRNAs. Putative target genes were identified using the publically available miRWalk (which combines information gathered from 8 established miRNA-target prediction programs i.e. DIANA-microT, miRanda, miRDB, PicTar, PITA, RNA22, RNAhybrid and TargetScan/TargetScanS) available at http://mirwalk.uni-hd.de (4). The validated targets module was used to give information about experimentally validated miRNAs’ interaction information associated to protein-coding genes. MiRNA target prediction module was used to predict non-experimentally validated gene targets for differentially expressed miRNAs in our analysis.

**Statistical methods**

**Microarray data analysis**

Data analysis includes the determination of detectable signals, calculation of signal intensities, and calculation of differential ratios (log2 transformed). The data process began with background subtraction, detectivity determination, and then P-value calculation.

**Detectivity determination**

A transcript to be listed as detectable must meet at least two conditions: signal intensity higher than 3_(standard deviation) and spot coefficient of variance (CV) < 0.5. CV is calculated as (standard deviation)/(signal intensity). When repeating probes are present on an array, a transcript is listed as detectable only if the signals from at least 50 % of the repeating probes are above detection level.

**T-test analysis**

T-test was done using Welch test, assuming no equivalence between the variables. The significant P-value used was <0.05.

**Results and Discussion**

Several candidate genes possibly associated with autism have been examined to date. These candidate genes were generally selected based on supportive linkage/cytogenetic evidence or the presence of certain findings at the clinical level in subjects with autism. One of the main techniques in screening these candidate genes was direct sequencing of gene exons using genomic DNA from subjects with autism compared to controls. The study of posttranscriptional regulation in autism by evaluating ncRNAs (e.g., miRNAs) can provide a link between autism and epigenetic factors (14). Several observations can be considered in support of the assumption that miRNAs are a source of molecules that would be reasonable to test in pathogenesis of brain disorders such as autism (10). Some miRNAs and other small RNAs show co-expression in whole blood and brain; there are brain enriched microRNA species also expressed in peripheral whole blood. Using µParaflo™ miRNA microarray assay we found that miR-486-3p is differentially expressed in blood with statistical significance (P = 0.0304) (Fig. 1).

The results of this study are in agreement with the basic hypothesis of Gilad et al. (6) that microRNAs are present in bodily fluids (here, whole blood) and represent useful clinical biomarkers. Additionally, this study demonstrates the
use of miRNA signatures as an important advance in autism research. We searched for potential microRNAs that might play a role in the regulation of autism susceptibility genes. Of all differentially expressed miRNAs we focused on miR486-3p. After compiling the list of candidate mRNAs, PubMed searches were performed, and peer-reviewed publications were reviewed to assure that these miRNAs actually had been implicated in autism susceptibility. This strategy offers an opportunity to take a first step in understanding the role of miRNAs and their target mRNAs in the etiology of autism (14). The hsa-miR-486-3p has many experimentally validated targets and several potential targets with many interesting neural active functions (Fig. 2).

**TABLE 1**

Demographic information about the autistic and control cohorts

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Autism (n = 30)</th>
<th>Control (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td>24 male; 6 female</td>
<td>20 male; 5 female</td>
</tr>
<tr>
<td>Mean age/years</td>
<td>8.133</td>
<td>7.96</td>
</tr>
<tr>
<td>Age range/years</td>
<td>3–20</td>
<td>3–20</td>
</tr>
</tbody>
</table>

![Fig. 1. Heat map displaying miRNA expression in whole blood samples from autistic pooled samples (AT) and healthy controls (ATC). Increased expression is shown in red; decreased one, in green; no/little changes, in black.](image)

![Fig. 2. Target analysis of miR-486-3p. Venn diagram of miRWalk-predicted and validated gene targets. The green circle shows validated gene targets; the lilac circle represents predicted sites in target genes and the blue circle represents all predicted target genes. ![Venn diagram image](image)](image)

For example, some of the previously validated mRNA targets like: SOX9, NRXN1, HEY1, PTEN, NLGN3 and PTCHD1, all of which are involved in nervous system development and function, were found to be down-regulated in a previous experiment (11). In a study of lymphoblastoid cell lines from patients with ASD, Yasuda et al. (16) observed a relationship between ASD and alterations in the mRNA expression levels of NLGN3 and SHANK3, which were significantly decreased in the individuals with ASD compared to the control group. Furthermore, several interesting results described recently (14) included the identification of autism susceptibility genes, such as MECP2, FMR1, NLGN3, PTEN, as well as neurexin genes (NRXN1 and NRXN3), among the validated and predicted targets for our differentially expressed miR486-3p. Specifically, they found that a subset of brain expressed miRNAs is also expressed in LCL, which enables the detection of brain-expressed miRNAs’ changes in the absence of access to a large number of autism brain and blood samples (11). Our analysis also identified a dysregulated miRNA – has-miR-128, which is in consistency with another study (1), where the same miRNA was derived from postmortem cerebelar cortex tissue and was found to be dysregulated as well.

Recent studies identify dysregulated genes and miRNA transcripts involved in ASD using lymphoblastoid cell-line derived total RNA (11). However, this might not be the most appropriate RNA source for a disorder which is generally considered to be brain-specific and occurs in the early developmental stages. Neural tissue is presumed to be the ideal material for studies in ASD, since previous approaches using lymphoblastoid cell lines for identifying genes, miRNA transcripts and networks of molecules, have some limitations because the material used does not show the best proximity. Additionally, cell line experiments do not reflect disease conditions in the appropriate time, which is one of the most important questions under consideration in the case of investigation of abnormal gene expression contributing to the disease. In our point of view, the approach used here is one of the most powerful, but underrated, tools for abnormal gene expression investigation. The prediction and future validation of putative mRNA targets for differentially expressed miRNAs, such as hsa-miR-486-3p, will help characterize the miRNAs involved in biological processes.

**Conclusions**

During the past decade, global gene expression profiling has become increasingly and more reliably used for obtaining disease-specific information from blood samples. Despite the accumulating evidence linking miRNAs to various diseases, very little is known about how these small RNAs contribute to the disease. Moreover, we have demonstrated that miRNA whole blood levels could reflect pathophysiological conditions, such as autism. As a whole, our study suggests that evaluation of miRNA expression may have the potential to identify biological pathways involving protein-coding genes implicated in autism.

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REFERENCES