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Williams' neural stem cells: new model for insight into microRNA dysregulation

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TABLE OF CONTENTS

1. ABSTRACT

Williams syndrome (WS) is a neurodevelopmental genetic disorder, due to a 7q11.23 hemizygous deletion. WS has a characteristic neurocognitive profile that includes intellectual disability (ID). Haploinsufficiency of some of the deleted genes is partially associated with the cognitive phenotype. The aim of this paper is to determine the differences in the microRNA (miRNA) expression in WS patients, using a neural cell model from the patient´s olfactory neuroepithelium (ONE), and to establish the relationship with those genes involved in neurodevelopment and neural function. To assess these goals, we made a comparative analysis of the miRNAs expression profile between WS patients and controls. Through an *in silico* analysis, we established potential pathways and targets associated with neural tissue. The expression profile shows 14 dysregulated miRNAs, including nervous system (NS)-rich miRNAs such as miR-125b, let-7c and miR-200. Most of these miRNAs have potential targets associated with NS functions while others have been reported to have specific neuronal functions. These data suggest that miRNAs widely contribute to the regulation of neurodevelopmental intrinsic processes, and that specific miRNAs could participate in WS neurobiology.

2. INTRODUCTION

Williams Syndrome (WS) (OMIM No. 194050) is a neurodevelopmental genetic disorder caused by hemizygous deletion of 1.5 to 1.8 Mb on chromosome 7q11.23, a region comprehending 28 genes, known as Williams region (WR) (1-4). The frequency of the WS has been reported 1 in 7500 live births (5), and is characterized by alterations in development and cognition with a characteristic cognitive-behavior profile, mild to moderate intellectual disability (ID, IQ ~50-70), impaired visuospatial constructive cognition and behavior, among others (6-11).

Haploinsufficiency of the WR genes generates a hypothetical gene dosage effect with a subsequent decrease of 50 percent in the RNA expression level of the deleted genes, the imbalance that causes WS phenotype (12). For the cognitive phenotype, *LIMK1* has been associated with alterations in visuospatial construction, alterations in learning and synaptic function (13-15), and *GTF2IRD1* and *GTF2I* with visuospatial construction deficits and neuroanatomical defects (16-23). *CLIP2* (also known *CYLN2*) has roles in dendro-dendritic synapses, it is associated with motor, spatial learning and brain disorders (23-24). Also, *STX1A* has been associated with the release of neurotransmitters (25,26), *EIF4H* with brain and neuronal alterations (27), and *FZD9* with visuospatial learning and memory (28). However, these associations have been limited and partial because they were derived from case studies with atypical deletions or from animal models (29-35).

Although there is no specific evidence on genes mediating this phenotype, these studies indicate that altered molecular pathways of several genes involved could mediate it. Besides ID, individuals with WS have various morphological and functional defects at the NS level, indicating that the associated genes are involved in neurodevelopment and should be investigated whether there is dysregulation at this level (36-41). Neurodevelopment is a complex process involving multiple genes and there is a high degree of spatial and temporal regulation, with a key role of regulatory molecules such as miRNAs. miRNAs are endogenous noncoding RNA molecules that negatively regulate the expression at post-transcriptional level, a fundamental role in the development and function of the NS (42-47). There is evidence of miRNAs involved in neurogenesis and NS pathologies including those with ID such as Fragile X syndrome (FXS) (48-51), Rett syndrome (RS) (52-54), Down syndrome (DS) (55-56) and DiGeorge syndrome (DGS) or 22q11 deletion (57). To study the pathways in which miRNAs are involved can be complex, since a single miRNA can affect up to 200 targets, and also a mRNA can be regulated by several miRNAs, considering that reciprocal regulation exists between them and their targets (58). As reported so far, of the WR genes regulated by miRNAs only *LIMK1* is a validated target of mir-124 (59) and miR-134 (60), although its expression is not known in individuals with WS. It is also unknown whether other WR genes are regulated by miRNAs and if these could have a role in neural cell function.

Due to ethical and methodological limitations, the cognitive phenotype associations with the WR genes have been generated from non-neural cell lines and animal models, so that inferences are biased. This creates the need to use a system that brings us closer to the patient's own neural tissue, as done in other diseases (61-64). The cells of the patient's olfactory neuroepithelium (ONE) can proliferate through neurosphere assays (NSA) into neural stem cells (NSC) (65-70). This is a better model to study neural biological processes of development, and it will allow us to make inferences closer to the neural tissue (64).

So far there are no reports about miRNAs expression on WS nor is it known whether the haploinsufficiency of genes in the region could dysregulate its expression in neural cells. It is important to determine whether other genes involved in the disease's biology could provide data on the pathogenesis of affected pathways in neurodevelopmental function or cognitive processes. The goal of this paper is to prove that the haploinsufficiency of WR genes could modify the expression of miRNAs that regulate them, and that these could be involved in the WS phenotype. Specifically, the purpose was to determine whether there are differences in the miRNAs expression in WS patients in neural cells derived from ONE and its relationship with genes involved in neurodevelopment or neural function. To assess these, we performed a comparative study of miRNAs expression by RT-qPCR array of miRNAs between patients with WS and controls without ID. The expression profile showed miRNAs differences with potential targets associated with NS development and function. To evaluate the possible role of these miRNAs in neural processes, the function of target mRNAs expressed in NS involved in neurodevelopment and / or neural pathology was determined *in silico*.

3. METHODS

3.1. Patients and controls

Four probands with WS and molecular deletion detected with fluorescent *in situ* hybridization (FISH), from the Hospital de Pediatria, Centro Medico Nacional Siglo XXI and Asociacion Nacional de Sindrome de Williams were included. Four healthy volunteers without neural pathology were used as controls. Samples were collected under the approved protocol by the Research and Ethical Committee of The Pediatrics Hospital, Centro Medico Nacional Siglo XXI, IMSS. All patients' families and controls agreed to participate and signed a written informed consent.

3.2. Sample collection and neurosphere assay

ONE was collected by nasal mucosa exfoliation technique and processed as previously reported by Benitez-King (71). Samples were processed through NSA. The cellular sample was immediately placed in DMEM/F12 culture medium. The cells were mechanically dissociated by thoroughly pipetting to get a homogeneous sample without clumps. After, the homogenate was centrifuged to

Table 1. Primers

Gene	Primer forward	Primer reverse
TUBB3	5′- AACGAGGCCTCTTCTCACAA -3′	5′- CCTCCGTGTAGTGACCCTTG -3′
SOX2	5′- ACACCAATCCCATCCACACT -3′	5′- GCAAACTTCCTGCAAAGCTC -3′
MSI1	5′- ACAGCCCAAGATGGTGACTC -3′	5′- CCACGATGTCCTCACTCTCA -3′
GFAP	5′- ACATCGAGATCGCCACCTAC -3′	5′- ATCTCCACGGTCTTCACCAC -3′
NES	5′- CAGGAGAAACAGGGCCTACA -3′	5′- TGGGAGCAAAGATCCAAGAC -3′
OMP	5′- TGTGTACCGCCTCAACTTCA -3′	5′- GTCGGCCTCATTCCAATCTA -3′
GAPDH	5′- TGATGACATCAAGAAGGTGGTGAAG -3′	5′- TCCTTGGAGGCCATGTGGGCCAT -3′

TUBB3: Tubulin beta-III, SOX2: Sox2, MSI1: Musashi, GFAP: Glial fibrillary acidic protein, NES: Nestin, OMP: Olfactory marker protein, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

500 g for 5 min. The cellular pellet was placed in serum free medium according to Pacey´s formulation (72) to obtain spherical clusters of cells in suspension. Cell passages were made every 5-8 days, until we obtained a suitable number of cells for analysis.

3.3. Neural cell phenotype characterization

The neural lineage was corroborated through the expression of polymerase chain reaction (PCR) and immunofluorescence analysis of specific neuronal molecular markers.

3.3.1. Polymerase chain reaction

Total RNA extraction of the neurosphere cultures was made using a miRVANA PARIS Kit (Applied Biosystems, Carlsbad, CA) according to manufacturer´s recommendations. 100 ng of total RNA were transcribed with MMLV Reverse Transcriptase kit (Cat. No. 28025-013 Invitrogen, Carlsbad, CA), using random hexamers, according to the recommended protocol. Primers were designed online using Primer3 (http://primer3.wi.mit.edu) to assess gene expression of Nestin (*NES*), Musashi (*MSI1*), Sox2 (*SOX2*), Glial fibrillary acidic protein (*GFAP*), Tubulin beta-III (*TUBB3*), olfactory marker protein (*OMP*) as negative control and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as internal control. Following total RNA extraction, target mRNA gene expression were measured by reverse transcriptase PCR (RT-PCR) with the primers enlisted in Table 1. PCR was performed in a thermocycler (ABI Geneamp 9700). The presence of the PCR products was analyzed on 2 percent agarose gels stained with SYBR green (Invitrogen, Carlsbad, CA).

