Molecular Neurobiology (2019) 56:3657–3663
https://doi.org/10.1007/s12035-018-1321-4

Altered Expression of Umbilical Cord Blood Levels of miR-181b and Its Downstream Target mUCH-L1 in Infants with Moderate and Severe Neonatal Hypoxic-Ischaemic Encephalopathy

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Received: 9 February 2018 / Accepted: 15 August 2018 / Published online: 3 September 2018

Abstract

Hypoxic-ischaemic encephalopathy (HIE) remains one of the leading causes of neurological disability worldwide. No blood biomarker capable of early detection and classification of injury severity in HIE has been identified. This study aimed to investigate the potential of miRNA-181b (miR-181b) and its downstream target, ubiquitin C-terminal hydrolase-L1 (UCH-L1), to predict the severity of HIE. Full-term infants with perinatal asphyxia were recruited at birth and observed for the development of HIE, along with healthy controls. Levels of miR-181b and messenger UCH-L1 (mUCH-L1) in umbilical cord blood were determined using qRT-PCR. In total, 131 infants; 40 control, 50 perinatal asphyxia without HIE (PA) and 41 HIE, recruited across two separate cohorts (discovery and validation) were included in this study. Significant and consistent downregulation of miR-181b was observed in infants with moderate/severe HIE compared to all other groups in both cohorts: discovery 0.25 (0.16–0.32) vs 0.61 (0.26–1.39), $p = 0.027$ and validation 0.33 (0.15–1.78) vs 1.2 (0.071–2.09), $p = 0.035$. mUCH-L1 showed increased expression in infants with HIE in both cohorts. The expression ratio of miR-181b to mUCH-L1 was reduced in those infants with moderate/severe HIE in both cohorts: discovery cohort 0.23 (0.06–0.44) vs 1.59 (0.46–2.54), $p = 0.01$ and validation cohort 0.41 (0.10–0.81) vs 1.38 (0.59–2.56) in all other infants, $p = 0.009$. We have validated consistent patterns of altered expression in miR-181b/mUCH-L1 in moderate/severe neonatal HIE which may have the potential to guide therapeutic intervention in HIE.

Keywords miRNA · |miR-181b · UCHL1 · Biomarker · Perinatal asphyxia · Hypoxic-ischaemic encephalopathy

Introduction

Hypoxic-ischaemic encephalopathy (HIE) in newborn infants remains a major cause of acute mortality and chronic neurologic morbidity [1]. It is estimated that 23% of the 4 million term newborn deaths each year are due to HIE, with the same number of infants surviving with disability [2]. Therapeutic hypothermia improves the long-term outcome, but to be effective must be commenced within 6 h after birth. This narrow treatment window has increased the need for early and accurate identification of infants with HIE.

Limited progress has been made over the last 20 years within the field of biomarker discovery to rapidly identify and classify the grade of severity of hypoxic-ischaemic (HI) injury [3, 4]. Recent advances in the field of miRNA discovery and function have led to an interest in their potential as biomarkers in the diagnosis of HIE. Novel miRNA may be identified through the examination of microarray signatures in affected cases compared to healthy controls [5]. Alternatively, they may be identified through the examination of upstream miRNAs which are known to regulate proteins associated with HIE. As miRNAs alteration may precede mRNA and protein alterations, they may be detected more rapidly [6, 7]. This alternative approach may reveal novel biomarkers, which will

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12035-018-1321-4) contains supplementary material, which is available to authorized users.

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be clinically useful for the rapid diagnosis and prediction of the long-term outcome in at risk infants, while also potentially revealing novel pathological mechanisms involved in the progression of injury during the different stages of HIE. Notably, during periods of sudden environmental change, such as a hypoxic insult, stress-induced factors such as p53 are known to modulate miRNA expression. In turn, the resultant loss of miRNA can alter the expression of target mRNA, potentially causing a profound physiological effect [6]. This mechanism suggests miRNA:mRNA pairings may also provide crucial diagnostic information regarding HIE.

One promising early biomarker of HIE is ubiquitin C-terminal hydrolase L1 (UCH-L1), a neuron-specific enzyme that is highly abundant in the brain [8]. UCH-L1 has been associated with traumatic brain injury and stroke in adults [9, 10]; and altered expression following brain injury has been reported as both indicative of injury severity and predictive of neurological outcome, including temporal changes [11–14]. In addition, altered expression of miR-181b, an upstream regulator of UCH-L1, has been observed in a mouse model of ischaemic injury [15].

