Identification of microRNAs Associated With Human Fragile X Syndrome Using Next Generation Sequencing

Maryam Sotoudeh Anvari (Dr_msotudeh@yahoo.com)
Tehran University of Medical Sciences
Hamed Vasei
Sharif university of technology
Hossein Najmabadi
University of Social Welfare and Rehabilitation Sciences
Reza Shervin Badv
Tehran University of Medical Sciences
Akram Gholipour
Islamic Azad University
Samira Mohammadi-Yeganeh
Shahid Beheshti University of Medical Sciences
Saeede Salehi
Tehran University of Medical Sciences
Mahmood Mohamadi
Tehran University of Medical Sciences
Hamidreza Goodarzynejad
Tehran University of Medical Sciences
Seyed Javad Mowla
Tarbiat Modares University

Research Article

Keywords: Blood miRNA, disease biomarker, expression profile, FMR1 gene, fragile X syndrome, high-throughput technologies

Posted Date: May 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-523429/v1

Abstract

Fragile X syndrome (FXS) is caused by a mutation in the FMR1 gene which can lead to a loss or shortage of the FMR1 protein. This protein interacts with specific miRNAs, and a change can cause a range of neurological disorders. Therefore, miRNAs could act as a novel class of potential biomarkers for common CNS diseases. The aim of this study was to test this theory by exploring the expression profiles of various miRNAs in Iranian FXS patients using deep sequencing-based technologies, and validate the miRNAs affecting expression of the FMR1 gene. Blood samples were taken from 15 patients with FXS (9 males, 6 females) and 12 controls. 25 miRNAs were differentially expressed in individuals with FXS compared to controls. Levels of 9 miRNAs were found to be significantly changed (3 upregulated and 6 downregulated). In FXS patients, the levels of hsa-miR-532-5p, hsa-miR-652-3p and hsa-miR-4797-3p were significantly upregulated while levels of hsa-miR-191-5p, hsa-miR-181-5p, hsa-miR-26a-5p, hsa-miR-30e-5p, hsa-miR-186-5p, and hsa-miR-4797-5p exhibited significant downregulation; and these dysregulations were confirmed by RT-qPCR. This study present altered miRNA expression in blood samples from FXS patients, which could be used for diagnostic, prognostic, and treatment purposes. Larger studies are required to confirm these preliminary results.

Introduction

Fragile X syndrome (FXS, OMIM 300624), a neurodevelopmental disorder characterized by intellectual disability and autism, is caused by a mutation in the fragile X mental retardation 1 (FMR1) gene located on the X chromosome at Xq27.3 wherein a DNA segment, known as the CGG triplet is expanded within the 5' untranslated region (5' UTR) of FMR1 gene. In people with FXS, CGG triplet is repeated more than 200 times (that is known as a “full” mutation); those with a premutation carry a CGG segment of intermediate size, with 55–200 of such repeats, while this DNA segment is normally repeated between 5 and 55 times. The carriers of premutation do not show an FXS phenotype, but are at risk of developing a neurodegenerative disorder in adulthood called fragile X tremor-ataxia syndrome (FXTAS). Full mutations cause the FMR1 gene to “turn off” resulting in shutting down its ability to produce a functional protein, fragile X mental retardation 1 protein (FMRP) [1]. FMRP is an RNA binding protein acting as a translational repressor of a variety of messenger RNA (mRNA) targets at the synapse that, though present in many tissues, is thought to play a pivotal role in synaptic maturation, functioning of neurons and their communication [2]. Therefore, its loss or shortage leads to FXS and induces a range of neurodevelopmental problems that include cognitive impairment, learning disabilities, and hyperactivity [3].

This protein associates not only with specific mRNAs and with microRNAs (miRNAs) but also with the components of the miRNA pathway such as the Dicer and Argonaute proteins [4]. The miRNAs, small (∼22-nucleotide) single-strand noncoding RNAs, negatively regulate target mRNA expression or activity of genes; they act as a guide by base-pairing with target mRNA. Type of silencing mechanism employed, i.e., translation inhibition or cleavage of target mRNA with subsequent degradation, is determined by the level of complementarity between the guide and mRNA target [5]. Many miRNAs have been shown to be engaged in neurodevelopmental disorders and disruption of miRNA, by impairing the communication between nerve cells, seems to contribute in the development of FXS as the primary cause of inherited intellectual disability [6]. In a twin study for identifying miRNA biomarkers of this disorder, the levels of miRNAs in the urine of a boy with FXS and his twin brother, who was a premutation carrier and had no clinical signs of fragile X, were compared [7]. Using next-generation sequencing, the investigators found twenty-eight miRNAs with different levels between the two twins. In the brother with fragile X, eight miRNAs had higher levels, the greatest increase (i.e., a 1.6-fold increase) was found in the levels of miR-125a compared to his twin brother [7]. This increase in miR-125a levels was also shown in two other sets of urine samples; ten Spanish FXS children aged 2–7 years and nine Finnish FXS children aged 4–17 years, as compared to healthy subjects but with a wide variation in urine miR-125a levels among young fragile X patients [7].

miRNAs could serve as a novel class of potential biomarker for the diagnosis and prognosis of common central nervous system (CNS) diseases including, neurodevelopmental disorders and offer novel therapeutics [8–10]. The aim of the present study was to explore the expression profiles of various miRNAs in FXS patients using deep sequencing-based technologies and validate the miRNAs that affect the expression of FMR1gene in a sets of blood sample among an Iranian population.

Methods And Materials

2.1 Study participants

Between November 2016 and June 2019, a total of 27 individuals, some of whom are related, were recruited for this study from several different cities across Iran. For this study, we recruited fifteen patients with different types of FXS from four families. Diagnoses confirmed via southern blot analysis at the University of Social Welfare and Rehabilitation Sciences (USWR), and the patients included nine males (between 25–36 years old) and six females (between 48–80 years old).

Twelve sex- and age-matched healthy individuals were taken as controls. All participants provided written informed consent, in agreement with the Declaration of Helsinki for research involving human subjects, explicitly gave permission for RNA analyses and gathering the relevant clinical data. The National Institute for Medical Research Development (NIMAD) ethics committee approved the study protocol and use of human blood for research (No. 957806). Participants were asked to fill out a questionnaire detailing their family history of FXS, associated disease, food habits, and medication history for the evaluation of miR interactions. Data regarding the result of food habits and medication history were not included in the analyses due to concerns about the accuracy of these data.

