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DOI: 10.1515/folmed-2017-0009

ORIGINAL ARTICLE, MEDICINE

Profiling of Circulating Serum MicroRNAs in Children with Autism Spectrum Disorder using Stem-loop qRT-PCR Assay

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Received: 21 Nov 2015
Accepted: 31 Aug 2016
Published Online: 30 Nov 2016
Published: 27 March 2017

Key words: autism spectrum disorder, miRNAs, stem-loop qRT-PCR, serum

Citation: Kichukova TM, Popov NT, Ivanov IS, Vachev TI. Profiling of circulating serum microRNAs in children with autism spectrum disorder using stem-loop qRT-PCR assay.
Folia Medica 2017;59(1):43-52.
doi: 10.1515/folmed-2017-0009

Background: Development of biomarkers for autism spectrum disorder (ASD) has still remained a challenge to date. Recently, alterations of the expression of microRNAs (miRNAs) in peripheral blood, serum and post-mortem brain tissue have been linked to ASD. miRNAs are known to be secreted by various cell types and can mediate transmission of information into recipient cells and to modulate their physiological functions. On this basis it is assumed that circulating miRNAs could be useful biomarkers for the diagnosis or prognosis of pathological conditions.

Aim: The aim of this study was to test whether circulating miRNAs display differential expression profile in serum of ASD patients.

Patients and methods: The relative expression levels of 42 miRNAs were analyzed by stem-loop qRT-PCR assay in the serum of ASD patients compared to healthy controls.

Results: The results indicated that 11 miRNAs in ASD patients were substantially higher expressed than these in control subjects, and 29 miRNAs were lower expressed, respectively. In addition, target gene analysis displayed that the altered serum miRNAs targeted some important genes like alpha 1C subunit of voltage-dependent calcium channel, L type, (*CACNA1C*), beta 1 subunit of voltage-dependent calcium channel (*CACNB1*) and other genes involved in epigenetic processes like dicer 1, coding ribonuclease type III (*DICER*).

Conclusion: Our results suggested that differentially expressed miRNAs in serum might be involved in ASD molecular pathways, and serum miR-424-5p, miR-197-5p, miR-328-3p, miR-500a-5p, miR-619-5p, miR-3135a, miR-664a-3p, and miR-365a-3p might be able to serve as potential biomarkers for ASD because they displayed significant alterations in the expression profile in children diagnosed with ASD.

BACKGROUND

Autism spectrum disorder (ASD) involves a range of early-onset neurodevelopmental conditions featured by impaired social interactions and communications, together with repetitive stereotypic behaviors (DSM5). In the last decades, there has been considerable increase in the prevalence of this pervasive developmental disorder, which may be due to the interplay between worsened environmental factors (prenatal, pollution, etc.) and undoubted heritable factors.

ASD does not meet the criteria of known models of inheritance. For most patients, the polygenic pattern of inheritance with many interacting genes is the most expected model. However, the etiology of ASD is not fully understood. So far, it has been hard to understand how various genetic susceptibilities translate to a clinical phenotype with many genomic loci resulting in heterogeneous functions.[1]

Because of the increasing social burden and the variability in clinical presentation, the search for biomarkers is a leading line in autism research expecting to enable early diagnosis and more successful therapeutic interventions. Until now, studies have centered more on genetic factors, but only a few studies have investigated the role of miRNA as epigenetic factors involved in ASD.[2-4] Epigenetic factors regulate heritable modifications in gene function or activity without changes of the DNA sequence.

MicroRNAs have recently became leading epigenetic regulators of various cellular processes. They are small, non-coding, single-stranded RNA molecules of around 22 nucleotides (nt) in length. MicroRNAs are themselves active molecules that affect gene function, and thus their expression may be associated with altered cell physiology. There is increasing evidence that has linked miRNAs with regulation of many biological pathways and processes like cell development, proliferation, differentiation, and apoptosis.[5] As a group of important endogenous molecules they modulate gene expression by recognizing complementary sequences in target messenger RNAs (mRNAs), implicated in normal cell function.[6] The dysregulation of miRNA expression can profoundly alter the gene expression pattern by means of post-transcriptional gene silencing including different mechanisms.[7]

MiRNAs, previously known to be expressed only in cells and tissues, have also been detected in extracellular body fluids such as serum, plasma, saliva, urine, breast milk, seminal plasma, tears, amniotic fluid, colostrum, bronchial lavage, cerebrospinal fluid, peritoneal fluid, and pleural fluid.[8,9]

Although the miRNAs transcriptomic connection between serum and brain is not well known, numerous lines of evidence suggest that examination in tissues that are not primarily involved in the disease process may reveal disease signatures. Several investigators have demonstrated differential expression of genes in peripheral white blood cells in disorders of the central nervous system.[10-13] The findings from that research may suggest that altered miRNA expression profiles target genes closely involved in neurological functions and disorders and, in turn, may lead to the pathophysiological conditions associated with autism. Altered expression of cellular and circulating miRNAs have been observed in autistic individuals compared to healthy controls. MicroRNAs are now being considered as potential targets for the development of novel therapeutic strategies for autism.

