



Downregulation of Umbilical Cord Blood Levels of miR-374a in Neonatal Hypoxic Ischemic Encephalopathy

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Objective To investigate the expression profile of microRNA (miRNA) in umbilical cord blood from infants with hypoxic ischemic encephalopathy (HIE).

Study design Full-term infants with perinatal asphyxia were identified under strict enrollment criteria. Degree of encephalopathy was defined using both continuous multichannel electroencephalogram in the first 24 hours of life and modified Sarnat score. Seventy infants (18 controls, 33 with perinatal asphyxia without HIE, and 19 infants with HIE [further graded as 13 mild, 2 moderate, and 4 severe]) were included in the study. MiRNA expression profiles were determined using a microarray assay and confirmed using quantitative real-time polymerase chain reaction.

Results Seventy miRNAs were differentially expressed between case and control groups. Of these hsa-miR-374a was the most significantly downregulated in infants with HIE vs controls. Validation of hsa-miR-374a expression using quantitative real-time polymerase chain reaction confirmed a significant reduction in expression among infants with HIE compared with those with perinatal asphyxia and healthy controls (mean relative quantification [SD] = 0.52 [0.37] vs 1.10 [1.52] vs 1.76 [1.69], $P < .02$).

Conclusions We have shown a significant step-wise downregulation of hsa-miR-374a expression in cord blood of infants with perinatal asphyxia and subsequent HIE. (*J Pediatr* 2015;167:269-73).

Despite continued advances in neonatal medicine, hypoxic ischemic encephalopathy (HIE) remains one of the leading causes of neonatal morbidity and mortality.¹ The current gold standard of treatment for this condition—therapeutic hypothermia—can reduce neonatal mortality and improve neonatal morbidity² when initiated within 6 hours after birth.^{3,4} Current screening methods used to predict HIE severity in the delivery room are unreliable.⁵ This has led to interest in laboratory-based biomarkers for the early diagnosis and grading of injury. To date, no definitive blood-based biomarker of HIE has been reported.

MicroRNAs (miRNAs) are small noncoding RNA molecules, approximately 22 nucleotides in length. These RNA fragments modulate gene expression by inhibiting translation of messenger RNA (mRNA) and as a result, subsequent protein synthesis.⁶ In this way, miRNAs are involved in crucial biological events such as cell differentiation, proliferation, death, and metabolism.⁷ Animal models have demonstrated a rapid alteration in the miRNome following transient focal cerebral ischaemia in the adult rat brain,⁸ highlighting the potential for the miRNA profile to aid in the diagnosis and prognosis of stroke,⁹ and to act as a potential therapeutic target in reducing cerebral oedema following an ischemic event.¹⁰ However, there are currently no reports demonstrating the use of miRNA as specific biomarkers for any neonatal condition.

The aim of this study was to profile the expression of miRNAs in umbilical cord blood from neonates. We hypothesized that changes in miRNA expression in cord blood might occur in infants with perinatal asphyxia and HIE.

Methods

This study was approved by the Clinical Ethics Committee of the Cork Teaching Hospitals. All study subjects were enrolled in the ongoing Biomarkers of Hypoxic Ischemic Encephalopathy Study between May 2009 and June 2011 according to strict recruitment criteria: (1) gestation >36 weeks; and (2) 1 or more of the following: cord pH <7.1, 5-minute Apgar score ≤6, and

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EEG	Electroencephalogram
HIE	Hypoxic ischemic encephalopathy
miRNA	MicroRNA
mRNA	Messenger RNA
qRT-PCR	Quantitative real-time polymerase chain reaction

intubation/cardiopulmonary resuscitation at birth. Parents of neonates meeting inclusion criteria were approached and written informed consent obtained. After enrollment, clinical and demographic details on all infants were recorded prospectively.

