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MiR-29b Is Associated with Perinatal Inflammation in Extremely Preterm Infants

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Abstract

Background: Inflammation is strongly associated with premature birth and neonatal morbidities. Increases in infant haptoglobin (Hp&HpRP) and IL-6 levels are indicators of intra-amniotic inflammation (IAI) and have been linked to poor neonatal outcomes. Inflammation causes epigenetic changes, specifically suppression of miR-29 expression. The current study sought to determine whether miR-29b levels in cord blood or neonatal venous blood are associated with IAI, identified by elevated IL-6 and haptoglobin, and subsequent clinical morbidities in the infant.

Methods: We tested 92 cord blood samples from premature newborns and 18 venous blood samples at 36 weeks corrected gestational age. MiR-29b, haptoglobin (Hp&HpRP), and IL-6 were measured by PCR and ELISA respectively.

Results: Decreased levels of miR-29b were observed in infants exposed to IAI with elevated Hp&HpRP and IL-6 levels and in infants delivered by spontaneous preterm birth. Lower miR-29 levels were also observed in women diagnosed with histological chorioamnionitis or funisitis and in infants with cerebral palsy. Higher levels of miR-29 were measured in infants small for gestational age (SGA) and in venous samples from older infants.

Conclusion: MiR-29 may be an additional biomarker of IAI and a potential therapeutic target for treating poor newborn outcomes resulting from antenatal exposure to IAI.

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INTRODUCTION

Premature birth before 37 weeks' gestational age (GA) has a global incidence of approximately 15 million per year, according to a World Health Organization report in 2018 (1). Moreover, the March of Dimes reported a preterm birth rate of 9.93% in 2017 in the United States (2). Despite increased survival of extremely premature infants related to advances in neonatal care, this subpopulation remains at high risk for significant short and long-term morbidities. These morbidities include necrotizing enterocolitis (NEC), bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), and neurodevelopmental sequelae such as cerebral palsy (CP), periventricular leukomalacia (PVL), and intraventricular hemorrhage (IVH) (3). The wide clinical spectrum of chronic disease and severity among this population suggest a multifactorial etiology, including prematurity, maternal and fetal inflammation, and exposure to the postnatal interventions required to compensate for organ system immaturity. These adverse exposures are thought to lead to epigenetic changes which predispose preterm infants to more severe disease in response to life-saving interventions after birth (4).

MicroRNAs (miRs) are small non-coding RNAs (~22 nucleotides) that act as epigenetic regulators of normal physiologic processes and are implicated in abnormal pathologic processes (4). MicroRNAs have been investigated in a variety of diseases, and changes in microRNA expression have been proposed as biomarkers of disease severity (5–8). MiR-29 regulates extracellular matrix deposition and has been implicated in cancers and fibrotic diseases (9–13). Inflammation has been shown to suppress miR29 expression via multiple pathways (14, 15). Given the significant role of maternal inflammation on preterm birth and health of the infant, miR-29 may be a significant modulator of the risk for prematurity-related neonatal complications.

Interleukin-6 (IL-6) is a well characterized pro-inflammatory cytokine and an activator of acute phase responses. Elevated IL-6 levels at birth are considered a risk factor for sepsis-induced disseminated intravascular coagulation, pneumonia, periventricular leukomalacia, and necrotizing enterocolitis (16–19). Haptoglobin (Hp), a well-characterized acute-phase reactant, is an abundant plasma protein synthesized primarily by the liver. Hp was previously considered to be nearly absent at birth, with an increase to adult levels throughout the first year of life (20, 21). Hp acts as a potent antioxidant which counters lipid peroxidation twenty-fold more effectively than vitamin E and has indirect antioxidant effects by binding plasma free hemoglobin with high affinity to inhibit its oxidative activity (22, 23). Buhimschi et al. revealed that the antenatal exposure to intra-amniotic infection and/or inflammation (IAI) induces a precocious “switch-on” of Hp expression in the cord blood of premature neonates and this could serve as a biomarker for the inflammatory context of preterm birth (23–25). Because the employed immunoassays do not discriminate Hp from the near-homologous Hp-related protein (HpRP) this cord blood biomarker is denoted as Hp&HpRP. Further studies identified that the sub-population of preterm neonates exposed to IAI who are unable to switch-on Hp&HpRP expression and thus remain an- or hypohaptoglobinemic despite elevated cord blood IL-6 had higher odds of the composite outcomes of cerebral palsy (CP) or death and grade III/IV intraventricular hemorrhage and/or death than those newborns with appropriate Hp production (22).

