

Original Research Article

Down regulation of miRNA let-7b-3p and let-7d-3p in the peripheral blood of children with autism spectrum disorder

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ABSTRACT

Keywords

autism spectrum disorder; miRNAs; miRNA expression profiling.

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder having both genetic and epigenetic etiological elements. Currently it is unclear how many genes have been associated with ASD and how strong the evidence is. MiRNAs are a widespread class of small non-coding RNAs that have the ability to silence gene expression through sequence complementarity to their targets. Owing to the dynamic nature of the whole blood transcriptome, understanding miRNAs gene expression profiling in autism is a promising tool for discovery of disease-related genes and biological pathways. The aim of this study was to identify miRNAs expression changes in children with ASD compared to general population controls. In the present study, to demonstrate the relevance of miRNAs expression changes in the autistic patients we examined miR let-7b-3p and let-7d-3p gene expression, applying custom made LC Science miRNA expression profiling. The involved miRNAs expression changes may contribute to define the etiology, genetics, and clinical phenotype. Further molecular analysis on miRNA gene expression changes will give a more detailed picture about the miRNA associated mechanism and processes common to ASD.

Introduction

ASD is a severe neurodevelopmental disorder with specific social and communication deficits and repetitive behaviors. Many etiologies have been suggested and numerous risk factors have

Autism is associated with a high degree of heredity, few specific genetic mutations have been identified accounting for a minority of cases (Sykes *et al.*, 2007, Stephan *et al.*, 2008, Wang *et al.*, 2009, so

et al., 1996, Zoghbi *et al.*, 2005). A genetic basis now is evidential by the greater than 70% concordance in monozygotic twins and elevated risk in siblings compared with the general population. (Bailey *et al.*, 1995, Zhao *et al.*, 2007). Recently, several *de novo* mutations were identified using exome sequencing of individuals with sporadic ASD (cases) and their parents, reasoning that these families would be enriched for *de novo* mutations of major effect. (Brian *et al.*, 2011). Additionally, multiple studies have confirmed the involvement of rare *de novo* copy number variations (CNV) to the risk for ASD (Sebatet *et al.*, 2007, Pinto *et al.*, 2010). Research on gene expression in autism has previously focused on identifying specific or a limited group of genes related to disease. (Enstrom *et al.*, 2009, Hu *et al.*, 2006). The idea that alterations at the global level of gene expression regulation might be important in mediating the risk for autism, as well as a novel tool for prognostic biomarker discovery has been largely underexplored. The identification of miRNAs has significantly expanded our knowledge of the regulatory mechanisms of gene expression. MicroRNAs (miRNAs) are evolutionary conserved small non-coding RNAs that have been shown to mediate the posttranscriptional regulation of at least one-third of protein-coding genes (Bartel 2004, Filipowicz *et al.*, 2008). In mammals, the greater part of endogenous miRNA genes are transcribed initially as primary transcripts (pri-miRNAs) that range from hundreds to thousands of nucleotides in length and contain one or more extended hairpin structures. (Du and Zamore 2005). MiRNA could also negatively regulate protein expression through targeting of mRNA coding regions (Tay *et al.*, 2008). Furthermore, miRNAs are found to upregulate the

translation of target mRNAs in a cell cycle-dependent manner, switching between translational suppression in proliferating cells to translational activation in quiescent cells (Vasudevan *et al.*, 2008.). The discovery of endogenous regulatory non-coding RNA, has led to wide spread interest because of the implication to several human diseases, including autism. Moreover, heterogeneous dysregulation of microRNAs across the autism spectrum were also reported in postmortem tissues from the cerebellum cortex of autistic patients (Abu-Elneel, *et al.*, 2008). Currently, there is increasing effort to develop promising prognostic and/or diagnostic molecular biomarkers that meet requirements like easy accessibility from peripheral blood, sufficiently high specificity and sensitivity, low costs and applicability by laboratories holding standard equipment. In this sense, microarray-based gene expression analysis of peripheral blood is a common strategy in the development of clinically relevant biomarker panels for a variety of human diseases. The aim of this study was to identify miRNA expression changes possibly involved in the etiology, genetics and clinical phenotype with potential prognostic characteristics, by applying custom made LC Science miRNA expression profiling.

Materials and Methods

Participants details

30 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. Approved written

informed consent form from the Medical University of Plovdiv Ethics Committee was taken from the parents. The control group included 25 healthy subjects matched by gender ($p=1,0$) and age ($p=0,839$). Patients and healthy controls did not receive any medication before blood sampling and they had received a standard breakfast. Patients and controls with other chronic medical illness were also excluded.

Blood collection

An aliquot of peripheral whole blood (2.5 ml) for each subject (autism and healthy controls) was collected directly into PAXgene blood RNA tubes (PreAnalytiX) according to the manufacture's instructions and stored at room temperature for minimum of 4 hours and then freezed at -20°C , as this method shows the biggest yield of RNA.

