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Case Report

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Detection and analysis of plasma lncRNA, miRNA and mRNA profile in preterm birth with intraventricular hemorrhage

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ABSTRACT

Intraventricular hemorrhage (IVH) is a cause of morbidity and mortality in preterm infants and is strongly associated with adverse neurological outcomes. The incidence of severe IVH (grade 3 or 4) has persisted despite the overall decline in IVH. IVH has been attributed to changes in cerebral blood flow to the immature germinal matrix microvasculature. The cascade of adverse events following IVH includes inflammation, white matter injury, and delayed oligodendrial maturation. In this study, we aimed to identify long non-coding RNA (lncRNA), microRNA (miRNA), and messenger RNA (mRNA) expression in the peripheral blood of preterm infants with IVH compared to normal controls, resulting in the finding of novel biomarkers for IVH. We conducted transcriptome sequencing and small RNA sequencing for identifying differential expression of RNA in preterm infants with IVH. We identified differentially expressed 47 lncRNAs, 95 miRNAs, and 1,370 mRNAs in preterm infants with IVH compared to normal control. Particularly, lncRNA H19 exhibited significantly high expression in preterm infants with IVH. The functional analysis revealed that differentially expressed RNAs in preterm infants with IVH were associated with ferroptosis, heme metabolism, and immune response such as lymphocyte activation and interferon response. In conclusion, these results demonstrate the potential of lncRNA, miRNA, mRNA as possible diagnostic and prognostic biomarkers for IVH.

Keywords: Cerebral Intraventricular Hemorrhage; Preterm Infants; Ferroptosis; H19 Long Non-Coding RNA

INTRODUCTION

Preterm infants are at an elevated risk of experiencing intraventricular hemorrhage (IVH) due to the underdeveloped nature of their germinal matrix, which renders their blood vessels more susceptible to rupture, leading to bleeding within the brain's ventricular cavity [1]. In extremely premature infants born before 27 weeks of gestation, IVH is a commonly occurring serious complication, and it continues to be a significant concern in very preterm infants born before 32 weeks of gestation [2]. The severity of IVH can result in a range of short-term and long-term neurological issues in these infants [1]. Although there have been notable

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advancements in obstetric and neonatal care over the past century, IVH remains a significant factor in the health challenges and mortality rates of premature infants, particularly those born before 27 weeks of gestation [1]. Therefore, it is important to discover new diagnostic biomarkers for the prevention and early predictive diagnosis of IVH in premature infants.

The RNA sequencing used to analyze transcriptome profiles is a good tool for searching therapeutic targets and clinical diagnostic markers and discovering the molecular mechanisms of diseases. RNA can be categorized into two groups based on their coding potential: coding RNAs, which typically include messenger RNAs (mRNAs) that encode proteins serving as various cellular components such as enzymes, cell structures, and signal transducers, and non-coding RNAs (ncRNAs) that function as cellular regulators without encoding proteins [3]. The two main categories of ncRNAs consist of extensively studied short microRNAs (miRNAs) and recently identified long non-coding RNAs (lncRNAs). miRNAs, which are highly conserved, small, single-stranded ncRNAs ranging from 17 to 25 nucleotides, while lncRNAs are larger transcripts (> 200 nucleotides in size) synthesized similarly to mRNAs but do not undergo translation into proteins; furthermore, they have been implicated in complex biological processes such as immune cell development and function, immune disorders, neural development, and neurological diseases [4].

In this study, we are screening differentially expressed lncRNAs, miRNAs, and mRNAs in the blood of preterm infants with IVH and normal infants. We performed functional analysis using gene ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of differentially expressed mRNAs and examined the correlation between differentially expressed miRNA and mRNA. Based on these things, the altered expression of lncRNAs, mRNAs, and miRNAs in the blood of preterm infants with IVH could potentially serve as promising candidates for the diagnosis and disease progression of IVH in preterm infants.

CASE REPORT

Sample collection

Two cases of preterm infants with IVH and two cases of normal infants were selected from the Department of Pediatrics, Ewha Womans University Mokdong Hospital. This study protocol was approved by the Institutional Review Board (IRB) of the Ewha Womans University Mokdong Hospital (IRB protocol number: 2016-06-009).

RNA extraction and RNA sequencing

Total RNA was extracted from peripheral blood samples of preterm infants with IVH and normal infants using TRIzol LS reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. We utilized total RNA with integrity, as indicated by an RNA Integrity Number (RIN) value greater than or equal to 8, assessed using 2100 Bioanalyzer instrument (Agilent, Santa Clara, CA, USA). For transcriptome sequencing, library preparation was performed using the TruSeq RNA kit (Illumina, San Diego, CA, USA). For Small RNA sequencing, library preparation was accomplished using the TruSeq Small RNA library Prep kit (Illumina). To verify the library size, we confirmed the size of polymerase chain reaction (PCR)-enriched fragments and checked the templated size distribution by running them on a 2100 bioanalyzer instrument using a DNA 1000 chip. Additionally, we quantified the prepared libraries using quantitative PCR according to the Illumina qPCR Quantification protocol guide to achieve ideal cluster densities across all lanes of each flow

cell. The Illumina HiSeq 2000 platform was used for transcriptome sequencing with 101-bp paired-end sequencing and small RNA sequencing with 51-bp single-end sequencing.

