Identification and bioinformatic analysis of dysregulated microRNAs in human oligodendroglial cells infected with borna disease virus

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Abstract. MicroRNAs (miRNAs) are recognized as important regulators of gene expression via translational depression or mRNA degradation. Previously, dysregulated miRNAs have been found in neurodegenerative and neuropsychiatric disorders. Borna disease virus (BDV) is a neurotropic, negative single-stranded RNA virus, which may be a cause of human neuropsychiatric disease. BDV is regarded as an ideal model to analyze the molecular mechanisms of mental disorders caused by viral infection. In the present study, 10 miRNAs were dysregulated in human oligodendrocytes (OL cells) infected with the BDV strain, Hu-H1 (OL/BDV). The predicted target genes of those different miRNAs were closely associated with DNA binding, receptor activity, cytoplasm and membrane, biopolymer metabolic process and signal transduction, which were ranked highest using Gene Ontology (GO) analysis, and were predominantly involved in 'Immune system and adaptive Immune system pathways' on pathway analysis. Reverse transcription-quantitative polymerase chain reaction analysis confirmed that seven miRNAs (miR-1290, miR-1908, miR-146a-5p, miR-424-5p, miR-3676-3p, miR-296-3p and miR-7-5p) were significantly downregulated in the OL/BDV cells, whereas two miRNAs (miR-1244 and miR-4521) showed no significant differences between the two groups. The present study revealed for the first time, to the best of our knowledge, the miRNA profile of BDV Hu-H1-infected human OL cells. Based on GO and pathway analyses, further investigation of the signaling processes in BDV-infected oligodendrocytes may offer particular promise in improving understanding of the neuropathogenesis of BDV.

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Introduction

Borna disease virus (BDV), a neurotropic, non-cytolytic, non-segmented RNA virus, is an enveloped virus of ~8.9 kb with six open reading frames (1,2), which infects a wide variety of mammalian species, including horses, sheep and dogs (3). BDV has been widely investigated in neuroscientific fields on account of its numerous unique attributes causing neurobehavioral diseases (4) and the ability to introduce its RNA transcripts into host genomes (5). Previous epidemiological studies have shown that there may be a latent association between BDV infection and human neuropsychiatric diseases (6), encephalitis and other brain diseases (3,7-12). In our previous study, BDV infection was reported in Chinese neuropsychiatric patients and health care professionals (13,14), which supported the hypothesis that BDV can infect humans and may be a pathogen in certain mental disorders, although the underlying molecular mechanism remains to be fully elucidated. However, certain studies have found no direct evidence of BDV infection in schizophrenia, bipolar disorder or major depressive disorder (15-17). The controversy requires resolution prior to use as a diagnostic method to ensure reliability. BDV-associated functional disturbances of neuron and glial cells have been evidenced (18-21) and its potential effects cannot be ignored.

MicroRNAs (miRNAs) are small (~22 nucleotides in length), non-coding, single-stranded RNAs (22). They regulate

gene expression by binding to the complementary sequence in the 3'-untranslated region (3'-UTR) of target mRNAs, resulting in inhibited protein synthesis or destabilizing of target mRNA translation at the post-transcriptional level. miRNAs have been shown to be pervasive in several biological processes, including cell death, cell proliferation, the function of immune cells, hematopoiesis and patterning of the nervous system (23). A wide range of studies have revealed that miRNAs are associated with several human diseases, including cancer, chronic inflammation and viral diseases (24-26). Previous studies have also indicated that miRNAs have an effect on neurodegenerative and neuropsychiatric disorders (27-30), which suggests the possibility to associate the mechanisms of BDV infection with miRNA dysregulation.

In the present study, miRNA arrays were used to identify the differences in miRNA expression between oligodendrocytes (OL cells) infected with the Hu-H1 BDV strain (OL/BDV cells) and non-infected OL cells. The differentially expressed miRNAs were then bioinformatically analyzed using Gene Ontology (GO) and pathway analyses, in order to determine their biological function and localization. Finally, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was performed to validate the expression of the differentially expressed miRNAs. The aim of the present study was to determine which miRNAs are dysregualted in OL/BDV cells, and to facilitate further investigation of the role of miRNAs in BDV infection.