Concurrently, a control population was recruited as part of an ongoing birth cohort study (The BASELINE [Babies After SCOPE: Evaluating the Longitudinal Impact on Neurological and Nutritional Endpoints] Study, www.baselinestudy.net), with antenatal parental consent. The control population were all full-term infants, with uneventful deliveries, normal neonatal examinations, and without admission to the neonatal intensive care unit. Controls were age, sex, and gestation matched throughout the study.

All case infants had continuous multichannel electroencephalogram (EEG) recorded, initiated within the first 24 hours of life. The EEG was recorded and reviewed as previously described.¹¹ Grade of encephalopathy, if any, was assigned at 24 hours after birth by a dedicated research fellow (B.W.), using the modified Sarnat score.¹² Standardized neurologic assessment was additionally performed on day 3 and at discharge.¹³ Case infants were divided into those with HIE (graded mild, moderate, or severe), and those with biochemical or clinical signs of perinatal asphyxia, but without clinical encephalopathy.

An initial exploratory study was performed in a subgroup of the total cohort (n = 24), allowing us to identify potential useful miRNAs through microarray. Specific results of this exploratory microarray were then confirmed using quantitative real-time polymerase chain reaction (qRT-PCR) prior to targeted miRNA analysis in the total cohort.

Sample Collection and RNA Isolation

Umbilical cord blood from all infants in the total cohort was processed within 3 hours of delivery; 3 mL of cord blood was placed into Tempus Blood RNA tubes (Applied Biosystems, Foster City, California) and biobanked at -80°C . Total RNA was isolated from the Tempus system using the MagMAX for Stabilized Blood Tubes RNA Isolation Kit as per the manufacturer's instructions (Ambion, Life Technologies, Austin, Texas). Isolated RNA was subsequently stored at -80°C until further processing. Total RNA concentration was quantified using a NanoDrop 8000 Spectrophotometer (ThermoScientific NanoDrop, Wilmington, Delaware). In addition, RNA quality of all samples in the exploratory cohort were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Agilent Laboratories, Santa Clara, California) to ensure purity of samples.

miRNA Microarray

To identify differentially expressed miRNAs during HIE, we used the Agilent Human miRNA Microarray v 3.0 (Agilent Technologies Inc) in the exploratory cohort. This system contains probes for 866 human miRNA from the Sanger miRBase 12.0 Release (<http://www.mirbase.org>).

miRNA Real-Time PCR

Four miRNAs were chosen for in-house follow-up analysis to confirm the microarray results. These miRNAs were chosen based on a significant fold change in expression between case and control samples in the exploratory microarray (log fold change $> \pm 1.3$) or because of biological plausibility based on previous literature.¹⁴ qRT-PCR was performed for hsa-miR-374a, hsa-miR-210, hsa-miR-451, and hsa-miR-148a. For this analysis, the miRCURY LNA Universal RT microRNA PCR (Exiqon, Woburn, Massachusetts) was used with predesigned primers (Exiqon) for the miRNAs of interest. The sequences (5'-3') of the amplified miRNA are as follows: UUAUAAUACAACCUGAUUAGUG (hsa-miR-374a), AGCCCCUGCCCACC GCACACUG (hsa-miR-210), AAACCGUUACCAUUAUAGAGUU (hsa-miR-451), and AAAGUUCUGAGACACUCCGACU (hsa-miR-148a). hsa-miR-223 (CGUGUAUUUGACAAGCUGAGUU) was used as a reference gene because of its stable expression in the initial microarray and subsequent experimental validation using qRT-PCR. All analysis was performed as per the manufacturer's protocols. All experimental samples were run in duplicate, cycle threshold values were recorded, and expression levels were calculated relative to controls using the $2^{-\Delta\Delta\text{cycle threshold}}$ method.¹⁵

Statistical and Bioinformatic Analyses

Statistical analysis was performed using PASW v 18, IBM SPSS Statistics 21 (SPSS Inc, Chicago, Illinois) or GraphPad Prism v 5 (GraphPad Software Inc, San Diego, California). Statistical comparisons of clinical data between cases and controls were performed using ANOVA, Tukey post hoc tests, Mann-Whitney test, and Kruskal-Wallis tests, as appropriate. The threshold of statistical significance was set at a *P* value of .05.