miRNA-mRNA integrative genomic analysis

We employed a novel approach consisting of four separate steps to identify candidate miRNA-mRNA networks. First, we extracted lists of differentially expressed miRNAs and mRNA using a significant cut-off of |fold change| ≥ 2 in both the miRNA and mRNA datasets. Second, we inferred a putative miRNA-mRNA regulatory relationship based on target information provided by miRDB [5]. Third, we confirmed the negative correlation between miRNA expression profiles and mRNA expression profiles. Finally, we categorized the distribution of miRNAs targeting mRNAs with a negative correlation in the sequencing data and the distribution of predicted target mRNAs from miRDB, and then conducted a hypergeometric test to assess the distribution of mRNAs regulated by miRNAs. Significant miRNAs were chosen as a statistically significant threshold ($p < 0.05$).

Functional analyses of differentially expressed mRNAs

To investigate the function of differentially expressed mRNAs in preterm infants with IVH and predicted mRNAs regulated by miRNAs, we performed Gene Ontology (GO) enrichment analysis, KEGG pathway, and Gene Set Enrichment Analysis (GSEA) [6]. The visualization of GO enrichment analysis and KEGG pathway was performed by ShinyGO [7]. GSEA was performed by applying human hallmark gene sets collections from MSigDB [8].

Clinical features

The patient group consisted of two extremely preterm infants with severe cerebral hemorrhage. One preterm infant was born at 25^{+1} weeks of gestation by caesarean section, with a birth weight of 790 g. Surfactant was administered through an intubation tube immediately after birth for lung expansion, and a mechanical ventilator was applied. On the next day, pulmonary hemorrhage developed. A blood test was performed, and disseminated intravascular coagulopathy was diagnosed. A pediatric radiologist conducted brain ultrasonography, revealing severe IVH. Despite treatment with inotropics, transfusions, and other interventions, the infant passed away on the fourth day of life. Another preterm infant in the group was born at 25^{+5} weeks of gestation by caesarean section, with a birth weight of 940 g. This infant experienced severe IVH starting from the second day of life and passed away on the seventh day of life. The control group included two healthy term infants. Their birth weights were 3.18 and 3.44 kg, respectively. They were hospitalized, one for jaundice and the other for testing due to exposure to tuberculosis. Blood samples were obtained with parental informed consent during hospitalization within the seventh day of life.

The transcriptome analysis identifies differentially expressed lncRNAs, miRNAs, and mRNAs between preterm infants with IVH and normal infants

We conducted transcriptome sequencing and small RNA sequencing to identify gene profiles in the blood of preterm infants with IVH and normal infants. In the transcriptome sequencing, genes with FPKM values of zero in at least one out of four total samples are excluded from the analysis. Out of a total of 25,906 genes, the analysis was conducted on 14,594 genes after excluding 11,312 genes. Of the 14,594 genes, protein coding genes (mRNAs) were 13,255, non-coding genes were 930, and pseudo genes were 409. The non-coding genes include various types of genes such as lncRNA, snoRNA, and tRNA, of which lncRNA was the most common (98.4%). In the small RNA sequencing, genes with RPM values of zero in at least one out of four total samples are excluded from the analysis. Out of

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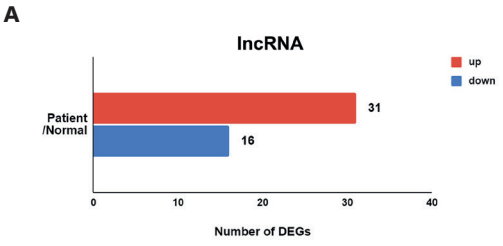
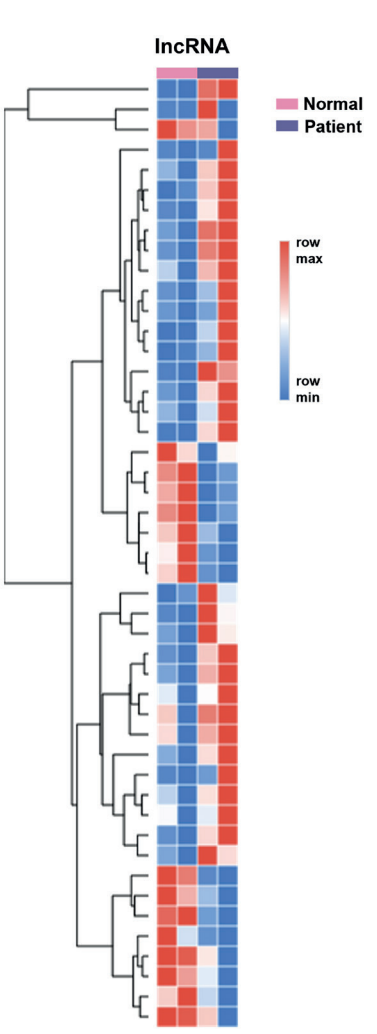
2,588 miRNAs, the analysis was conducted on 1,001 miRNAs after excluding 1,587 miRNAs. In total, 47 lncRNAs, 95 miRNAs, and 1,370 mRNAs were identified according to the criteria of the expression levels with 2-fold changes. Among them, 31 lncRNAs were up-regulated and 16 lncRNAs were down-regulated, 45 miRNAs were up-regulated and 50 miRNAs were down-regulated, and 858 mRNAs were up-regulated and 512 mRNAs were down-regulated in preterm infants with IVH compared to normal infants (**Figs. 1A**, **2A**, and **3A**). Hierarchical clustering analysis displayed the expression of differentially expressed lncRNAs, miRNAs, and mRNAs between preterm infants with IVH and normal infants (**Figs. 1B**, **2B**, and **3B**). We displayed the lncRNAs, miRNAs, and mRNAs list of the top 10 of the up-regulated and down-regulated in preterm infants with IVH (**Figs. 1C**, **2C**, and **3C**).