Using two separate prospectively recruited cohorts of infants [16], we wished to investigate the expression of both miR-181b and UCH-L1 messenger RNA (mRNA) in umbilical cord blood from infants with well-defined HIE, infants with perinatal asphyxia without HIE (PA) and healthy controls to establish their use as indicators of injury severity.

Methods

Study Population

Our biomarker discovery cohort was collected in the Cork University Maternity Hospital, Ireland, between May 2009 and June 2011 as part of the BiHIVE study (biomarkers of HIE). Prior to commencement, the local hospital ethics committees approved this study's recruitment, consenting and sample processing procedures prior to commencement. The recruitment and classification of all infants within this study, both cases and controls, have previously been described in detail [5]. Briefly, term infants (> 36 weeks gestation) were recruited to the study if a cord pH < 7.1 and/or Apgar score ≤ 6 at 5 min of life and/or a requirement for intubation or cardiopulmonary resuscitation were observed at the time of delivery. A control population was recruited as part of a contemporaneous birth cohort study; The BASELINE Study (www.baselinestudy.net). All control infants in this study were full term, had an uneventful pregnancy ending in an uncomplicated delivery and did not require admission to the neonatal intensive care unit.

A validation cohort of infants was recruited between March 2013 and August 2015 (The BiHiVE 2 study, www.medscinet.net/BIHIVE, NCT02019147), using identical criteria to the discovery cohort, recruiting infants with perinatal asphyxia (with and without HIE) across two clinical sites; Cork University Hospital and Karolinska University Hospital. The BiHiVE2 study also included recruitment of healthy control infants who had umbilical cord blood biobanked at birth using identical collection, processing and storage procedures. In both cohorts, infants with confirmed sepsis, suspected inborn errors of metabolism or coexisting congenital abnormalities were excluded from analysis.

Assignment of HIE Grade

All infants in both cohorts with clinically suspected HIE had continuous EEG monitoring which was commenced within the first 24 h of age as previously described [16]. The grade of HIE was assigned using a modified Sarnat score and was later confirmed by analysis of EEG recordings by a neonatal neurophysiologist (GB).

Sample Collection and RNA Extraction

Umbilical cord blood was drawn for all infants (case and control, discovery and validation cohorts) and processed within 3 h of delivery. Three milligrams of umbilical cord whole blood was placed into Tempus™ Blood RNA tubes (Applied Biosystems, Foster City, CA). The tubes were then agitated for 15 s to ensure the stabilising reagent made uniform contact with the sample, before being biobanked at − 80 °C. RNA was extracted from the Tempus system using the MagMAX™ for Stabilised Blood Tubes RNA Isolation Kit as per the manufacturer's instructions (Ambion, Life Technologies, Austin, Tx) and subsequently stored at − 80 °C.

miR-181b and mUCH-L1 Analysis

For the analysis of miR-181b, the miRCURY LNA™ Universal RT microRNA PCR kit (Exiqon, Woburn, MA) was used according to manufacturer instructions using hsa-miR-223-3p as a reference gene. Commercially available primers for hsa-miR-181-5p (AACAUUCAUUGCUGUCGGUGGGU) and hsa-miR-223-3p (UGUCAGUUUGUCAAAUACCCCA) were used (Exiqon, Woburn, MA). miR-223-3p was used as a reference gene due to its stable expression in previous analysis [5]. UCH-L1 mRNA (mUCH-L1) expression was analysed using the TaqMan® Gene Expression Assay (Applied Biosystems, Life Technologies, Paisley, UK), using 18 s as a reference gene. All samples were run in duplicate, cycle threshold (Ct) values were recorded and expression levels were calculated relative to controls using the $2^{-\Delta\Delta CT}$ method [17].

Finally, as miR-181b is an upstream regulator of mUCH-L1, reductions in miR-181b are expected to produce

upregulation of mUCH-L1 [15]. To look at this relationship further, we examined the ratio of expression levels of miR-181b to mUCH-L1 in all infants calculated as miR-181b/mUCH-L1.

Statistical Analysis

All statistical analysis was carried out using IBM SPSS Statistics 22 (SPSS Inc., USA). Parametric data were analysed using Student's *t* tests or one-way ANOVA followed by Tukey's b post hoc tests. Results are reported as mean (standard deviation). Non-parametric data were analysed using Mann-Whitney *U* and Kruskal-Wallis tests; results are reported as median (IQR) or median (min-max). Spearman's rank correlation coefficients were calculated to test for dependence between miR-181b and UCH-L1. Statistical significance was set at $p < 0.05$.