Using the combination of increased IL-6 levels and “switch-on” haptoglobin as the indicator of exposure to intrauterine inflammation, the current study sought to determine whether miR-29b levels in cord blood or neonatal blood are associated with antenatal exposure to IAI and subsequent clinical morbidities. By identifying the population of premature infants at the greatest risk for long-term disease, we can target our interventions to the infants most likely to benefit.

METHODS

Sample population and Study Design

The study used bio-banked cord blood specimens obtained at delivery and infant blood samples obtained at 36–40 weeks corrected GA. Mothers were recruited at The Ohio State University Wexner Medical Center and samples obtained as part of a biorepository (Maternal Fetal Medicine Preterm Birth Repository (IRB #17–0079)). Inclusion criteria were infants born 32 weeks’ gestation with no genetic or anatomic anomalies. Informed consent was obtained from the mother. For the purpose of this study, gestational age selection for infants that were born less than or equal to 30 weeks, to include the subpopulation at highest risk for morbidities associated with prematurity, and had available cord blood samples was incorporated. These criteria resulted in 92 individual samples. A total of 88 placentas from these patients were sent for pathologic analysis

In addition, venous blood samples obtained at 36–40 weeks postmenstrual age were identified through the Perinatal Research Repository at Nationwide Children’s Hospital (Perinatal Research Repository, IRB# 10–00035) for 18 of these same infants. Both repositories contained detailed fetal and postnatal data for the infant as well as for the mother, including cytokine and biomarker analysis. Clinical outcomes of the newborns and the results of placental pathology were obtained through retrospective chart review.

Analysis of IL-6, Hp&HpRP, and miR-29b

Umbilical cord blood was collected immediately after delivery and neonatal venous blood (36–40 weeks) was collected in the Neonatal Intensive Care Unit. All blood samples were separated within 45–120 minutes, plasma frozen within 12 hours of blood collection, and stored at –80°C until analysis. Interleukin-6 and Hp&HpRP were measured on 92 cord blood samples as previously described (24). Hp was measured as Hp&HpRP because the antibody employed in ELISA does not discriminate between Hp and the closely related haptoglobin-related protein (HpRP) in cord blood.

MiR-29b levels were measured using RT-PCR and normalized to the internal expression of SP2 for the 92 cord blood samples and 18 36–40 weeks’ GA plasma samples. A RNeasy Mini kit (Qiagen; Hilden, Germany) was used to isolate total RNAs from plasma samples. cDNA was synthesized using a Maxima First Strand cDNA Synthesis Kit for RT-Quantitative PCR (K1642, Thermo Fisher; Waltham, MA). A MasterCycler egradient RealPlex RT-PCR Detection System (Eppendorf, Hamburg, Germany) was used for quantitative real-time PCR analyses with Maxima SYBR Green/ROX qPCR Master Mix (K0221, Thermo Fisher; Waltham, MA). Quantitative realtime PCR analyses for miR-29b

were performed using the miRCURY LNA RT Kit (339340, Qiagen; Maryland) for cDNA synthesis and the RNA Spike-In Kit (339347, Qiagen; Maryland).