2.2 Specimen collection and DNA/RNA extraction

Each participant provided 10-15ml cubital vein blood samples that placed in ethylenediamine tetra-acetic acid (EDTA)-containing (lavender top) tubes. The genomic DNA was isolated from peripheral blood leukocytes using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions by using the spin column extraction method. Erythrocytes were lysed in Erythrocyte-Lysis-Buffer (Buffer EL, Qiagen) and total RNA including small RNAs, was extracted using miRNeasy Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol.

2.3 CGG Repeat Primed PCR

Molecular analysis of the FMR1gene CGG repeat locus was performed in all participants to reconfirm diagnosis and to provide accurate sizing of alleles. To assess the number of CGG repeats in the 5' UTR of theFMR1, a three-primer CGG Repeat Primed PCR [RP-PCR] was carried out on the purified genomic DNA. Samples were PCR-amplified by an Eppendorf Mastercycler gradient PCR system (Eppendorf, Hamburg, Germany) using the AmplideX® PCR/CE FMR1 kit (Asuragen, Austin, TX) and AmplideX® PCR/CEFMR1reagents (cat. no. 49402), following the manufacturer’s instructions. PCR products were then separated in a 3310XL capillary electrophoresis system [Applied Biosystems Genetic Analyzer, Foster City, CA, USA] based on conditions described in the kit manual. GeneMapper software (Applied Biosystems) was used to analyze and convert the separated PCR products into CGG repeat length using. In accordance with the current ACMG Guidelines, the CGG triplets that repeated 45–54 and 55–200 times were considered as intermediate and premutation respectively. Those with more than 200 repeats were defined as full mutation. Samples with both premutation and full mutation alleles were identified as full mutation mosaics.

2.4 Total RNA quantity and quality control

All isolated RNA samples were eluted in RNase-free water and RNA concentrations were determined with Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA quantity was evaluated by calculating absorbance at λ = 260 nm, and the quality was assessed by a ratio of λ = 260/280 nm being close to 2.0 -2.3. The RNA concentration of each sample was more than 50 ng. The integrity of the RNA, as a key feature that affects the performance of sequencing and RT-qPCR, was assessed via two methods: First, by running extracted RNA through 1% agarose gel and then staining with ethidium bromide to observe the 28S ribosomal RNA band at 4.5 kb, and the 18S rRNA band at 1.9 kb. Second, by using the Agilent 4200 TapeStation System (Agilent Technologies, Santa Clara, CA, G2991A) to assess the electrophoretic profile of the 18S and 28S RNA and generating an RNA Integrity Number (RIN). All RNA samples revealed RIN values of greater than eight and the miRNA extractions were stored at − 80◦C until processing.

2.5 Library preparation for next-generation sequencing

The TruSeq Small RNA Library Preparation Kit (Illumina, San Diego, USA) was used for generating miRNA sequencing libraries directly from total RNA as per manufacturer’s protocol for this kit (TruSeq® Small RNA Library Prep Reference Guide 15004197 v02). Briefly, after ligation of the 5' and 3' RNA adapters using T4 RNA ligase, reverse transcription was performed to generate cDNA; cDNA libraries were subsequently amplified by PCR. The products were then purified. After acrylamide gel purification, eight libraries were pooled in equimolar amount to create one lane and validation was compatible with multiplexed sequencing. Finally, the libraries were checked and normalized according to protocol.

2.6 miRNA profiling through next generation sequencing (miRNA-seq) and analyses

The miRNA cDNA libraries were sequenced on an Illumina MiniSeq platform in the Pars gene company, Dr Faghihi’s Genetics Laboratory, Shiraz, Iran. With this platform, DNA fragments of the libraries go through clonal amplification by bridge PCR followed by sequencing using reversible terminator. It consists of sequencing by synthesis (SBS) technology using only two-channel (i.e., red and green) which needs only two images for determination of all four base calls reducing number of cycles, cost and time required for data processing and yet delivering high accuracy and quality [11]. Within every cycle of sequencing, for each cluster, base calls were created by an inbuilt real-time analysis software and raw data were stored in the format of individual base call files (*.bcl). The BCL files were converted to standard FASTQ file formats (a text-based sequencing data file format that stores both raw sequence data and quality scores) for downstream analysis.

In the next step, the output data were streamed into Illumina's BaseSpace Sequence Hub for cloud-based data management and analysis. FASTQ files were cleaned by adapter removal using CutAdapt 1.6. The Phred numerical quality scoring system was used as base call quality filter [12, 13]; reads with Q scores of < 33 (checked both before and after adapter trimming) were removed. After removal, the adaptors and filtering out low quality sequences, the processed reads were aligned against miRBase database and human genome hg19 by using version 2.2.3 of Short Read Mapping Package (SHRiMP). Mapped files were then sorted and indexed as binary format (BAM) files. To perform differential expression analysis on aligned RNA samples from FXS patients and controls, we used DESeq2 R and Rankprod packages.

2.7 Relative quantification of miRNA by reverse-transcription PCR analyses

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) is a well-established method for miRNA profiling with the highest sensitivity and accuracy and with the widest dynamic range [14]. We used stem-loop RT-PCR miRNA assay for quantification of miRNA expression levels [15] using a commercially available qRT-PCR miRNA detection kit and primer sets (Zist Fanavari Pishgam Company, Tehran, Iran). To normalize the expression data, a commonly-used endogenous reference gene, U6 small nuclear RNA (U6-snRNA), was adopted [16]. Real-time PCR data with use of a LightCycler® 96 instrument (Roche Applied Science, Indianapolis, IN, USA), were analyzed by $2^{-\Delta\Delta CT}$ method using the following equation: ΔΔCT= ((Ct miRNA of concern − Ct U6-snRNA) in patients) − ((Ct miRNA of concern − Ct U6-snRNA) in controls) [17]. These relative gene expression data analyses were double checked by using relative expression software (REST) tool [18].

To evaluate the degree of similarity between RT-qPCR and miRNA-seq results, we compared miRNAs' expression data generated through both approaches. Downregulation was defined when

$2^{-\Delta\Delta CT}$ was less than zero and upregulation presented as more than zeao result of $2^{-\Delta\Delta CT}$

Result

3.1 CGG allele sizing and Clinical characteristics of studied subjects

The clinical and demographic findings of the studied patients are listed in Table 1. The specific genotype category results, characterized as full mutation (n = 10), premutation (n = 3) and full mutation mosaic (n = 2). In this study all (100%) available full mutation and full mutation mosaic FXS patients presented with ASD and one third (33%) had ADHD.