Results

Cohort Demographics

A total of 131 infants were included in the two phases of this study; 59 within the discovery cohort and 72 in the validation cohort. The discovery cohort consisted of 18 control, 25 PA and 16 HIE infants; 10 mild HIE, 3 moderate and 3 severe grade of HIE. The validation cohort consisted of 22 control, 25 PA and 25 HIE infants; 17 mild and 8 moderate HIE infants. Total population demographics are shown in Table 1.

Umbilical Cord Blood miR-181b Expression

Levels of miR-181b were first analysed in the discovery cohort. Of the 59 infants in this phase, 57 had measurable levels of miR-181b on qRT-PCR, 18 control, 23 PA and 16 HIE infants. On analysis, a trend to reduced expression, but no significant difference in expression was detected between the control, PA and HIE groups; median RQ expression = 0.76 (IQR = 0.22–1.36) vs 0.58 (0.27–2.20) vs 0.34 (0.21–0.088) respectively, $p = 0.241$, or between grades of HIE grade (mild vs moderate vs severe); 0.61 (0.27–1.38) vs 0.28 (0.04–0.28) vs 0.22 (0.20–0.22), $p = 0.085$ (Supplementary Figure 1). When infants were grouped into those who would meet the current criteria for therapeutic hypothermia (i.e., those with moderate or severe HIE, $n = 6$), and compared to those who would not (control, PA and mild HIE, $n = 51$), miR-181b levels were found to be significantly downregulated in infants eligible for therapeutic hypothermia, 0.25 (0.16–0.32) vs 0.61 (0.26–1.39) respectively, $p = 0.029$.

Similar patterns of expression of miR-181b were observed in the validation cohort, from which 65 of the 72 infants had measurable miR-181b expression levels. A significant reduction in expression of miR-181b was seen in those with HIE and in particular those with moderate encephalopathy. Control infants ($n = 18$) vs PA ($n = 23$) vs HIE ($n = 24$):1.0 (0.83–1.32) vs 1.91 (0.87–2.88) vs 0.79 (0.24–1.20) respectively, $p = 0.043$. Within the HIE group, the greatest reduction was seen in the moderate group = 0.33 (0.15–1.78). When those eligible for therapeutic hypothermia ($n = 8$) were compared to all other infants ($n = 57$), there was again significant downregulation in the TH group = 0.33 (0.15–1.78) vs 1.2 (0.71–2.09), $p = 0.035$ (Fig. 1).

Table 1 Discovery and validation cohort demographics. *Represents $p < 0.001$ between groups within discovery and within validation calculated using Kruskal-Wallis or Mann-Whitney *U* tests

	Control		Perinatal asphyxia		HIE	
	Validation $n = 18$	Discovery $n = 22$	Validation $n = 25$	Discovery $n = 25$	Validation $n = 16$	Discovery $n = 25$
Gestation (weeks+days)	40 + 2(39 + 4–41 + 1)	40 + 4(39 + 1–41 + 1)	40 + 5(39 + 1–41 + 1	40 + 2(39 + 2–41 + 1	40 + 3(39 + 4–41 + 2)	40 + 4(39 + 2–41 + 2)
Birth weight (g)	3400 (3200–3653)	3895 (3498–4090)	3490 (3135–3990)	3735 (3460–4003)	3465 (3270–4108)	3860 (3359–4149)
Gender (male/female)	10/8	14/8	17/8	15/10	12/4	16/9
Mode of delivery						
Spontaneous vaginal delivery	11(61%)	13(59%)	7(28%)	9(36%)	5(31%)	4(16%)
Instrumental delivery	3(17%)	7 (32%)	12 (48%)	13 (52%)	4 (25%)	17 (68%)
Elective caesarian section	3 (17%)	–	1 (4%)	–	–	–
Emergency caesarian section	1 (5%)	2 (9%)	5 (20%)	3 (12%)	7 (44%)	4 (16%)
1 min Apgar*	9 (9–9)	9 (9–9)	4 (3–6)	7 (5–9)	2 (1–3)	2 (1–4)
5 min Apgar*	10 (10–10)	10 (9–10)	8 (6–9)	9 (9–10)	5 (3–7)	4 (4–6)
10 min Apgar*	–	10 (10–10)	–	10 (10–10)	–	7 (6–9)
Cord pH	–	7.27 (7.20–7.32)	7.15 (7.07–7.25)	7.03 (7.00–7.08)	7.09 (6.99–7.28)	6.98 (6.91–7.14)