Serum miRNAs which may be collected from circulating blood cells are known particularly to be very stable, reproducible and resistant to the actions of RNase,[14] suggesting efficacy as noninvasive biomarkers for ASD.

AIM

The aim of this study was to test whether circulating miRNAs display differential expression profile in serum of ASD patients in comparison with typically developing children.

PATIENTS AND METHODS

Ethics Statement

This study's design and the Informed Consent Form (ICF) were approved by the Ethics Committee of Plovdiv Medical University. The University Institutional Review Board approved the use of the samples for this study. Written ICFs were obtained from the parents of the probands and the children of the control group after the objectives of the study had been explained and confidentiality ensured.

Patients

A total of 30 subjects (24 male and 6 female) with ASD aged 3 to 11 years (mean age of the sample, 6.86 years), and 30 healthy children age- and sex-matched to the patient's group were recruited between March 2013 and February 2014. All probands were randomly selected from the Pediatric Department of the Plovdiv Medical University Hospital Database. Parents of the participants were interviewed by experienced psychiatrists and the diagnose of ASD was made by clinical examination, ADI-R[15], CARS and GARS[16], adhering to the DSM V criteria. The control group was assigned from several family practices in the Plovdiv region aiming to be matched by age and sex to the probands' group. All children in the control group were examined for absence of autistic features by clinical examination and CARS. All participants were Bulgarians. None of the participants had received any medications before blood sampling.

Blood collection, serum processing and RNA extraction

Blood samples (4 ml) were drawn, collected in EDTA containing tubes and stored at 4°C before centrifugation. The samples were separated into serum and blood cells by centrifuging at 1600 g for 10 min at 4°C. A volume of 500 µl serum was re-centrifuged at 16 000 g for 10 min at 4°C to remove any residual blood cells. The clear supernatant was collected into RNase/DNase-free microfuge tubes in 300 µl aliquots and stored at -80°C until use. Total serum RNA including miRNAs was extracted from the samples, where a 5 µl synthetic spike-in control, *Caenorhabditis elegans* miR-39 (100nM), were previously added for internal normalization. Total serum RNA extraction were performed by using the PAXgene blood miRNA kit (PreAnalytiX), according to the "Manual Purification of Total RNA,

Including miRNA protocol" recommended by the manufacturer with some modifications including starting the purification at step 4 and elution in 30 µl of BR5 buffer.

Stem-loop real-time PCR quantification of serum microRNAs

Five microliters of each RNA sample was used for miRNA specific cDNA synthesis using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) and miRNA-specific stem-loop primers. Reverse transcription reactions contained 8 µl of total RNA samples, 4 µl miRNA specific cDNA synthesis SL primer mix (100 µm each), 4 µl Maxima Enzyme Mix, 4 µl 5X Reaction Mix and nuclease free water to a final volume of 20 µl. MicroRNA-specific stem-loop (SL) and forward primers (For) used in this study are summarized in **Table 1**.

Five microliters of each miRNA specific cDNA were subjected to pre-amplification prior to the real-time PCR step with peqGOLD Taq DNA Polymerase to potentially enhance sensitivity of the assay. qRT-PCR was performed using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) with ABI 7500 system (Applied Biosystems). Each sample was normalized using spiked-in synthetic *C. elegans* miRNAs as controls. Experiments were performed in duplicate. The expression levels of miRNAs for qRT-PCR were normalized to cel-miR-39, and relative quantification (RQ) was calculated utilizing the $2^{-\Delta\Delta Ct}$ method.

Stem-loop QRT-PCR pooled expression analysis of serum microRNAs in ASD

Individual serum samples from ASD (30) and healthy controls (30) were analyzed using stem-loop qRT-PCR pooling assay. Pooled samples were created by physically adding an equivalent amount of serum volume from each individual ASD sample and healthy controls and finally adjusted to 300 µl

