Research Article

Expression Profiles of Exosomal MicroRNAs Derived from Cerebrospinal Fluid in Patients with Congenital Hydrocephalus Determined by MicroRNA Sequencing

Shiyu Chen, Hao Li, Jicui Zheng, Lili Hao, Tianrui Jing, Peixuan Wu, Bowen Zhang, Duan Ma, Jing Zhang, and Jing Ma

1Institutes of Biomedical Sciences, Fudan University, Shanghai, China
2Children’s Hospital of Fudan University, Shanghai, China
3School of Basic Medical Sciences, Fudan University, Shanghai, China
4ENT Institute, Department of Facial Plastic and Reconstructive Surgery, Eye & ENT Hospital, Fudan University, Shanghai, China

Correspondence should be addressed to Duan Ma; duanma@fudan.edu.cn, Jing Zhang; 13816653516@139.com, and Jing Ma; 14111010077@fudan.edu.cn

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Purpose. Congenital hydrocephalus is one of the most common birth defects worldwide. Exosomal microRNAs (miRNAs) in body fluids have been implicated in many diseases. However, their involvement in cerebrospinal fluid from congenital hydrocephalus is not well understood. This study is aimed at investigating the role of dysregulated exosomal miRNAs in congenital hydrocephalus.

Methods. We collected cerebrospinal fluid samples from 15 congenital hydrocephalus patients and 21 control subjects. We used miRNA sequencing to generate exosomal miRNA expression profiles in three pairs of samples. We identified 31 differentially expressed exosomal miRNAs in congenital hydrocephalus and predicted their target miRNAs.

Results. Three microRNAs (hsa-miR-130b-3p, hsa-miR-501-5p, and hsa-miR-2113) were selected according to their fold changes and the function of their target miRNAs, and only hsa-miR-130b-3p and hsa-miR-501-5p were confirmed their expression levels in all samples. Moreover, upregulated hsa-miR-130b-3p might mediate the downregulation of the phosphatase and tensin homolog gene (PTEN), which has been associated with hydrocephalus, via binding to its 3’-untranslated region by dual-luciferase reporter assay.

Conclusion. This study implicates that abnormally expressed exosomal miRNAs in cerebrospinal fluid may be involved in the pathomechanism of congenital hydrocephalus.

1. Introduction

Congenital hydrocephalus (CH) is among the top five birth defects worldwide, with a prevalence of 4.65 in 10,000 births [1]. As one of the most common congenital central nervous system anomalies, CH results from the accumulation of cerebrospinal fluid in the brain ventricles, leading to severe neurological damage. The main clinical manifestations are ventriculomegaly, increased intracranial pressure, and brain dysfunction, which may lead to dysgnosia, while ventriculomegaly can critically impair the developmental processes affecting various anatomical and functional aspects of brain maturation [2]. Ventriculoperitoneal shunts are common treatments for CH; however, intracranial pressure may be normal or even low in some patients, including those with other structural brain abnormalities, and surgical shunting may fail to improve the neurological impairment in those patients [2, 3]. Furthermore, there is a high risk of postoperative complications, such as intracranial infection. It is therefore important to explore the etiology and pathogenesis of CH to improve its prevention and treatment.