Materials and methods

Cell line and preparation of BDV Hu-H1 strain solution. The BDV Hu-H1 strain (passages 75-76 in OL cells), originally isolated from PBMCs of a patient with bipolar disorder, and a human fetal-derived OL cell line, were provided by Professor Hanns Ludwig (Free University of Berlin, Berlin, Germany) (31). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin solution, phosphate-buffered saline (PBS), 0.25% trypsin-EDTA and L-glutamine were purchased from GE Healthcare Life Sciences (Logan, UT, USA). The human OL cell line infected with the BDV Hu-H1 strain was cultured with DMEM in 10% FBS and 100 U/ml penicillin/streptomycin in a humidified incubator (5% CO_2; 37˚C). The preparation and viral titration of the BDV Hu-H1 solution were performed, as described previously (32). The cells in 20 10-cm dishes (density, 10^7) were washed twice with PBS, and 1 ml fresh growth medium was added when the cells in the dishes reached 90% confluence. The cell solution was then frozen (-80˚C) and thawed (25˚C) for 15 min, and repeated three times. The lysate was then centrifuged at 3,000 x g for 10 min at room temperature. The resulting supernatant, which contained infectious viral particles, was used as the stock viral solution.

The OL cells were seeded into 96-well plates ($3x10^4$ cells/well). At 8 h post-adherence, the medium was removed and 100 μl viral solutions were added to each well. The stock viral solution was serially diluted 10-fold five times, with four replicates for each concentration. The cells were cultured for 7 days in DMEM/2% FBS, during which the cell medium was replaced once every 2 days to maintain the extracellular environment. The viral titration was assessed using immunohistochemistry. The BDV-infected OL cells were fixed in 96-well plates for 30 min at room temperature with 4% paraformaldehyde, followed by permeabilization for 10 min in 0.25% Triton X-100. The cells were then rinsed three times with PBS (5 min each time) and blocked with 5% (w/v) skimmed milk solution for 1 h at 37˚C. The cells were then incubated overnight with mouse anti-BDV-specific nuclear-protein (p40) antigen primary monoclonal antibody (provided by Professor Ludwig Hanns, 1:1,000 diluted with PBS) (33) at 4˚C, followed by incubation for 1 h with secondary goat anti-mouse antibody (cat. no. A0216; 1:5,000; Beyotime Institute of Biotechnogy, Shanghai, China) at room temperature. Immunofluorescence was detected using a phase-contrast microscope following three PBS washes (32).

BDV infection of OL cells. A total of 10^5 non-infected OL cells were seeded into four separate 6-well plates (total 24 wells) with 10% FBS in DMEM. Half of these wells were infected with Hu-H1 stock solution (as above) at a multiplicity of infection of 1.0. Specifically, following adherence of the OL cells, the medium was removed, and 150 μl Hu-H1 strain solution was added per well to produce BDV-infected OL (OL/BDV) cells. The cells were stored in a humidified incubator (5% CO_2 at 37˚C) for 1.5-2 h. Next, the excess viral solution was removed by suction at the edge of the plated, and the cells were cultured in fresh medium. The remaining 12 wells of OL cells were maintained as non-infected control OL cells. The two cell groups were incubated under the same conditions for the remainder of the experiment. The BDV infection was detected and observed using an immunouorescence assay, as described previously (32,34).

miRNA arrays. On day 14 post-infection, six wells of the OL and OL/BDV cells were used for miRNA arrays, respectively. Fluorescent miRNA targets were prepared from 1 or 2.5 μg total RNA samples, which were extracted from the OL/BDV and non-infected OL cells using an OneArray® Amino Allyl miRNA Amplification kit (Phalanx Biotech Group, Hsinchu, Taiwan) and Cy5 dyes (GE Healthcare, Piscataway, NJ, USA). Fluorescent targets were hybridized to the Human Whole Genome OneArray® using a Phalanx hybridization buffer on the Phalanx miRNA OneArray® Hybridization system (Phalanx Biotech Group). Following 16 h of hybridization at 50˚C, non-specific binding targets were removed through three washing steps (42˚C for 5 min, 42˚C for 5 min and 25˚C for 5 min, followed by rinsing 20 times), and the slides were dried by centrifugation at 1,000 x g for 3 min at room temperature and scanned using an Axon 4000B scanner (Molecular Devices LLC, Sunnyvale, CA, USA). The intensities of each probe were obtained using GenePix 4.1 software (Molecular Devices LLC). The probes with a $\log_2$ ratio ≥ 0.58 or ≤ -0.58, and $P<0.05$ were defined as differential genes for further pathway enrichment analysis. Each experiment was repeated three times.