Table 1
Clinical characteristics of studied subjects

Family no	Case no.	Relationship	Gender	Age/year	CGG repeats	Phenotype	Genotype	ADHD	Seizure	Autism
1	1	Grand mother	Female	80	68.66/68.66	Normal looking	Premutation	N	N	N
	2	Daughter	Female	48	312/30.2	Affected	Full mutation	N	P	P
	3	Son	Male	44	311.8	Affected	Full mutation	P	P	P
	4	Daughter (mother of cases 5 and 6)	Female	54	104.4/104.4	Normal looking	Premutation	N	N	N
	5	Grand son	Male	25	310.8	Affected	Full mutation	P	N	P
	6	Grand son	Male	27	310.8	Affected	Full mutation	P	P	P
2	7	Son	Male	36	312.36	Affected	Full mutation	Not available	Not available	Not available
	8	Son	Male	34	319.4	Affected	Full mutation	Not available	Not available	Not available
	9	Mother of cases7 and 8	Female	62	318/22	Normal looking	Full mutation mosaic	Not available	Not available	Not available
3	10	Son	Male	29	317	Affected	Full mutation	P	N	P
	11	Son	Male	32	318	Affected	Full mutation	N	N	P
	12	Mother of cases10 and 11	Female	57	88/22	Normal looking	Premutation	N	N	N
4	13	Son	Male	28	316	Affected	Full mutation	P	N	P
	14	Son	Male	26	316	affected	Full mutation	N	N	P
	15	Mother of cases 13and 14	Female	59	227.2/30	Normal looking	Full mutation mosaic	N	N	P

ADHD: attention deficit hyperactivity disorder, P: Positive, N: Negative.

3.2 Differentially expressed deep sequencing of miRNA

In terms of selecting miRNAs using NGS data, although p-values can be corrected for multiple hypothesis testing in order to control for type I errors (i.e., a false positive result), even most restrictive methods for correcting p-values such as Bonferroni correction [3] leaves us with hundreds of candidate miRNAs. A reasonable way of choosing miRNAs for further exploration, would be choosing miRNAs with the lowest p-values. The number of chosen miRNAs depends on what type of further exploration we are going to apply and its cost. However, because the lowest p-values vary significantly by adding or removing some samples, they are too sensitive to noise, and their differences are too small, we used a slightly different approach. In this study, to determine differentially expressed miRNAs, we conducted differential expression analysis using four different software tools, namely, DESeq2 [4], edgeR [5], limma-trend [6] and limma-voom [6]. We picked 30 miRNAs with the lowest p-values in all four methods and computed intersections of these 30 candidates. Interestingly, the agreement among the methods in calling differentially expressed miRNAs was high, despite their differences in logic algorithm and also in the order and magnitude of their resulted p-values. These intersections consist of 25 miRNAs which showed noteworthy agreement between different methods (Table 2), but with different orders using different methods. We then sorted this list based on overall mean expression level for all cases and controls and chose the first nine highly expressed miRNAs. The intuition behind this selection was that we expected these miRNAs present more powerful signals with lower noises as opposed to miRNAs with lower level of expressions. miR-125, though was not in 30 highly dysregulated candidate

miRNAs in this study, was also analyzed due to its importance in urine in Putkonen et al. [7] study and the existence of small number of NGS studies about FXS. As seen in Table 3, all members of miR-125 family were found to be downregulated in patients with FXS.

Table 2
Twenty-five proposed miRs in deep sequencing using four differential expression analyses.

miR no.	miR name	Regulation mode in case samples	Overall mean expression	Case mean expression	Control mean expression	Log fold change
1	hsa-miR-191-5p	down	8876.373051	1276.631887	18376.0495	3.940923422
2	hsa-miR-26a-5p	down	7813.136539	1442.80566	15776.05014	3.487235906
3	hsa-miR-181a-5p	down	6185.741177	1183.864533	12438.08698	3.702044447
4	hsa-miR-30e-5p	down	3199.723878	357.0752659	6753.034643	4.141018947
5	hsa-miR-186-5p	down	1701.390164	559.7558769	3128.433024	2.572602978
6	hsa-miR-532-5p	up	442.5755252	711.2084902	106.7843188	-2.843199798
7	hsa-miR-652-3p	up	167.1577007	258.3405092	53.17919022	-2.189509984
8	hsa-miR-4797-3p	up	101.9033055	176.4535552	8.715493351	-3.969467534
9	hsa-miR-4797-5p	up	101.3007798	175.4015655	8.674797652	-3.96298818
10	hsa-miR-139-5p	up	68.4866255	114.8116871	10.58029852	-3.162100671
11	hsa-miR-210-3p	up	57.60665272	96.72746231	8.705640726	-3.349322696
12	hsa-miR-339-5p	up	54.82137012	86.19747425	15.60123996	-2.301431168
13	hsa-miR-548av-5p	down	23.67638935	2.193939122	50.52945214	3.943386072
14	hsa-miR-548k	down	23.65788696	2.193939122	50.48782175	3.942268815
15	hsa-miR-324-5p	up	20.9344738	33.52838669	5.192082674	-2.508217082
16	hsa-miR-148a-5p	down	20.2027045	4.392828836	39.96504909	3.084674792
17	hsa-miR-148b-5p	down	20.13664435	3.12648513	41.39934337	3.425508366
18	hsa-miR-24-2-5p	down	15.03578544	5.367524883	27.12111113	2.4804667
19	hsa-miR-4485-3p	up	14.65427465	24.97665627	1.751297614	-3.515081463
20	hsa-miR-30d-3p	down	8.531824974	2.279761135	16.34690477	2.904832494
21	hsa-miR-548av-3p	down	8.264467723	1.266091684	17.01243777	3.266046604
22	hsa-miR-141-3p	down	8.06856032	0.45925296	17.58019452	3.776080468
23	hsa-miR-548f-5p	down	7.741525273	0.151715543	17.22878743	4.027732978
24	hsa-miR-548o-3p	down	7.364951245	0.930963985	15.40743532	3.212444121
25	hsa-miR-559	down	7.351270372	0.773720351	15.5732079	3.335571389

Table 3
hsa-miR-125 family in deep sequencing using four different methods for differential expression analysis.