The underlying causes of CH are currently not well understood, but it is generally believed to be a multifactorial disease involving genetic and environmental interactions. CH may be associated with chromosomal (e.g., abnormality of chromosome 6q and Xp22.33) or single gene
abnormalities [1], and mutations of at least 43 genes/loci have been associated with CH in animal models or patients [4]. In addition, gene expression disorders may also be involved in the etiology of CH [5]. Among the factors regulating gene expression, microRNAs (miRNAs; miR) are responsible for modulating nearly one-third of genes and thus regulating a variety of physiological processes [6]. Although miRNAs are found widely both inside and outside cells, about 70% of all miRNAs are stably distributed in exosomes, instead of in their host cells [7]. Exosomes are phospholipid bilayer vesicles, with a diameter of 40–100 nm. They are secreted by cells and form a cell-to-cell information transmission system by carrying various biological molecules (protein, DNA, mRNA, and miRNA), which in turn affect the physiological state of the cells and are closely related to the occurrence and process of a variety of diseases [8]. Exosomes can cross the blood-brain barrier and are readily accessible in various human biofluids, including cerebrospinal fluid [9, 10]. The characteristics of exosomes with a substantial miRNA content ensure to act as promising biomarkers in some diseases. For instance, exosomal miR-181-5p from blood samples was identified as a potential diagnostic biomarker for early-stage non-small-cell lung cancer [8], and exosomal miR-150 and miR-21 from blood samples were indicated as biomarkers for the early detection of colorectal cancer [11]. These exosomal miRNAs can be delivered to recipient cells to exert their functions [12, 13] by affecting the translation or stability of their target miRNAs via direct interactions [9]. The existence and importance of exosomal miRNAs in cerebrospinal fluid have been confirmed in several neurological disorders, such as Alzheimer’s disease [14], Parkinson’s disease [15], and intraventricular hemorrhage (IVH) in preterm infants [16]. However, the role of exosomal miRNAs in the cerebrospinal fluid in CH remains unknown.

In the current study, we characterized the exosomal miRNA profile of cerebrospinal fluid from patients with CH using miRNA sequencing and bioinformatics analysis and focused on three miRNAs (miR-130b-3p, miR-501-5p, and miR-2113). We verified the differential expression of the above three miRNAs in patients with CH, and we also investigated the expression of phosphatase and tensin homolog gene (PTEN), which was associated with hydrocephalus in previous reports [17] via the mechanism of these miRNAs.

### 2. Materials and Methods

#### 2.1. Patients and Samples

Cerebrospinal fluid samples were obtained from the age-sex-matched 15 CH patients and 21
control subjects (CS) from the Children's Hospital of Fudan University. The diagnosis of CH was carried out at the Children's Hospital of Fudan University and excluded trauma, tumor, secondary hydrocephalus, and infection. The CS were the patients diagnosed with three types of secondary hydrocephalus (intracranial space-occupying lesions, intracranial hemorrhage, and congenital tethered cord syndrome). The cerebrospinal fluid samples were collected and filtered through a 0.22 μm filter to remove additional cellular fragments. Exosomes were isolated and purified from the supernatant using an exoEasy Maxi Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Total exosomal RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA quality and concentration were evaluated based on the optical density 260/280 and 260/230 ratios using a Nano Drop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was assessed by agarose gel electrophoresis.

2.2. Exosome Isolation and RNA Extraction. The cerebrospinal fluid samples were centrifuged for 30 min at 900 g and 4°C to remove cells and large debris. The supernatant was collected and filtered through a 0.22 μm filter to remove additional cellular fragments. Exosomes were isolated and purified from the supernatant using an exoEasy Maxi Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. Total exosomal RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions. The RNA quality and concentration were evaluated based on the optical density 260/280 and 260/230 ratios using a Nano Drop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was assessed by agarose gel electrophoresis.

2.3. miRNA Sequencing and Data Analysis. Exosomal miRNA libraries were constructed using total RNA samples from exosomes and assessed using an Agilent 2100 Bioanalyzer (Agilent, California, USA). The 3' and 5' adapters were ligated to total RNA samples, respectively, and cDNAs were synthesized with the adapter-ligated miRNAs and used as templates for polymerase chain reaction (PCR) amplification. Amplified fragments of 135-155 base pairs were selected to construct miRNA libraries, which were then denatured with 0.1 M NaOH and sequenced using a TruSeq Rapid SR Cluster Kit (Illumina, CA, USA) with Illumina NextSeq 500, according to the manufacturer’s instructions. Raw reads were subjected to quality control to assess the suitability of the raw data for subsequent analysis. Trimmed data were obtained by removing the 3' adapters and shorter reads (≤15 base pairs) from the raw data. The subsequent reads were aligned with the human reference genome annotated with miRNA to generate an miRNA expression value (counts per million reads; CPM) using miRDeep2 [17]. The detected miRNAs were determined based on an average CPM value > 1.