Prediction of target genes and bioinformatic analysis. GO analysis (www.geneontology.org) was applied to determine the functions of the intersecting genes on the basis of molecular function, cellular component and biological process. To ensure understanding of the gene expression information,

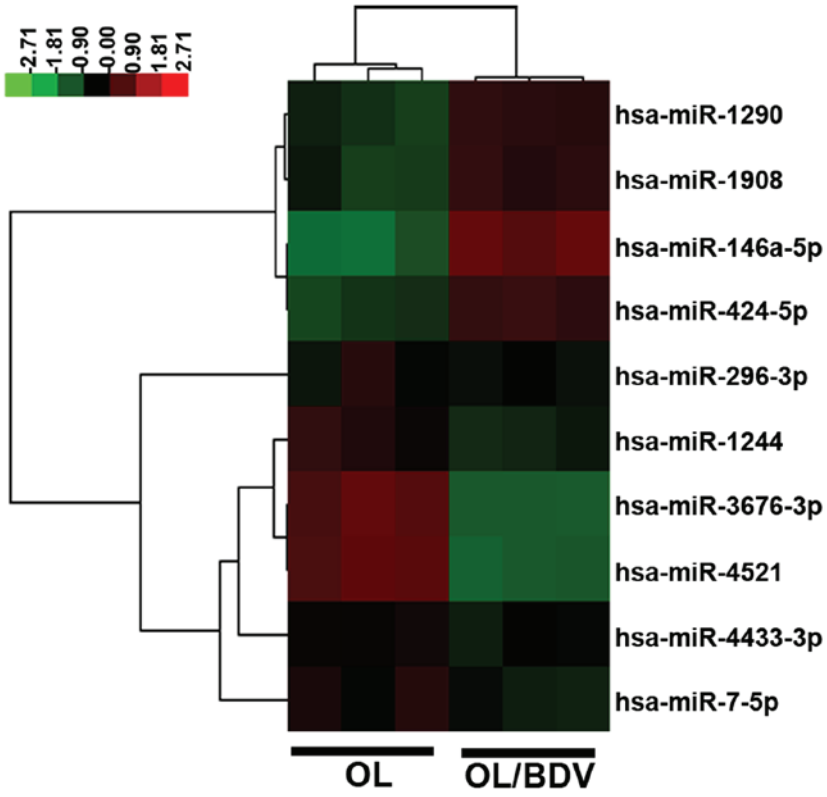

Figure 1. Differentially expressed miRNAs in BDV-infected OL cells (OL/BDV) and non-infected OL cells. A total of 10 miRNAs were differentially expressed between the OL/BDV and non-infected OL cells. The color scale (top) shows the grading of relative expression: Red denotes a higher relative expression level, green denotes a lower relative expression level. miRNA/miR, microRNA; OL, oligodendrocytes; BDV, borna disease virus.

the pathways (www.reactome.org/ReactomeGWT/entrypoint.html and www.genome.jp/kegg/) of the target genes of the different miRNA were also analyzed.

RT-qPCR analysis. On day 14 post-infection, the remaining six wells of OL and OL/BDV cells were used for RT-qPCR assays, respectively. Total RNA was extracted using the miRNEasy Mini kit (cat. no.217004; Qiagen, Hilden, Germany). RT-qPCR was performed on a Corbett Research Rotor-Gene 6000 thermocycler (Corbett Life sciences, Sydney, Australia). The All-in-OneTM First-Strand cDNA Synthesis kit (cat. no. AOPT-0020) and All-in-One™ miRNA qPCR Detection kit (cat. no. AOPR-0200) was purchased from GeneCopoeia, Inc. (GeneCopoeia Inc., MD, USA). All 10 pairs of miRNA primers and the U6 primers for the RT-qPCR were purchased from GeneCopoeia, Inc. (GeneCopoeia, Inc. Guangzhou, China). Briefly, the volume used for RT was 25 μl, comprising 5 μl 5X RT buffer, 1 μl 2.5 U/μl PolyA polymerase, 1 μl RTase Mix, 2,000 ng total RNA template and RNase-free water. RT was performed using a Gene Amp PCR system 9700 (Applied Biosystems Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C for 60 min and 85˚C for 5 min.