3.3.2 Immunofluorescence

Cell neurospheres in suspension were recovered and fixed with 4 percent paraformaldehyde in phosphate-buffered saline (PBS) solution for 15 minute. Then cells were washed with PBS and permeabilized for 15 min with Tris buffered saline with Tween 20 (TBST), and blocked with protein block serum-free solution (DAKO, Carpinteria, CA) for 1 hour at 25ºC (in a humid chamber). The slides were washed twice with PBS-T (PBS with Tween-20, 0.1 percent) for 5 minutes. Slides were processed for immunofluorescence labeling using the following primary antibodies (Millipore, Billerica, MA): mouse anti-Nestin (1:200), rabbit anti-Sox2 (1:500), rabbit anti-Musashi (1:200), and mouse monoclonal anti-Tubulin beta-3 chain (1:500). Primary antibodies were incubated overnight at 4ºC. Secondary antibodies corresponding to each primary antibody were prepared: for Sox2 and Musashi, anti-rabbit IgG–Alexa Fluor 594 (Life Technologies, Carlsbad, CA) at a dilution of 1:50 in PBS; for Nestin and anti-Tubulin beta-3 chain, bovine anti-mouse IgG-FITC (Santa Cruz Biotechnology, Dallas, TX) at a 1:100 dilution with antibody diluent (Dako-Cytomation Carpinteria, CA). They were incubated for one hour in a dark room at room temperature. The slides were then washed with PBS-T twice for 5 minutes. Vectashield mounting media was placed (Vector Laboratories, Burlingame, CA) with the selected nuclei labeling fluorochrome (propidium iodide or DRAQ7, Biostatus UK). The slides were analyzed with confocal laser scanning microscope (Zeiss). Assays were performed in triplicate. As negative controls, parallel sections were processed similarly without the respective primary antibody for each condition.

3.4. microRNA expression assay

For miRNAs profiling, we used qPCR with TaqMan low-density array (TLDA) microfluidic cards (Human miR v2.0, Applied Biosystems Foster City, CA). Two plates were defined in this system: plate A, containing well characterized and widely expressed miRNAs, while plate B had less characterized miRNAs. The combined plates evaluated the expression of 667 unique human specific miRNAs (V14 of the Sanger miRBase) (http://www.mirbase.org) in parallel. Briefly, 50 ng of total RNA with Megaplex RT stem-loop primers was used to obtain a cDNA template. A pre-amplification step was included in order to increase the original material concentration and to detect miRNAs that are expressed at low levels. The preamplified product was loaded into the TLDA and amplification signal detection was carried out using the 7900 FAST real time thermal cycler (ABI).

3.4.1. Data analysis

The expression was assessed using the qbasePlus software (http:///www.qbaseplus.com).. Median global was selected for normalization of miRNA expression data, using the equation $2^{-\Delta\Delta Ct}$ (73). The Cq raw data (fractional cycles numbers at which the fluorescence crosses the threshold) was determined using an automatic baseline and a threshold of 0.2. Samples with a Cq value greater than 36 cycles were excluded from the analyses and the controls cell samples were used as calibrators. Less than 0.5 or greater than 1.5 fold-change (FC) value obtained by the comparative Ct method ($2^{-\Delta\Delta Ct}$) and consistent expression in at least 75 percent of the samples was used to determine the differentially expressed miRNAs. Significance differences in the Cq values between controls and patients were evaluated with two tails Students t-test. miRNAs with p-values of 0.05. The RT-qPCR assays were performed

Table 2. Patients' clinical data, IQ score and controls data

Patients				Controls				
Id	**Sex**	**Age**	**Phenotype**	**IQ**	**Id**	**Sex**	**Age**	**IQ**
W1	F	9	Characteristic facies, CVa hypersociability, anxiety, hyperacusia, growth retard	Mild	C1	F	35	N
W2	F	25	Characteristic facies, CVa, hypersociability, anxiety, depression, hypracusia, Growth retard	Mild	C2	F	23	N
W3	F	15	Characteristic facies, CVa,b, hypersociability, Growth retard	Mild	C3	M	30	N
W4	F	16	Characteristic facies, CVa, hypersociability, growth retard	Mild	C4	M	35	N

CVa: Cardiovascular alteration without Supravalvular aortic stenosis, CVb: Cardiovascular alteration with Supravalvular aortic stenosis; CVAb: CVa and CVb, IQ: Intellect Quotient, F: Female, M: Male N: Normal

according to the MIQE guide lines (http://www.rdml.org/miqe) (74).

3.4.2 Hierarchical Clustering and principal component analysis (PCA)

Unsupervised clustering analysis was performed with significantly differentially expressed miRNAs using Euclidean correlation and average linkage to identify sub-groups according to differences in miRNA expression. dChip software was used for clustering (http://www.hsph.harvard.edu/cli/complab/dchip) and R language in Java's platform for PCA.

3.5. Preliminary functional analyses of miRNA's targets

Recognized and validated mRNAs targeted by differentially expressed miRNAs were identified using miRECORDs (http://mirecords.biolead.org) (75) and TarBase v5.c (http://microrna.gr/tarbase) (76). Potential target gene predictions were based on the integrative evaluation through miRDIP (http://ophid.utoronto.ca/mirDIP/) (77) with different algorithms: TargetScan V 6.2 (http://www.targetscan.org) (78), PicTar (http://pictar.mdc-berlin.de) (79), PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html) (80), miRanda(http://www.microrna.org/) (81), Diana MicroT v4.0 , (http://diana.cslab.ece.ntua.gr/microT) (82), with preference to TargetScan Non-Conserved Targets, PicTar-5 way and PITA-TOP according to Shirdel (77).

3.6. Identification of possibly disrupted biological functions by dysregulated miRNAs

In order to gain insight into biological functions that may be altered in neural cells as a consequence of altered miRNA expression, significantly differentially expressed miRNAs were evaluated using KEGG Database (http://www.genome.jp/kegg/pathway.html) through MirWalk (http://mirwalk.uni-hd.de) (83). Significant biological functions in pathways and diseases highly represented in the networks were selected according to p-value minor than 0.05.

4. RESULTS

4.1. Patients and controls

Four female WS patients were included in this study, with ages from 9-25 years and 4 controls between 23 to 35 years old, 2 male and 2 female. Table 2 shows the main clinical characteristics of each group.

4.2. Neurosphere cultures

ONE samples were cultured by neurosphere assay, and neural lineage cells were corroborated by expression of specific markers. RT-PCR showed expression of neural stem cell markers *NES*, *MSI1*, and *SOX2*; early neuronal marker *TUBB3* and astrocyte marker *GFAP* (Figure 1). Cells did not express *OMP*, a mature olfactory neuron marker thus ruling out the presence of mature neural cells (Figure 1). Furthermore neural phenotype was determined according to the expression by immunofluorescence of NES, MUS, SOX2 and TUBB3 (Figure 2).

4.3. Differential expression analysis of miRNAs

From the 667 analyzed miRNAs, 320 were expressed in WS patients' neurospheres and 363 in the controls, corresponding to 48 and 55 percent respectively. Ct ($2^{-\Delta\Delta Ct}$) comparative analysis between controls and patients expression levels show that 14 miRNAs were significantly differentially expressed (Table 3). We also found miR-7f-2 *, 7g * and miR-206 not expressed in any WS patient, only in the controls (Table 4).

From the 14 miRNAs with significant differential expression, 11 of them are over-expressed and 3 under-expressed. These miRNAs and their corresponding ratios for WS over controls are shown in table 3 and Figure 3. Cluster analysis of these miRNAs allowed distinguishing different expression patterns between the two groups in the unsupervised hierarchical cluster analysis (Figure 4a). PCA was used to reduce the dimensionality of the data of the miRNAs in the same group; Figure 4b shows the separation of the two groups (patients and controls).

4.4. Potential and validated mRNA targets

Target mRNAs analysis shows 163 validated targets (Tables 5 and 7), and over a thousand potential ones (tables 6 and 8). From these, *CLIP2* is target for miR-200c and *VPS37D* for miR-744 codified in WR. Tables 5 and 6 show the potential and validated targets for this group of miRNAs.

As for the targeted mRNAs of the miRNAs without expression in WS patients, there is poor information regarding let-7f-2 * and let-7g * as well as a small number of targets compared to miR-206 which is further studied and the analysis shows a greater number of targets (table 7-8).