2.4. Differential Expression of Exosomal miRNAs. Differentially expressed exosomal miRNAs were identified using edgeR with a threshold fold change (FC) > 1.5 and P value ≤ 0.05. We used the CPM value of significantly expressed exosomal miRNAs to perform hierarchical clustering analysis and reveal the expression patterns of the exosomal miRNAs and samples. Scatter plots were generated to assess the...
Figure 1: Differential expression profiles of exosomal miRNAs in CH patients and CS. (a) Hierarchical clustering analysis of exosomal miRNAs between the CH patients (CH1, CH2, and CH3) and CS (CS1, CS2, and CS3). Expression values are represented by red and green shades, indicating expressions above and below the median expression level across all samples, respectively. (b) The scatter plot of 910 exosomal miRNAs. Pearson’s correlation coefficient was 0.849. The red dots indicate upregulated genes, the green dots indicate downregulated genes, and the black dots indicate nondifferentially expressed genes. (c) The volcano plot of 910 exosomal miRNAs. The fold change threshold is 1.5 and P value ≤ 0.05. The red dots indicate upregulated genes, the green dots indicate downregulated genes, and the black dots indicate nondifferentially expressed genes.
Table 4: Upregulated and downregulated miRNAs in the volcano plot.

| miRNA ID     | Log₂FC | miRNA ID     | Log₂FC |
|--------------|---------|--------------|---------|
| hsa-miR-2113 | 7.910966974 | hsa-miR-501-5p | -6.405186884 |
| hsa-miR-302d-3p | 7.613506656 | hsa-let-7e-3p | -3.172106369 |
| hsa-miR-137-5p | 3.781667006 | hsa-miR-29c-5p | -2.72261609 |
| hsa-miR-320e | 3.453737277 | hsa-miR-223-5p | -1.647303056 |
| hsa-miR-320c | 3.379260772 | hsa-miR-584-5p | -1.59751174 |
| hsa-miR-320c | 3.373467094 | hsa-miR-320b | 2.229398228 |
| hsa-miR-129-5p | 3.194153868 | hsa-miR-129-5p | 3.194153868 |
| hsa-miR-129-5p | 3.194153868 | c | |
| hsa-miR-320b | 3.143871008 | | |
| hsa-miR-130b-3p | 3.037326563 | | |
| hsa-miR-442 | 3.015750545 | | |
| hsa-miR-320d | 3.00870049 | | |
| hsa-miR-320d | 2.93745457 | | |
| hsa-miR-412-5p | 2.780774639 | | |
| hsa-miR-296-3p | 2.607140495 | | |
| hsa-miR-708-3p | 2.537461519 | | |
| hsa-miR-320a-3p | 2.480146691 | | |
| hsa-miR-1224-5p | 2.413574833 | | |
| hsa-miR-134-5p | 2.381827533 | | |
| hsa-miR-1298-5p | 2.24115725 | | |
| hsa-miR-760 | 2.091967229 | | |
| hsa-miR-136-5p | 2.0757691 | | |
| hsa-miR-181a-3p | 2.051552079 | | |
| hsa-miR-193a-5p | 1.813698773 | | |
| hsa-miR-7704 | 1.784297108 | | |

FC: fold change.

distribution trends of the miRNAs in the CS and CH patients. Differentially expressed exosomal miRNAs were screened based on a \( \log_{10}FC \) and \(-\log_{10}P\) value to generate volcano plots demonstrating the relationship between the FC of differential expression and statistical significance.

2.5. The Target Genes of miRNA Prediction, Functional Annotation, and Pathway Enrichment. The miRDB and TargetScan algorithms were used to predict the target genes of exosomal miRNAs that were differentially expressed between the CS and CH patients. Functional enrichment of the target genes was then determined by Gene Ontology (GO) (http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg) analyses.

2.6. Quantitative Polymerase Chain Reaction (qPCR). cDNAs were synthesized from 1 \( \mu \)g total RNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Tokyo, Japan), and qPCR was conducted with SYBR Premix Ex Taq™ (Takara) on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). The relative expression levels of miRNAs and mRNAs were normalized to the housekeeping gene U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively, and were calculated by the relative quantification method (\( 2^{\Delta\Delta Ct} \)).