	Mir	U/D	LFC	Limma-Trend	LFC	Limma-Voom	LFC	edgeR	LFC	DESeq2
1	125a-5p	Down	2.27	4.66E-06	2.3	2.72E-06	1.97	2.67E-06	1.54	0.00030171
2	125b-2-3p	Down	0.6	2.42E-05	0.82	0.0540679	3.81	2.60E-06	1.9	0.01564258
3	125a-3p	Down	0.4	0.00922126	0.3	0.43746489	1.43	0.01400016	1.01	0.13573104
4	125b-5p	Down	0.31	0.29037969	0.29	0.4493121	0.2	0.58630045	-1.27	0.95661198
U/D: Up /Down LFC: log fold change										

3.3 Proposed miR reconfirmation by RT-PCR

The schematic heat map shows expression-related changes in miRNA transcriptome based on three groups of full mutations, permutation, full mutation mosaic. (Fig. 1). Data regarding the most expressed miRNAs in ten full mutation samples are given in Table 4. hsa-miR-181a-5p downregulated in 90% of patients and the highest upregulated miRs were hsa-miR-4797-3p and hsa-miR-652-3p. Through RT-PCR, a significant downregulation of miR-181a-5p, miR-26a-5p, and miR-30e-5p as well as miR-191-5p and miR-146a was observed in all premutation patients (n = 3), while miR-652-3p was upregulated in all three premutation patients. In two cases identified as full mutation mosaics, five human (Homo sapiens) miRNAs, i.e., miR-181a-5p, miR-26a-5p, miR-532-5p miR-191-5p and miR-4797-5p, revealed to be downregulated, but no common upregulated miRNA was found.

Table 4
List of differentially expressed miRNAs among the full mutation group

MicroRNA	Dysregulation	Percent of affected patients (%)
hsa-miR-181a-5p	Downregulated	90
hsa-miR-26a-5p	Downregulated	80
hsa-miR-4797-5p	Downregulated	70
hsa-miR-186-5p	Downregulated	60
hsa-miR-191-5p	Downregulated	60
hsa-miR-4797-3p	Upregulated	50
hsa-miR-652-3p	Upregulated	50
hsa-miR-30e-5p	Up/Down*	40/40
* 40% of patients expressed downregulation and 40% upregulation.		

Some miRNAs including miR-191-5p, miR-26a-5p, and miR-181-5p were downregulated, whereas miR-652-3p was upregulated in all six females. These miRNAs seemed to be gender dependent. Similarly, age dependency was seen in those aged over 55 years with miR-191-5p, miR-26a-5p, and miR-181-5p. In addition, no significant correlation was existed between any miRNAs and ADHD. The presence of seizure disorder was documented in three patients that all showed downregulation of miR-26a-5p and miR-186-5p. Autism spectrum disorder was significantly present in patients with downregulated miR-181-5p. (p-value < 0.05). Finally, the end result of relative expression with $2^{-\Delta\Delta CT}$ calculation are summarized in Table 5 and Fig. 2 presents diagram of normalized CT comparison in cases and controls. In search in targetscan7.7 database, (http://www.targetscan.org/vert_72/) for prediction of miRNA target, no target was found for miR-181-5p.

Table 5
Relative quantification of 9 miRs confirmed by RT-PCR

Case	hsa-miR-191-5p	hsa-miR-30e-5p	hsa-miR-4797-3p	hsa-miR-4797-5p	hsa-miR-532-5p	hsa-miR-26a-5p	hsa-miR-652-3p	hsa-miR-186-5p	hsa-miR-181a-5p	ADHD	Seizure	Autism	Genotype
1	down	down	up	up	up	down	up	down	down	neg	neg	neg	Premutation
2	down	down	up	up	up	down	up	down	down	neg	pos	pos	Full mutation
3	down	no diff	up	up	no diff	down	up	down	down	pos	pos	pos	Full mutation
4	down	down	up	up	up	down	up	up	down	neg	neg	neg	Premutation
5	down	up	up	down	up	down	up	no diff	down	pos	neg	pos	Full mutation
6	up	up	down	down	down	down	no diff	down	up	pos	pos	pos	Full mutation
7	up	no diff	no diff	no diff	up	up	up	no diff	down	N/A	N/A	N/A	Full mutation
8	down	no diff	up	down	down	down	down	down	down	N/A	N/A	N/A	Full mutation
9	down	down	up	down	down	down	up	down	down	N/A	N/A	N/A	Full mutation mosaic
10	up	up	down	down	up	up	up	up	down	pos	neg	pos	Full mutation
11	down	up	no diff	down	up	down	down	down	down	neg	neg	pos	Full mutation
12	down	down	no diff	down	down	down	up	no diff	down	neg	neg	neg	Premutation
13	down	down	no diff	down	no diff	down	no diff	down	down	pos	neg	pos	Full mutation
14	no diff	down	up	down	down	down	down	no diff	down	neg	neg	pos	Full mutation
15	down	no diff	no diff	down	down	down	no diff	no diff	down	neg	neg	pos	Full mutation mosaic

ADHD, Attention deficit hyperactivity disorder N/A, not available; no diff, no differences; down, downregulated; up, upregulated; neg, negative; pos, positive

Discussion

The current study identified FXS-specific changes in miRNAs among Iranian blood samples. We identified twenty-five differentially expressed miRNAs sequenced in blood of individuals with FXS compared to the controls, and we found minor downregulation of miR-125a-5p. The main finding of this study is that levels of three miRNAs (i.e., hsa-miR-532-5p, hsa-miR-652-3p and hsa-miR-4797-3p) were significantly upregulated in FXS group versus healthy controls while levels of six miRNAs (i.e., hsa-miR-191-5p, hsa-miR-181-5p, hsa-miR-26a-5p, hsa-miR-30e-5p, and hsa-miR-186-5p, hsa-miR-4797-5p) exhibited significant downregulation in FXS patients compared to controls; and these dysregulations were confirmed by RT-qPCR.

MicroRNAs regulate mRNAs at the post-transcriptional level and therefore affecting protein translation [19]. Changed miRNA expression patterns epigenetically affect almost every aspect of CNS function (i.e., in neurogenesis, synaptogenesis and neuronal migration) and its development [20–22]. For instance, miR-532, is reliably expressed in the human brain, localized as distinct granules in distal axons and growth cones, and proposed to play a role in axon growth and guidance [23]. ZFHX3 gene, among 5 target genes of the hsa-miR-532-5p from the MiRTarBase microRNA Targets dataset, encodes a transcription factor that regulates neuronal differentiation [24]. Hence, unsurprisingly, in many neuropsychiatric disorders it has been demonstrated that the dysregulation of miRNAs is associated with changes in behaviour,

learning, and memory [25]. Understanding the miRNA-mediated translational regulation mechanism(s) whereby FMRP modulate the translation of its mRNA ligands would help in understanding of the molecular pathogenesis of FXS and also of converging mechanisms shared by FXS and its related disorders [26]. FXS is a well-known monogenic cause of autism spectrum disorder (ASD) [27]; list of other related disorders to FXS may include but not limited to FXTAS, Rett syndrome, Down syndrome, attention deficit hyperactivity disorder (ADHD), and schizophrenia.