4.5. Potentially altered pathways

In order to reduce the number of mRNAs, we evaluated the expression of altered miRNAs with functions or units already validated in several pathways involving neural tissue expression in both neurodevelopment and

Table 3. MicroRNAs with the highest differential expression between controls and WS patients

	microRNA	Locus	Fold Change	p
1	miR-32	9q31.3	3.32	0.04035
2	miR-125b	11q24.1/ 21q21.1	2.24	0.01614
3	let-7c	21q21.1	2.03	0.01885
4	miR-200c	12p13.31	0.54	0.03786
5	miR-151-3p	8q24.3	0.41	0.00996
6	miR-744	17p12	0.39	0.01733
7	miR-345	14q32.2	0.35	0.02887
8	miR-934	Xq26.3	0.33	0.00530
9	miR-452	Xq26	0.27	0.03763
10	miR-149	2q37.3	0.23	0.00249
11	miR-378*	5q32	0.23	0.03437
12	miR-589*	7p22.1	0.20	0.03240
13	miR-15a*	13q14.2	0.19	0.02790
14	miR-489	7q21.3	0.16	0.03864

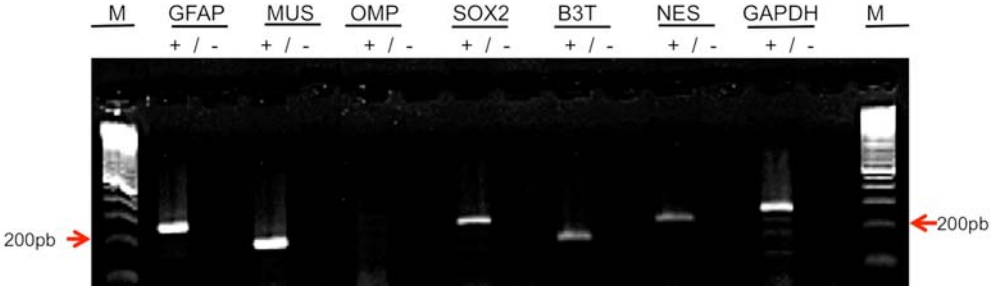

Figure 1. Characterization of the neural cell markers expression by RT-PCR. GFAP: Glial Fibrillar Acidic Protein, MSI1: Musashi, OMP: Olfactory Marker Protein, TUBB3: Tubulin beta-III, NES: Nestin, GAPDH: Glyceraldehyde Phosphate Dehydrogenase, M: bp marker (+ lanes are samples with RNA. - lanes are without RNA).

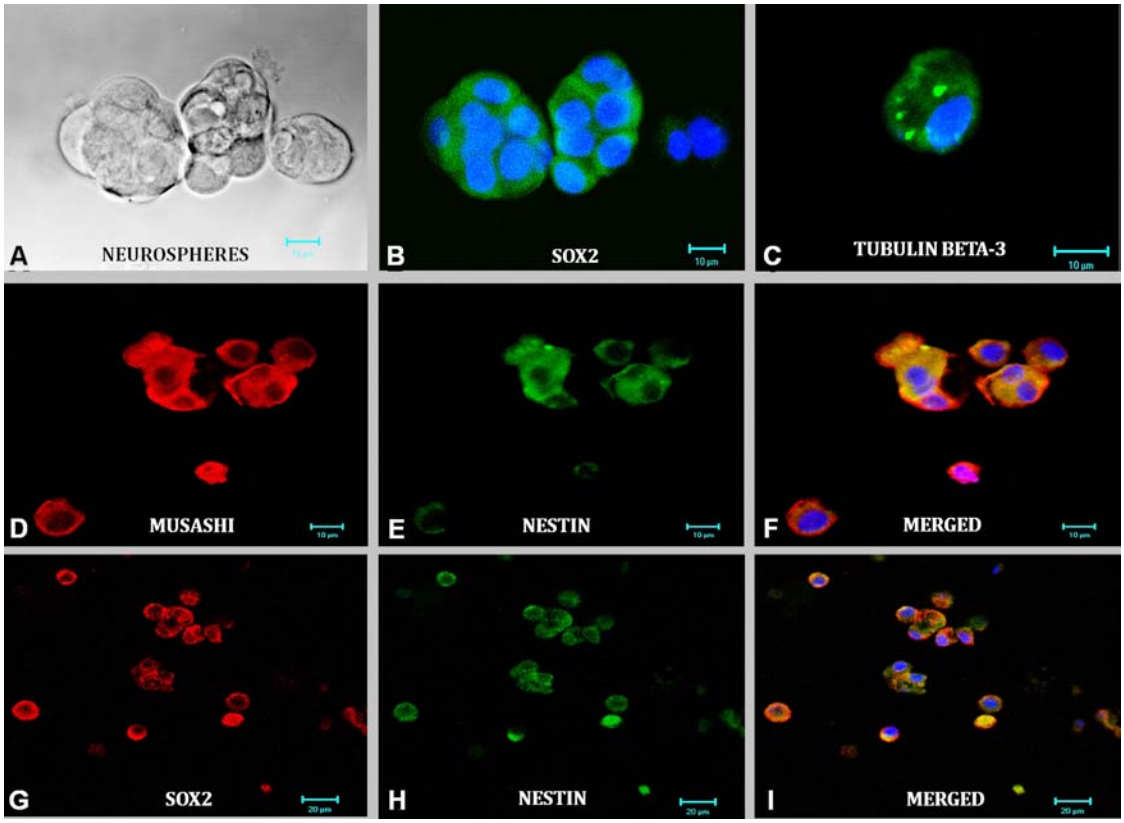

Figure 2. Characterization of the neural stem cell markers expression by immunofluorescence. Nuclei were stained with DRAQ7 (blue). A: Characteristic neurospheres in DIC. B and C Positive cells to SOX2 and Tubulin beta-III chain (merged green and blue channel) D-E: Positive cells to Musashi (red) and Nestin (green). E: merged D, E and blue channel. G-H: Positive cells to SOX2 (red), Nestin (green). I: merged G, H and blue channel. Bar A-F shows 10 µm, bar G-I 20 µm.

Table 4. MicroRNAs without expression in WS patients

microRNA	Locus	Expression C/W	Cq* Control	Cq* SW
let-7f-2*	Xp11.22	3C/0W	32.7	NE
let-7g*	3p21.1	3C/0W	31.9	NE
miR-206	6p12.2	3C/0W	31.7	NE

C: Controls W: WS patients NE: No expression, Cq: Quantification cycle.

Table 5. Validated targets for microRNAs with differential expression

miRNA	Database	mRNA target (valid)	Total
let-7c	A, B	*TRIM71 MYC, HMGA2, TGFBR1, EIF3S1, MED28, RTCD1*	7
miR-125b	A, B	*LIN28, ERBB8, ERBB3, LIN28, BAK1, gag-pol, NTRK3, C10orf104, H3F3B, ADAMTS1, PERP, B3GALT4, HOMER2, GPR160, CASP7, CEBPG, CBLN2, HIST1H4A, LOC92, 270, CBX7, UTG2B15, UBE2I, RBM8A, FLJ41484, IL1RN, UGT2B28, CASP6, DIO3, ID2, PCDHB10, SGPL1, CYP1A1, CLU, IGFBP3, PIGR, UGT2B17, TSPAN8, MAN1A1, ODZ2, JARID2, ID3, ID1, FAM19A1, SMO, CDKN2A, ST18, DICER1, KRT, CDK6, CDC25A, HuR, BMPR1B, BMF, TP53INP1, kB-Ras2, E2F3, Bak1, Cdc25c, Ppp1ca, Ppp2ca, Prkra, PUMA, Tdg, TP53, Zac1, 4E-BP1*	67
miR-200c	A, B	*SIP1, ZEB1, ERRFI1, ZEB1, JAG1*	5
miR-32	A, B	*PFV-1*	2
miR-378*	A, B	*TUSC2, SUFU, VEGFA*	3
miR-15a	A, B	*DMTF1, BCL2, H3F3B, PDCD4, MCL1, VEGFA, RAB21, CADM1, SKAP2, WT1, BCL2, RAB9B, ACTR1A, TPI1, ASXL2, C10orf104, C14orf109, CARD8, CDC14B, CENPJ, CEP63, CREBL2, ECHDC1, CCDC76, NPAL2, C4orf27, C2orf43, CCDC111, GOLGA5, GOLPH3L, GTF2H1, HACE1, HDHD2, HERC6, C17orf80, HRSP12, HSDL2, HSPA1A, JUN, PWWP2A, FAM122C, LOC339804, FAM69A, MSH2, NT5DC1, OMA1, OSGEPL1, PDCD6IP, PHKB, PMS1, PNN, PRIM1, RAD51C, RHOT1, RNASEL, SLC35A1, SLC35B3, TIA1, HSP90B1, UGDH, UGP2, VPS45, WIPF1, ZNF559, BMI1, MYB, CCND1*	67
miR-489	B	*GCA, PEX7*	2

None: miR-149, miR-151-3p, miR-345, miR-452, miR-744 miR-934,. Database A: Tarbase; B: miRecords.