The primers used were as follows: hsa-miR-2113-GSP: GGGGATTTGTGCTTGGCTGC, hsa-miR-2113-R: GTGCGTGCTTGAGGTAGCTG, hsa-miR-130b-3p-GSP: GGGACGGTCAATGATGAGAA, hsa-miR-130b-3p-R: GTGCGTGTCGTGGAGGTCG, hsa-miR-501-5p-GSP: GGAGAATTCTTGTCCCTGG, hsa-miR-501-5p-R: GTGCGTGTCGTGGAGGTCG, U6-F: GCTTCGCCAGCACTATACTAAAAAT, U6-R: CGCTTCACAGAATTTCGCTGTCAT; PTEN-F: ACACGACGGGAAGACAAGTT, PTEN-R: CTGGTCCTG TGATGAGAAATG; and GAPDH-F: GGGAAAACTGTGGCGTGAT, GAPDH-R: GAGTGGGTTGCGCTGGTGA.

2.7. Cell Culture. Human embryonic kidney 293 (HEK293T) cells were seeded in Dulbecco’s modified Eagle’s medium (Biological Industries, Kibbutz Beit HaEmek) with 10% fetal bovine serum (Biological Industries) at 37°C in 5% CO₂. All cell culture dishes and culture plates were purchased from Hangzhou Xinyou Biotechnology Co., Ltd.

2.8. Dual-Luciferase Reporter Assay. The recombinant plasmid pGL3-promoter-PTEN-WT (wild-type PTEN 3'-untranslated region (UTR)) and pGL3-promoter-PTEN-Del (deleted PTEN 3'-untranslated region (UTR)) were constructed. Mimics and NC oligonucleotides for hsa-miR-130b-3p were obtained from RiboBio Co., Ltd. (China). HEK293T cells (Cell Bank, Shanghai, China) were seeded in 96-well plates at \( 1 \times 10^4 \) cells per well and incubated overnight at 37°C. The respective mimics and NC oligonucleotides were cotransfected into HEK293T cells with pGL3-promoter-PTEN-WT/pGL3-promoter-PTEN-Del and pGL3- Renilla using Lipofectamine 3000 (Invitrogen). Cells were then harvested 48 h after transfection. Both firefly and Renilla luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega, USA), and the firefly luciferase activities were normalized to Renilla luciferase activities.

The primers used were as follows: PTEN-3utr-Xba1-F: GCTCTAGAGCGgctgccttttctagcaccaatatgc, PTEN-3utr-Xba1-R: GCTCTAGAGCTgctgccttttctagcaccaatatgc.

2.9. Statistical Analysis. All experiments were repeated three times. All statistical analyses were performed by paired two-tailed Student’s t-tests using GraphPad Software (GraphPad Inc., La Jolla, CA, USA). A value of \( P < 0.05 \) was considered significant.

3. Results

3.1. Analysis of Differentially Expressed Exosomal miRNAs in CH Patients and CS. Differential expression of miRNAs in cerebrospinal fluid exosomes from three CH patients and three CS was analyzed using edgeR. The criteria for differential miRNA expression were an FC threshold of 1.5, \( P \) value \( \leq 0.05 \), and mean CPM \( \geq 1 \). \( \log_{10}FC \) was calculated to represent differential miRNA expression, with a positive value
Figure 2. Continued.