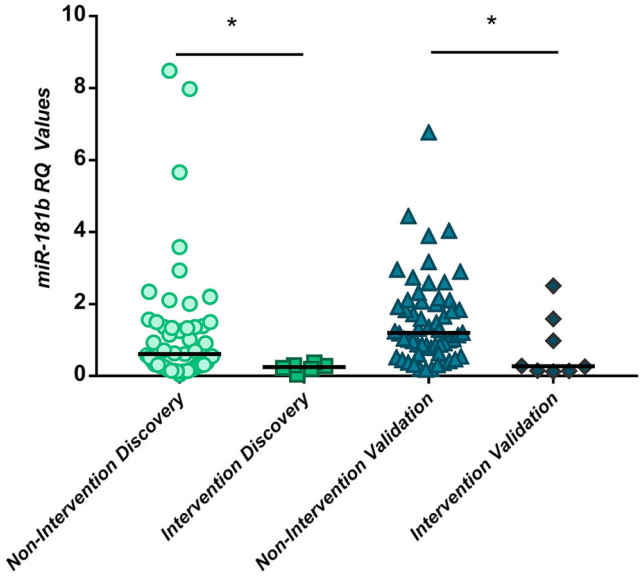

Fig 1 miR-181b expression in discovery and validation cohorts. A statistically significant downregulation of miR-181b was observed in the discovery ($n = 57$) and validation cohort ($n = 65$) when comparing infants who would currently not meet the criteria for intervention in the form of therapeutic hypothermia ($n = 51$ and $n = 57$, respectively) with those who would, i.e., moderate/severe HIE ($n = 6$ and $n = 8$, respectively). *Represents statistical significance value of $p < 0.05$

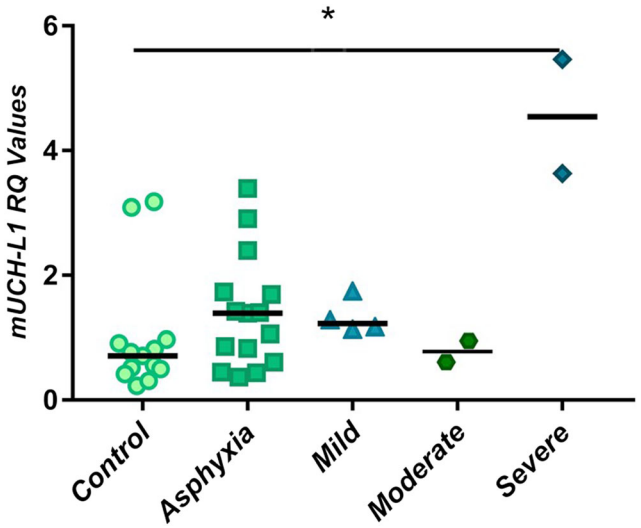

Fig 2 UCH-L1 mRNA expression in healthy controls ($n = 13$), infants with perinatal asphyxia ($n = 15$), mild HIE ($n = 4$), moderate HIE (n = 2) and severe HIE ($n = 2$). A significant elevation of UCH-L1 mRNA expression was observed between the control, PA and HIE groups ($p = 0.05$). *Represents a statistically significant value of $p < 0.05$

UCH-L1 mRNA Expression

Discovery Cohort

Following previous work [5] and miR-181b analysis, only 36 infants from the discovery cohort had sufficient RNA available for UCH-L1 mRNA qRT-PCR. This included 13 controls, 15 infants with PA and 8 infants with HIE (mild ($n = 4$), moderate ($n = 2$) and severe ($n = 2$)). Following qRT-PCR analysis, a statistically significant elevation in UCH-L1 mRNA was detected between the control, PA and HIE groups: 0.71 (0.46–0.94) vs 1.39 (0.62–1.73) vs 1.23 (0.99–3.16) respectively, $p = 0.047$. Significant elevation of mUCH-L1 was also observed across all grades of HIE with the greatest alteration seen in the severe group; 1.23 (1.14–1.63) in mild HIE vs 0.78 (0.61–0.78) in moderate HIE vs 4.55 (3.63–4.55) in severe HIE, $p = 0.05$ (Fig. 2). No difference in expression was observed between infants not currently eligible for therapeutic hypothermia ($n = 32$) compared to those with moderate or severe HIE ($n = 4$); 0.97 (0.54–1.71) vs 0.95 (0.61–0.95), $p = 0.19$.