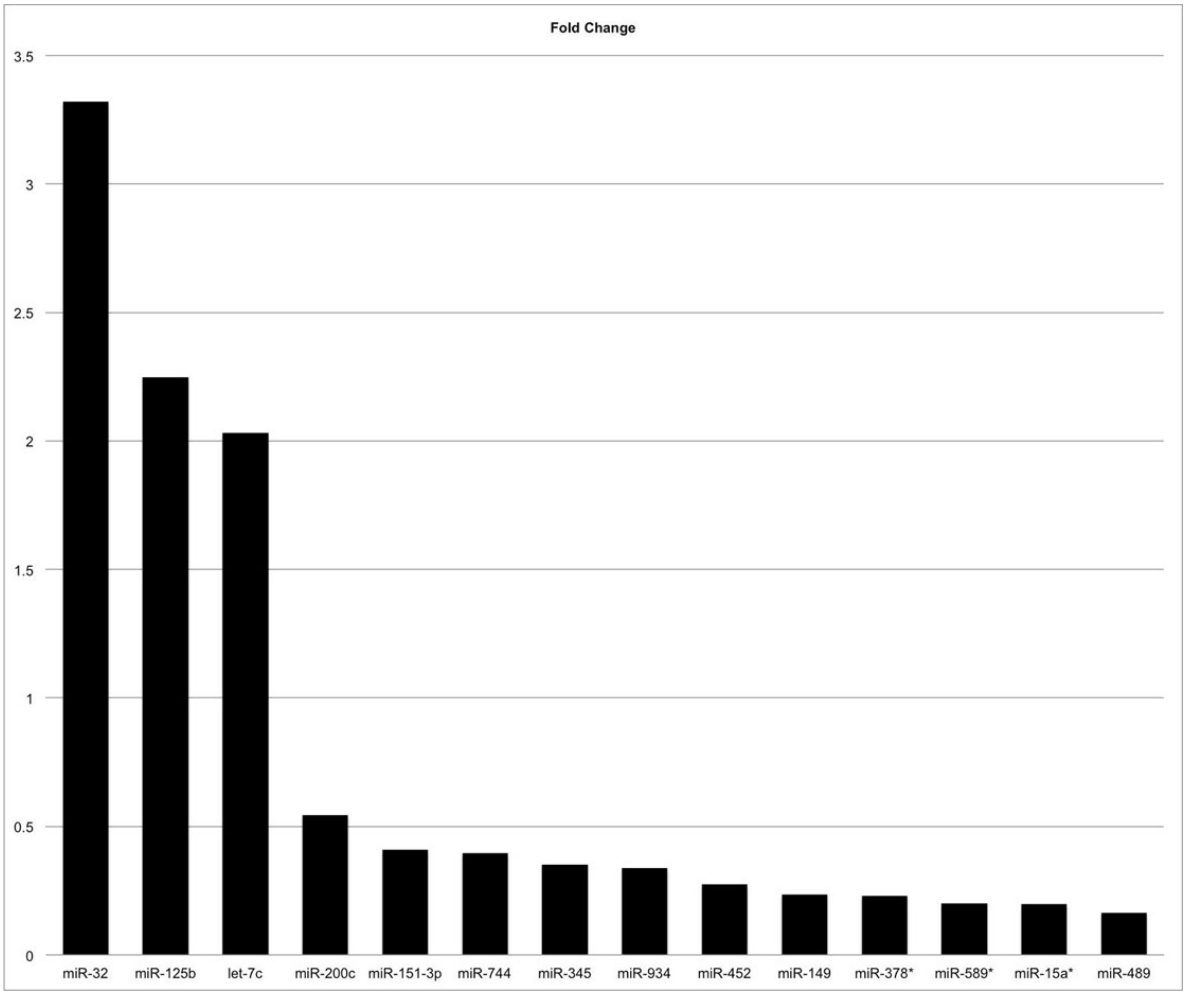

Figure 3. Fold-change histogram of microRNAs expression with statistically significant differential expression.