Bioinformatic analysis was also performed. All samples in the initial exploratory study were analyzed for miRNA expression using human miRNA microarray 8×15 K release 14.0 (029297) arrays. The data from the microarrays were normalized using variance stabilizing normalization.¹⁶ The data were filtered to remove the control probes and those that did not pass the quality filters (ie, those that were undetectable or were not expressed higher than the negative controls in greater than 75% of the samples). After filtering, 259 miRNAs out of 866 were retained for differential expression analysis. All data processing and differential expression analysis was carried out in R using the AgiMicroRNA¹⁷ and LIMMA¹⁸ packages from Bioconductor (www.bioconductor.org).¹⁹ *P* values were corrected for multiple testing using the Benjamini-Hochberg method.²⁰

Potential molecular targets and signalling pathways of hsa-miR-374a were identified using miRecords (<http://mirecords.biolead.org/>). Additional enrichment analyses were carried out using WebGestalt (<http://bioinfo.vanderbilt.edu/webgestalt/>).

Table. Population demographics

	Control		Perinatal asphyxia		HIE	
	Exploratory n = 9	Total n = 18	Exploratory n = 8	Total n = 33	Exploratory n = 7	Total n = 19
Gestation (wk)	40.6 (39.6-41.1)	39.6 (39.1-40.3)	40.0 (39.0-41.1)	40.5 (39.3-41.1)	41.1 (40.3-41.4)	40.3 (40-41.2)
Birth weight (g)	3550 (3380-3900)	3340 (3488-3458)	3595 (3350-3910)	3565 (3150-4025)	3500 (3405-4035)	3500 (3325-3920)
Sex (M \ F)	7/2	10/8	6/2	21/12	5/2	14/5
Mode of delivery						
SVD	9	12	3	11	2	5
Instrumental	-	2	2	15	2	7
LSCS	-	4	3	7	3	7
1 min Apgar*	9 (9-9)	9 (9-9)	4 (3-6)	5 (3-6)	1 (0-2)	3 (1-5)
5 min Apgar*	10 (10-10)	10 (9-10)	8 (6-9)	8 (8-9)	3 (2-4)	6 (4-7)
Cord pH	-	-	7.01 (6.92-7.04)	7.03 (6.96-7.07)	7.03 (6.98-7.09)	7.02 (6.95-7.10)
Initial lactate†	-	-	8.5 (8.1-9.5)	7.6 (5.6-8.5)	10.6 (9.1-11.9)	9.9 (8.3-10.6)

F, female; LSCS, lower segment cesarean section; M, male; SVD, spontaneous vaginal delivery.

Data expressed as median (IQR).

*Represents a *P* value of <.001 between groups calculated using Kruskal-Wallis or Mann Whitney U tests.

†Represents a *P* value of <.05 between groups calculated using Kruskal-Wallis or Mann Whitney U tests.

Results

In total, 73 infants were recruited for this study. The initial exploratory study included 15 cases (8 perinatal asphyxia, 3 moderate HIE, and 4 severe HIE) and 9 controls. Following exploratory work, 3 samples had to be excluded from the final cohort because of an insufficient amount of sample. In the total cohort we performed qRT-PCR in 70 infants, consisting of 33 with perinatal asphyxia (no HIE), 19 with HIE, and 18 controls. In the HIE group, 6 had moderate-severe HIE (2 moderate and 4 severe), and 13 had mild HIE. Demographic details of both groups are in the [Table](#). There was a strong correlation between clinical assessment and EEG grade by 24 hours after birth ($r = 0.875$, $P = .004$).

