Peer Review File

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Reviewer A

Jin et al reported their study (2015-2016) on the blood mi-RHA 210 levels were elevated in asphyxiated neonates, associated with mode of delivery, cord pH, and correlated significantly with Apgar Scores at 1 min and clinical symptoms. This was a prospectively designed single centred study with 53 subjects included. Blood 5 ml was collected within 1 hour after birth with informed parental consents. The authors further studied the significance of mi-RNA 210 and found that target genes were associated with autism and epilepsy based on GO and KEGG.
This reviewer has the following concerns:

1. Significant overlaps of blood mi-RNA 210 levels between asphyxiated and normal neonates, although they found significant differences between groups; The ROC with area 0.671 supports the modest value of blood miRNA-210

Reply 1: Thanks very much for your comments. After statistical analysis, there was a significant difference in blood mi-RNA 210 levels between asphyxiated and normal neonates, even if there was significant overlap in blood mi-RNA 210 levels, it did not affect the difference.
Changes in the text: We have modified our text as advised (see Page 6, line 826-827).

2. Despite significant correlation between 1 min Apgar and miRNA-210, the r was not clinically significant and there was no significant correlation with 5 min or 10 min Apgar scores.

Reply 2: Thank you for your proposal. The results were indeed as suggested by the reviewers. We have revised this part of the results in the revised manuscript, as follows: we also analyzed the relationship between miR-210 expression and the pathological features, and found that miR-210 expression in the HIE group was not related to the 1-, 5-, and 10-min Apgar scores, and the possible reason for this might be the insufficient sample size (Figure 2B). The reason for such results may be that we do not have enough sample size. Serum miR-210 can only be applied as a potential indicator marker associated with hypoxia. Vast literatures have reported that miR-210 is associated with hypoxia(1-4). In contrast, Apgar scores are based on many factors.
Changes in the text: We have modified our text as advised (see Page 6, line 1035-1036).

3. Although the target genes of miRNA-210 were associated with autism and epilepsy, there was no clinical evidence in the study groups.

Reply 3: Thanks very much for your comments. The target genes of miR-210 related to autism and epilepsy were predicted based on the database, which shows potential correlations. To examine the correlation, long-term follow-up is needed. In this study we were unable to provide clinical data related to autism and epilepsy. And we put this restriction in the discussion section.
Changes in the text: We have modified our text as advised (see Page 10, line 1487-1491).

4. The findings in Table 2 seem to deviate from the main study with the results from 29 out of 53 subjects, and elevation in respiratory distress.

Reply 4: Thank you for your proposal. There were 26 patients in the normal group and 27 patients in the asphyxia group. We are very sorry, the number of cases in Form 2 was filled in incorrectly. There were 10 cases in miR-210 low-expression group; there were 17 cases in miR-210 high-expression group. The miR-210 high expression group was higher than the average value of the control group, and the miR-210 low expression group was lower than the average value

Changes in the text: We have modified our text as advised (see Page 14, line 1621-1622; Page 15, line1629).

Other concerns included:

1. Lack of details and specificity in the abstract

Reply 1: Thanks very much for your comments. We have modified the abstract.

Changes in the text: We have modified our text as advised (see Page 1, line 22-68).

2. Details in methods regarding the consenting procedure, subgroup analyses (Table 2)

Reply 2: Thanks very much for your comments. We have added the details in methods (Page 3, line 367-371). The asphyxia subgroup was defined as the high expression group when the asphyxia subgroup was higher than the average value of the normal group, and the low expression group when it was lower than the average value.

Changes in the text: We have modified our text as advised (see Page 3, line 367-371; Page 15, line1629).

3. Demographic and clinical characteristics between asphyxiated and normal neonates

Reply 3: Thank you for your proposal. We have added the demographic and clinical characteristics between asphyxiated and normal neonates.

Changes in the text: We have modified our text as advised (see Page 3, line 367-371).

4. Minor grammatical and typo-graphical errors despite language edits.

Reply 4: Thank you for your proposal. Our manuscript has been revised with the help of native speaker regarding the deficiencies in English grammar, spelling, and sentence structure.

Changes in the text: We have modified our text as advised (Each page of the manuscript was revised).

Reviewer B

In this study, the authors sought to investigate the correlation between miR-210 and diagnosis of HIE in neonates. Although an interesting concept, the study uses a small sample size of neonates with uncertain diagnosis of HIE, lacks important information to understand the relation between each of the figures, and the conclusions that the authors describe are not supported by the data that is presented.

1. The authors describe inclusion and exclusion criteria, but no information is provided on number of screened patients, included and excluded patients. Since blood was collected within the first hour of life, potentially some patients were found to not have HIE. A flow chart would be needed.