Table 6. Potential targets for microRNAs with differential expression

miRNA	Data base	Potential Gene Targets	Total
let-7c	A, B, C	*ABCC5, ACVR1B, ACVR1C, ADAMTS5, AKAP6, ARID3B, BACH1, BZW1, BZW2, C15orf29, CCNJ, CDV3, CHD4, COL1A2, COL24A1, COL3A1, COL5A2, CPEB2, DCUN1D3, DDX19B, DMD, DYRK1A, E2F5, ELOVL4, FNDC3A, GALNT1, GATM, GDF6, GNAL, GNPTAB, GOLGA7, GOLT1B, HIC2, HMGA2, IGF2BP2, IGF2BP3, IL13, IRS2, KLF9, LCOR,L LGR4, LIN28B, LRIG2, LRIG3, MAP4K3c, MAP4K4, MGAT4A, NLKc, NOVA1, OSBPL3, PAPPA, PBX3, PCGF3, PRPF38B, RAB11FIP4, RDH10, RDX, RUFY3, SLC4A4, SLC6A1, MARCAD1, SNX16, TGFBR1, TMED5, TUSC2, UHRF2, ULK2, USP32, USP38, USP6, WDR37, ZNF644*	72
miR-15a*	B	*DNAJA2, ARPP-21, ADAMTS5, UBR3, CPEB2, CRKL, SNF1LK, E2F3, EDA, FGF2, KIF1B, LPHN2, JARID2, ARHGDIA, LRP6, MAP3K3, PAFAH1B1, PAPPA, PLXNA2, PHIP, SOBP, PELI2, SLC24A3, SIPA1L2, BCL2, KCNAB1, ATP13A3, C1orf21, SYNJ1, MTMR4, DCLK1, SOCS6, CPEB2, EDA, HOXA10, MAP3K3, CSNK1G1, RCOR3, MAP2K4, KCNAB1, YTHDC1, PDIA6, NEBL, RAB10, PRDM4, C10orf46, RASGEF1B, ADAMTS18, DYNC1LI2, DYRK1A, EYA1, CPEB3, NLGN1, ELL2, SATB2, KDSR, MAP3K7IP3, LRIG1, ERC2, GHR, GLUD1, HOXC8, KCNJ2, ZC3H6, KPNA3 ,PBX3, CRIM1, CAB39, CMPK1, DLL4, RELN, ARHGAP20, PTPN3, RAP2C, BACH2, SLC20A2, TLE4, CHD9, PPM1D, RANBP3, CCND2, QKI, N4BP1, TSC22D2, USP15, CAPN3, AKT3, TSPAN5, FRY, RBM6, CTDSPL, UBE4B, FERMT2, WWP1, IL1RAPL1, COL12A1, E2F7, MIPOL1, THSD3, CCNYL1, C5orf41, RNF217, CAMSAP1, DYNC1I1, DYRK1A, EIF2C4, EFNB2, EIF4E, EYA, BPTF, FGF7, HSPA4L, COBLL1, RAB11FIP2, PPM1E, WDR47, WAPAL, ZNF423, DCUN1D4, RTF1, FBXO33, BACE2, HECTD1, MOBKL3, KBTBD2, GORASP2, WIPI2, WSB1, SERBP1, LATS2, SALL3, SESN1, PDCD4, KCTD1, DLL1, C3orf23, C9orf150, C16orf72, GRM7, USP25, NXPH1, HAS2, HOXA3, LAMC1, SMAD7, MAP3K4, MYB, NFATC3, NFE2L1, OMG, PAM, PCDH9, PCTK2, WBP11, PIM1, PLAG1, LRP1B, LRRN3, RNF111, SNRK, ZCCHC2, PID1, PPP2R5C, PPP6C, TMEM55A, KIF21A, CDC37L1, MAP2K1, PCDHA6, PCDHA6, KCNQ5, RELN, PTCH1, RAF1, RARB, RBBP6, PLEKHA1, CCND1, TGIF2, BDNF, ATXN2, SNX16, SGK1, SH3GL2, SMURF2, SIAH1, SLC12A2, BMPR1A, SOX5, STXBP3, WEE1, YWHAH, ZMYM2, CSDE1, FSD1, BHLHB3, SEMA6D, NARG1, GPR63, AXIN2, RASSF5, TFAP2D, RECK, CUL2, RSPO3, RANBP3, RUNX1T1, EIF3A, SLC4A4, CD164, NRP2, CDK5R1, BTRC, CCNE1, CCNT2, MAP7, USP14, ACVR2A, LRRFIP2, COPS2, QKI, ADAMTS3, TBPL1, TLK1, DYRK1A, PPM1A, C16orf7, TLK2, KANK1, SYNE1, HOXA3, HOXA10, PPM1A, PCDHAC2, PCDHAC1, PCDHA10, PCDHA5, PCDHA3, PCDHA2, PTHLH, PTHLH, RAB1A, BCL9, BDNF, C14orf4, SFRS5, HMGA2, SEH1L, CAPN3, SETD3, PCDHA9, CDC42*	252
miR-125b	A, B, C	*ABCC5, ANTXR2, ATP13A3, CCNJ, CPSF6, DICER1, DOCK3, DUS1L, EIF2C2, EIF5A2, ELOVL6, ENTPD4, ETV6, FLJ36031, FNDC3B, FUT4, GALNT7, GPC4, ITGA9, KCNH7, KCNS3, KIAA0174, KLF13, LBH, LIFR, LNPEP, MAMDC2, MTUS1, MXD4, MYT1, OSBPL9, PAFAH1B1, PPP2R5C, PRDM1, RAPGEF5, RBM7, SEMA4D, SH3BP4, SLC39A9, SLC4A10, SLITRK6, SMG1, SULT4A1, TRPS1, UBE2W, USP37, ZFYVE1*	47
miR-149	A, C	*AFF2 ARHGAP19 ARPC4 BAI2 BBC3 BCL2L11 BCL2L2 BHLHB5, BRPF3 CACHD1 CACNA1C CBX1 CCDC6 CCNI, CD47 CD72 CLCN5 CNNM1 CNTNAP2 CROP, CRTC2, DCLRE1B, DUSP16, EDA, EDNRA, EIF5, EPHB3, EXT1, FBXL16, FCHSD1, FMO2, GIT1, GRIA3, HM13, ID4, IGF2BP1, IGFBP5, KIAA0152, KLHL24, KLHL3, LMBR1L, LOC91461, MARCKSL1, MLL2, MSI1, MSRB3, MYST2, NCDN, NDST1, NFIB, NFIX, NNAT, NRP2, OXSR1, PCDH19, PCTK2, PHC1, PHLDB1, PHLPPL, PLAG1, POU2F2, PRKAR2A, PURB, PVRL1, RAP1A, RAP1B, RIMS4, RNF121, RNF2, RSBN1L, SEC24C, SEMA4G, SERF2, SH3PXD2A, SHB, SLC11A2, SLC24A3, SLC4A4, SLCO3A1, SNCAIP, SNCB, SOCS6, SP1, SPTLC2, SRC, SRF, TCAP, TNFRSF19, TNKS, TOP1, UPF2, YWHAZ*	92
miR-200c	A, B, C	*ABAT, ACACA, ACVR1C, ACVR2A, ADAMTS3, ADCY2, ADD3, ADIPOR2, AFF3, AHNAK, AKAP2, AKT2, ALS2CR8, AM, AMOTL2, ANKH, ANKRD28, ANKRD52, ANLN, AP152M APAF1, APLP2, ARHGAP19, ARHGAP26, ARHGDIA, ARHGEF17, ARHGEF3, ARID4B, ARIH2, ASF1A, ASH1L, ATAD2B, ATP1B, ATRX, ATXN1, AUTS2, B3GNT1, BACH2, BAG5, BAP1, BASP1, BAZ2B, BCL11B, BCL2, BNC2, BRDW1, BTF3L4, C10orf26, C10orf46, C14orf100, C14orf147, C17orf71, C3orf23, C5orf1, C6orf6, C9orf25, CAB39, CACNA1C, CALU, CASC4, CASR, CASZ1, CBL, CBX4, CCNE2, CCNJ, CDC14B, CDH11, CDKN1B, CDR2, CDR2L, CDYL, CECR2, CEP350, CFL2, CHD1, CHD2, CHMP5, CHN2, CITED2, CLASP1, CLASP2, CLIC4, CNN3, CNOT4, CNOT6, CNTFR, CNTN4, COPS8, CORO1C, CREB5, CREBBP, CRHBP, CRKL, CRKRS, CRTAP, CSNK1G3, DACH1, DCBLD2, DCUN1D4, DDEF1, DDX26B, DDX3X, DDX3Y, DGKA, DIXDC1, DCL1, DLGAP2, DNAJB5, DNAJC5, DNMT3B, DOCK4, DPY19L1, DTNA, DUSP1, DZIP1, E2F3, EFNA1, EFNB2, EGLN1, EGR3, EIF2S1, EIF5B, ELAVL2, ELF2, ELL2, EPS15,EPS8, ERBB4, ERLIN1, ERRFI1, ESRRG, ETS1, ETS2, ETV5, EVI5, FAM118B, FAM120C, FAM19A, FAM60A, FAM76B, FAM80B, FARP1, FAT3, FBX033, FBXW11, FBXW7, FEZ2, FGD1, FIGN, FLI1, FNBP4, FNDC3B, FOXF1, FOXF2, FOXK1, FOXP1, FREQ, FRMD4A, FRMD4B, FRS2, FSTL1, FUBP1, FUBP3, FXR1, FXR2, G6PC, GABBR2, GABPA, GALNT2, GATA2, GATA4, GEM, GIT2, GLCCI1, GLI3, GLIS2, GLT8D3, GNAI3, GNPDA1, GOLGA1, GTF2E1, HDAC4, HLF, HMBOX1, HMGB3, HNF1B, HOOK1, HOXA5, HRB, HRNBP3, HS2ST1, HS3ST1, HSPA9, ICA1L, ICK, IER5, IGF2R, IGSF3, IHPK1, IKZF2, INTS6, INTS8, JAG2, JAZF1, JUN, KBTBD6, KBTBD8, KCTD15, KDR, KIAA0101, KIAA0152, KIAA0182, KIAA0256, KIAA0355, KIAA0423, KIAA1012, KIAA1432, KIAA1462, KIAA2018, KLF12, KLF3, KLF4, KLF9, KLHL14, LAMC1, LASS6, LATS2, LEMD3, LFNG, LFHP, LIN7B, LM07, LOC153364, LOC26010, LRP1, LRP1B, LRP4, LRRC8A, LRRTM3, LYSMD3, KLHL3, KRAS, MAFG, MAMDC2, MAP2, MAP3K1, MAP3K5, MAP4K3, MAP4K4, MAPRE1, MARCKS, MATR3, MCC, MCFFD2, MED1, MEF2D, MGA, MGAT2, MGC24039, MLL3, MLL5, MMD, MMP16, MSL2L1, MSN, MTFR1, MYB, MYCN, MYLK, MYT1, NAB1, NAPB, NBR1, NCAM1, NCOA2, NCOA3, NCOA7, NCOR2, NDN, NDST1, NEGR1, NEO1, NFASC, NFIB, NFYA, NIN, NLGN4X, NOG, NOTCH1, NOVA1, NPC1, NPTX1, NR2C2, NR5A2, NRBF2, NRBP1, NRIP1, NTF3, NUDT4, NUFIP2, NUMB, NUP153, ODZ1, ORMDL3, OSBPL11, OTUD4, OXR1, P15RS, PAG1, PAIP2, PAK6, PAK7, PALM2, PAN3, PAPD5, PARD6B, PBX3, PCDH8, PCMTD1, PCNX, PCSK2*	321
miR-32	A, B, C	*ADAM10, ADAMTSL3, ARID1B, ARMC1, ARRDC3, ATP2A2, ATRX, B3GALT2, BAZ2B, BCL11A, BCL2L11, C6orf62, CBFA2T3, CBLN4, CDKN1C, COL1A2, CPEB2, CPEB3, CPEB4, CSMD3, CXXC5, DAB2IP, DLGAP2, DMXL1, DOCK9, DPP10, DUSP10, DUSP5, EDEM1, EXOC5, FAM126B, FAM19A1, FBXW7, FMR1, FNDC3B, FRY, FZD10, GAP43, GATA6, GFPT2, GOLGA3, GOLGA4, GPR180, GRHL1, GRIA1, GRIA3, HAND2, HIVEP1, HNF1B, INSIG1, IQGAP2, ITPR1, JARID2, JOSD1, KIF5B, KLF4, LATS2, MAN2A1, MAP2K4, MARK1, MYCBP2, MYLIP, MYO1B, N-PAC, NEFH, NEFM, NFIA, NFIB, NLK, NOVA1, NOX4, NPTN, NR4A3, NSMAF, PAX3, PCGF3, PDZD2, PER2, PHTF2, PIK3R3, PITPNA, PLEKHA1, POLS, PPP1R12A, PRKCE, RAB14, RAB23, RAD21, RAP1B, REV3L, RGS3, RNF38, RNF44, RSBN1, SCUBE3, SDC2, SESN3, SIM2, SLC12A5, SLC17A6, SLC24A3, SLC32A1, SMAD6, SMAD7, SNN, SOX4, SRPR, SSFA2, SUV420H1, SYN2, SYNJ1, TACC2, TEAD1, TMEM87A, TOB1, TRAF3, TRAK2, TRIO, TSC1, TULP4, UBE2W,*	127