C

	lncRNA	Fold change
Up-regulated lncRNAs (Top 10 out of 31)	H19	356.020131
	LINC02506	9.608132
	SNHG10	7.418723
	MEG3	4.776339
	PINK1-AS	4.697982
	MIR22HG	3.996334
	BGLT3	3.837953
	MAFA-AS1	3.570395
	SLC2A1-DT	3.505060
	LOC101927272	3.058741
Down-regulated lncRNAs (Top 10 out of 16)	ITGB2-AS1	-4.389747
	IRF1-AS1	-3.012056
	ASMTL-AS1	-2.694130
	HCP5	-2.437003
	DBH-AS1	-2.368115
	HCG26	-2.350338
	AATBC	-2.291786
	PTOV1-AS2	-2.258312
	LOC100506076	-2.245345
	LINC00861	-2.207045

Figure 1. Altered lncRNA expression in preterm infants with IVH. (A) Counts of lncRNAs were up- and down-regulated in preterm infants with IVH. (B) Hierarchical clustering visualizes the 47 lncRNAs in preterm infants with IVH (patient) and normal infants (normal). (C) The top 10 most differentially up- and down-regulated lncRNAs in preterm infants with IVH.
lncRNA, long non-coding RNA; IVH, intraventricular hemorrhage.

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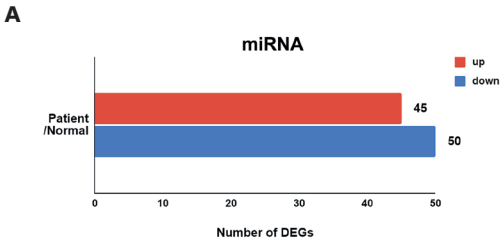

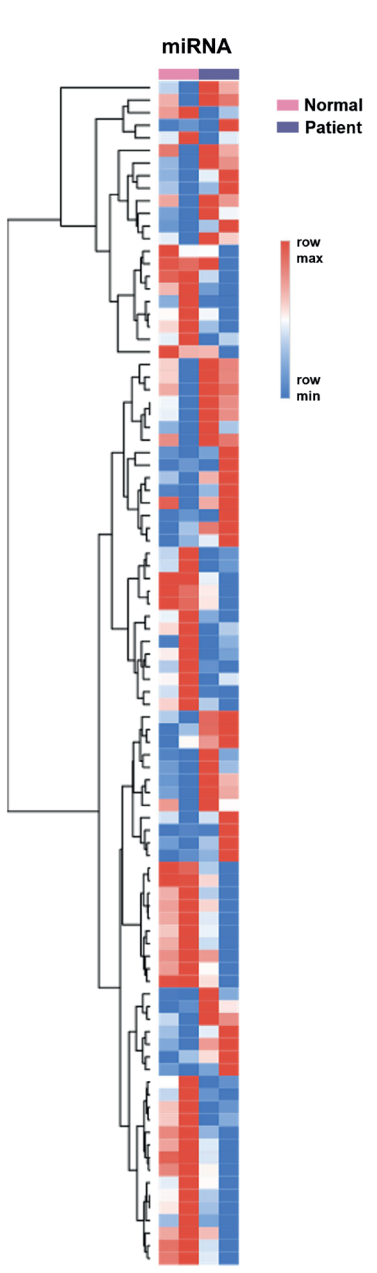

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	miRNA	Fold change
Up-regulated miRNAs (Top 10 out of 45)	hsa-let-7b-5p	15.247731
	hsa-miR-96-5p	14.955730
	hsa-miR-19b-3p	11.249604
	hsa-miR-100-5p	10.979845
	hsa-miR-10b-5p	9.816356
	hsa-miR-19a-3p	9.552620
	hsa-miR-183-5p	8.150743
	hsa-miR-182-5p	5.448478
	hsa-miR-3200-3p	4.612196
	hsa-miR-10a-5p	4.308704
Down-regulated miRNAs (Top 10 out of 50)	hsa-miR-127-3p	-7.191470
	hsa-miR-411-5p	-7.079316
	hsa-miR-381-3p	-5.679153
	hsa-miR-369-3p	-5.561772
	hsa-miR-487b-3p	-4.961056
	hsa-miR-410-3p	-4.837758
	hsa-miR-493-3p	-4.150935
	hsa-miR-1180-3p	-4.115849
	hsa-miR-493-5p	-3.816181
	hsa-miR-33b-5p	-3.481847