Microarray Results

The microarray revealed altered levels of expression in 70 miRNAs between HIE and control samples. Of these, 67 miRNAs were downregulated, and 3 miRNA were upregulated in the HIE group. [Figure 1](#) (available at www.jpeds.com) displays the log fold change in expression of the 70 miRNA. Hsa-miR-374a had the greatest expression change, being significantly downregulated in HIE vs control infants (log fold change of -2.231 , $P < .001$). No miRNA data from the microarray results had a statistically significant change in expression between the HIE and perinatal asphyxia group, or between the perinatal asphyxia and control groups.

qRT-PCR Results

Three miRNA were selected for validation of the results of the microarray assay using qRT-PCR: hsa-mir-374a, hsa-mir-451, and hsa-mir-148a. An additional miRNA, hsa-mir-210, was selected due to previous descriptions of its role in acute tissue hypoxia.¹⁴ Of these 4, hsa-mir-374a was the only miRNA whose expression remained significantly altered, demonstrating a significant decrease between HIE vs controls ($P < .003$; [Figure 2](#)).

Total Cohort Results

Subsequent qRT-PCRs using the total cohort ($n = 70$) confirmed a decrease in hsa-miR-374a expression across all 3 groups. A 1-way ANOVA revealed a significant reduction in hsa-miR-374a expression when comparing control, perinatal asphyxia, and HIE groups ($P < .02$). Post hoc analysis showed a significant alteration in expression between all groups (reduction in expression between; control vs perinatal asphyxia groups [$P < .05$]; control vs HIE groups [$P < .01$]); and asphyxia vs HIE groups ($P < .05$). However, when the HIE group was further divided into cases of mild, moderate, and severe HIE, no significant difference was observed (median relative quantification values, 0.25, 0.52, and 0.24, respectively) ([Figure 3](#)). There were no correlations

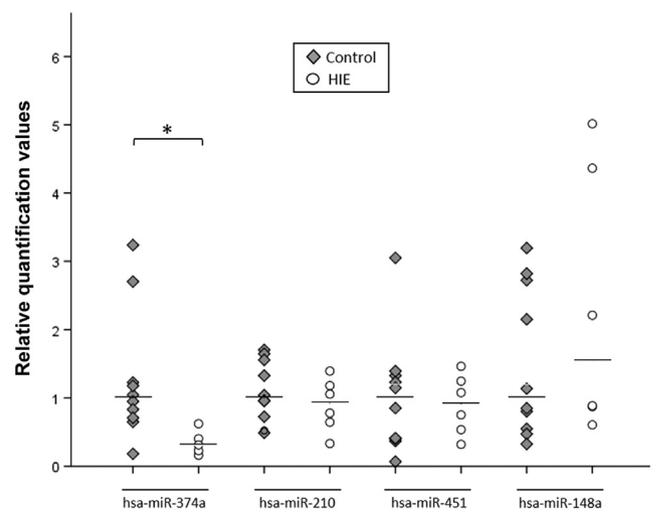


Figure 2. Comparison of qRT-PCR miRNA expression between controls and HIE cases in exploratory cohort for hsa-miR-374a, hsa-miR-210, hsa-miR-451, and hsa-miR-148a. *Represents a statistically significant *P* value of <.05. Each dot represents an individual infant, median value of each group is indicated.