Sig GO terms of target genes of upregulated miRNA

Enrichment score

(a) Intracellular organelle [GO:00444299]
Nucleus [GO:00056340]
Cell [GO:00056231]
Intracellular membrane-bound [GO:00442311]
Nucleolus [GO:00056430]
Intracellular [GO:00056622]
Membrane-bounded organelle [GO:00442277]
Early endosome [GO:00057690]
Cytoplasm [GO:00057373]
Double-stranded DNA binding [GO:0006907]
RNA_polymerase_II_regulator [GO:00009777]
RNA_polymerase_II_regulator [GO:00009712]
Transcription regulator_lex [GO:0001012]
Calcium ion binding [GO:00057373]
Transcription regulator_lex [GO:0001012]
Metal ion binding [GO:0046872]
Sequence-specifc double-strand [GO:0001012]
Cation binding [GO:0046872]
RNA_polymerase_II_transcrip [GO:00009777]
Response to water deprivation [GO:0009414]
Tube morphogenesis [GO:0035239]
Roof of mouth development [GO:0060021]
Skeletal system morphogenesis [GO:0048705]
Protein import [GO:0017038]
Organ growth [GO:0035265]
Amide binding [GO:0033218]
GTP binding [GO:0005525]
Protein transporter activity [GO:0008565]
Purine nucleoside binding [GO:0001883]
Ribonucleoside binding [GO:0032549]
Nucleoside binding [GO:0001882]
Guanyl ribonucleotide binding [GO:0032561]
Endosome [GO:0005768]
Dendritic_sha/f_t [GO:0043198]
Integral component of organism [GO:0031301]
Vesicle [GO:0031982]
Cytoplasm [GO:0005737]
Axon terminus [GO:0043679]
Neuron projection terminus [GO:0044306]
Endosome membrane [GO:0010008]
HMG box domain binding [GO:0071837]
Protein binding [GO:0005515]
Peptide binding [GO:0042277]
Embryonic skeletal system morphogenesis [GO:0048704]
Cartilage development [GO:0051216]
Tube development [GO:0035295]
Positive regulation of protein metabolic process [GO:0070863]
Dendritic spine [GO:0043197]
Neuron spine [GO:0044309]

**Figure 2: Continued.**

(b) GO Biological Process Classification

Metabolic_process [261]
Regulation_of_cellular_process [310]
Cellular_process [380]
Primary_metabolic_process [248]
Regulation_of_nitrogen_compound_metabolic_process [208]
Nitrogen_compound_metabolic_process [236]
Macromolecule_metabolic_process [221]
Cellular_nitrogen_compound_metabolic_process [232]
Regulation_of_metabolic_process [221]
Figure 2: Continued.
indicating upregulation and a negative value indicating downregulation. We identified thousands of differentially expressed human miRNAs.

We performed differential expression analyses of three miRNAs by hierarchical clustering, scatter plots, and volcano plots, respectively. Hierarchical clustering analysis of exosomal miRNA signal intensities revealed evidence of significant differential expression of exosomal miRNAs between the CH patients and CS (Figure 1(a)). There were 910 miRNAs in the scatter plot, of which 314 and 274 were upregulated and downregulated, respectively (Figure 1(b)). Pearson’s correlation coefficient was 0.849. Among these miRNAs, 26 and 5 were significantly upregulated and downregulated in the volcano plot, respectively (Figure 1(c), Table 4).

3.2. Target Gene Prediction for Differentially Expressed Exosomal miRNAs. The top ten most upregulated exosomal miRNAs (hsa-miR-129-5p, hsa-miR-130b-3p, hsa-miR-2113, hsa-miR-302d-3p, hsa-miR-320b, hsa-miR-320c, hsa-miR-320d, hsa-miR-320e, hsa-miR-4429, and hsa-miR-137-5p) and the top four most downregulated exosomal miRNAs (hsa-let-7e-3p, hsa-miR-223-5p, hsa-miR-501-5p, and hsa-miR-584-5p) were selected for evaluation. Target genes were predicted using TargetScan and miRDB, generating 4640 potential target genes, including 3542 genes for upregulated and 1098 genes for downregulated miRNAs.