Figure 2. Altered miRNA expression in preterm infants with IVH. (A) Counts of miRNAs were up- and down-regulated in preterm infants with IVH. (B) Hierarchical clustering visualizes the 47 miRNAs in preterm infants with IVH (patient) and normal infants (normal). (C) The top 10 most differentially up- and down-regulated miRNAs in preterm infants with IVH.
miRNA, microRNA; IVH, intraventricular hemorrhage.

Bioinformatics analyses of significantly expressed mRNAs in preterm infants with IVH

Next, to understand the functions of the differentially expressed mRNAs in preterm infants with IVH, we conducted GO, KEGG pathway, and GSEA analyses. GO analysis by including significantly up-regulated mRNAs revealed that neutrophil degranulation, neutrophil activation involved in immune response, and neutrophil activation were mainly enriched in the biological process (**Fig. 4A**). Meanwhiles, GO analysis by including significantly down-

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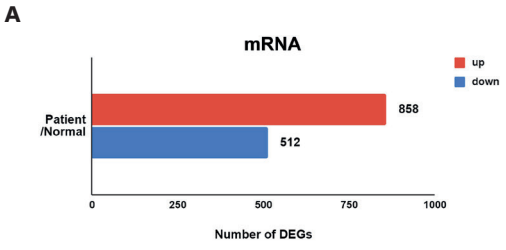

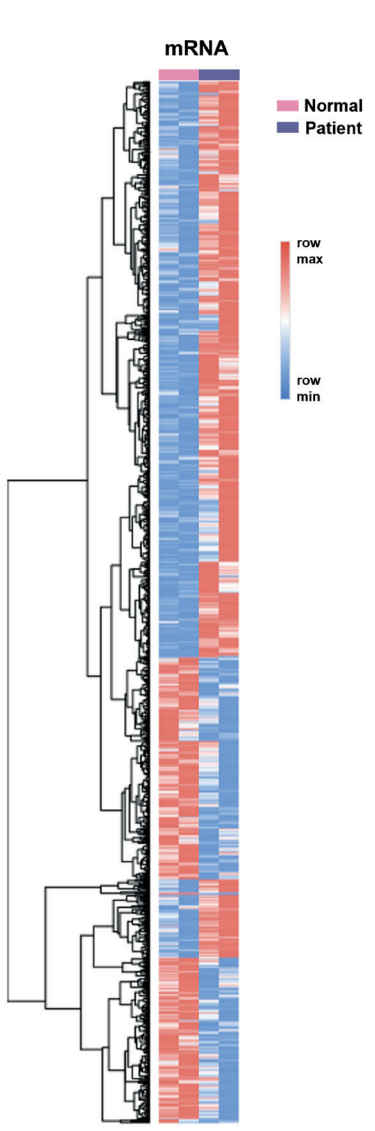

C

	mRNA	Fold change
Up-regulated mRNAs (Top 10 out of 858)	IL1R2	44.528577
	SMIM1	31.840600
	RUNDC3A	27.049684
	PRTN3	26.037507
	CD177	25.217803
	AHSP	22.125995
	WFDC1	21.333409
	GYPA	19.807694
	TSPO2	18.811453
	PRDX2	17.419663
Down-regulated mRNAs (Top 10 out of 512)	IFI27	-11.531907
	GZMH	-9.746278
	IFI6	-9.179086
	FGFBP2	-8.616652
	GNLY	-7.684768
	LAIR2	-7.460609
	IFI44	-7.048937
	MYOM2	-6.993450
	CX3CR1	-6.964836
	GZMB	-6.885817

Figure 3. Altered mRNA expression in preterm infants with IVH. (A) Counts of mRNAs were up- and down-regulated in preterm infants with IVH. (B) Hierarchical clustering visualizes the 47 mRNAs in preterm infants with IVH (patient) and normal infants (normal). (C) The top 10 most differentially up- and down-regulated mRNAs in preterm infants with IVH.
mRNA, messenger RNA; IVH, intraventricular hemorrhage.

regulated mRNAs in preterm infants with IVH revealed that T cell activation, lymphocyte activation, immune effector process were enriched in the biological process (**Fig. 4A**). For the molecular function of up-regulated mRNAs, hemoglobin binding, haptoglobin binding, and peroxidase activity were enriched (**Fig. 4B**). The molecular function with the highest enrichment of down-regulated mRNAs were 2–5-oligoadenylate synthetase activity, MHC class II protein binding, and cytokine receptor activity (**Fig. 4B**). The cellular component with the highest enrichment of up-regulated mRNAs were specific granule lumen, tertiary granule lumen, and platelet alpha granule (**Fig. 4C**). In the cellular component, down-regulated mRNAs were enriched in alpha-beta T cell receptor complex and T cell receptor complex (**Fig. 4C**). The KEGG pathway analysis showed that up-regulated mRNAs were significantly enriched in ferroptosis, mitophagy, and complement and coagulation cascades (**Fig. 5A**).