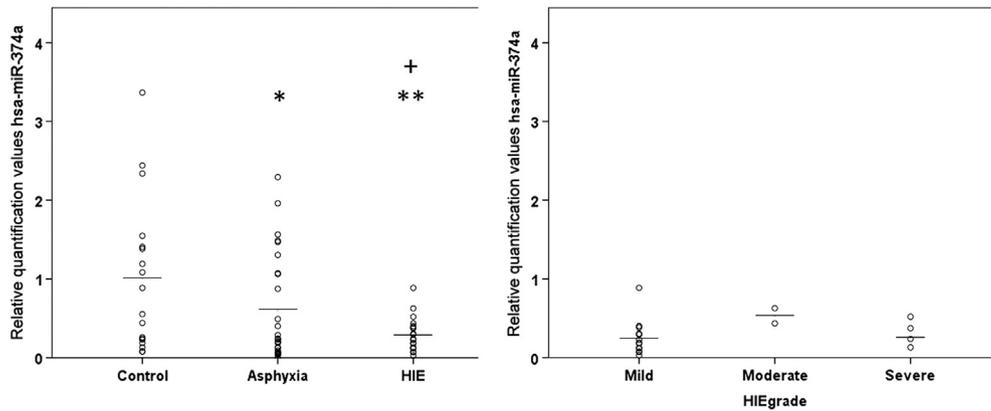


Figure 3. hsa-miR-374a Expression in total population (n = 70). *Represents a statistically significant *P* value of <.05 between control and asphyxia. **Represents a statistically significant *P* value of <.01 between control and HIE. +Represents a statistically significant *P* value of <.05 between asphyxia and HIE. Each dot represents an individual infant, median value of each group is indicated.

between hsa-miR-374a and any clinical variables tested (gestation, birth weight, sex, Apgar score 1 and 5 minute, and cord pH).

In Silico Analysis of hsa-miR-374a Targets

miRecords was used to create a list of putative hsa-miR-374a mRNA targets.²¹ We performed a search of the “Validated Targets” and “Predicted Targets” components of miRecords and compiled a list of 1457 mRNA targets of hsa-miR-374a, predicted by at least 3 target prediction programs. To identify functional categories and pathways over-represented in these genes, we performed a number of enrichment analyses using WebGestalt.^{22,23} Gene ontology enrichment analysis was used to identify the top 10 most statistically significant (after multiple testing for false discovery rate) gene ontologies associated with biological processes (Figure 4; available at www.jpeds.com).

Discussion

We have investigated miRNA expression in umbilical cord blood of infants with perinatal asphyxia and HIE. Seventy miRNA had altered expression between our control and HIE groups. The most significantly altered miRNA was hsa-miR-374a. The altered expression remained significant after validation using an alternate method of analysis (qRT-PCR) and in a larger cohort of infants. To our knowledge, hsa-miR-374a has not previously been linked to hypoxia or to HIE.

Alterations in hsa-miR-374a expression have been reported in studies of various cancers. These studies have reported upregulation and a strong correlation between increased levels of the miRNA and overall survival rate.²⁴⁻²⁶ No clear mechanism of action for this miRNA has been described, however, our target analysis identified specific pathways and biological processes that may be affected by

or linked to neurologic injury associated with HIE. These findings support further investigation into the alteration in expression in hsa-miR-374a following HIE injury.

The ideal biomarker for HIE will not only distinguish an infant with perinatal asphyxia from an infant with HIE but will also rapidly determine grade of HIE. Specifically, it should distinguish between infants with mild HIE who do not need hypothermia therapy and infants with moderate HIE who would benefit from therapeutic intervention. Within this current study, hsa-miR-374a does not fit this criteria, but we believe further investigation is warranted to determine the role this miRNA may play in the pathogenesis of hypoxic ischemic injury. We intend to further validate our current findings in a larger cohort, which is currently being recruited (ClinicalTrials.gov: NCT02019147).

Expression of the hsa-miR-374a is altered in the umbilical cord blood samples of infants with perinatal asphyxia and is further downregulated in infants with clinical and electrographically confirmed HIE. ■