Table 1. MicroRNA specific qRT- PCR primer sets

MiRNAs	Primer sequence 5'- 3'
miR-424-5p SL	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTTCAAAAC
miR-424-5p For	ACACTCCAGCTGGGCAGCAGCAATTCATGT
miR-197-5p SL	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCTCCCAC
miR-197-5p For	ACACTCCAGCTGGGCGGGTAGAGAGGGCAGT
miR-328-3p SL	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACGGAAGG
miR-328-3p For	ACACTCCAGCTGGGCTGGCCCTCTCTCTGCCC
miR-500a-5p SL	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCTCACCC
miR-500a-5p For	ACACTCCAGCTGGGTAATCCTTGCTACCTGG
miR-619-5p SL	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGCTCATG
miR-619-5p For	ACACTCCAGCTGGGGCTGGGATTACAGGCA
miR-3135a SL	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCACTGCAG
miR-3135a For	ACACTCCAGCTGGGTGCCTAAGGCTGAGACT
miR-664a-3p SL	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGTAGGCT
miR-664a-3p For	ACACTCCAGCTGGGTATTCATTTATCCCCAG
miR-365a-3p SL	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATAAGGAT
miR-365a-3p For	ACACTCCAGCTGGGTAATGCCCCTAAAAAT
Cel-miR-39 Spike-in	UCACCGGGUGUAAAUUA
Cel-miR-39 SL	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCAAGCTGA
Cel-miR-39 For	ACACTCCAGCTGGGTCACCGGGTGTAAATC
Universal Rev	GTCGGCAATTCAGTTGAG

total volume. Pooled serum samples were subjected to RNA extraction. The pooled RNA samples that had been extracted were subjected to stem-loop qRT-PCR for 42 cell-free serum miRNAs and Cel-miR-39 as exogenous control. All samples were measured in duplicates and the mean values were used for analysis.

Validated microRNA target gene analysis

To study the potential modulation in gene expression that may be associated with specific serum miRNAs changes, miRNA validated target studies were carried out using the publicly available database, miRWalk - Database.[17] The validated targets module hosted experimentally verified miRNA interaction information associated with target genes were used.

RESULTS

Pooled expression analysis of serum microRNAs in children with ASD

In the preliminary expression experiments 42 miRNA molecules were selected as candidate biomarkers for initial analysis. We found that 29 miRNAs were downregulated (miR-589-3p, -6849-3p, -3135a, -15a-5p, -328-3p, -183-5p, -3674, -96-5p, -3687, -6799-3p, -587-3p, -504-5p, -576-5p, -486-3p, -3909, -let-7i-3p, -29c-5p, -301a-3p, -3064-5p, -145-5p, -424-5p, -193b-3p, -487b-3p, -197-5p, -500a-5p, -664b-3p, -20b-3p, -671-3p and -199a-5p) and 11 were upregulated (miR-4489, -8052, -106b-5p, -142-3p, -3620-3p, -365a-3p, -664a-3p, -374b-5p, -18b-3p, -619-5p, and 210-5p), while two of the selected 42 miRNAs did not show any expression changes in children with ASD compared to general population controls (**Fig. 1**). Bars represent the fold change in subjects with ASD as compared to controls.

We further confirmed the expression of the eight most dysregulated serum miRNAs using qRT-PCR pooled analysis. The results showed that the serum relative levels of only three miRNAs (miR-365a-