FXS is the first neurodevelopmental disease found to be linked to the dysfunction of miRNA pathway [28]. The in vivo evidence of miRNA involvement in FXS pathogenesis was first provided in a study of the zebrafish model by identifying and isolating numerous miRNAs, including miR-fmr1-27 and miR-fmr1-42 in this model [29]. Subsequent studies in the Fmr1 KO mouse models found that disruption of the regulating of miR-125a, miR-125b, and miR-132 causes early neural development and synaptic physiology [30] and that there is an interaction between miR-34b, miR-340, and miR-148a with the Met 3' UTR of the FMR1 gene [31]. Moreover, by isolating mesenchymal stem cells from peripheral blood and differentiating these cells into neuronal cells, Fazeli et al. [32] recently analyzed the expression of miR-510 by qPCR method. The authors reported an enhanced expression of miR-510, located on chromosome X in the 27.3Xq region, flanking to a fragile X site, in the female carriers of FMR1 full mutation [32].

There are twelve brain miRNAs identified to interact with FMRP in mouse brain including, miR-125a, miR-125b, and miR-132 [30]. To the best of our knowledge, there is only one published study on miRNA expression profiling in FXS patients using deep sequencing [7]. In a most recent study Putkonen et al. [7] showed upregulation of miR-125a in urine from children with FXS. The investigators did not examine differential miRNA expression changes in FXS blood samples or the correlations of miR-125a levels in urine with those of in the cell-free circulation (i.e., in serum and plasma) and other body fluids [7]. In our study, we found a minor downregulation of miR-125a-5p in blood of individuals with FXS. One preliminary hypothesis for this finding is that due to urinary secretion or of miR-125a-5p, its blood level expression decreased similar to what happens for blood-urine balance of electrolytes.

In line with our results in Alvarez-Mora MI study on FXTAS patients using deep sequencing, the authors also observed a slight but not significant reduction of miR-125a-5p in blood of FXTAS patients [33]. Mundalil Vasu et al. [34], found thirteen differentially expressed serum miRNAs in individuals with autism spectrum disorder (ASD) compared to the controls and miR-125a was not among the dysregulated miRNAs.

Even though larger studies are needed to confirm our results and investigate the effect of other miRNAs, the changes in miRNAs seen among our patients provide evidence that these miRNAs could have roles in developmental processes, nervous system homeostasis, and the function of nerve cells in those with FXS. Our finding demonstrates significant involvement of hsa-miR-30e-5p in FXS, which was found to be the most significantly upregulated miRNA in FXS patients compared with controls. miR-30 family play a major regulating role in the tissue and organ development and the pathogenesis of various clinical diseases [35]. Several studies have shown that hsa-miR-30e-5p among other miRNAs might be associated with the onset and progression of Parkinson's disease and schizophrenia [36–38]. Sun et al. [39] observed significant increase in expression of has-miR-30e in both plasma samples and peripheral blood mononuclear cells (PBMC) samples amongst schizophrenia patients.

Our results also showed deregulated hsa-miR-191-5p. Although, as far as we are aware of, no evidence for hsa-miR-191-5p contribution to FXS has been reported so far, alterations in expression level of hsa-miR-191-5p has earlier been found in patients with neuropsychiatric disorders sharing genetic overlap with FXS, including ASD, ADHD, schizophrenia, bipolar disorder, and major depressive disorder [40–43].

Moreover, in concordance with our results, the association between autistic traits and X-linked SNPs in the gene family linked with FXS, is likely to be owing to a disruption in the recognition between has-miR-181 and the corresponding seed match sequences in these genes [44]; miR-181d and FMRP cooperatively regulate the axon elongation process [10, 45]. Altered expression pattern for miR-181 and miR-191 in hippocampal neuron development has shown to occur [46].

In one recent study, 13 miRNAs were differentially expressed in maternal plasma samples from pregnant women with fetal Down syndrome versus healthy control subjects; among the others, hsa-miR-191 was upregulated and hsa-miR30e downregulated [48]. In another study, miR-26b-5p, miR-185-5p, and miR-191-5p were identified as potential biomarkers for ADHD in peripheral blood mononuclear cells [40]. Altered expression of miR-26a and miR-26b have been shown in peripheral blood of major depressive patients during antidepressant therapy, in Alzheimer's disease and in Parkinson's disease [41]. Finally, hsa-miR-532-5p and hsa-miR-652-3p have been shown to be upregulated in schizophrenia [48, 49] .

It is noteworthy that despite large number of miRNAs associated with FXS and its related disorders that have been identified in multiple expression studies, only a few miRNAs are common between various studies. This discrepancy can be explained in part by the polygenic and complex nature of neuropsychiatric disorders [50]. The expression profiles of the miRNAs in our study confirm some existing findings but

conflict with others. For example, our result regarding expression level of has-miR-30e in FXS patients is consistent Sun et al. [39] report that miR-30e was upregulated in PBMCs from patients with schizophrenia. In contrast, Perkins et al. [37] have found that miR-30e is downregulated in the prefrontal cortex of subjects with schizophrenia compared with healthy subjects. The exact reason for these conflicting results remains to be determined but it may be because of differences in screening standards (i.e., patients' ethnicity, geographical region, and screening criteria), techniques used for miRNA detection and profiling, and experimental design. Furthermore, as previously mentioned by Alvarez-Mora et al. [34], it is documented that the expressions of miRNAs are tissue-specific and/or temporally regulated which may partially explain the differences seen between the findings of different studies.

Several limitations to our study should be addressed. First, the sample size is relatively small. Despite this, it is the first evidence of altered miRNA expressions in blood samples from FXS patients. Second, although patients consisted of individuals from all across the country, they were only Iranian in origin. Third, we examined miRNA expression changes in non-neuronal cells due to the fact that neuronal tissue is not easily accessible. However, it has been shown that miRNA expression changes in the peripheral circulation are highly correlated with those of neuronal tissue from patients with various neuropsychiatric disorders [20].

Conclusions

Our study is among the first to present the characterization of the miRNA's expression profiles in blood samples of FXS patients using deep sequencing-based technologies. Levels of nine miRNAs were found to be changed (i.e., 3 upregulated and 6 downregulated) in FXS blood. Altered peripheral miRNA levels have been identified in numerous neuropsychiatric disorders, including FXTAS, ASD, ADHD, Down syndrome, depression, and schizophrenia. Our results provide a new perspective for the role of miRNA profiling in the pathophysiology of FXS, but larger studies are required to confirm these preliminary results and explore the influence of the other dysregulated miRNAs. If confirmed, it could open the possibility of using miRNAs as novel non-invasive FXS biomarkers or broad-spectrum therapeutic agents.

Declarations

Conflict of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgements

The authors acknowledge Ms Samaneh Mahmoudian , Ms Sanaz Arzhangi and Mr Mohammad Silawi and Dr Dr. Mohammad Ali Faghihi for their valuable technical assistance.