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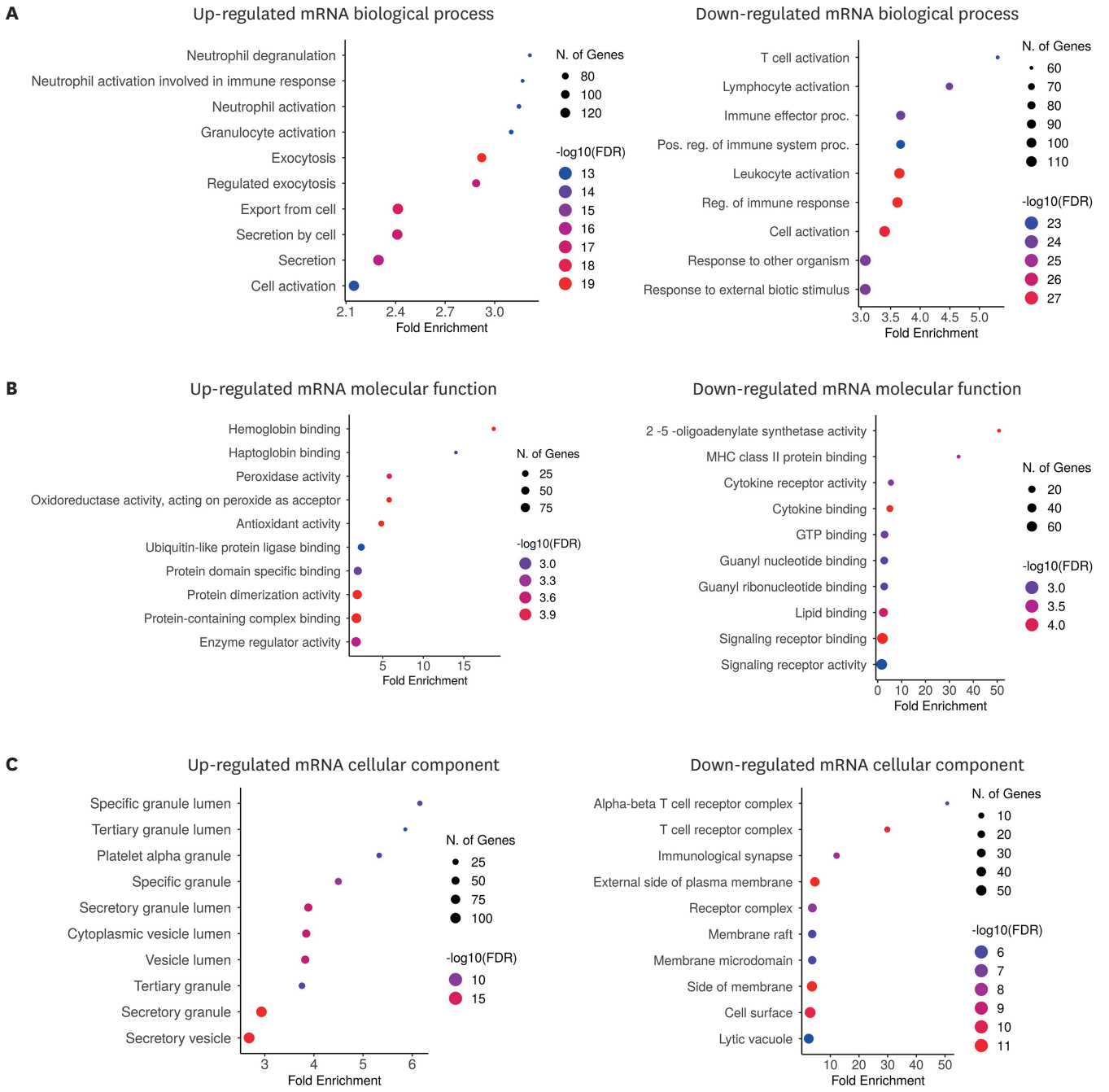

Figure 4. GO terms analysis of differentially expressed mRNAs. The enrichment analysis in biological process (A), molecular function (B), cellular component (C) of significantly up- and down-regulated mRNAs in preterm infants with IVH.
GO, Gene Ontology; mRNA, messenger RNA; IVH, intraventricular hemorrhage.

The KEGG pathway analysis showed that down-regulated mRNAs were mainly enriched in natural killer cell mediated cytotoxicity, hematopoietic cell lineage, and Th1, Th2, and Th17 cell differentiation (**Fig. 5A**). In addition, we performed GSEA on the mRNAs expressed in preterm infants with IVH. We identified that up-regulated mRNAs were enriched in heme metabolism and reactive oxygen species pathway and down-regulated mRNAs were enriched in interferon gamma response and interferon alpha response (**Fig. 5B and C**).