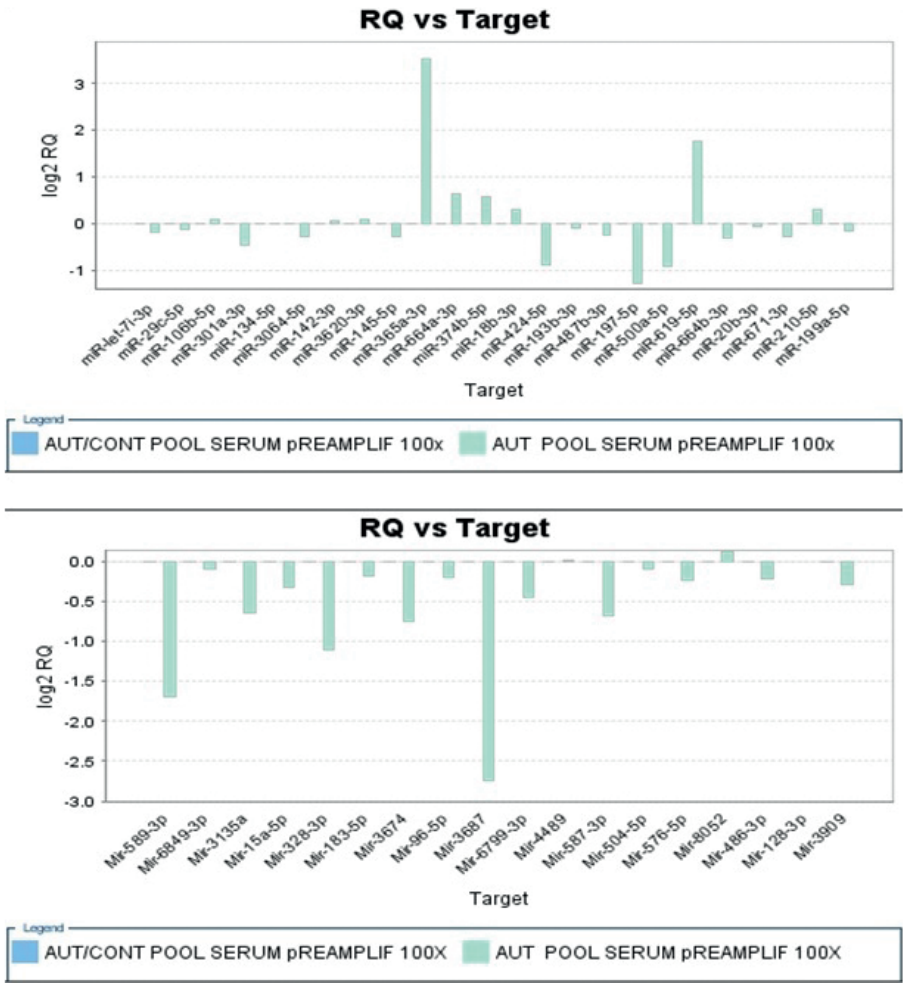

Figure 1. Results from preliminary expression experiments. The expression levels of 42 selected single miRNAs were determined using stem-loop qRT-PCR in testing pool sets from cohort of 30 ASD patients and 30 healthy controls.

3p, miR-619-5p and miR-664a-3p) in ASD patients were markedly higher than these in control patients, and the serum relative levels of other five miR-3135a, miR-328-3p, miR-197-5p, miR-500a-5p, and miR-424-5p in ASD patients were lower than that in control patients (**Fig. 2**). Fold changes in the expression of microRNAs in serum from patients with ASD compared with healthy control individuals are summarized (**Table 2**). PCR amplification of the specific products corresponding to all miRNAs amplicons was confirmed by monitoring the dissociation curve (Melting curve analysis) (**Fig. 3**).

Target gene analysis of dysregulated miRNAs in ASD

A list of validated protein coding gene targets for the dysregulated miRNAs (miR-424-5p, miR-197-5p, miR-328-3p, miR-500a-5p, miR-619-5p, miR-3135a, miR-664a-3p, and miR-365a-3p) was generated by data extraction from miRWalk database (**Table 3**). Specific downstream targets of the dysregulated miRNAs miR-328-3p and miR-619-5p include some genes related to mediation of the influx of calcium ions into the cell upon membrane polarization (calcium channel, voltage-dependent, L type, alpha 1C subunit (*CACNA1C*) and calcium channel, voltage-dependent, beta 1 subunit (*CACNB1*) as well as other genes involved in epigenetic processes like dicer 1, ribonuclease type III (*DICER*). We also identify other miR-424-5p validated target gene like *RNASEN* (drosha, ribonuclease type III).

DISCUSSION

The benefits of miRNAs as biomarkers for various diseases has been intensively investigated and some promising potential candidates have been explored. The advantages of circulating miRNAs in contrast to other nucleic acid molecules are that they show an unexpected stability in different body fluids. MiRNAs can stay stable not only in the RNase-rich blood environment, but also in extreme conditions, including boiling temperatures, high or low pH, long storage, and freeze-thaw cycles.[18] Most