The RT-qPCR assays used a total volume of 20 μl, according to protocol, comprising 10 μl 2X All-in-One qPCR mix, 2 μl All-in-OneTM miRNAqPCR primer (2 μM), 2 μl Universal Adaptor PCR primer (2 μM) and 2 μl cDNA (diluted 1:5). All reactions were run in a Corbett Research Rotor-Gene 6000 thermocycler for 40 cycles, which consisted of 94˚C for 5 min, followed by 35 cycles of 94˚C for 30 sec, 58˚C for 30 sec and 68˚C for 30 sec. The melting analysis of the PCR products was performed as follows: Temperature was increased between 50 and 99˚C (1˚C increase at each step), with a 90 sec period of pre-melt conditioning in the first step, and 5 sec for each subsequent step. Each experiment was repeated three times. Values were normalized against the expression levels of U6, and ΔΔCq values were calculated. The relative abundance of each miRNA was calculated using the $2^{-\Delta\Delta Cq}$ method (35-37).

Statistical analysis. For all miRNA quantification experiments, quantification cycle (Cq) values >35 were excluded. Values were normalized against the expression levels of U6, and ΔΔCq values were calculated. Statistical analysis was performed using SPSS 19.9 software (IBM SPSS, Armonk, NY, USA). Student's *t*-test was used to analyze the differences in miRNA expression between the OL/BDV and non-infected OL cells. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

Results

miRNA expression profiling. To evaluate the different miRNAs between the OL/BDV cells and non-infected OL cells, the present study profiled the expression levels of 657 miRNAs in the two groups using an miRNA array. Compared with the non-infected OL cells, a total of 10 miRNAs were differentially expressed in the OL/BDV cells (four upregulated; six down-regulated). miR-146a-5p showed the highest expression level in the OL/BDV cells, whereas miR-4521 and miR-3676-3p showed the lowest expression levels (Fig. 1).

Table I. Gene Ontology analyses and pathway analyses of microRNA target genes.

Gene function	Genes (n)	P-value
Molecular function		
DNA binding	602	3.53E-13
Receptor activity	583	8.94E-14
Transferase activity, transferring phosphorus-containing groups	424	6.75E-21
Transmembrane receptor activity	419	1.34E-08
Substrate-specific transporter activity	392	1.22E-05
Cellular component		
Cytoplasm	2,131	1.88E-43
Membrane	1,994	9.85E-30
Membrane part	1,670	6.37E-25
Nucleus	1,430	1.37E-25
Plasma membrane	1,426	6.07E-21
Biological process		
Biopolymer metabolic process	1,684	1.98E-40
Signal transduction	1,634	1.46E-36
Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	1,244	5.47E-21
Protein metabolic process	1,231	1.89E-32
Cellular macromolecule metabolic process	1,131	6.78E-31
Pathway analyses		
Immune system	933	5.06E-16
Adaptive immune system	539	1.03E-14
Metabolism of lipids and lipoproteins	478	2.37E-11
Hemostasis	466	3.29E-11
Developmental biology	396	1.52E-17

GO and pathway analyses of miRNA target genes. The target genes of the differentially expressed miRNAs were predicted using the online database (http://targetscan.org/ and http://www.mirbase.org/), and then submitted for GO functional classification and pathway analysis (http://www.broadinstitute.org/gsea/msigdb/annotate.jsp). The molecular functions of the target genes were predominantly associated with 'DNA binding and receptor activity', and involved in certain biological process, including 'biopolymer metabolic process' and 'signal transduction' (Fig. 2A-C; Table I). In terms of pathway analysis, the immune system and adaptive immune system were the most significant pathways (Fig. 2D; Table I).