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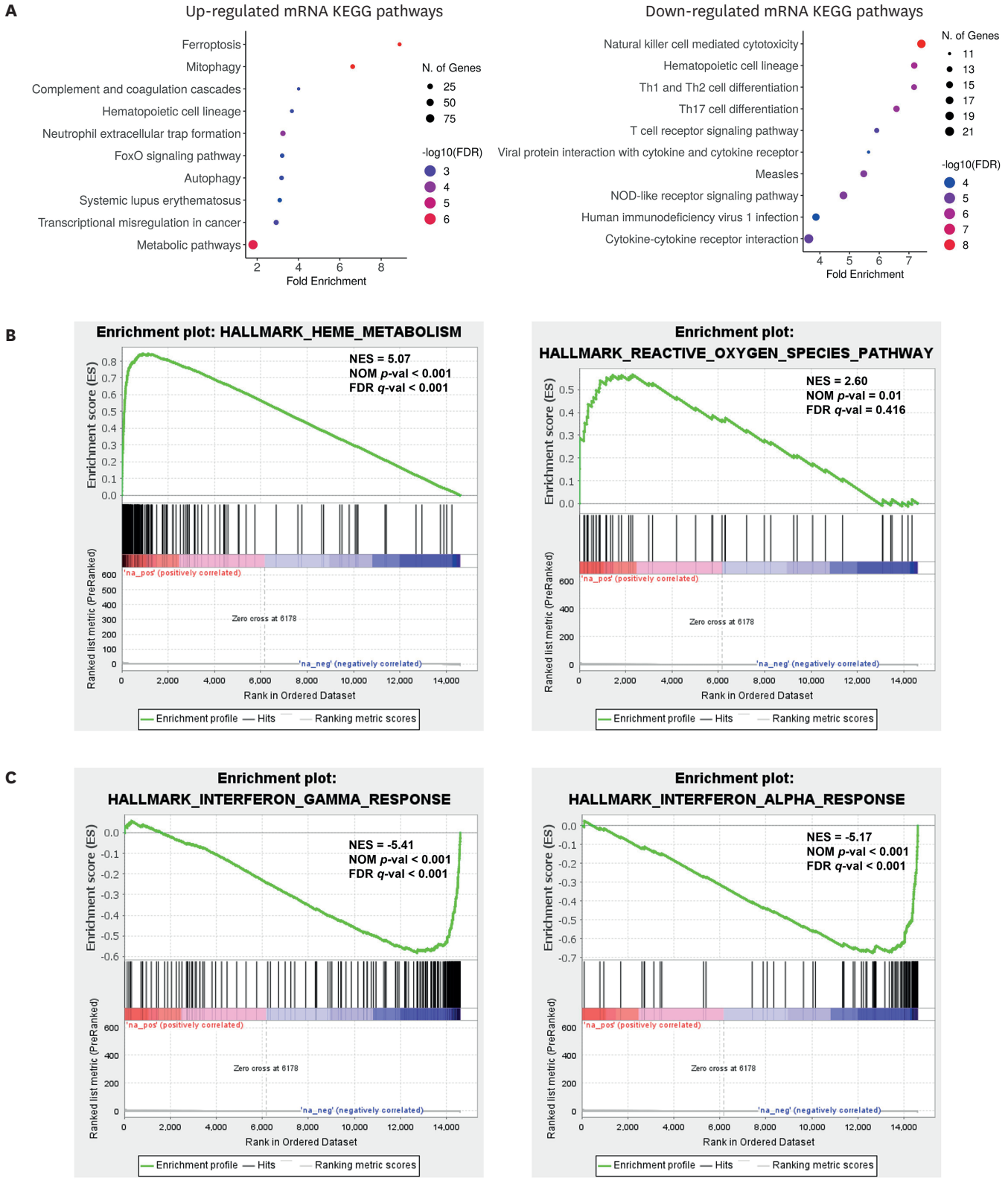

Figure 5. KEGG pathway and GSEA analyses of differentially expressed mRNAs. (A) KEGG pathway analysis of up- and down-regulated mRNAs. (B) GSEA analysis of up-regulated mRNAs. (C) GSEA analysis of down-regulated mRNAs.
KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, Gene Set Enrichment Analysis; mRNA, messenger RNA.

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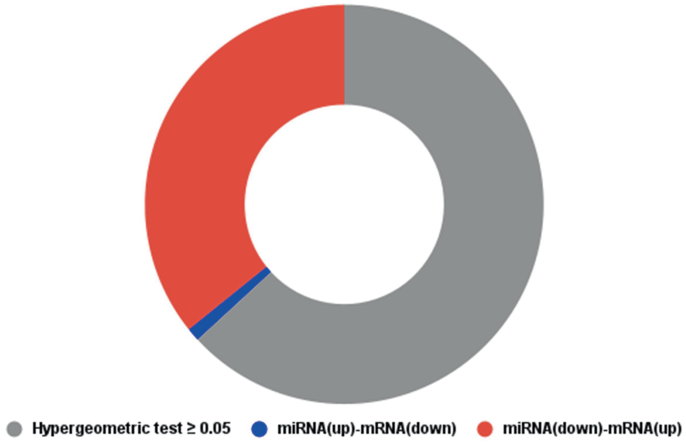
Integrative genomic analysis of miRNA-mRNA negative correlation in blood of preterm infants with IVH