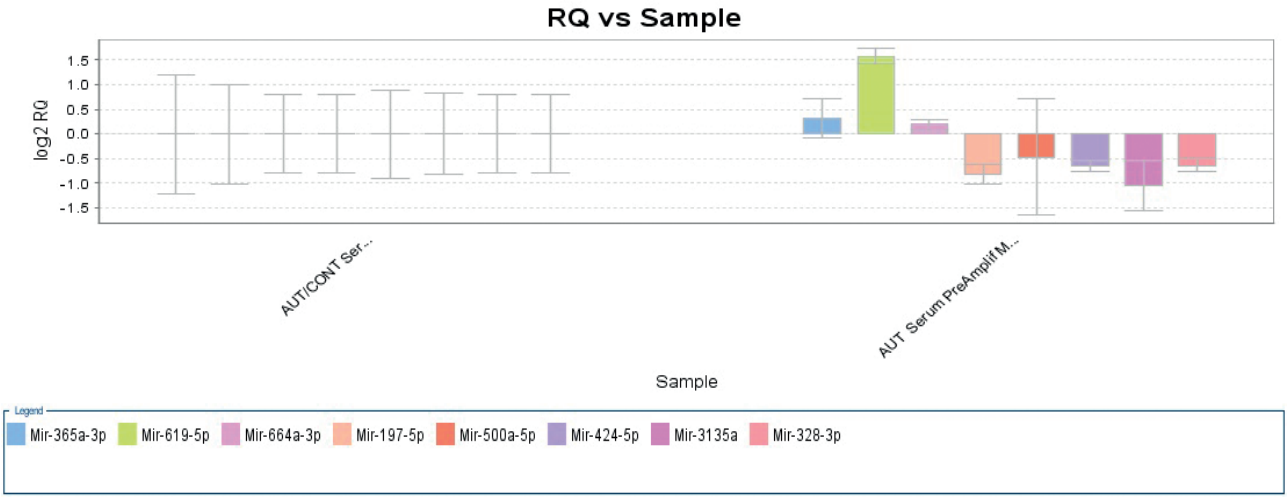

Figure 2. Expression of the eight dysregulated miRNAs using qRT-PCR in pooled analysis. The circulating serum miRNAs signatures identified by stem-loop qRT-PCR analysis of several miRNAs differentially expressed in case and control groups. Expression levels of the analyzed miRNAs were normalized to spiked-in Cel-miR-39 control and expressed in relation to controls.

Table 2. Fold changes in the expression of microRNAs in serum from patients with ASD compared with healthy control individuals

Up-regulated microRNAs	Fold change (RQ)	Down-regulated microRNAs	Fold change (RQ)
miR-365a-3p	1.248	miR-328-3p	0.641
miR-664a-3p	1.141	miR-500a-5p	0.722
miR-619-5p	2.983	miR-3135a	0.646
		miR-197-5p	0.569
		miR-424-5p	0.639

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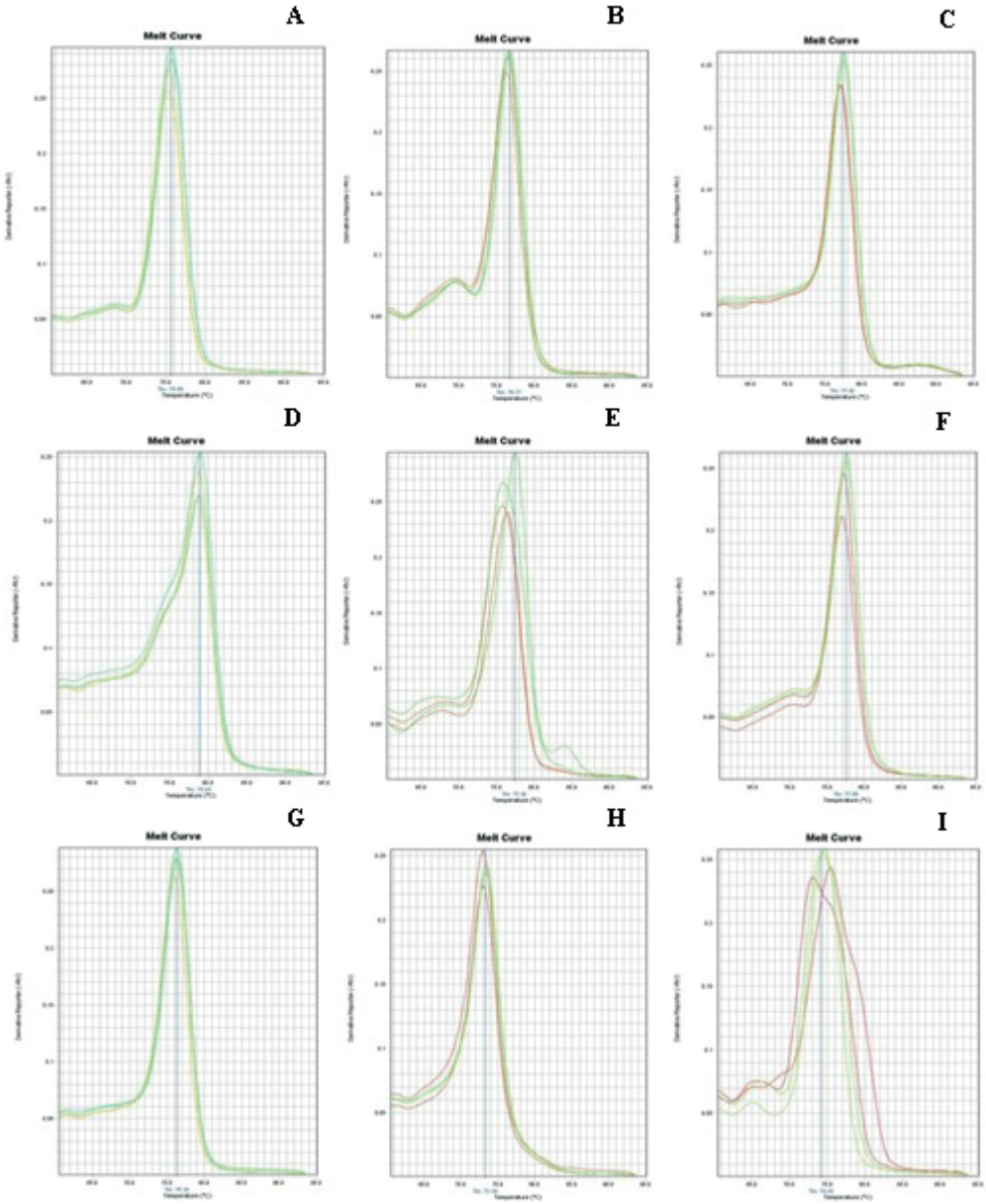