Author Contributions

M.S.A, H.N, and S.J.M. designed the project; H.N, R.S.B and M.M. selected FXS cases and samples; S.S, M.S.A, H.N and Bayan gen company performed the tests; H.V, M.S.A and A.G. analyzed and interpreted the data; S.M.Y designed primers. H.V prepared all figures; M.S.A and H.R.G wrote and revised the paper.

References

1. Tassone, F. Advanced technologies for the molecular diagnosis of fragile X syndrome. Expert Rev. Mol. Diagn. **15**(11), 1465-1473 (2015).
2. Darnell, J.C., Klann, E. The translation of translational control by FMRP: therapeutic targets for FXS. Nat. Neurosci. **16**(11), 1530-1536 (2013).
3. Zhan, X., Asmara, H., Cheng, N., Sahu, G., Sanchez, E., Zhang ,F.X., et al. FMRP(1-297)-tat restores ion channel and synaptic function in a model of Fragile X syndrome. Nat. Commun.**11**(1), 1-16 (2020).
4. Duan, R., Jin, P. Identification of messenger RNAs and microRNAs associated with fragile X mental retardation protein. Methods. Mol. Biol.**342**, 267-276 (2006).
5. Macfarlane, L.A., Murphy, P.R. MicroRNA: Biogenesis, Function and Role in Cancer. Curr. Genom. **11**(7), 537-561(2010).
6. Zhang, S.F., Gao, J., Liu, C.M. The Role of Non-Coding RNAs in Neurodevelopmental Disorders. Front. genet. **10**, 1033 (2019).
7. Putkonen, N., Laiho ,A., Ethell ,D., Pursiheimo, J., Anttonen, A.K., Pitkonen, J., et al. Urine microRNA Profiling Displays miR-125a Dysregulation in Children with Fragile X Syndrome. Cells. **9**(2), 289 (2020).
8. Jin, X.F., Wu ,N., Wang, L., Li ,J. Circulating microRNAs: a novel class of potential biomarkers for diagnosing and prognosing central nervous system diseases. Cell. Mol. Neurobiol. **33**(5), 601-613 (2013).

9. Gurwitz, D. Genomics and the future of psychopharmacology: MicroRNAs offer novel therapeutics. Dialogues. Clin. Neurosci. **21**(2), 131-148 (2019).
10. Zhou, Y., Hu ,Y., Sun ,Q., Xie, N. Non-coding RNA in Fragile X Syndrome and Converging Mechanisms Shared by Related Disorders. Front. genet.**10**, 139 (2019).
11. Ambardar, S., Gupta, R., Trakroo, D., Lal, R., Vakhlu, J. High Throughput Sequencing: An Overview of Sequencing Chemistry. Indian. J. Appl. Microbiol. **56**(4), 394-404 (2016).
12. Ewing, B., Hillier, L., Wendl, M.C., Green, P. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome. Res. **8**(3), 175-185 (1998).
13. Ewing, B., Green, P. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome. Res. **8**(3), 186-194 (1998).
14. Pritchard, C.C., Cheng, H.H., Tewari, M. MicroRNA profiling: approaches and considerations. Nat. Rev. Genet. **13**(5), 358-369 (2012).
15. Varkonyi-Gasic, E., Wu, R., Wood, M., Walton, E.F., Hellens, R.P. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. Plant. methods. **3**,1-12 (2007).
16. Schwarzenbach, H., da Silva, A.M., Calin, G., Pantel, K. Data Normalization Strategies for MicroRNA Quantification. Clin. Chem. **61**(11), 1333-1342 (2015).
17. Livak, K.J., Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. **25**(4), 402-408 (2002).
18. Pfaffl, M.W., Horgan, G.W., Dempfle, L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic. Acids. Res.**30**(9), e36-e36. (2002).
19. Guo, H., Ingoliam N.T., Weissman, J.S., Bartel, D.P. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature. **466**(7308), 835-840 (2010).
20. Roy, B., Yoshino, Y., Allen, L., Prall, K., Schell, G., Dwivedi, Y. Exploiting Circulating MicroRNAs as Biomarkers in Psychiatric Disorders. Mol. Diagn. Ther. **24**(3), 279-298 (2020).
21. Blandford, S.N., Galloway, D.A., Moore, C.S. The roles of extracellular vesicle microRNAs in the central nervous system. Glia. **66**(11), 2267-2278 (2018).
22. Follert, P., Cremer, H., Béclin, C. MicroRNAs in brain development and function: a matter of flexibility and stability. Front. Mol. Neurosci. **7**, 5 (2014).
23. Sasaki, Y., Gross, C., Xing, L., Goshima, Y., Bassell, GJ. Identification of axon-enriched microRNAs localized to growth cones of cortical neurons. Dev. Neurobiol. **74**(3), 397-406 (2014).
24. Miura, Y., Tam, T., Ido, A., Morinaga, T., Miki, T., Hashimoto, T., et al. Cloning and characterization of an ATBF1 isoform that expresses in a neuronal differentiation-dependent manner. J. Biol. Chem. **270**(45), 26840-26848 (1995).
25. Wang, W., Kwon, E.J., Tsai, L.H. MicroRNAs in learning, memory, and neurological diseases. Learn. Mem. **19**(9), 359-368 (2012).
26. Duan, R., Jin, P. Identification of messenger RNAs and microRNAs associated with fragile X mental retardation protein. MicroRNA Protocols: Springer, 267-276.(2006)
27. Wang, T., Bray, S.M., Warren, S.T. New perspectives on the biology of fragile X syndrome. Curr. Opin. Genet. Dev. **22**(3), 256-263 (2012).
28. Jin, P., Alisch, R.S., Warren, S.T. RNA and microRNAs in fragile X mental retardation. Nat. Cell Biol. **6**(11), 1048-1053 (2004).
29. Lin, S.L., Chang, S.J., Ying, S.Y. First in vivo evidence of microRNA-induced fragile X mental retardation syndrome. Mol. Psychiatry.**11**(7), 616-617 (2006).
30. Edbauer, D., Neilson, J.R., Foster, K.A., Wang, C.F., Seeburg, D.P., Batterton, M.N., et al. Regulation of synaptic struecture and function by FMRP-associated microRNAs miR-125b and miR-132. Neuron. **65**(3), 373-384 (2010).
31. Muddashetty, R.S., Nalavadi, V.C., Gross, C., Yao ,X., Xing, L., Laur, O., et al. Reversible inhibition of PSD-95 mRNA translation by miR-125a, FMRP phosphorylation, and mGluR signaling. Mol. Cell. **42**(5), 673-688 (2011).
32. Fazeli, Z., Ghaderian, S.M.H., Najmabadi,H., Omrani, MD. High expression of miR-510 was associated with CGG expansion located at upstream of FMR1 into full mutation. J. Cell. Biochem. **120**(2), 1916-1923 (2018).
33. Alvarez-Mora, M.I., Rodriguez-Revenga, L., Madrigal, I., Torres-Silva,F., Mateu-Huertas ,E., Lizano, E., et al. MicroRNA expression profiling in blood from fragile X-associated tremor/ataxia syndrome patients. Genes. Brain. Behav.**12**(6), 595-603 (2013).
34. Mundalil Vasu, M., Anitha, A., Thanseem, I., Suzuki, K., Yamada, K., Takahashi, T., et al. Serum microRNA profiles in children with autism. Mol. Autism. **5**(1), 1-9 (2014).
35. Mao, L., Liu, S., Hu, L., Jia, L., Wang, H., Guo, M., et al. miR-30 Family: A Promising Regulator in Development and Disease. Biomed Res. Int. (2018)