The hypergeometric test analysis was used to investigate the correlation between differentially expressed miRNAs and mRNAs in comparison to preterm infants with IVH and normal infants. As a result, miRNAs that showed more than a 2-fold difference in expression exhibited at least one negative correlation pair with mRNAs (**Fig. 6A**). Also, 35 miRNAs

A

Result Directory (Comparison)	# of sig miRNA (|fc|>=2)	# of sig mRNA (|fc|>=2)	# of miRNA (at least 1 miRNA-mRNA negative regulated pairs in sig mRNAs)	# of miRNA (at negative relationship)	
				hypergeometric test < 0.05	hypergeometric test ≥ 0.05
Patient vs. Normal	95	1370	95	**35**	60

B

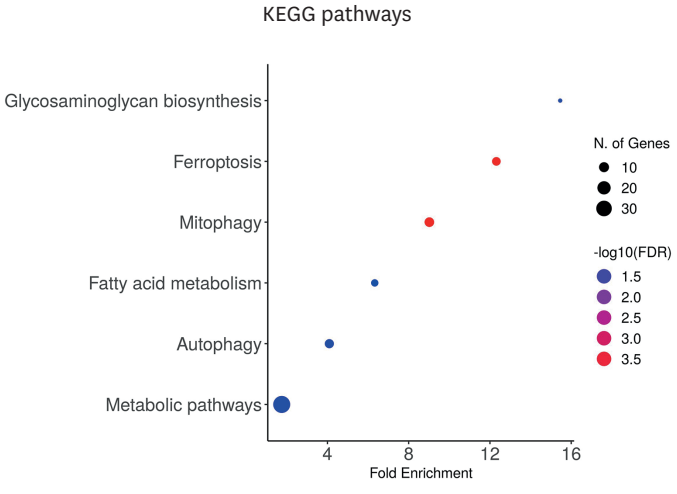


Hypergeometric test ≥ 0.05
miRNA : 60
mRNA : 130
miRNA-mRNA pair :273

miRNA(up)-mRNA(down)
miRNA : 1
mRNA : 2
miRNA-mRNA pairs : 2

miRNA(down)-mRNA(up)
miRNA : 34
mRNA: 344
miRNA-mRNA pairs : 624

C



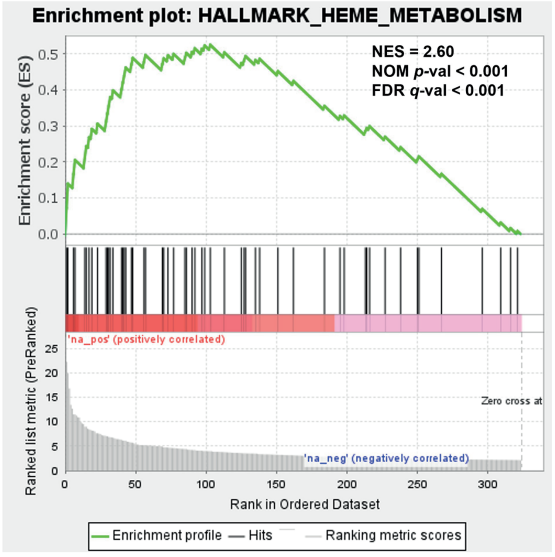


Figure 6. Significant miRNA-mRNA correlation pairs and the functional analysis of miRNA-associated mRNAs. (A) Summary of miRNA-mRNA negative genomic analysis. (B) A circle chart shows the results of the hypergeometric test. (C) KEGG pathway and GSEA analyses of miRNA-associated mRNAs.
miRNA, microRNA; mRNA, messenger RNA; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, Gene Set Enrichment Analysis.

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showed a significant correlation ($p < 0.05$), resulting in 1 up-regulated miRNA was found to correlate with 2 down-regulated mRNAs, and 34 down-regulated miRNAs were associated with 344 up-regulated mRNAs (**Fig. 6B**). To understand the function of 344 up-regulated mRNAs associated with 34 down-regulated miRNAs, we conducted KEGG pathway and GSEA analyses. As for KEGG pathway, we observed that 344 up-regulated mRNAs are involved in glycosaminoglycan biosynthesis, ferroptosis, and mitophagy (**Fig. 6C**). Notably, 344 up-regulated mRNAs were significantly enriched in heme metabolism, which is consistent with the enrichment of up-regulated mRNAs in preterm infants with IVH (**Fig. 6C**).