3.3. Functional Analysis of Differentially Expressed Exosomal miRNA Target Genes. To further highlight the functional features of exosomal miRNAs, the target genes were annotated using GO terms. The target genes of upregulated miRNAs were mainly enriched in "regulation of nitrogen compound metabolic process" (208 genes, P = 3.14E-12), "nuclear lumen" (145 genes, P = 6.31E-10), "double-stranded DNA binding" (47 genes, P = 4.34E-07), and so on (Figures 2(a), 2(c), 2(d), and 2(e), Table S1). The target genes of downregulated miRNAs were mainly enriched in "response to water deprivation" (two genes, P = 6.25E-04), "dendritic_spine" (four genes, P = 7.63E-03), "HMG_box_
Sig pathway of target genes of upregulated miRNA

| Pathway                          | Genes |
|----------------------------------|-------|
| MTOR_signaling_pathway [hsa04150]| 12    |
| Adrenergic_signaling_in_car...   | 11    |
| TGF-beta_signaling_pathway [hsa04350]| 8     |
| Endocytosis [hsa04144]          |       |
| Autophagy-animal [hsa04140]     | 9     |
| Longevity_regulating_pathway [hsa04211]| 7    |
| PS3_signaling_pathway [hsa04115]| 6     |
| Axon_guidance [hsa04360]        | 10    |
| Phosphatidylinositol_signal...   | 7     |
| Oocyte_meiosis [hsa04114]       | 8     |

Sig pathway of target genes of downregulated miRNA

| Pathway                          | Genes |
|----------------------------------|-------|
| Spliceosome [hsa03040]           | 3     |
| Apelin_signaling_pathway [hsa04371]| 3    |
| Phospholipase_D_signaling_p...   | 3     |

**Figure 3: Continued.**
Sig pathway of target genes of upregulated miRNA

- MTOR_signaling_pathway [hsa04150]
- Adrenergic_signaling_in_cardiovascular_system [hsa04261]
- TGF-beta_signaling_pathway [hsa04350]
- Endocytosis [hsa04144]
- Autophagy-animal [hsa04140]
- Longevity_regulating_pathway [hsa04211]
- P53_signaling_pathway [hsa04115]
- Axon_guidance [hsa04360]
- Phosphatidylinositol_signaling [hsa04070]
- Oocyte_meiosis [hsa04114]

Enrichment score (-log 10 (p-value))

Count
- 6
- 8
- 10
- 12
- 14

(c)

Figure 3: Continued.
domain_binding” (two genes, $P = 3.18 \times 10^{-03}$), and so on (Figures 2(b), 2(f), 2(g), and 2(h), Table S2).

KEGG enrichment analysis demonstrated that target genes were significantly enriched in 54 signaling pathways, of which “mTOR signaling_pathway” (12 genes, $P = 1.83 \times 10^{-04}$) was the most significantly enriched pathway of upregulated miRNA target genes (Figures 3(a) and 3(c), Table S3), and “spliceosome” (three genes, $P = 2.62 \times 10^{-02}$) was the most significantly enriched pathway of downregulated miRNA target genes (Figures 3(b) and 3(d), Table S3). These results suggest that CH has various genetic and phenotypic characteristics.

3.4. Real-Time qPCR Validation of Differentially Expressed Exosomal miRNAs. Among these signaling pathways, we selected PTEN as a target gene in “nervous system development”, because this has been related to hydrocephalus in previous reports [17] (Figure 4(a), Figure S1). The miRNA corresponding to PTEN was hsa-miR-130b-3p. In addition, hsa-miR-2113 and hsa-miR-501-5p were noticeably differentially expressed in the CH patients (Table 4). Future studies should be carried out focusing on larger samples at an individual level. We performed real-time qPCR validation of these three miRNAs in cerebrospinal fluid exosomes from 15 CH patients and 21 CS and revealed that hsa-miR-130b-3p was upregulated, while hsa-miR-501-5p was downregulated, in CH patients compared with CS (Figures 4(b) and 4(c)). However, there was no significant difference in hsa-miR-2113 expression between CH patients and CS (Figure 4(d)). The trends in expression levels of these two miRNAs according to qPCR were in accordance with the miRNA sequencing results.