Data Analysis

Log transformation was applied to the biomarkers (i.e., Hp and IL-6, mir-29). We performed a sensitivity analysis to assess if the inclusion of twins changed the analysis outcomes due to the familial effect (i.e., shared genetics and environment). No imputation of missing data was performed. Categorical by categorical relationships were assessed using Barnard's test on the contingency tables as a uniformly more powerful test than Fisher's exact test (26, 27). Categorical by quantitative relationships were tested using the Kruskal-Wallis rank sum test (28). Comparisons with p-values < 0.05 were considered statistically significant. R version 3.6.0 (<https://CRAN.R-project.org>) was used for testing, tabulations and to assess data.

RESULTS

Maternal and infant demographics

Demographics from the gestational age selected cohort are presented in Table 1. The average infant gestational age was 27.3 weeks and the average birth weight was approximately 1,000 grams. Racial distribution matched the distribution of the geographical area. All mothers received antenatal corticosteroids and the majority (66.3%) were delivered by C-section. Approximately 14% were multiple gestations and sensitivity analysis was performed to assess if newborn relatedness affected results. Preterm birth (PTB) was defined as spontaneous (i.e. due to spontaneous preterm labor or preterm pre-labor rupture of membranes) or medically-indicated (i.e. due to maternal or fetal indications including preeclampsia). Overall, there were twice as many spontaneous as medical PTBs in the dataset.

Non-exposed vs Exposed to IAI

IL-6 and Hp&HpRP were measured on all 92 cord blood plasma samples and the results were segregated by "non-exposed" and "exposed" status as previously described [22]. Briefly, "non-exposed" to IAI status was assigned in those samples with Hp&HpRP levels < 2,000 ng/mL and IL-6 levels <100 pg/mL. All samples with Hp&HpRP levels ≥ 2,000 ng/mL were subjected to western blot to confirm switch-on status (by presence of Hp beta band). Newborns with switch-on Hp status (visible Hp beta band) were assigned as "exposed" irrespective of IL-6 levels. Newborns with switch-off status (absent beta band) by western blot were assigned as non-exposed if IL-6 levels were <100 pg/mL and as exposed if IL-6 levels ≥ 100 pg/mL (24). There were only 3 exposed hypohaptoglobimemic newborns in this dataset. Clinical outcomes data were analyzed for differences between non-exposed and exposed status. Biochemical criteria, rather than histologic placenta examination for chorioamnionitis and/or funisitis were chosen to distinguish exposed vs non-exposed because placental pathology was not performed for all patients. In addition, intraamniotic infection is generally a clinical, rather than histological, diagnosis (29). Both placental pathologies analyzed, funisitis and chorioamnionitis, demonstrated significant differences in diagnoses between exposed and non-exposed status (Table 2). On the other hand, there was a significantly higher rate of early-onset neonatal sepsis in the non-exposed group. This was

potentially due to maternal intrapartum antibiotic exposure, with a significantly higher proportion of the “exposed” group receiving antibiotics around the time of labor and delivery (81.6% vs. 51.9%, Barnard test, $S=-2.93$, $p=0.002$). No other differences were observed in neonatal outcomes.

MiR-29b and clinical outcomes.

Expression of miR-29b was not normally distributed, so Kruskal-Wallis test was applied to assess miR-29b with categorical clinical outcomes. MiR-29b levels were measured on all 92 cord blood plasma samples and tested for correlations between miR-29b levels and placental pathology, specifically funisitis and chorioamnionitis, as well as fetal and neonatal outcomes: fetal growth restriction, necrotizing enterocolitis, retinopathy of prematurity, intraventricular hemorrhage, periventricular leukomalacia, cerebral palsy, and bronchopulmonary dysplasia (Table 3). MiR-29b CT values were higher in cases considered “exposed” (mean=11.51, SD=2.53) than in “non-exposed” (mean=10.97, SD=1.52) (Kruskal-Wallis rank sum test, $df=1$, $\chi^2=5.29$, $p=0.02$) indicating that there is a greater number of miR-29b transcripts in the non-exposed group. We also observed that miR-29b CT levels were greater in infants born by spontaneous PTB (mean=11.25, SD=1.76) than those whose PTB was medically indicated (mean=10.51, SD=2.42) (Kruskal-Wallis rank sum test, $df=3$, $\chi^2=10.28$, $p=0.02$) again indicating fewer miR-29b transcripts in the spontaneous PTB group. Similar findings were observed with umbilical cord funisitis, with higher CT values in the funisitis group compared to the non-funisitis group indicating lower levels of miR-29b transcript in the group with diagnosed funisitis (Kruskal-Wallis rank sum test, $df=1$, $\chi^2=4.17$, $p=0.04$).