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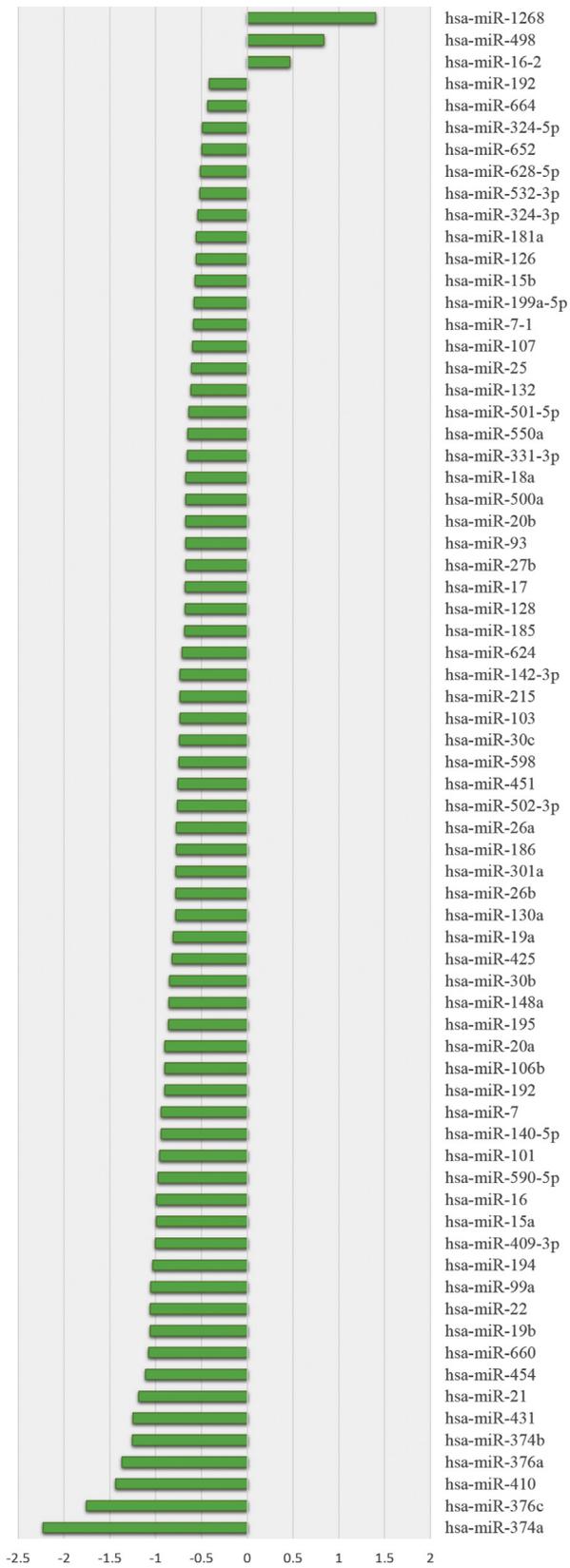


Figure 1. Fold change plot of altered miRNA in microarray in exploratory study.