3.5. Upregulation of hsa-miR-130b-3p Decreased Expression of PTEN via the Predicted Binding Site. PTEN is a potential target gene of hsa-miR-130b-3p. PTEN was downregulated in the 15 CH patients compared with 21 CS, according to the real-time qPCR results, in contrast to the trend for hsa-miR-130b-3p (Figure 5(a)). The TargetScan showed the predicted binding sites in the 3′-UTR of PTEN with hsa-miR-130b-3p (Figure 5(b)). hsa-miR-130b-3p mimics significantly suppressed luciferase reporter activity compared with NC mimics, after transfection with pGL3-promoter-PTEN-3′-UTR (Figure 5(c)). To avoid unspecific binding, the binding site in pGL3-promoter-PTEN-3′-UTR was deleted. Transfection of hsa-miR-130b-3p mimics significantly inhibited pGL3-promoter-PTEN-3′-UTR-WT

**Figure 3:** KEGG pathway significantly enriched in the predicted target genes of differentially expressed exosomal miRNAs in CH patients. (a, c) Enriched top 5 pathways of differentially upregulated miRNA target genes in CH patients compared with CS. (b, d) Enriched top 3 pathways of differentially downregulated miRNA target genes in CH patients compared with CS. Size and color of the bubble represented the amount of differentially expressed genes enriched in the pathway and enrichment significance, respectively.

| Enrichment score ($-\log 10 (p\_value)$) | Count | Pathway Name | Enrichment score ($-\log 10 (p\_value)$) | Count | Pathway Name |
|---------------------------------------|-------|--------------|---------------------------------------|-------|--------------|
| 1.50                                  | 3     | Spliceosome  | 1.52                                  | 3     | Apelin_signaling_pathway |
| 1.54                                  | 3     | Phospholipase_D_signaling_pathway | 1.56 | 3     | |
| 1.58                                  | 3     | |

**Figure 4:**

- **(a)** Enriched top 5 pathways of differentially upregulated miRNA target genes in CH patients compared with CS.
- **(b)** Enriched top 3 pathways of differentially downregulated miRNA target genes in CH patients compared with CS. Size and color of the bubble represented the amount of differentially expressed genes enriched in the pathway and enrichment significance, respectively.
activity, but had no effect on pGL3-promoter-PTEN-3′-UTR-Del activity (Figure 5(d)). These results show that upregulation of hsa-miR-130b-3p regulated downregulation of PTEN via the predicted binding site.

4. Discussion

Extracellular circulating miRNAs exist in most human body fluids, including cerebrospinal fluid, and are highly stable [18]. The delivery of miRNAs to recipient cells in circulating exosomes provides a novel method of intercellular communication. Dysregulation of exosomal miRNAs is an emerging element in a number of diseases, which reveals the important roles of exosomal miRNAs in both physiological and pathological pathways [9]. Several studies have implicated the function of exosomal miRNAs in some kinds of hydrocephalus. For example, Spaull et al. provided the first evidence for exosomes and exosomal miRNA expression in

![Graph](image-url)
the cerebrospinal fluid in patients with posthemorrhagic hydrocephalus, and the increase in miR-1991-5P following the development of posthemorrhagic hydrocephalus made this an interesting potential biomarker [19]. hsa-miR-4274 was identified as a potential cerebrospinal fluid biomarker for idiopathic normal pressure hydrocephalus, with diagnostic potential, as well as the ability to predict the response to shunt treatment [20]. A prolonged elevation was shown in grade IV vs. grade III of intraventricular hemorrhage with higher miR-155 and miR-181b expression in cerebrospinal fluid at days 41-60 after intraventricular hemorrhage. These alterations may contribute to the development of later clinical complications in this clinical condition [21]. However, the role of exosomal miRNAs in the cerebrospinal fluid of patients with CH remains unclear. We therefore sequenced exosomal miRNAs in cerebrospinal fluid samples from three CH patients and three CS to compare the miRNA expression profiles and explore their functions in CH. As it has been reported that age and gender differences affect the expression pattern of miRNA in exosomes, the CH patients were compared with the age-sex-matched CS in our study [22, 23]. We identified 31 significantly expressed exosomal miRNAs in CH, including 26 that were upregulated and 5 that were downregulated. Among these differentially expressed miRNAs, hsa-miR-2113 and hsa-miR-501-5p were the most significantly upregulated and downregulated, respectively. Previous studies indicated that hsa-miR-2113 was associated with epithelial-mesenchymal transition in diabetes [24] and hepatocellular carcinoma [25], while hsa-miR-501-5p played an important role in modulating tumor progression, e.g., in hepatocellular carcinoma [26], gastric cancer [27], and colorectal cancer [28]. However, their roles in CH have not been investigated.