36. Acharya, S., Salgado-Somoza, A., Stefanizzi, F.M., Lumley, A.I., Zhang, L., Glaab, E., et al. Non-Coding RNAs in the Brain-Heart Axis: The Case of Parkinson's Disease. Int. J. Mol. Sci. **21**(18), 6513 (2020).
37. Perkins, D.O., Jeffries, C.D., Jarskog, L.F., Thomson, J.M., Woods, K., Newman, M.A., et al. microRNA expression in the prefrontal cortex of individuals with schizophrenia and schizoaffective disorder. Genome Biol. **8**(2), 1-11(2007).
38. Liu, S., Zhang, F., Shugart, Y.Y., Yang, L., Li, X., Liu, Z, et al. The early growth response protein 1-miR-30a-5p-neurogenic differentiation factor 1 axis as a novel biomarker for schizophrenia diagnosis and treatment monitoring. Transl. Psychiatry. **7**(1), e998-e998 (2017).
39. Sun, X.Y., Lu, J., Zhang, L., Song, H/T., Zhao, L., Fan, H.M., et al. Aberrant microRNA expression in peripheral plasma and mononuclear cells as specific blood-based biomarkers in schizophrenia patients. J. Clin. Neurosci. **22**(3), 570-574 (2015).
40. Sánchez-Mora, C., Soler Artigas, M., Garcia-Martínez, I., Pagerols, M., Rovira, P., Richarte, V., et al. Epigenetic signature for attention-deficit/hyperactivity disorder: identification of miR-26b-5p, miR-185-5p, and miR-191-5p as potential biomarkers in peripheral blood mononuclear cells. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. **44**(5), 890-897 (2019).
41. Maffioletti, E., Tardito, D., Gennarelli, M., Bocchio-Chiavetto, L. Micro spies from the brain to the periphery: new clues from studies on microRNAs in neuropsychiatric disorders. Front. Cell. Neurosci.**8**, 75 (2014).
42. Zhao, D., Lin, M., Chen, J., Pedrosa, E., Hrabovsky, A., Fourcade, H.M., et al. MicroRNA Profiling of Neurons Generated Using Induced Pluripotent Stem Cells Derived from Patients with Schizophrenia and Schizoaffective Disorder, and 22q11.2 Del. PLoS One.**10**(7), e0132387 (2015).
43. Hicks, SD., Middleton, F.A. A Comparative Review of microRNA Expression Patterns in Autism Spectrum Disorder. Front. Psychiatry. **7**, 176 (2016).
44. Stepniak, B., Kästner, A., Poggi, G., Mitjans, M., Begemann, M., Hartmann, A., et al. Accumulated common variants in the broader fragile X gene family modulate autistic phenotypes. EMBO Mol. Med.**7**(12), 1565-1579 (2015).
45. Wang, B., Pan, L., Wei, M., Wang, Q., Liu, W.W., Wang, N., et al. FMRP-Mediated Axonal Delivery of miR-181d Regulates Axon Elongation by Locally Targeting Map1b and Calm1. Cell Rep. **13**(12), 2794-2807 (2015).
46. van Spronsen, M., van Battum, E.Y., Kuijpers, M., Vangoor, V.R., Rietman, M.L., Potho,f .J., 'et al.'. Developmental and activity-dependent miRNA expression profiling in primary hippocampal neuron cultures. PLoS One. **8**(10), e74907 (2013).
47. Zbucka-Kretowska, M., Niemira, M., Paczkowska-Abdulsalam, M., Bielska, A., Szalkowska, A., Parfieniuk, E., et al. Prenatal circulating microRNA signatures of foetal Down syndrome. Sci. Rep. **9**(1), 1-6. (2019).
48. Santarelli, D.M., Beveridge, N.J., Tooney, P.A., Cairns, M.J. Upregulation of dicer and microRNA expression in the dorsolateral prefrontal cortex Brodmann area 46 in schizophrenia. Biol. Psychiatry. **69**(2), 180-187 (2011).
49. Khavari, B., Cairns, M.J. Epigenomic Dysregulation in Schizophrenia: In Search of Disease Etiology and Biomarkers. Cells. **9**(8), 1837 (2020).
50. Alural, B., Genc, S., Haggarty, S.J. Diagnostic and therapeutic potential of microRNAs in neuropsychiatric disorders: Past, present, and future. Prog. Neuropsychopharmacol. Biol. Psychiatry. **73**, 87-103 (2017).