For neonatal outcomes, high CT values for miR-29b indicating lower transcript numbers were observed for infants diagnosed with cerebral palsy (mean=11.93, SD=0.57) than those who were not (mean=10.92, SD=2.12) (t-test, $df=12$, $t=2.34$, $p=0.02$) (Table 3). A reverse correlation was observed for infants with small for gestational age (SGA) with lower miR-29b CT values and thus higher transcript numbers in the infants diagnosed with SGA (Kruskal-Wallis rank sum test, $df=1$, $\chi^2=6.38$, $p=0.01$) (Table 3).

Venous blood samples collected at 36–40 weeks postmenstrual age from 18 of the original subjects were also analyzed ($n=18$ samples). As shown in Table 4, lower miR-29b CT values and higher transcript levels in these samples were associated with IVH for cases with (mean=4.27, SD=3.27) compared to cases without (mean=8.19, SD=1.56) (Kruskal-Wallis rank sum test, $\chi^2=4.00$, $p=0.04$). No other morbidities tested were associated with miR-29b levels at this time point, including bronchopulmonary dysplasia

DISCUSSION

More than 50% of deliveries at <30 weeks gestation are associated with intrauterine or maternal inflammation (30). In addition, these infants are at risk for increased incidence of neonatal morbidities and poor long-term outcomes (31–33). Those who survive beyond infancy are at greatest risk for developing adult disease, in fact, infection-related PTB has been associated with the development of early onset sepsis and neonatal morbidities

including necrotizing enterocolitis, retinopathy of prematurity, intraventricular hemorrhage, periventricular leukomalacia, cerebral palsy, and bronchopulmonary dysplasia (34–36)

Previous studies to identify epigenetic changes that occur due to prematurity and/or inflammation have found an association between miR-29b levels and development of bronchopulmonary dysplasia in human neonates. Specifically, decreased circulating miR-29b levels from plasma in the first week of life are found in those infants who are subsequently diagnosed with BPD at 36-weeks corrected gestational age. A significant inverse association was demonstrated between BPD severity and miR-29b level shortly after birth, suggesting that decreased miR-29b early in life may predict or contribute to disease severity (4). Similar findings were recapitulated in animal models (4, 12)

This current investigation was designed to identify whether cord blood miR-29b levels were associated with perinatal/neonatal inflammation and thus increased risk for neonatal morbidities. Using measures of Hp&HpRP and IL-6, infants were designated as “exposed” vs “non-exposed” to antenatal inflammation as described in prior studies from our group (22, 24, 37). This current study identified a negative correlation with miR-29b levels and exposed infants. Similarly, we observed a negative correlation between miR-29b levels and births classified as spontaneous preterm birth. Both criteria, “exposed” status and spontaneous nature of preterm birth, have been linked to intrauterine inflammation and agree with our previous findings of decreased miR-29b in response to inflammation (4).

Prior studies have reported correlations between amniotic fluid or blood levels of IL-6 and the relative severity of intrauterine inflammation (37). Negative correlations were identified between miR-29b and IL-6 levels in the infant cord blood and clinical inflammatory conditions including funisitis. Buhimschi et al. has reported associations between maternal funisitis and infant sepsis (38). Our findings further support the hypothesis that miR-29b levels are suppressed by intrauterine inflammation and early suppression may be involved in altering developmental pathways.