Validation of differential miRNA expression using RT-qPCR analysis. RT-qPCR analysis was performed to validate the expression levels of the 10 differentially expressed miRNAs in the OL/BDV cells. The relative expression levels of miRNAs were normalized against the expression levels of U6. Of the 10 miRNAs, seven exhibited significantly lower levels of expression in the OL/BDV cells: miR-1908, miR-3676-3p, miR-296-3p, miR-146a-5p, miR-1290, miR-424-5p and miR-7-5p (Fig. 3). No differences in expression were found in miR-1244 or miR-4521 between the two groups (Fig. 3), and only one miRNA (miR-4433-3p) was undetected in the RT-qPCR analysis. Of note, RT-qPCR showed that the expression of miR-146a-5p, which was upregulated in the miRNA array, was downregulated in the OL/BDV cells.

Discussion

BDV is a neurotropic virus, which can cause central nervous system dysfunction in several mammalian species, including humans (3,36). BDV Hu-H1, originally derived from a human bipolar patient (31), can induce apoptosis and metabolic dysfunction in human OL cells *in vitro* (32,38). Proteomic analyses have indicated that BDV Hu-H1 can activate the downstream extracellular signal-regulated kinase (ERK)-ribosomal S6 kinase complex of the Raf/mitogen-activated protein kinase (MAPK) kinase/ERK signaling cascade in human OL cells (39). Additionally, BDV Hu-H1 can result in brain metabolic dysfunction in Sprague-Dawley rats (40). Despite these findings, the mechanisms underlying BDV Hu-H1 infection in the human brain remain to be fully elucidated. miRNAs may provide a novel approach to addressing remaining question. Thus, the present study profiled and analyzed miRNA expression in BDV Hu-H1-infected human OL cells.