Validation Cohort

Expression levels of mUCH-L1 were next analysed in the validation cohort. A total of 70 samples were available for qRT-PCR; control ($n = 22$), PA ($n = 24$) and HIE ($n = 24$), consisting of 16 mild and 8 moderate grades. Similar trends to those in the discovery cohort were observed, although statistical significance was not achieved; control ($n = 22$) vs PA ($n = 24$) vs HIE ($n = 24$); 0.98 (0.56–1.91) in controls vs 0.75 (0.61–1.32) in PA vs 1.19 (0.57–1.89) in HIE, $p = 0.655$. Within the HIE group, the median RQ (IQR) expression was not significantly different in the moderate vs mild group; 1.25 (1.15–1.96) vs 1.03 (0.46–1.69) respectively, $p = 0.462$.

Ratio of Expression miR-181b/mUCH-L1

Discovery Cohort

A ratio of expression was calculated for each infant = miR181b/mUCH-L1. On analysis, this ratio was significantly

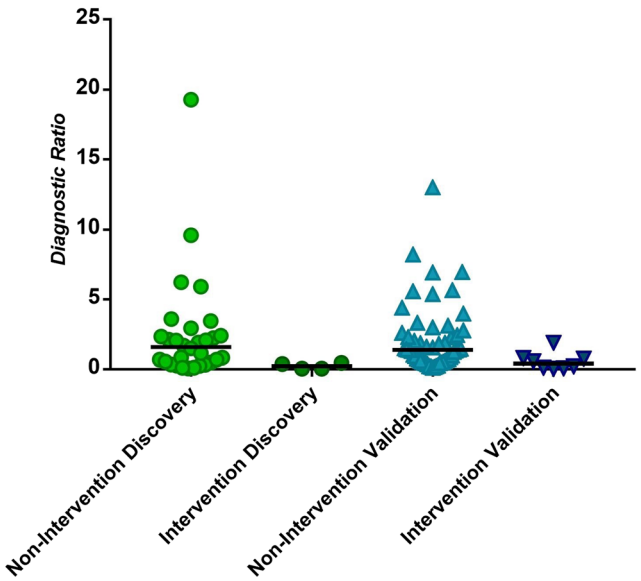

Fig 3 Scatter plot is presenting diagnostic ratio levels—discovery and validation cohorts

reduced in infants with HIE compared those in the PA or control groups; 0.42 (0.07–0.79), 2.08 (0.51–3.53) and 1.7 (0.42–2.67) respectively, $p = 0.036$. Additionally, when infants with HIE were grouped as those who would currently receive therapeutic hypothermia ($n = 4$) versus those who would not meet the criteria ($n = 30$), this ratio revealed a significant difference between the two groups; 0.23 (0.06–0.44) vs 1.59 (0.46–2.54), $p = 0.01$ (Fig. 3).

Validation Cohort

Again, in the validation cohort, the ratio of miR-181b/mUCH-L1 was significantly decreased in those with HIE, particularly in the moderate group. Control ($n = 18$) vs PA ($n = 23$) vs HIE ($n = 23$); 0.95 (0.53–2.09) vs 1.54 (0.74–3.97) vs 0.82 (0.23–1.61) respectively, $p = 0.025$. The median (IQR) level in those with moderate HIE ($n = 8$) was 0.41 (0.10–0.81) compared to a level of 1.38 (0.59–2.56) in all other infants ($n = 56$), $p = 0.009$ (Fig. 3).

Discussion

In this study, we have investigated the use of miR-181b and its validated downstream target mUCH-L1 as potential biomarkers of injury severity in HIE. We have reported a significant downregulation in the expression of miR-181b following HI injury, specifically within those infants who would currently be eligible for clinical intervention with therapeutic hypothermia (infants with moderate and severe HIE). While capable of identifying infants with moderate or severe HIE when grouped together, a statistically significant difference was not observed between the three different grades of HIE when examined individually. Secondly, a statistically significant elevation in mUCH-L1 levels was observed when comparing healthy controls, infants with PA and infants who developed HIE. This elevation was highest in infants with severe HIE. Finally, as both miR-181b and UCH-L1 mRNA were measured by relative quantification within this study, we have created a "diagnostic ratio" combining miR-181b and UCH-L1 in order to examine the relationship between the two markers and to provide a potential novel biomarker of HIE severity.