Our earlier publication reported a strong association between decreased miR-29b levels and development of BPD in infant blood samples obtained during the first week of life (4). We did not observe a similar correlation in cord blood samples. This may be due to the timing of development of BPD, as BPD is not clinically diagnosed until 36 weeks postmenstrual age. Moreover, postnatal rather than antenatal factors may be important determinants of risk for BPD than for neurological abnormalities (39). We did observe associations between miR-29b levels in cord blood and neurological morbidities, specifically cerebral palsy. In addition, miR-29b levels in the older infants (~36–40 weeks postmenstrual age) were also associated with diagnosis of neurological morbidities, specifically intraventricular hemorrhage. The vast majority of intraventricular hemorrhages occur in the first three days of life, so the increased miR-29b levels at 36–40 weeks post menstrual age do not play a role in diagnosis of IVH, but may represent a biomarker of previous injury (40). A previous study revealed elevated plasma levels of miR-29b in patients diagnosed with intracerebral hemorrhage, compared to controls (41).

MiR-based therapies are being explored as novel approaches to treatment for other diseases, including metabolic disorders, cardiovascular disease, cancer, and infections (42, 43). Using our murine model of perinatal inflammation, we demonstrated improved alveolarization and attenuated defects in matrix protein expression and localization by supplementing miR-29b on postnatal day 3 (4). Our data indicate that miR-29b is associated with inflammation in the infants, and that further investigation of the pathways associated with miR-29b may provide an avenue for therapeutic development.

CONCLUSION

Lower miR-29b levels in cord blood correlate with clinical and biochemical markers of inflammation including IL-6 and haptoglobin. We found an association between miR-29b levels with neurologic morbidities, including IVH and cerebral palsy. There was no significant association between miR-29b levels and BPD at the time points tested. These data provide promising results that further investigation of miR-29b in the fetus and neonate will allow for early diagnosis or therapeutic intervention for those at highest risk for morbidities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Impact:

- Decreases in miR-29b are associated with intra-uterine inflammation
- Hp&HbRP increases may be associated with decreased miR-29b
- MiR-29b may be an additional biomarker for neonatal outcomes and a potential therapeutic target for intra-uterine inflammation.

Informed consent was obtained from the mother of the infants included in this study.

Table 1.

Demographics and clinical variables of the newborns.

| Variable | Mean (SD) or N (%) |
|--|--------------------|
| Gestational age (weeks) | 27.3 (1.7) |
| Birth weight (grams) | 1023.8 (265.8) |
| Race | |
| Caucasian | 68 (73.9%) |
| African American | 18 (19.6%) |
| Asian | 1 (1.1 %) |
| Other | 4 (4.3%) |
| Male sex | 51 (55.4%) |
| Clinical characteristics | |
| Maternal GBS positive | 27 (29.3%) |
| Maternal intrapartum antibiotics | 59 (64.1%) |
| Full course of antenatal corticosteroids | 83 (90.2% %) |
| Multiple gestation | 13 (14.1%) |
| PPROM | 34 (36.9%) |
| SGA | 3 (3.3%) |
| Cesarean delivery | 61 (66.3%) |
| Spontaneous PTB | 66 (71.7%) |
| Medically-indicated PTB | 26 (28.3%) |
| Pre-eclampsia | 19 (20.7%) |
| Preterm labor | 40 (43.5%) |

N=92

Abbreviation: GBS, group B Streptococcus; PPRM, preterm prelabor rupture of membranes; SGA; small for gestational age (below 10th percentile); PTB, preterm birth

Table 2.