RNA extraction

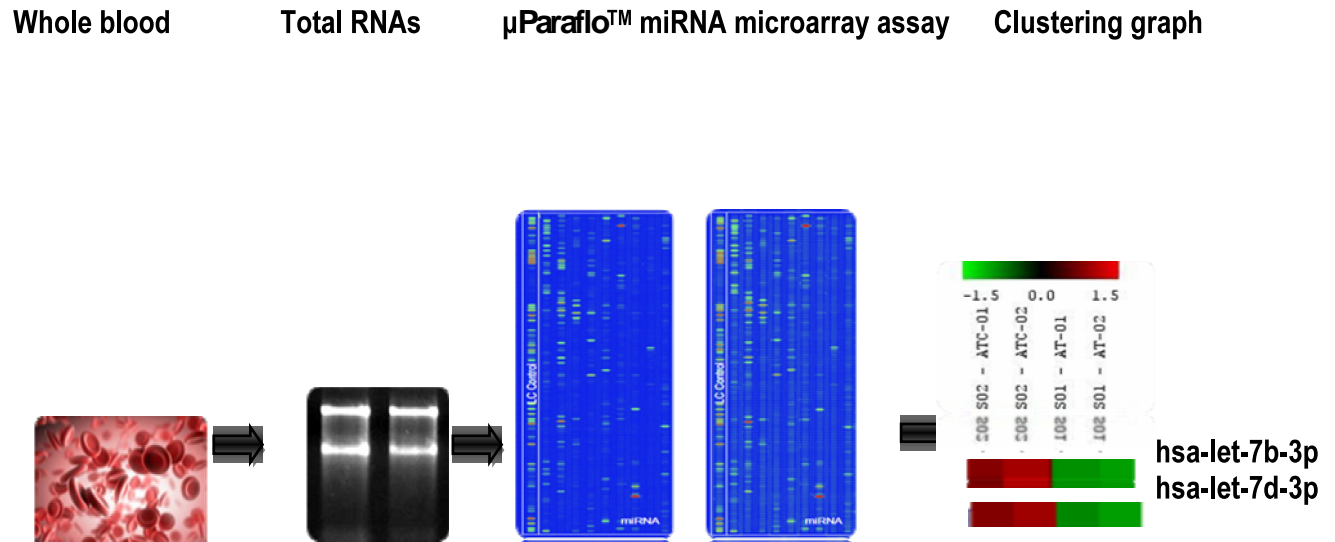
After collection of all samples, total RNA containing small RNA fraction was isolated using the PAXgene blood miRNA kit (PreAnalytiX, Hombrechtikon, Switzerland), according to the manufacturer's protocol. Assesment of A260/A280 ratios revealed that all RNA samples are with sufficient quality for microarray analysis (1.93 - 2.10). RNA quality and purity were analyzed by Epoch Micro-Volume Spectrophotometer System (BioTek), and additionally RNA integrity was analyzed by agarose gel-electrophoresis. Pooled samples were created by adding an equivalent amount of total RNA from each individual sample from autistic and healthy controls to final concentration of 5 μg total RNA samples. RNA integrity of pooled samples (autistic and healthy controls) were checked by agarose gel- electrophoresis followed by

assessment with Agilent 2100 Bioanalyzer. For microRNA expression profiling purpose pooled RNA samples were precipitated according to the service requirements. Briefly, each 5 μg pooled RNA samples collected from autistic and healthy controls were mixed with 1/10th volume of 3M NaOAc, pH 5.2 and 3 volume 100% ethanol, to the final volume of 400 μl and mix well by vortexing. Aliquots of RNAs were prepared and frozen at -80°C until shipment on dry ice to miRNA microarray service provider LC Sciences, Houston, TX.

MicroRNA Expression Profiling (LC Science)

The $\mu\text{Paraflo}^{\text{TM}}$ 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://microrna.sanger.ac.uk/sequences>. designed to detect 1898 unique mature human miRNA sequences. The assay required 5 μg total RNA sample, which was size fractionated using a YM-100 Microcon centrifugal filter (Millipore, Billerica, MA) and the small RNAs (300 nt) isolated 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 $\mu\text{Paraflo}$ microfluidic chip using a microcirculation pump (Atactic Technologies, Houston, TX). Internal control probes were used for quality control of chip production, sample labeling, and assay conditions. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100

Figure.1 Experimental workflow of the μ Paraflo™ miRNA microarray assay



ml 6xSSPE buffer (0.90M NaCl, 60mM Na₂HPO₄, 6mM EDTA, pH 6.8) containing 25% formamide at 34°C. After RNA hybridization, tag-conjugating Cy5 dye was circulated through the microfluidic chip for dye staining. Fluorescence images were collected using a GenePix 4000B laser scanner (Molecular Device, Sunnyvale, CA) and digitized using Array-Pro image analysis software (Media Cybernetics, Bethesda, MD).

Results and Discussion

To date, several candidate genes have been examined to evaluate their possible associations with ASD. The study of posttranscriptional regulation in ASD by evaluating ncRNAs (e.g., miRNAs) can provide a link between ASD and epigenetic factors. Several observations can be used in support of the assumption that microRNAs are a class of molecules that would be reasonable to analyse in

pathogenesis of brain disorders such as ASD. Some microRNAs and other small RNAs show co-expression in whole blood and brain; there are brain enriched microRNA species also expressed in peripheral whole blood; there is many micro RNAs with validated targets including some of candidate genes for ASD. Using μ Paraflo™ miRNA microarray assay we found that miR let-7b-3p and miR let-7d-3p are differentially expressed in whole blood with statistical significance $p = 0,010$ ($\log_2 -1,05$) and $p = 0,038$ ($\log_2 -0,72$), respectively (Fig. 1). The results of this study clearly support our basic hypothesis that microRNAs present in body fluids as whole blood could represent useful prognostic clinical biomarkers. Additionally, this study demonstrates the use of microRNA signatures as an important advance in autism research. Further studies should also examine the physiological correlations of ASD-related social

impairment with prognostic blood-based biomarkers like identified miRNAs.

The prediction and future validation of currently predicted protein coding mRNA targets for differentially expressed miRNAs, such as let-7d-3p and let-7b-3p will support to characterize the miRNAs involved in biological processes including ASD.

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