Figure 3. DNA melting profile results for amplification of the specific single miRNAs products in stem-loop qRT-PCR analysis. Specific single products corresponding to each microRNA amplicon in ASD and healthy control patients (**Panel A** spiked-in *Cel-miR*-39 control), **Panel B** miR-424-5p, **Panel C** miR-197-5p, **Panel D** miR-328-3p, **Panel E** miR-500a-5p, **Panel F** miR-619-5p, **Panel G** miR-3135a, **Panel H** miR-664a-3p, and miR-365a-3pamplicon **Pane I**, respectively was confirmed by monitoring the dissociation curve (Melting curve analysis).

miRNAs sequences are specific among different species and have been detected in various tissues, and miRNAs can easily be detected using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Also miRNAs expression is tissue- or cell-specific and is changed during the pathophysiologic processes. It is very important that altered miRNAs expression profiles in circulation reflect the changes in diseased tissues. Furthermore, serum miRNAs extraction is a noninvasive, simple process and besides miRNAs have a low complexity which enables exploration. These advantages demonstrate that circulating miRNAs can be useful candidates for diagnosis and other clinical applications in human diseases. Still, there are several challenges that have to be overcome in order to successfully use this potential biomarker. Because one miRNA can target many proteins and one protein can be regulated by many miRNAs, there is intrinsic complexity in the relation between a single miRNA and a pathogenic protein. Thus, analyzing combinations of multiple miRNAs rather than a single miRNA may increase

Table 3. List of validated target genes of dysregulated serum miRNAs

MiRNAs	Validated target genes
miR-424-5p	ATF6, BMPER, INPPL1, KIF23, ENDOGL1, MBD4, USF2, NFKB1, CCND3, BCR, EIF2C1, FGF2, CDC25A, MAP2K1, E2F1, YY1, NOS2A, CCNE1, SETD2, PAK3, CAMTA1, NIT1, CEBPB, ESR1, RUNX1, PTGS2, CREB1, HIF1A, ITGB1, MED1, KCNH8, NUDT6, DDX20, SMAD3, OPRS1, PDXP, AKT1, CUL2, POU2F2, CXCL12, MAPK9, CREB1, FGFR1, EIF2C2, MYB, CHD4, IL6, JUN, LATS2, BCL6, RUNX2, MAP2K1, ATF2, KDR, EGFR, RARA, AP2M1, EPHB2, CCND1, MYB, WEE1, CBFB, RASA1, E2F3, VEGFA, LGALS3, RXRA, DDX5, ITGAL, PLAG1, SIAH1, CDC14A, CD44, TAF10, HOXA4, MUC1, GTF2IRD1, FGFR1, ITGAM, CDKN1A, ADM, CCNE1, PDXP, ARPC5L, NFE2L1, CD4, CDK6, CDC25A, NCOA6, CHEK1, MAP2K1, CCNF, AADAC, PIAS1, NFE2L2, CD8A, RNASEN, FLT3, CCND1, FOSB, ETS1, CCNE2, IBSP, SYNGAP1, SP3, COX8A, CCND1, SPINK1
miR-197-5p	No validated target genes
miR-328-3p	BBS9, ABL1, CACNA1C, CACNB1, PIM1, DICER1, GNG7, BCR, ABCG2, SYNE1, PRKCA, CD44, BACE1, SLC39A5, BACE1, EGFR, ABCB1, ABCG2, KRAS, BCR, CYP3A4, ATP8A2, ABCG2, VDR, ABCG2, CEBPA
miR-500a-5p	No validated target genes
miR-619-5p	*DICER1*
miR-3135a	No validated target genes
miR-664a-3p	No validated target genes
miR-365a-3p	CCND1, BCL2, IL6, BAX, NCOA4, IMPA1, SCARB2, TUBB4B, SRP68, CALU, OSBP, VANGL1, ZCCHC7, KLHL12, HDLBP, NOLC1, TRIM16L, ANKRD12, AIMP2, STK16, MCMBP, TMEM167B, PPP2R5E, KLHL15, GPBP1, GPBP1, GPBP1, LIMA1, PRKACA, NUFIP2, GATAD2B, MTA2, POLR1B, USP22

the sensitivity and specificity of the diagnosis. In addition, because biofluids contain very low amount of circulating miRNAs, extracting miRNA from serum or plasma is technically challenging.