		UGP2, USP28, VPS54, WWP2, YIPF4, ZDHHC5	
miR-345	B, C	*40787, ADAMTS4, BBC3, BSN, C1orf144, CCDC4, CNBP, EPS8, FBXW11, FCHO2, FRMD6, FZD5, GABRA4, GALNT17, GPR3, HSPA12A, KIAA1539, KIF1B, KITLG, LIMD2, LOC606495, MAGIX, MED8, MLL3, MMP16, OLFM3, PGAP1, RAB39B, RAB3B, RC3H1, RPA1, SLC11A2, SLC4A5, SLC7A6, SMAD1, SORBS1, SSBP2, TOB1, TRPS1, USP15, USP54*	41
miR-378*	D,E	*KCNE3, BCL2L11, SLC17A4, ABCC5, ABCC5, HUWE1, INADL, STX6, ADAR, ADARB1, VAT1, NEBL, SLC34A2, SORBS1, CDC42EP3, TGOLN2, IGF2BP1, CAMKK2, CAMKK2, ADCY1, NFAT5, HBS1L, AHCYL1, TSPAN9, LILRB3, RAB31, TMEM115, DIDO1, FOXN3, PTPRT, LDB3, LZTS1, GALNT6, ZHX1, CHRNA4, SIGLEC11, TUBGCP5, FHAD1, WDR31, ZNF554, CISH, LRRC58, FOXP4, RAB3IP, KRT74, C14orf79, CCR4, LRRC28, SPATA2L, ZNF491, SHE, CNP, B3GALT6, TYW3, C1orf93, MYOM3, UBXD3, COL4A4, TBC1D20, EMID1, ANKRD54, COL9A2, ICA1L, GALM, IL17RE, CMBL, SHROOM1, COX15, MYO3B, CRX, ZBTB46, SMCR8, KRT72, ZNF280B,STK35, PRIMA1, CDAN1, GSG1L, TMC8, ZNF599, C19orf55, ZNF362, IL23R, CLDN19, CLDN19, CCDC117, LOC150383, FBXO41, CYB561, PRELID2, AMOT, AMZ1, ADRBK2, CYP2E1, RDH10, CYP8B1, CYP11B2, AP2A2, DBT, DCX, WBP2NL, LONRF2, DDX6, SPIN3, SYNPO2, DHCR24, DLX1, DNASE1L1, DYRK1A, E2F2, EFNB3, C1orf69, PIP5K3, DHFRL1, DNAJC18, ENSA, C9orf91, ZNF449, EPHA8, CLN8, ERBB4, ERG, ETV6, F2RL1, C10orf56, ORAOV1, C18orf24, HNRPA3, LOC221091, OPN5, FOXK1, MMD2, TMEM130, SLC29A4,, FGF2, FGR, VASH1, ICK, BTBD3, PDCD11, UNC13A, NMNAT2, GGA2, SETX, ERC1, ZC3H13, PHF8, EPB41L3, CEP68, MESDC2, JMJD6, CLEC16A, C22orf9, SLC9A8, ARHGEF12, PPP1R13B, NUDCD3, GPR161, LEPROTL1, TTC9, KCTD2, SEC14L2, ZNF346, DAPK2, KPNA6, KPNA6 C9orf5, FUT2, KDSR, SLC25A30, RNF169, FAM26C, GK5, TMEM184B, ZNF473, HSPA12A, ODZ4, ZNF500, C1orf144, GTPBP5, GMEB2*	528
miR.589*	D, E	*ZNF313 MAPK1 ANKH, RP5-1022P6.2, PSAP PRDM10 CDC42SE2 C12orf5 PLXDC1 KIAA1199, VANGL2, KIAA1244, ZNF304, KIAA1161, CNOT6, KIAA1211, AHRR, MKL2, PCDH19, MIB1 ZNF398 KLHDC5 SEMA6A SYT13 PITPNM2 DIP2B RNF213 KIAA1609 NOPE NCOA5 PTPRE, PTPRJ, PXN, MS4A7, PRUNE, RBM25, RAC1, PLEKHA1, HRH4, PRPH2, RFXAP, RGS16, RP1, BCR, RPS6KA2, BDH1, RPS23, ATXN1, SR,L CCL5, NAPB, NPAS3, C10orf54, XYLT1, MAP2K4, NECAB1, SFRP1, SFRS6, NOM1, SGCD, BNC1, MOSC1, SIAH2, DDX31, SKP1, SLC1A4 SLC1A5, SLC2A3, SLC6A6, SLC7A1, BMPR1A, RASL11B, SNX1, SNTB2, SOX10, SOX11, SRP72, SS18, STK3, MED22, SUV39H1, TBL1X, TEAD3, TFAP2B, THRB, TIMP2, TNNI1, TPD52, TPM3, NR2C2, TRPS1, TSN, WHSC1, WNT5A, XDH, YES1, YWHAB, C18orf1, ZFX, ZNF192, CACNA1C, ZNF229, BTG2, FZD5, TMEM109, C20orf121, WDR32, ZXDC, BHLHB3, PGBD5, CCNJL, LRRK1, PRKRIP1, C12orf49, ALG9, C10orf81, GRHL2, SVEP1, WWC2, C2orf37, NAT13, WDR26, C16orf70, CCDC6 CD276 NDFIP1 CALM3 KIAA1712 SH3BP5L ST8SIA2 CAB39L ITM2C NUAK2VANGL1, SPRY4, DGCR14, CASP2, TLN2, ITCH, FRMD8, SYT15, MRO, PIP4K2B, SPOP, ZNRF3, MLSTD2, NICN1, CASP10, TMEM101, DYRK2, MEGF11, MEGF10, CUL4B, PARD6G, PARD6B, USP32, GPT2, UNC119B, USP30, NFATC2IP, C9orf100, ZNF587, PPP1R15B, ZNRF1, PIK3R3, GAS7, DGKD, SELI, CDC14B, PDXK, RUNX2, CBFA2T3, CBL, RNMT, WDR22, HDAC3, EIF2S2, PRPF4B, PHOX2B, LIMD1, CDKL2, TSPAN18, MPZL1, SNX21, SEMA5A, C1orf201, HN1L, CD1A, USP6, DERL3, ISX, SYT12, VAPA, UBTD2, DLGAP2, MOBKL1A, PSCD1, BCL7B, MED14, ZNF235, MGC21874, ZNF264, MAP4K4, AKAP6, MED20, CHST10, GOSR1, BAG5, H6PD, CD47, ZNF592, PPM1F, HS2ST1, IQCB1, CD59, SLC25A44, EDEM1, SNPH, KIAA0513, RASSF2, RAPGEF5, BCLAF1, TATDN2, MRPL19, KIAA0141, GINS1, LBA1, NUAK1, SRGAP3, FAM20B, KBTBD11, ZBTB40, XYLB, SLC23A2, MED13, SLC12A6, HDAC5, BCL2L11, ABCC5, CTDSP2, FAM13A1, WASF2, EFS, NMUR1, SIRPB1, ABCA9, RAPGEF3, SMC2, IVNS1ABP, IGF2BP3, RRAGA, TRAF3IP2, ADCY2, SEC24A, SDCCAG3, C5orf3, PDE10A, CEACAM8, EHMT2, COPS8, KIF2C, B3GNT1, POLS, DUSP14, BVES, LZTS1, SEC23IP, ZHX1, VPS26B, BTBD14B, SLCO2B1, CBX3, C20orf108, OLIG1, MED12L, SSX2IP, LOH12CR1, ZMYND17, ZNF641, ADFP, C17orf57, KRT222P, NR2C2AP, LYPLAL1, IQGAP3, COL4A3, MBOAT2*	1153
miR-744	B, C	*CSMD2, GRIN2D, JUNB, LRP3, VPS37D*	5
miR-934	B, C	*ADNP, ATP6V1C1, BCLAF1, DCUN1D3, EAF1, EML1, EPB41L1, ESR1, EVI5, FAM120C, FAM45A, FBXL17, GPHN, HELZ, KCNMA1, LMO4, LOC399947, LRRN1, LRRN3, MEX3A, MLL3, NAV2, NEGR1, PGRMC1, PICALM, RANBP9, RERE, RUNX1T1, SBK1, SHC1, SLC1A2, SLC35F1,UBE2E1, WAC, YWHAZ, ZFAND5, ZNF217*	36

None: let-7g*, let-7f-2*, miR-151-3p. A: PITA top targets; B: picTar; C: Targetscan, D: miRanda, E: Diana MicroT

Table 7. Validated targets for microRNAs without expression in WS.

miRNA	mRNA target	Database
Let-7g*	IGF2BP1 / IMP-1, HMGA2	A
miR-206	GJA1, TAC1, ESR1, MET, UTRN, GJA1, FSTL1, ESR1	A y B

none let-7f-2*. Database A: miRecords, B: Tarbase.