Exposed status and neonatal clinical outcomes.

| | Non-Exposed (n=54) | Exposed (n=38) | P-value |
|------------------------------|-------------------------------|---------------------------|------------------|
| Placental pathology | | | |
| Placenta weight (grams) | 240.5 (172.0) | 219.5 (62.1) | 0.59 |
| Funisitis | 3 (5.6%) | 17 (44.7%) | <0.001 |
| Chorioamnionitis | 13 (24.1%) | 32 (84.2%) | <0.001 |
| Abruption | 1 (1.9%) | 3 (7.9%) | 0.25 |
| Neonatal Outcomes | | | |
| Male sex | 30 (55.6%) | 21 (55.3%) | >0.99 |
| Birth weight (grams) | 1002.9 (273.1) | 1053.6 (255.8) | 0.33 |
| Gestational age (weeks) | 27.5 (1.7) | 27.1 (1.6) | 0.33 |
| Small for gestational age | 3 (5.6%) | 1 (2.6%) | 0.68 |
| Early-onset neonatal sepsis | 12 (22.2%) | 1 (2.6%) | 0.01 |
| Necrotizing enterocolitis | 5 (9.3%) | 5 (13.2%) | 0.66 |
| Retinopathy of prematurity | 23 (42.6%) | 22 (57.9%) | 0.14 |
| Intraventricular hemorrhage | 21 (38.9%) | 14 (36.8%) | 0.80 |
| Periventricular leukomalacia | 6 (11.1%) | 9 (23.7%) | 0.11 |
| Cerebral palsy | 4 (7.4%) | 3 (7.9%) | 0.98 |
| Bronchopulmonary dysplasia | 24 (44.4%) | 17 (44.7%) | 0.95 |

Mean (SD) or n (%). P values in bold font are considered statistically significant at $p < 0.05$.

Table 3.

Cord blood miR-29b CT values and clinical outcomes total sample.

| Clinical Outcomes | Present | Not present | |
|---------------------------------|--------------------|--------------------|--------|
| Placental pathology* | | | |
| Funisitis | 11.79 (1.16, n=19) | 10.80 (2.17, n=69) | p=0.04 |
| Chorioamnionitis | 11.26 (1.67, n=44) | 10.78 (2.30, n=44) | p=0.36 |
| Fetal/neonatal Outcomes# | | | |
| Small for gestational age | 8.00 (3.67, n=4) | 11.15 (1.80, n=88) | p=0.01 |
| Necrotizing enterocolitis | 11.08 (1.25, n=9) | 11.06 (2.08, n=83) | p=0.93 |
| Retinopathy of prematurity | 10.90 (2.33, n=44) | 11.14 (1.68, n=42) | p=0.94 |
| Intraventricular hemorrhage | 10.95 (2.21, n=33) | 11.14 (2.04, n=59) | p=0.51 |
| Periventricular leukomalacia | 10.54 (2.59, n=14) | 11.13 (1.89, n=74) | p=0.40 |
| Cerebral palsy | 11.93 (0.57, n=7) | 10.92 (2.12, n=77) | p=0.02 |
| Bronchopulmonary dysplasia | 10.91 (2.25, n=40) | 11.12 (1.83, n=47) | p=0.70 |

Mean (SD, n). P values in bold font are considered statistically significant at $p < 0.05$.

* n=88; four placentas were not sent for pathology

the n's that don't add up to 92 are due to transfer or death before time to diagnose or due to inadequate follow up after discharge

Table 4.

CT values and neonatal outcomes at 36 weeks.

| Neonatal Clinical Outcomes | Present | Not present | |
|------------------------------|-------------------|-------------------|---------------|
| Necrotizing enterocolitis | 8.22 (n/a, n=2) | 5.92 (3.18, n=16) | p=0.66 |
| Retinopathy of prematurity | 10.90 (2.33, n=9) | 6.61 (2.99, n=9) | p=0.58 |
| Intraventricular hemorrhage | 4.27 (3.27, n=7) | 8.19 (1.56, n=11) | p=0.04 |
| Periventricular leukomalacia | 1.7 (n/a, n=3) | 6.51 (2.91, n=15) | p=0.31 |
| Cerebral palsy | 4.67 (5.02, n=3) | 6.40 (2.90, n=15) | p=0.39 |
| Bronchopulmonary dysplasia | 5.59 (3.39, n=12) | 7.65 (1.53, n=6) | p=0.52 |

All comparisons using Kruskal-Wallis test. P values in bold font are considered statistically significant at $p < 0.05$.