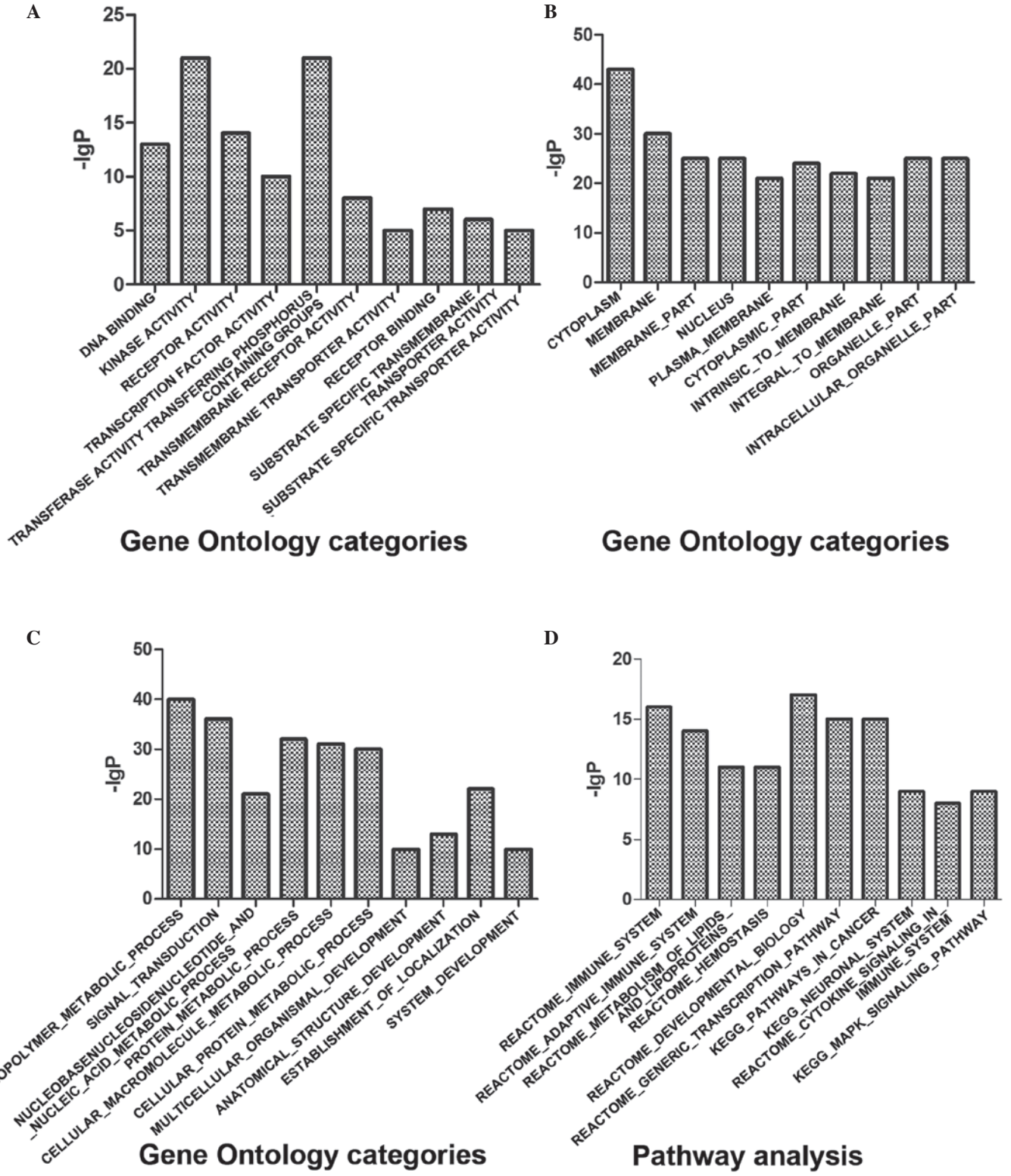

Figure 2. GO and pathway analysis of the differentially expressed miRNAs. The horizontal axes show the top 10 targeted GENE SET name by GO analysis and Pathway analysis: (A) molecular function, (B) cellular component and (C) biological process. (D) pathway analysis. Horizontal axes showed the targeted genes overlapped in Gene Set names of different miRNAs. miRNA, microRNA; GO, Gene Ontology.

An miRNA array is a high throughput and versatile screening tool for analyzing the expression of miRNA, however, its false positives cannot be ignored. Therefore, microarray data requires validation using RT-qPCR analysis, which has higher specificity and provides reliable quantity. It was reported in previous studies that preliminary results were inconsistent with the results of RT-qPCR analysis (41,42). Similarly, in the present study, miR-146a-5p, miR-1290, miR-1908 and miR-424-5p showed downregulation in expression levels using RT-qPCR analysis, which was inconsistent with the results of