Table 8. Potential targets of miRNAs without expression in WS.

miRNA	**RNA target**	**Database**
miR-206	ADPGK, AP3D1, API5, ARCN1, AZIN1, BACH2, BCL11A, BCL7A, BDNF, C11orf1, C20orf112, C7orf23, CLCN3, CLTC, CNN3, CORO1C, CPEB1, CREM, CTTNBP2NL, DDX5, DHX15, E2F5, EDN1, EFNB2, EIF1AX, EIF4E, ETS1, EYA4, FBXO33, FNDC3A, FNDC3B, FOXP1,FUBP1, FZD7, GDAP1L1, GJA1, GNPDA2, HDAC4, HIAT1, HIVEP2, HS3ST3B1, HSP90B1, HSPD1, JARID2, JOSD1, KIF2A, KTN1, LPPR4, MAB21L1, MAP4K3, MATR3, MEIS1, MIPOL1, MLL5, MMD, MNT, MPP5, MYLK, NADK, NR4A2, NRP1, PAFAH1B1, PAX3, PDCD10, PTPLAD1, PTPRG, RASA1, RNF38, RNGTT, RRBP1, RSBN1, RSBN1L, RSPO3, SEC63, SEMA6D, SFRS1, SLC25A25, SLC44A1, SMEK2, SNAI2, SNAP25, SNX2, SPRED1, TBC1D15, TBP, THBS1, TIMP3, TMCC1, TNKS2, TRIM2, UBE2H, USP33, VAMP4, VGLL4, WDR1, WDR48, YWHAZ, ZFP36L1	A, B y C

None: let-7f-2*, let-7g*. A: PITA top targets; B: picTar 5-way; C: Targetscan conserved targets.

Table 9. KEGG potential pathways associated to microRNAs with or without differential expression

Pathway	let-7c	miR-125b	miR-149	miR-151-3p	miR-200c	miR-345	miR-452	miR-489	miR-744	miR-934	miR-206	mir-15a*	miR-32	mir-378*	miR-589*
Axon guide	■	■	■	■	■	■	■	■	■	■		■		■	■
Glutamato metabolism	■	■	■			■				■		■	■	■	
HedgeHog Pathway	■		■		■	■	■				■	■	■		■
Neuroactive Ligand Receptor Interaction	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Neurodegenerative disease	■				■	■				■		■	■	■	■
Neurotransmitters Pathway										■					
Notch signalling pathway	■	■	■	■	■	■	■			■				■	■
Olfactory Transduction					■					■	■		■		
Regulation of actine citoskeleton	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SHH pathway							■					■			■
Snare transport Interactions in vesicular transport	■	■	■	■	■						■	■	■	■	■

None: Let-7g*, let-7f-2* Based in KEGG Database. Filled cells with p value less than 0.05. SHH: sonic Hedgehog.

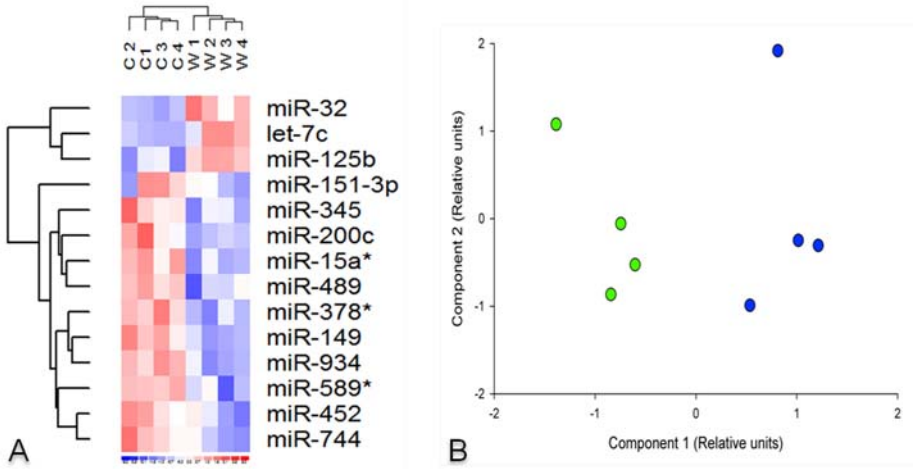

Figure 4. Unsupervised hierarchical cluster analysis and principal component analysis of significantly differentially expressed miRNAs. (a) Unsupervised hierarchical cluster analysis of 14 miRNAs based on the variation of miRNA abundance demonstrates different patterns between WS individuals and controls corresponding to distinct miRNA expression pattern of the two groups (P minor than 0.05). Normalized CT for each assay was transformed into **Δ**CT against the average CT of all assays examined and clustered without centering the data. Blue denotes down-regulated expression and red up-regulated expression compared to the mean A pseudocolor scale outlines the CT values represented C1-C4 depicts controls and W1-W41–30 individual patients (Table 2). (b) Principal component analysis (PCA) using the values in panel B shows the clear separation between the WS patients (green circles) and the controls (blue circles)

adults, or related diseases affecting this tissue according to KEGG database (table 9).

5. DISCUSSION

In an effort to identify possible mechanisms involved in the pathogenesis associated with the WS neurocognitive phenotype, we compared the expression of miRNAs in neural cells derived from the patient's own ONE against individuals without intellectual disabilities. We first established a NSA as culture of exfoliated cells from human ONE. In our study, suspended neurospheres were fixed to slides until immunofluorescence.

The presence of, Nestin, SOX2 and Musashi, corroborate the cells multipotent character and Tubulin beta-III the neuronal progenitor markers. The expression is higher for Nestin, SOX-2 and Musashi and lower for Tubulin beta-III, indicating that multipotential cells predominate over the progenitors. This could imply that the cells are in a very early stage of differentiation, since Tubulin beta-III is an early neuronal marker. To corroborate this, cells were cultured in an adherent manner with fetal bovine serum, which resulted in an increased expression of Nestin (data not shown). Most immunofluorescence reports have neuroespheres that grow by attachment (62-64), but in our study they were suspended and fixed. Therefore we believe that the miRNAs expression profile corresponds to cellular events in early neuronal stages of WS, being this first study to provide data on this regard.

Our analysis showed differential expression of 14 miRNAs and the absence of expression of 3 miRNAs in

individuals with WS compared to controls (table 3). This study provides information on dysregulated miRNAs and their possible involvement in regulatory pathways associated to WS. From significant miRNAs, at least three (miR-125b, let-7c and miR-200c) are brain-rich, and these are related to neural tissue or involved in neural differentiation (44,58,84-87).

miR-125b, whose expression was increased (FC equal to 2.2) , is a miRNA expressed in various tissues and its expression is high in the nervous system. An association with cell cycle regulation and apoptosis has been reported and is described in various types of cancer and also in embryonic development (88-90). In neural tissue it is associated with the development and growth regulation of neural extensions as well as in the regulation of neuronal differentiation via Nestin (91). Moreover miR-125b has also been associated with DS (55-56) and FXS (92-94). DS had over-expression of this miRNA, due to increased gene dosage since it is located on chromosome 21 (55).