Another major challenge for the analysis is the choice of suitable endogenous controls. Data normalization is another crucial question. Therefore, it is of vital importance to develop standardization methods for quantifying the circulating miRNA. Regardless of these obstacles, the application value of circulating miRNAs in different diseases is gradually being revealed.

In this study, we have performed miRNA specific stem loop qRT-PCR assay in order to identify microRNAs involved in ASD. Here, we intend to identify serum microRNAs that are specific and can be used to discriminate ASD patients from healthy controls. In a preliminary step of our study (**Fig. 1**) we identified 11 microRNAs that are upregulated and 29 that are downregulated in ASD patients compared with group of healthy controls. Among the differentially expressed miRNAs, 8 displayed highly dysregulated expression profile. Our results confirm the findings of M.M. Vasu et al., (2015) showing the same expression profile of miRNAs (miR-106b-5p and miR-328).[21] Additionally, we detected some dysregulated miRNAs that have not been previously reported (miR-365a-3p, miR-3135a, miR-619-5p, miR-664-3p, miR-197-5p, miR-500a-5, miR-424-5p). Some of the above mentioned miRNAs were analyzed in previous reports (**Table 4**).[19]

So far, three studies have identified expression of miRNAs in a post-mortem brain – cerebellar cortex, prefrontal cortex and temporal cortex of ASD patients.[3,20,21] Bradley P. Ander et al. observed alterations in miRNA expression and their functional relation to the nervous system and canonical signaling pathways implicated in ASD. It was interesting that hsa-miR 664a-3p that we identified as differentially expressed in our study has been described in Bradley P. Ander et al. study of post-mortem temporal cortex of ASD subjects and has the same alteration in the expression pattern.[21] This makes miRNAs such as miR 664a-3p interesting potential

biomarker for ASD.

Many differentially expressed miRNAs, such as the eight microRNAs analyzed in this study using qRT-PCR, are involved in various biological processes including proliferation, migration, differentiation and apoptosis.

The study of the differential expression of miRNA molecules is very important since alterations in the expression levels of miRNA molecules as a class (regulatory molecules) are expected to influence the transcription/translation of a number of protein-encoding genes. Using available bioinformatics strategies developed to predict the potential target sites in the 3' UTR of protein-coding genes a number of genes have been identified as potential targets of specific micro RNA molecules. Some of the potential target genes discussed in this study are DICER and RNASEN.

DICER gene encodes a protein possessing an RNA helicase motif containing a DEXH box in its

Table 4. Comparison of the results of the current study and previous serum miRNA ASD research.[21]

MicroRNAs	Present result	Previous report	Type of sample
hsa-miR-106b-5p	↑	↑	Serum
hsa-miR-181b-5p	No data	↓	Serum
hsa-miR-195-5p	No data	↑	Serum
hsa-miR-19b-3p	No data	↑	Serum
hsa-miR-320a	No data	↓	Serum
hsa-miR-328	↓	↓	Serum
hsa-miR-663a	No data	↓	Serum
hsa-miR-151a-3p	No data	↓	Serum
hsa-miR-433	No data	↓	Serum
hsa-miR-489	No data	↓	Serum
hsa-miR-572	No data	↓	Serum
hsa-miR-101-3p	No data	↑	Serum
hsa-miR-130a-3p	No data	↑	Serum
hsa-miR-27a-3p	No data	↑	Serum
hsa-miR-589-3p	↓	No data	Serum
hsa-miR-6849-3p	↓	No data	Serum
hsa-miR-3135a	↓	No data	Serum
hsa-miR-15a-5p	↓	No data	Serum
hsa-miR-328-3p	↓	No data	Serum
hsa-miR-183-5p	↓	No data	Serum
hsa-miR-3674	↓	No data	Serum
hsa-miR-96-5p	↓	No data	Serum
hsa-miR-3687	↓	No data	Serum
hsa-miR-6799-3p	↓	No data	Serum
hsa-miR-587-3p	↓	No data	Serum
hsa-miR-504-5p	↓	No data	Serum
hsa-miR-576-5p	↓	No data	Serum
hsa-miR-486-3p	↓	No data	Serum
hsa-miR-3909	↓	No data	Serum
hsa-miR-let-7i-3p	↓	No data	Serum
hsa-miR-29c-5p	↓	No data	Serum
hsa-miR-301a-3p	↓	No data	Serum
hsa-miR-3064-5p	↓	No data	Serum
hsa-miR-145-5p	↓	No data	Serum
hsa-miR-424-5p	↓	No data	Serum
has-miR-193b-3p	↓	No data	Serum
hsa-miR-487b-3p	↓	No data	Serum
hsa-miR-197-5p	↓	No data	Serum
has-miR-500a-5p	↓	No data	Serum
hsa-miR-664b-3p	↓	No data	Serum
hsa-miR-20b-3p	↓	No data	Serum
has-miR-671-3p	↓	No data	Serum
hsa-miR-199a-5p	↓	No data	Serum
hsa-miR-4489	↑	No data	Serum
has-miR-8052	↑	No data	Serum
hsa-miR-142-3p	↑	No data	Serum
hsa-miR-3620-3p	↑	No data	Serum
has-miR-365a-3p	↑	No data	Serum
hsa-miR-664a-3p	↑	No data	Serum
hsa-miR-374b-5p	↑	No data	Serum
hsa-miR-18b-3p	↑	No data	Serum
hsa-miR-619-5p	↑	No data	Serum
hsa-miR-210-5p	↑	No data	Serum