Figures

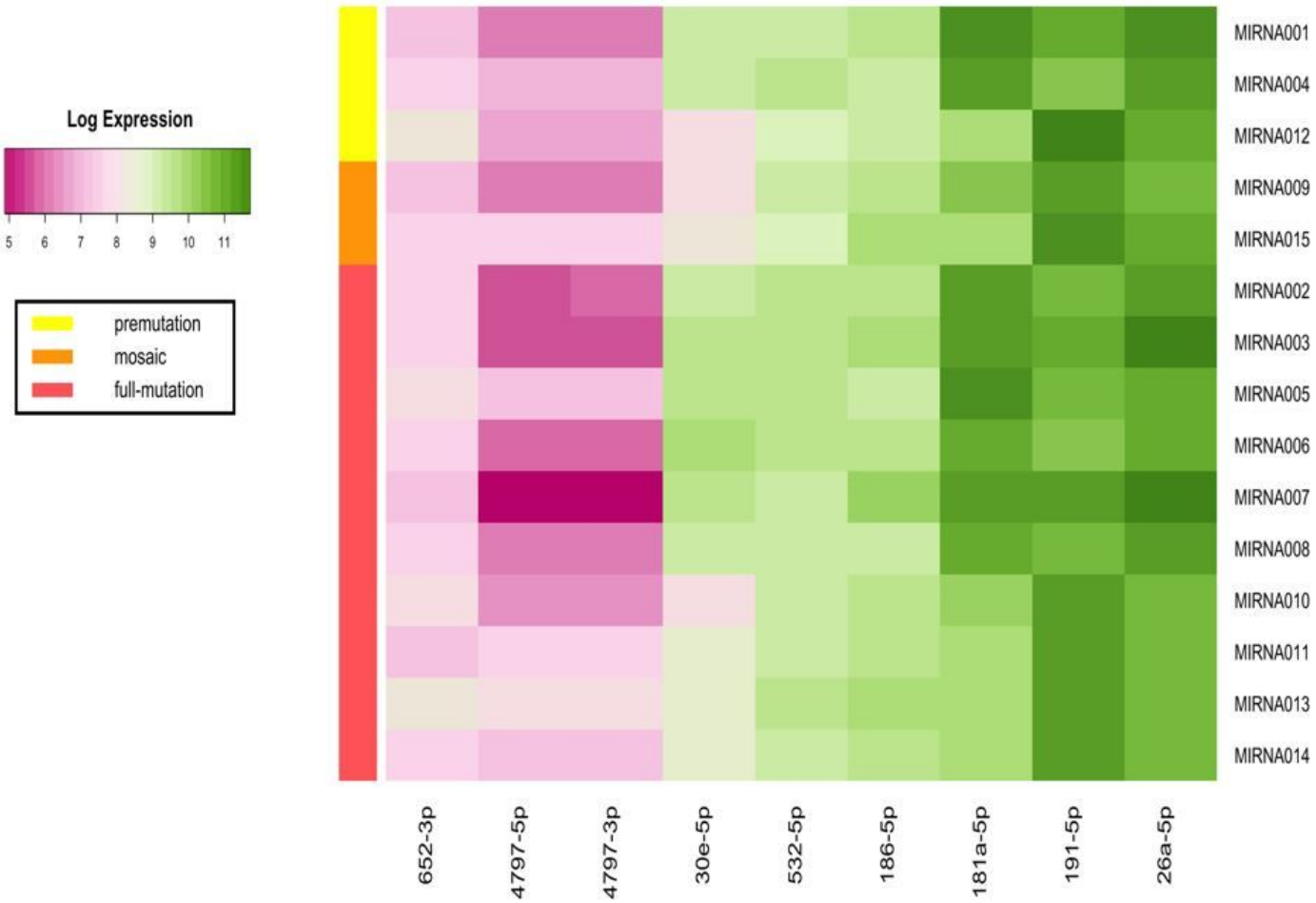

Figure 1

Expression-related heat map based on three groups of full mutation, permutation, full mutation mosaic.

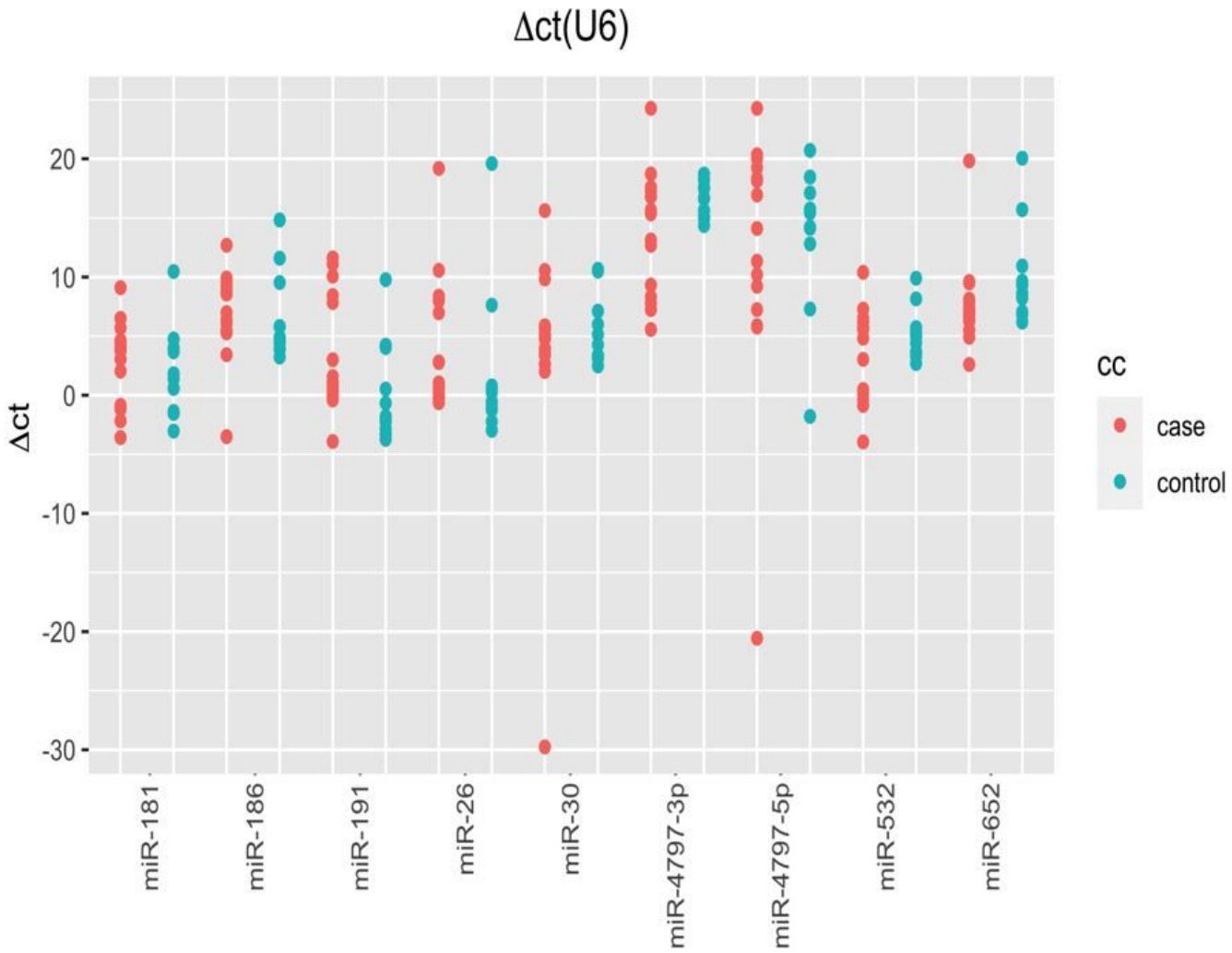

Figure 2

Normalization of miRs cycle threshold using U6.