There are two miR-125b with equal mature sequences (miR125b-1 and miR-125b-2), which makes them indistinguishable from each other. Outstandingly, miRNAs let-7c and miR-125b2 have the same orientation in the *C21orf34* gene intron 1 and are separated only by 50000 bp (92). miR-125b and let7c expressions are increased by at least 50 percent in fetal hippocampus and heart of DS(56). They are also over-expressed at least 15 times in the frontal cortex neurons of the fetal, child, adolescent and adult brains with DS together with other miRNAs located on chromosome 21 (55). Other reports indicate that let-7c and miR125b correspond to miRNAs highly expressed in human brain in the prefrontal cortex (95). In our study, let-7c has a similar expression pattern to miR-125b, which suggest the mir-125b upregulation corresponds to mir-125b2 sequence and a possible co-expression with let-7 because of their closeness.

miR-125b was also associated to fragile mental retard protein (FMRP). Its expression is abundant in places where this protein is localized (in mice brain). Its overexpression causes thinner and longer neuronal extensions, so that one of its functions is to regulate the size and shape of the dendrites (93). However, to produce this effect it requires the presence of FMRP, and therefore may be associated with FXS. On the other hand, the decreased expression of miR-125b increases the protrusions width. With these data, it is assumed that miR-125b regulates the morphology of dendritic spines and therefore has a role regulating the synapses structure and function. With regards to the data reported on miR-125b targets, its involvement in neuronal differentiation and cytoskeletal organization is suggested. (91). In our study, we identified potential neural pathways of development and of differentiated cells, similar to what has been reported. These predictions together with its different roles in neuronal cells highlight the possible role of this miRNA in WS neurobiology (table 9).

let-7c, over-expressed in our results (FC equal to 2), was one of the first miRNAs associated with embryonic development, it directly regulates lin-28, considered a pluripotency factor (96-98). As miR-125b, let-7c is also associated with FXS and DS. In FXS, it is associated with FMRP and in DS it is over-expressed in the fetal, child and adult brain (55-56,94). Besides these functions, it is associated with neuroprotection through complement C1q and neurotrophic factors and to myasthenia gravis through IL-10 (99-100). According to our results, some of the potential neural pathways in which let-7c participates, as axon guide, HH pathway (Hedge-Hog), neuroactive ligand receptor interaction, notch signaling, regulation of actine cytoskeleton and interactions in vesicular transport. Therefore, we believe that this miRNA is involved in regulating neuronal development.

These roles in neural function and development caused by over-expression of miR-125b and let-7c (91-94, 96-98), point to their possible involvement in WS neural pathogenesis in the same sense since our results show it is upregulated. On the other hand these miRNAs expression is increased in the temporal cortex of Alzheimer (101-102) and schizophrenia patients (103-104), and it is decreased in myotonic dystrophy type 2 (miR-125b-5p) (105); thus supporting that they also have important role in adult neural tissue.

Interestingly, miR-32 is the most upregulated miRNA in our results (FC equal to 3.32), which could be associated with NS structural defects in WS. Neural tissue has a high miR -32 expression, predominantly in white matter, specially in myelin rich brain regions and also in mature oligodendrocytes (106-108). Although its roles are not well defined, dysregulation has been detected in medulloblastomas, glioblastomas and temporal cortex of epileptic patients (109-112). It also causes deficits in myelin proliferation, migration and production, as well as defects in oligodendrocyte differentiation (124). In the gray matter an increase in miR-32 has been reported in patients with Alzheimer's disease (102). It's worth noting that WS diminished cognitive ability is associated to a decrease in cerebral gray matter (11 percent) and white matter volumes (18 percent) (37). The volume reduction could be related to alterations in white matter and this could be associated to miR-32 dysregulation. Therefore miR-32 could be associated to ID.

Considering downregulated miRNAs in our results, miR-15a has a mean expression in the NS, although there are differences between gray and white matter (102, 112-114). An over-expression has been reported in the cortex of individuals with schizophrenia and miR-15 family was predicted as being involved in axonal guidance, longer potentiation and the WNT pathway (104). Besides, our prediction results show the potential involvement in axonal growth, the HH pathway, neuroreceptor interactions, vesicular transport, and their possible association in neurodegenerative diseases. These data support their importance at neural level and the possible alteration of these mechanisms in WS.

miR-452 is the miRNA with highest expression in neural crest cells during a specific embryonic period in a mouse (7 times more compared to non-neural crest cells) (115). One of its documented roles is to regulate Wnt5a, a

signaling molecule for mesenchyme-epithelial interactions. Although its expression is lower in non-neural cells, alteration leads to defects in craniofacial development. It is known that disorders of neural crest development result in defects as DGS, entity that shares many features with WS, like cardiac defects and developmental delay (116). In DGS, the deleted region comprises the *DGSCR8* gene (DGS critical region gene 8) encoding the subunit microprocessor complex that mediates miRNA biogenesis (pri-miRNA of a pre-miRNA). Deletion of this gene results in dysregulation of several miRNAs in the brain, which supports the participation of several miRNAs in regulating various brain processes and therefore their involvement in the pathogenesis of cognitive deficiency (117-118).

According to this paper's hypothesis on the possible alteration of the miRNAs expression caused by hemizygous deletion of WR genes, it was found that CLIP2 (CYLN2) is a potential target of miR-200c and VPS37D for miR-744. CLIP-2 belongs to the CLIP proteins family and has high homology with CLIP-170, encoding a CAP-GLY-domain-containing linker protein 2 (CLIP-115) that regulates microtubule dynamics and has high expression in the NS, predominantly in the hippocampus, amygdala and cerebellum (119-120). CLIP proteins bind to microtubules during growth and have a role in regulating the dynamics of the cytoskeleton network, so CLIP-115 has been associated with WS neurological profile (15, 23-24). Furthermore, CLIP proteins also participate in the bidirectional transport mediated by microtubules through dynein and dynactin. It is assumed that the deletion of this gene alters proper cytoskeletal formation, transport and morphogenesis as well as transportation and growth of neural cell bodies and extensions (119-121). These data are supported by neurological defects present in knockout or heterozygous mice for this gene similar to those identified in WS (24). In this study the miR-200c expression was decreased. We think the results suggest that miRNA sub-expression is a compensation for CLIP115 haploinssuficiency to reduce the degree of structural and functional alterations of the nervous system. Moreover, these structural changes could explain deficiencies of cognitive skills There is a report of a patient with an atypical deletion, which doesn't include *CLIP2*, who had a better cognitive and motor function, compared with the classic WS phenotype, suggesting a possible effect of this gene on the WS (16). Another case reported two healthy brothers, with a pure *CLIP2* hemizygous deletion (122). This demonstrates that this haploinsufficiency is not necessary to cause a WS cognitive profile or neurological deficits. These data could contradict *CLIP2* involvement with the WS cognitive profile. However, we believe that it is possible that miR-200c regulation is more complex due to the likely involvement of different molecules or gene dependent mechanisms contained in the WR.

The *VPS37D* (vacuolar protein sorting 37 homolog D) gene alias *WBSCR24* is a potential target of miR-744. It encodes a vacuolar protein sorting that forms part of the ESCRT-I (endosomal sorting required for transport) (123). Although its role in humans is not well characterized, ESCRT complexes are involved in lisosomal function and trafficking in neural cells, possibly regulating neuronal morphogenesis, endosomal trafficking in neuronal synapsis, neuronal survival and proper signaling (124-129). ESCRT defects are associated to neurodegenerative diseases like amyotrophic lateral sclerosis, autosomic dominant frontotemporal dementia and Huntington's disease (130-133). ESCR-I is involved in several processes in cell growth and differentiation (128-129). Although *VPS37D* is contained in the WR, there are no previous reports focused on alterations that might be associated with the deletion of this gene in humans. The miR-744 downregulation in this study may imply a compensatory mechanism of the hemizygous deletion in order to increase the expression of this gene. However, the diversity of functions in neural tissue suggests that it has an important role in neural tissue.

According to neural pathway predictions, although most dysregulated miRNAs participate, they are less involved in neurotransmitters and olfactory transduction pathways. This supports their possible role in early neural stem cell development disorders, and therefore it provides data of early dysregulated miRNAs in WS.

As we have shown, some of the dysregulated miRNAs in our study have already been associated with diseases such as FXS, DGS and DS. This indicates that the molecular mechanisms involved may converge at some point. However, the differences or particular nuances for each disease depend on its etiology. This is the first study linking WS with a possible involvement of miRNAs as another entity that may present miRNAs dysregulation.

In conclusion, we found alterations in several miRNAs in neural cells derived from ONE, most of them involved in neural development, fetal and adult brain function, and diseases with ID. The dysregulation of these miRNAs may alter the expression of several target mRNAs, although functional test are required to determine it, in addition to assessing whether they affect the structure or function of neuronal cells. This would help to better understand the mechanisms underlying neural dysfunction in WS.

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Abbreviations: WS: Williams Syndrome; ID: Intellectual disability; ONE: olfactory neuroepithelium; miRNA microRNA; NS: Nervous system; WR: Williams region; IQ Intellectual quotient; NSA: Neurosphere assay; FISH: Fluorescent *in situ* hybridization; PCA: Principal Componente Analysis; FC: Fold Change; DS: Down syndrome; FXS: Fragile X Syndrome; FMRP: Fragile Mental Retard Protein; DGS: DiGeorge Syndrome

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