(↑), upregulated, (↓), downregulated

amino terminus and an RNA motif in the carboxy terminus. The encoded protein functions as a ribonuclease and is required by the RNA interference and small temporal RNA (stRNA) pathways to produce the active small RNA component that represses gene expression. RNASEN (drosha, ribonuclease type III) - the RNase III Drosha is the core nuclease that executes the initiation step of microRNA (miRNA) processing in the nucleus.

Additional benefit of this study is that the samples came from ASD patients aged between 3 and 11 years in comparison with previous report from Vasu et al. where serum miRNAs profile were analyzed in older individuals ranging from 6 to 16 years of age.

In conclusion, our findings supports the fact that levels of circulating miRNAs can be dysregulated under certain disease conditions. We found that miR-365a-3p, miR-619-5p, miR-664a-3p are the most upregulated and miR-3135a, miR-328-3p, miR-197-5p, miR-424-5p, miR-500a-5p are downregulated in the serum of patients with ASD. We suggest that this could reflect a metabolic imbalance of this miRNA *in vivo* and these miRNAs could be used as biomarkers of ASD. A greater sample size of patients with ASD is still needed in order to support these conclusions.

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Профилирование циркулирующих сывороточных микро РНК у детей с расстройством аутистического спектра при помощи шпилечного qRT-PCR анализа

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Дата получения: 21 ноября 2015
Дата приемки: 31 августа 2016
Дата онлайн публикации: 30 ноября 2016
Дата публикации: 27 марта 2017

Ключевые слова: расстройство аутистического спектра, miRNA, шпилечный qRT-PCR , сыворотка

Образец цитирования: Kichukova TM, Popov NT, Ivanov IS, Vachev TI. Profiling of circulating serum microRNAs in children with autism spectrum disorder using stem-loop qRT-PCR assay.
Folia Medica 2017;59(1):43-52.
doi: 10.1515/folmed-2017-0009

Введение: Разработка биомаркеров для расстройства аутистического спектра (ASD) по прежнему остается вызывающей проблемой и в наши дни. В последнее время изменения в экспрессии микроРНКН (miRNA) в периферической крови, сыворотке и мозговой ткани после смерти связаны с ASD. Известно, что miRNA секретируются разными видами клеток и могут опосредовать передачу информации в клетки-реципиенты и модулировать их физиологические функции. На этой базе принимается, что циркулирующие miRNA были бы полезни биомаркерами для диагноза и прогноза патологических состояний.

Цель: Целью настоящей работы является исследование возможности циркулирующих miRNA проявлять дифференцированный экспрессивный профиль в сыворотке пациентов с ASD.

Методы: Сравнительные уровни экспрессии 42 miRNA были исследованы при помощи шпилечного qRT-PCR (количественной полимеразной цепной реакции в „реальном времени") анализа в сыворотке пациентов с ASD.

Результаты: Результаты показывают, что у 11 miRNA пациентов с ASD значительно более высокая экспрессия по сравнению с экспрессией в контрольных объектах и соответственно у 29 miRNA более низкая экспрессия. Кроме того, анализ таргетного гена показывает, что miRNA с измененной сывороткой направляются к таким важным генам как альфа 1C субъединица вольтаж-зависимого кальциевого канала L типа (CACNA1C), бета 1 субъединица вольтаж-зависимого кальциевого канала (CACNB1) и другим генам, включенным в такие эпигенетические процессы как dicer 1, кодирующая рибонуклеазу типа III (DICER).

Заключение: Наши результаты показывают, что дифференциально экспрессированные miRNA в сыворотке могут быть включеными в ASD молекулярные пути, а сывороточные miR-424-5p, miR-197-5p, miR-328-3p, miR-500a-5p, miR-619-5p, miR-3135a, miR-664a-3p и miR-365a-3p могут использоваться в качестве потенциальных биомаркеров для ASD, так как они проявляют значительне изменения в экспрессивном профиле детей, диагностированых с ASD.