Reply 1: Thank you for your proposal. The inclusion and exclusion criteria have been included in the patient information in the Methods section, as follows: To be eligible for inclusion in this study, the patients had to meet the following diagnostic criteria for neonatal asphyxia jointly formulated by the American Academy of Pediatrics and the College of Obstetrics and Gynecology in 1996: (I) showing severe metabolic mixed acidosis (pH <7.00) in umbilical artery blood; (II) have an Apgar score 0–3 points, and a duration >5 minutes; (III) have neonatal early neurological manifestations, such as convulsions, coma, or hypotonia; (IV) show evidence of multiple organ dysfunction in premature delivery. The patients who met the above criteria were diagnosed with asphyxia. Above combined with multi-organ (3 or more organs) damage and (or) hypoxic-ischemic encephalopathy was diagnosed as severe asphyxia.

Patients were excluded from the study if they met any of the following exclusion criteria: (I) had another serious disease that might have affected the comparability of the present study, such as a congenital genetic metabolic disease, neonatal hemorrhage, neonatal hemolytic disease, septic shock, and birth trauma to intracranial hemorrhage; and/or (II) a diagnosis of hypoxic-ischemic encephalopathy (HIE) was excluded after 2 weeks of treatment and observation, and based on the laboratory results and head magnetic resonance imaging (MRI). Besides, HIE was diagnosed based on the diagnostic criteria, clinical symptoms, Apgar score and PH of umbilical cord blood at delivery. A flow chart was also shown in Figure 1.

Changes in the text: We have modified our text as advised (see Page 4, line 579-581; Page 11, line 1625-1627).

2. The authors say that parents were consented for participation, but blood was collected in the first hour of life, so it is difficult to understand how this worked logistically

Reply 2: Thanks very much for your comments. Blood sample collection from newborns is routine. We have signed an informed consent form with the parents during the waiting period (newborns are included in the experimental group if they are diagnosed with hypoxia after birth).

Changes in the text: We have modified our text as advised (see Page 3, line 315-318).

3. One major potential confounder is whether any of these neonates with HIE underwent therapeutic hypothermia. This is a critical point that is missing.

Reply 3: Thank for your very good advice. None of the patients selected for this experiment were treated with mild hypothermia.

Changes in the text: We have modified our text as advised (see Page 3, line 317-318).

4. The data on miR-210 and Apgar scores and the ROC curve are not convincing – the spread of data points in Figure 1 is significant and the outliers seem to be the driving force behind the statistical significance. This indicates that sample size was likely too small to detect a true difference in miR-210 between HIE and Controls. Furthermore, in Figure 1B, the correlations appear very weak and mostly driven by outliers. Figure 2 shows an ROC curve that is barely better than chance and does not support the authors claim that miR-210 could contribute to diagnosis of HIE.

Reply 4: Thank for your very good advice. Despite the limited sample size, the results of the difference analysis met statistical requirements. At the same time, the repeated experiments were performed for miR-210 detection in all samples, and there were no outliers.

5. Table 1 lacks many relevant clinical variables, particularly those related to maternal risk factors. Patients diagnosed with HIE had an average cord pH of 7.21 and 1-min Apgar score of 7 which is not consistent with the typical parameters used to indicate an acute perinatal event in the diagnosis of HIE. No information is provided on degree of encephalopathy – mild, moderate, severe.

Reply 5: Thank for your very good advice. The scope of this study is neonatal asphyxia, some of which is severe enough to develop or is proven to meet the criteria for HIE. Diagnostic and grading criteria for HIE are provided in the Appendix. Maternal risk factors are a favorable supporting basis but not a necessary condition. Some mothers have no risk factors, but their infants have asphyxia. This non-proportional correlation is the clinical question and difficulty. In our future study, we will also analyze more relevant clinical variables

Changes in the text: We have modified our text as advised (see Page 10, line 1487-1491).

6. Figures 3-6 relating miR-210 to genes associated with neurodevelopmental and cardiovascular diseases are not well explained and it is unclear how they fit into the study as it related to neonates with HIE.

Reply 6: Thanks very much for your comments. In this study, miR-210-related data were predicted based on a database, similar to a bioinformatics analysis. The aim is to show the potential relevance of miR-210 to neurodevelopmental and cardiovascular diseases， which can provide ideas for subsequent in-depth research on miR-210 and developmental diseases.

Besides, it has been shown that miR-210 can promote neurological repair in ischemic areas to some extent(5, 6). However, its regulatory mechanism is still not fully understood. miR-210 is a hypoxia-specific miRNA. miR-210 expression is stably upregulated under hypoxic environment in vitro(7). In vitro hypoxic stimulation can upregulate miRNA-210 expression in human umbilical vein endothelial cells (HUVE-12), which in turn promotes HUVE-12 angiogenesis (4). Hypoxia is the main pathophysiological basis of post-ischemia brain. Whether miR-210 is involved in the regulation of angiogenesis after cerebral ischemia is interesting.