DISCUSSION

We investigated the expression profiles of lncRNAs, miRNAs, and mRNAs using transcriptome sequencing and small RNA sequencing in the blood of preterm infants with IVH in order to find potential biomarkers for diagnosis and disease progression. The analysis revealed that 47 lncRNAs, 95 miRNAs, and 1,370 mRNAs were significantly expressed in the blood of preterm infants with IVH. 31 lncRNAs, 45 miRNAs, and 858 mRNAs were significantly up-regulated in preterm infants with IVH, meanwhiles 16 lncRNAs, 50 miRNAs, and 512 mRNAs were significantly down-regulated in preterm infants with IVH compared to normal infants.

H19 is the most up-regulated lncRNA from preterm infants with IVH (356-fold). H19 is the first discovered lncRNA and is correlated with central nervous system diseases such as cerebral ischemia, cerebral hemorrhage, brain tumors, and Alzheimer's disease [9]. Researchers recently reported that H19 is highly expressed following ICH injury in both in vivo and in vitro models [10,11]. We confirmed elevated H19 expression in the blood of preterm infants with IVH. Although ICH and IVH represent different types of brain hemorrhages, based on the results of both our study and previous research, H19 shows a high potential as a biomarker associated with hemorrhage.

Previous studies have demonstrated miRNA expression in the cerebrospinal fluid (CSF) of preterm infants with IVH. To the best of our knowledge, we are the first to identify miRNA expression in the blood of preterm infants with IVH. The expression of miR-145 and miR-223 has previously been reported to be significantly increased in the CSF of preterm infants with IVH, and our miRNA profiles also revealed elevated levels of both miRNAs (miR-145, 3.3-fold; miR-223, 2.1-fold) [12,13]. Therefore, these miRNAs could potentially serve as a biomarker for IVH in preterm infants.

The up-regulated mRNA expression in preterm infants with IVH was associated with neutrophil activation in biological processes in GO, and KEGG pathway and GSEA analyses revealed that up-regulated mRNAs were associated with ferroptosis and heme metabolism. Additionally, miRNA-mRNA negative correlation analysis showed that 344 mRNAs regulated by 34 miRNAs were associated with ferroptosis and heme metabolism. Ferroptosis, a recently discovered form of cell death, typically involves significant iron accumulation and lipid peroxidation during its progression and has been associated with various diseases, such as tumors, nervous system disorders, and ischemia-reperfusion injury [14]. Although we did not conduct further studies on the function of lncRNAs in this research, there have been several reports regarding the relationship between H19 and ferroptosis [11,15]. Furthermore, among the crucial regulatory genes of ferroptosis, including GPX4, NRF4, ACSL4, and NCOA4, it is known that NCOA4, as a nuclear receptor coactivator 4, is involved in iron metabolism

and autophagy [14]. In this study, NCOA4 expression was found to be increased by 7.4-fold in preterm infants with IVH. Further study will be needed, but it can be predicted that interactions of H19, down-regulated miRNAs, and NCOA4 may be functional mechanisms in preterm infants with IVH.

The down-regulated mRNA expression in preterm infants with IVH was associated with T cell and lymphocyte activation in biological processes in GO, and KEGG pathway and GSEA analyses revealed that down-regulated mRNAs were associated with NK cell-mediated cytotoxicity and interferon response. After IVH, the lysis of red blood cells and oxidation of hemoglobin (Hb), which leads to the release of heme, can trigger neuroinflammatory response [16]. Considering the decreased expression of mRNAs related to the immune system such as lymphocyte activation and interferon response, it can be inferred that preterm infants with IVH may have impaired immune system functions.

In this study, we conducted an analysis of blood transcriptome sequencing in 2 cases of grade 4 severe IVH using peripheral blood bulk RNA sequencing. Next-generation sequencing is increasingly becoming the preferred method for RNA profiling. Over the past few years, miRNAs have emerged as a new class of potential biomarkers for various diseases in tissues and biofluids, with blood-based biomarkers, in particular, offering utility due to the ease with which serum or plasma can be readily obtained and sampled regularly [17]. We anticipate that the utilization of RNA sequencing on the blood of preterm infants with IVH will contribute to the development of biomarkers, thereby aiding in the future assessment and diagnosis of diseases in preterm infants.

We divided the analysis into preterm infants with IVH and normal groups; however, due to a limited number of hospitalized preterm infants, we had to rely on samples from two patients. Due to the insufficient sample size per group, it was challenging to establish statistical significance; nevertheless, we identified RNAs with significant expression differences between the groups. Currently, in Korea, the prevalence of severe IVH (grade 3 or 4) has decreased rapidly due to the development and improvement in neonatal intensive care, making it difficult to meet the number of subjects required for analysis using single-center data. Although the overall incidence of IVH decreased in preterm infants, prediction and prevention of IVH still continue to be challenging in the care of preterm infants because of high mortality and severe neurological sequelae. Therefore, it is important to consider ways to generalize and discover powerful predictive and diagnostic biomarkers for severe IVH, and further experimental studies should be conducted through additional multicenter studies.

Overall, we identified the expression of lncRNA, miRNA, and mRNA in preterm infants with IVH that might be associated with ferroptosis, heme metabolism, and immune response. The results of the comprehensive analysis will contribute to the development of biomarker for diagnosis and disease progression and to helpful preventive and treatment strategies for preterm infants with IVH.

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