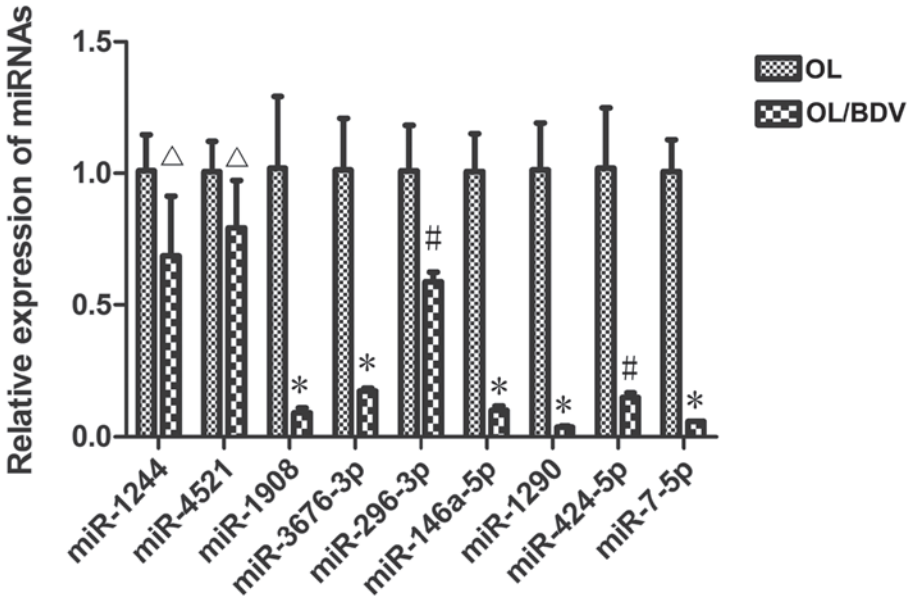

Figure 3. Validation of differential miRNA expression using RT-qPCR analysis. Of the 10 miRNAs, seven (miR-1908, miR-3676-3p, miR-296-3p, miR-146a-5p, miR-424-5p, miR-7-5p and miR-1290) were expressed at significantly lower levels in the BDV-infected OL (OL/BDV) cells, compared with the non-infected OL cells. No significant differences were found in two of the miRNAs (miR-1244 and miR-4521) between the two cell groups. The remaining miRNA was not detected using RT-qPCR. Data are expressed as the mean ± standard deviation. *P<0.01, $^{\#}$P<0.05 and $^{\Delta}$P>0.05, vs. OL group. miRNA/miR, microRNA; OL, oligodendrocyte; BDV, borna disease virus; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

the miRNA array. In addition, miR-4433-3p was not detected using RT-qPCR analysis, and no differences were found in the expression of miR-1244 or miR-4521 between the BDV-infected and non-infected OL cells. However, three consistently downregulated miRNAs were found: miR-7-5p, miR-296-3p and miR-3676-3p.

In the dysregulated miRNAs, the present study focused on miR-7-5p, miR-424-5p and miR-296-3p, which are closely associated with neural cell proliferation and apoptosis. miR-7-5p has been reported in a wide range of signaling pathways, including the MAPK and phosphoinositide 3-kinase (PI3K)/Akt pathway (43,44). miR-7-5p can inhibit vascular endothelial cell proliferation via directly targeting the 3'-UTR of RAF1, an upstream element of the Ras-Raf-MAPK pathway, which has a key effect on nervous system function. However, miR-7-5p is frequently downregulated in glioblastoma microvasculature (43). miR-7 can efficiently affect cell proliferation and metastasis in hepatocellular carcinoma, through regulation of the PI3K/AKT pathway by suppressing PIK3CD, mammalian target of rapamycin and p70S6 K (44). In addition, miRNA-7-5p can inhibit melanoma cell migration and invasion by regulating insulin receptor substrate-2 (45). The functions of miR-7-5p have also been reported in other types of cancer by targeting different signaling pathways (46-50). These results provide novel information for further investigating the function of miR-7-5p in BDV-infected nervous cells.

miR-424-5p is upregulated and modulates the ERK1/2 signaling pathway by targeting suppressor of cytokine induced signaling 6 in pancreatic cancer (51). However, its downregulation can lead to the progression of liver cancer, and regulate the resistance to anoikis and epithelial mesenchymal transition during the metastatic process of hepatocellular carcinoma cells by targeting inhibitor of β-catenin and T cell factor (52). In addition, previous studies have shown that activation of the ERK1/2 pathway may hinder nerve growth factor-induced cell differentiation in BDV-infected PC12 cells (19). Therefore, whether dysregulated miR-424-5p affects BDV-infected nervous cells through the ERK1/2 signaling pathway requires further investigation. Although reports of miR-296-3p are limited, miR-296-3p has been found to regulate cell growth by targeting the potassium channel, EAG1 (53), in human glioblastoma, which presents a novel view in understanding the pathogenic mechanisms of BDV infection.

Previous studies have reported that human OL cells infected with the BDV H1766 strain, isolated from a horse, revealed downregulated expression levels of miR-122 and miR-155 (54,55), which differed from the results of the BDV Hu-H1 infected and non-infected OL cells in the present study. Of note, miRNA expression profiling may be unique due to the possible divergent mechanisms of human OL cells infected with different BDV strains; this requires further investigation.

In conclusion, the present study screened 10 of 657 dysregulated miRNAs in BDV Hu-H1-infected human OL cells using an miRNA array. Of the 10 miRNAs validated using RT-qPCR, the expression levels of seven were significantly downregulated: miR-1908, miR-3676-3p, miR-296-3p, miR-146a-5p, miR-424-5p, miR-7-5p and miR-1290. The biological functions of the dysregulated miRNAs require further investigation, however, based on GO and pathway analyses, further investigation of BDV-infected OL cells offers promise in improving current understanding of the neuropathogenesis of BDV.

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