Because the main mechanism of miRNA is that it can recognize the target mRNA through base complementary pairing and guide the silencing complex to degrade the target mRNA or block the translation of the target mRNA according to the degree of complementarity, we predicted the target mRNAs of differentially expressed exosomal miRNAs and carried out GO and KEGG pathway enrichment analyses to reveal the biological processes and functions of the target mRNAs. Then, we found hsa-miR-130b-3p was predicted to be combined with PTEN, which was enriched in the "nervous system development" pathway. hsa-miR-130b-3p has been reported to have critical roles in CH; PTEN is located on 10q23.3 and encodes a lipid phosphatase with important roles in intracellular signal transduction through dephosphorylation of substrates such as Akt and S6 kinase [30]. Previous studies have suggested that PTEN was required for brain formation, and that dysregulation of PTEN resulted in abnormal brain development and progressive hydrocephalus [17]. A novel germline mutation of the PTEN gene is associated with VATER hydrocephalus syndrome [31]. And hsa-miR-130b-3p has been reported to...
negatively regulate PTEN by binding to the 3′-UTR in PTEN [29–31]. These suggested that hsa-miR-130b-3p was likely to play a pivotal role in the development of CH by targeting PTEN.

We therefore selected these three miRNAs (hsa-miR-2113, hsa-miR-501-5p, and hsa-miR-130b-3p) and PTEN for real-time qPCR validation. Because the cerebrospinal fluid samples were difficult to collect and susceptible to infection, we only collected 15 CH patients and 21 CS for real-time qPCR validation. hsa-miR-130b-3p and hsa-miR-501-5p were upregulated and downregulated, respectively, in CH patients compared with CS, in accordance with the miRNA sequencing results, while there was no significant difference for hsa-miR-2113. miRNA sequencing is a screening method in small samples, and the results of comparison between groups only suggest the possible differences and the false positive results also exist. Furthermore, real-time qPCR showed that PTEN was downregulated in CH patients, in contrast to the trend for hsa-miR-130b-3p. Dual-luciferase reporter assay showed that hsa-miR-130b-3p regulated the expression of PTEN by binding to the predicted site on the 3′-UTR. Therefore, upregulation of hsa-miR-130b-3p may be involved in the development of CH via interacting with PTEN and mediating its downregulation.

To the best of our knowledge, this study provides the first report of the expression profiles of exosomal miRNAs in CH. Exosomal hsa-miR-130b-3p and hsa-miR-501-5p may be involved in the development of CH. The mechanism of hsa-miR-130b-3p in CH has also been partly revealed. These findings will help to provide new diagnostic and therapeutic targets for CH.

Data Availability
The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Ethical Approval
This study was approved by the Institutional Research Ethics Committee of the Children’s Hospital of Fudan University, Shanghai, China (2016-121).

Conflicts of Interest
The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors’ Contributions
J.M., J.Z., and D.M. were responsible for the idea, project design, and concept of the paper. S.C. performed bioinformatics analysis. S.C., L.H., T.J., and J.Z. collected the clinical samples and information. S.C., P.W., L.H., and B.Z. performed the clinical sample detection and in vitro experiments. J.M., S.C., J.Z., and D.M. wrote, edited, and revised the manuscript. All authors read and approved the manuscript. Shiyu Chen, Hao Li, and Jicui Zheng contributed equally to this work.

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Supplementary Materials

Supplementary 1. Figure S1: the target gene PTEN in the significantly enriched KEGG pathway (mTOR signaling pathway).

Supplementary 2. Table S1: the mainly enriched pathways of target genes of upregulated miRNAs in GO analysis. Table S2: the mainly enriched pathway of target genes of downregulated miRNA in GO analysis. Table S3: the mainly enriched pathways of target genes of dysregulated miRNAs in KEGG analysis.

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