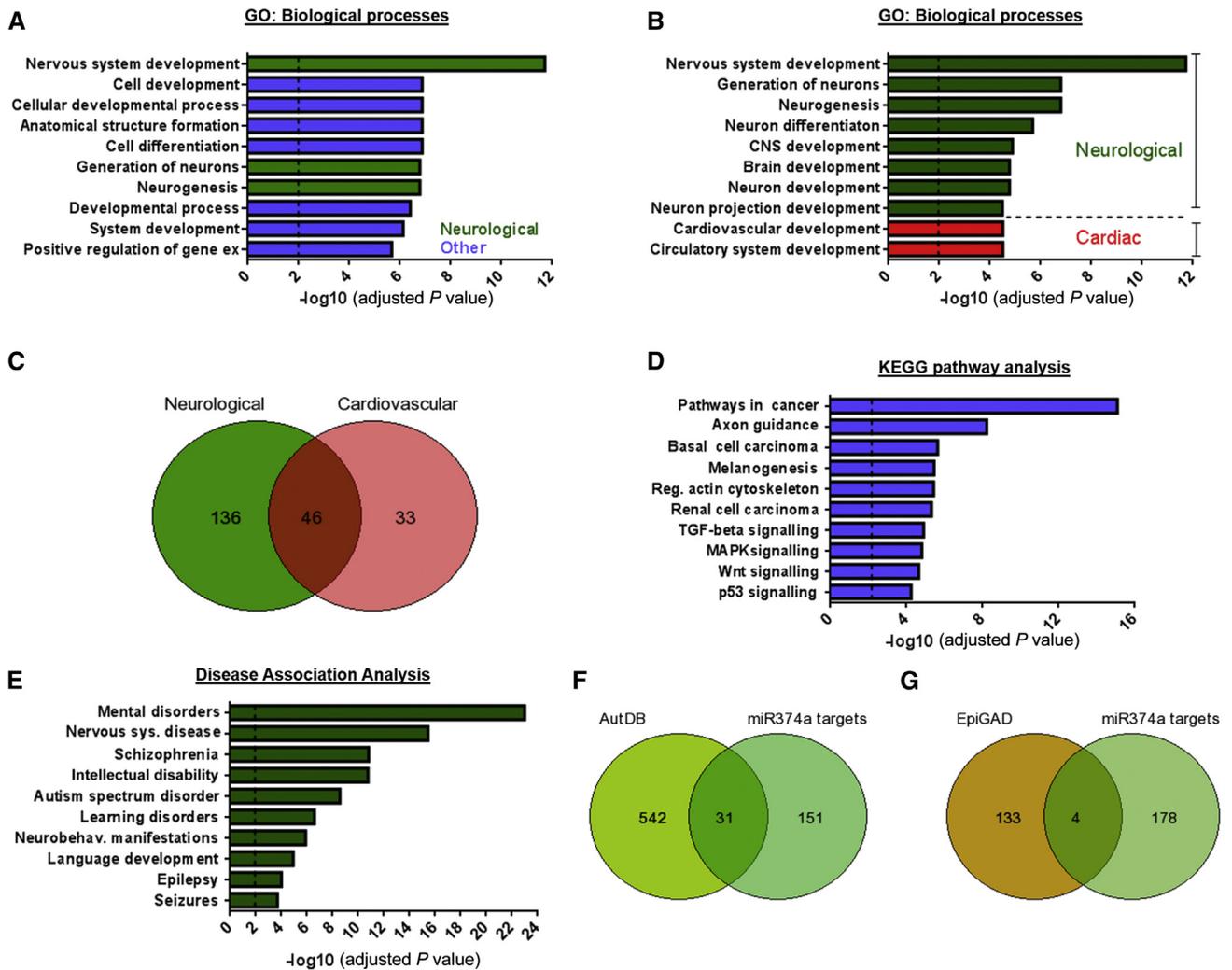


Figure 4. Gene ontology (GO) enrichment analysis of miR-374a predicted genes target genes 1023 of the predicted miR-374a targets mapped unambiguously to unique Entrez gene IDs, which were analysed using a range of enrichment approaches using the Webgestalt platform. **A**, These analyses revealed a significant enrichment in multiple GO “biological processes” categories in the top 10 most statistically enriched categories that were associated with brain development and **B**, “cardiovascular development,” when analyses at an false delivery rate of $P > .01$. **C**, Venn diagram showing that many miR-374a target genes are implicated in both neurologic and cardiovascular related processes, indicating a potential shared molecular overlap between these systems. **D**, Kyoto Encyclopedia of Genes and Genomes pathway analysis revealed multiple pathways with known involvement in brain development and brain injury. **E**, A “disease association analysis” revealed that predicted miR-374a targets had a statistically significant association with many disorders of the nervous system. **F** and **G**, Venn diagrams showing the degree of overlap between the 182 predicted miR-374a targets in the GO category “nervous system development” and genes in the autism spectrum disorder (AutDB) and epilepsy (EpiGAD) databases. CNS, central nervous system; TGF, transforming growth factor; MAPK, mitogen-activated protein kinases; Wnt, Wingless/integrase 1.