7. There are many English language errors throughout.

Reply: Thank for your very good advice. Our manuscript has been revised with the help of native speaker regarding the deficiencies in English grammar, spelling, and sentence structure.

Changes in the text: We have modified our text as advised (Each page of the manuscript was revised).

Reviewer C

The problem raised in the work is timely and interesting. Presentation and language need a major overhaul/amendments.

The title of the work must be precise/refined, you should add in the peripheral blood serum.

Line 40 is PH probably it should be pH.
Line 58 add citation after China. Add consent from the bioethics committee, i.e. the number and date of its issue. Precise criteria for the selection of newborns should be provided. In addition, clinical characteristics of the studied groups should be presented, e.g. age, gestational age, birth weight, Apgar score, gasometry. A picture of brain changes in NMR would be welcome. Also, a description of the control group should be provided, e.g. what diseases the newborns had.
Reply 1: Thank for your very good advice. We have provided the consent from the bioethics committee (2022-KYSB-014) in the attachment (see Page 3, line99-100). Clinical information on newborns has also been added in the Methods section (see Page 3, line94-97). The neonates in the control group had no disease (see Page 3, line 312-313).

Line 120 is "Peripheral blood (5ml.) was collected…" - it is a big volume like on newborn.
Line 156 what tests were really used, see figures.
Line 204, 286 first time used abbreviations should be defined (e.g. BP).
Line 253 - What it is MiRNA? Line 327 What it is PPI?
Reply 2: Thank for your very good advice. We have corrected the above errors in the revised manuscript.

Figure 1 is too small. It is mean±SD? And the number of samples? Figures 3 and 4 too small and the number of samples. Figure 5 the number of samples? Figure 6 has nice colors but is illegible.
Reply 3: Thanks very much for your comments. We have modified Figure 1 and Figure 6 to make it clearer. Figures 3, 4, and 5 are bioinformatics predictions without applying the samples of this study.

Tables 1, 2 and 3 for corrections, the number of newborns tested does not match, there is one digit in the tables, and in another place the mean + SD at the end what tests were used?
Reply 4: Thanks very much for your comments. By inspection, there was an error in the number of cases in the table. We have made a change. In our study, the used tests were mean + SD, which has been modified in the tables.

References

1. Marwarha G, Røsand Ø, Scrimgeour N, et al. miR-210 Regulates Apoptotic Cell Death during Cellular Hypoxia and Reoxygenation in a Diametrically Opposite Manner. Biomedicines. 2021;10(1).
2. Wu G, Ding X, Quan G, et al. Hypoxia-Induced miR-210 Promotes Endothelial Cell Permeability and Angiogenesis via Exosomes in Pancreatic Ductal Adenocarcinoma. Biochem Res Int. 2022;2022:7752277.
3. Zaccagnini G, Greco S, Longo M, et al. Hypoxia-induced miR-210 modulates the inflammatory response and fibrosis upon acute ischemia. Cell Death Dis. 2021;12(5):435.

4. Fasanaro P, D'Alessandra Y, Di Stefano V, et al. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. Journal of biological chemistry. 2008;283(23):15878-83.
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6. Zhang H, Wu J, Wu J, et al. Exosome-mediated targeted delivery of miR-210 for angiogenic therapy after cerebral ischemia in mice. J Nanobiotechnology. 2019;17(1):29.
7. Zeng L, Liu J, Wang Y, et al. MicroRNA-210 as a novel blood biomarker in acute cerebral ischemia. Frontiers in Bioscience-Elite. 2011;3(4):1265-72.

Reviewer D

1. Please also define "**" in Figure 2 legends.
Response: Added.

2. Please define "*, **" in Table 1 footnote.
Response: Added.

3. How were those data presented in Table 1? Please either define them inside the table or in table footnote.

Cord PH**	7.40±0.04	7.21±0.15	t=5.964	0
1-min Apgar**	9.76±0.56	7.08±2.38	t=5.523	0
5-min Apgar**	10	8.58±1.55	t=4.470	0
10-min Apgar**	10	9.04±1.18	t=4.146	0
miR-210 expression level*	0.61±0.44	0.99±0.80	t=−2.194	0.034

Response: Added in the table.

4. Table 3: Please check if they are correct, you got two numbers in one blank.

asphyxia

Project	Group	Number	miR-210	Z/t	P
NBNA [M (Q)]	Normal	21	0.74 (0.93)	−1.233	0.218
	Abnormal	6	1.04 (1.81)		
Low pH [M (Q)]	Normal	12	1.17 (0.92)	−0.659	0.510
	Abnormal	15	0.76 (0.75)		
Image inspection (mean + SD)	Normal	12	1.01±0.65	0.276	0.786
		7 (no data)			

Response: We have changed it. Here the total number in Normal group is 19, however, only 12 of them performed the Image inspection.