Although the sample numbers are small, this diagnostic ratio showed very similar patterns of expression in both the discovery and validation cohorts, with improved differentiation particularly of the infants with moderate or severe HIE. Hence, this diagnostic ratio may prove more useful than either miR-181b or mUCH-L1 expression alone. We have previously reported the potential benefits of using miRNA and their downstream targets as biomarkers of injury and disease state [5].

UCH-L1, previously known as PGP 9.5, is a cytoplasmic enzyme, found primarily in neurons and neuroendocrine cells with very high levels in perikarya, axons and dendrites, making it attractive as a biomarker specific to brain injury [8, 18]. It is involved in protein ubiquitination and elimination via the ubiquitin-proteasome system (UPS), which plays an important role in the removal of damaged misfolded proteins in the cells. The UPS process is essential to maintain cell homeostasis, and when dysfunctional can lead to disorders of both the central and peripheral nervous system [19–21].

UCH-L1 is a brain soluble protein that does not occur in plasma or erythrocytes [22]. Previous small clinical studies have found higher levels of serum UCH-L1 in HIE and proposed that its release into the blood may represent cortical damage [13]. It has been suggested that the small protein size and compact globular shape of UCH-L1 assists blood-based stability and in crossing the blood-brain barrier [23].

Reactive lipid species produced following cerebral ischaemia can cause covalent modification of the cysteine 152 of UCH-L1, unfolding the protein resulting in a build-up of UCH-L1 [24]. miR-181b is found to be upregulated under hypoxic conditions in retinoblastoma cells and increased significantly in nerve cells under oxygen-glucose deprivation (OGD) [25, 26]. In a separate study, UCH-L1 was shown to be downregulated during OGD but could be restored following reoxygenation [27]. Peng et al. examined both miR-181 and UCH-L1 after OGD using a luciferase assay and found that miR-181b could bind to UCH-L1 mRNA and repress it during translation, implicating miR-181b as a negative regulator of UCH-L1 [15]. Our study has attempted to investigate the expression of both the miRNA and mRNA elements in this regulator-target relationship and determining if alterations in expression are representative of HIE severity.

Previous studies have proposed UCH-L1 as an indicator of HIE grade [9, 12, 13] but the numbers studied were small, and the findings have not been validated in subsequent cohorts. As we have confirmed that both miR-181b and mUCH-L1 are altered in a validation cohort, we believe this miRNA: UCH-L1 pair may hold genuine potential as biomarkers of HIE severity. The investigation of miRNAs as potential biomarkers of HIE is a novel field, one in which preliminary work must be carried out to determine the validity of this research. Although our sample numbers in each cohort are limited, collectively they represent one of the largest neonatal study cohorts used. Additionally, these infants were all recruited as part of a contemporaneous cohort of inborn infants, under strict enrolment criteria within tertiary hospitals. These infants represent a well-defined cohort of infants with HIE, graded both clinically and electrographically.

Importantly, our cohort also encompasses healthy controls and infants with PA who did not develop HIE. This is necessary as in clinical practice, the difficulty is not in

differentiating infants with HIE from normal controls, the greatest difficulty is assessing which infants with clinical and biochemical signs of perinatal asphyxia at birth will require therapeutic hypothermia. Including these infants allows us to gain a true indication of the efficacy of miRNA as prognostic markers. This work will need to be validated once more in a large prospective clinical trial prior to moving into bedside use. The technology to enable rapid bedside analysis with portable next-generation sequencing (NGS) and rapid PCR is now available and utilised in many centres for other diseases where the therapeutic window is narrow. The greatest strength of this study lies in our ability to validate our results in a second cohort recruited with identical recruitment and sampling procedures. This gives us the confidence to report these novel findings, which we hope that others will replicate in larger cohorts.

Conclusion

This study of miR-181b and mUCH-L1 expression in the umbilical cord blood ofinfants with PA and HIE has highlighted a novel combination of markers which may prove clinically useful in grading HIE injury, particularly in the identification of infants with moderate or severe HIE, who are most likely to benefit from therapeutic hypothermia.

Funding This research was funded by the National Children's Research Centre, Crumlin (NCRC; B/14/1), the Health Research Board (HRB; CSA/2012/40), and a Science Foundation Research Centre Award (INFANT; 12/RC/2272).

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