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A proteome signature of umbilical cord serum associated with congenital diaphragmatic hernia

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ABSTRACT

Congenital diaphragmatic hernia (CDH) is a congenital anomaly characterized by a defect in the diaphragm. Despite the recent improvements in its treatment, CDH is associated with a high rate of neonatal mortality, which is often related to pulmonary hypoplasia (PH) as well as pulmonary hypertension. A better understanding of the underlying pathological mechanisms of PH in CDH could help establish a new treatment to improve its prognosis. In this study, we investigated serum biological profiles in neonates with CDH. For comprehensive investigation, umbilical cord serum samples were collected from isolated CDH cases (n = 4) and matched healthy controls (n = 4). Samples were analyzed using liquid chromatography–tandem mass spectrometry. A total of 697 proteins were detected; of them, 98 were identified as differentially expressed proteins. Among these differentially expressed proteins, complement C1q subcomponent showed the largest fold change, followed by complement C5. In the pathway enrichment analysis, the complement and coagulation cascades expressed the most significant enrichment ($p = 2.4 \times 10^{-26}$). Thus, the complement pathway might play some role in the pathophysiology of CDH.

Keywords: pulmonary hypertension, Liquid chromatography–tandem mass spectrometry, Complement and coagulation cascade

Abbreviations:
CDH: Congenital diaphragmatic hernia
C1q: Complement C1q
C5: Complement C5
PH: Pulmonary hypertension
LC–MS/MS: Liquid chromatography–tandem mass spectrometry

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INTRODUCTION

The worldwide incidence of congenital diaphragmatic hernia (CDH) is 1 in 4000 births.[1] The main feature of CDH is a defect in the diaphragm, which allows the intra-abdominal organs to migrate into the chest, preventing the lungs from growing normally due to the limited space and resulting in pulmonary hypoplasia. Pulmonary hypoplasia is accompanied by structural alterations of pulmonary vessel walls, which is thought to lead to pulmonary hypertension (PH).[2] Despite the recent progress, CDH is associated with a high rate of mortality rate (approximately 30% – 40%).[3] In CDH, PH is the main cause of death during neonatal period.[4] After birth, the therapeutic strategies include high-frequency oscillatory ventilation, inhaled nitric oxide, extracorporeal membrane oxygenation, and the use of corticosteroids and/or surfactants to improve the function of the hypoplastic lungs in PH.[5,6,7] However, the optimal management of PH in CDH remains controversial.[5] Recently, as an antenatal surgical intervention for severe CDH, fetoscopic endoluminal tracheal occlusion has been reported to increase neonatal survival rate by promoting pulmonary development, but it is reported to increase the risk of preterm birth.[8] Further studies are needed to establish a standard therapy to improve the prognosis of severe CDH.

Vascular abnormalities and remodeling caused by endothelial dysfunction is known to occur in patients with PH as part of its pathophysiology.[9] Pharmacotherapies for PH often target the vasoactive mediators, but poor response of patients with CDH to these treatments remains a problem.[10] Accumulating more knowledge regarding the pathophysiology of PH in infants with CDH will have important implications in future therapeutic designs for neonates with severe CDH.

The aim of this study was to describe proteomic profiling in isolated left-sided CDH using umbilical cord serum analyzed via liquid chromatography–tandem mass spectrometry (LC–MS/MS).

MATERIALS AND METHODS

Patient and sample collection

Study participants comprised healthy pregnant women who had singleton delivery at term via cesarean section at the Nagoya University Hospital from April 2012 to August 2018. Isolated left-sided CDH cases (CDH, n = 4) and normal healthy controls (Control, n = 4) were matched by gestational age at birth, parity, and sex of babies.

The serum samples from umbilical vein and clinical data of CDH and Control groups were obtained. The specimens were immediately stored in vacuum blood collection tubes at 4°C until isolation. The serum was isolated 16–24 h after blood collection, following which the samples were stored at −80°C.

The study was approved by the Ethics Committee at the Nagoya University Graduate School of Medicine (approval number: 2018-0145). Pre-existing samples and medical records were used. Thus, we provided disclosure of information on the methods of this study and gave participants an opportunity to reject the enrollment in this study, in accordance with the ethical guidelines established by the Japanese Ministry of Health, Labour and Welfare.

Mass spectrometry and data analysis

The procedure was performed in accordance with previous reports.[11,12] The total protein concentration of serum samples was assessed using the bicinchoninic acid assay (Pierce™ BCA Protein Assay kit; Thermo Fisher Scientific Inc., Waltham, MA) in accordance with the manufacturer's instruction. The proteins were trypsin-digested for 16 h at 37°C after reduction

with dithiothreitol (final concentration, 5 mM) for 30 min at room temperature and alkylation with iodoacetamide (final concentration, 10 mM) for 60 min at room temperature in the dark. The peptides were analyzed using LC–MS/MS using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific Inc.) coupled to an UltiMate3000 RSLC nano-LC system (Dionex Co., Amsterdam, The Netherlands) with a nano-HPLC capillary column, 150 mm × 75 μm i.d (Nikkyo Technos Co., Japan) via a nano-electrospray ion source. Reversed-phase chromatography was performed with a linear gradient (0 min, 5% B; 100 min, 40% B) of solvent A (2% acetonitrile with 0.1% formic acid) and solvent B (95% acetonitrile with 0.1% formic acid) at an estimated flow rate of 300 nL/min. A precursor ion scan was performed with a 400–1600 mass to charge ratio (m/z) prior to MS/MS analysis. Furthermore, tandem MS was performed by isolation at 0.8 Th with quadrupole, high-energy collision dissociation fragmentation with normalized collision energy of 35% and a rapid scan MS analysis in the ion trap. Only precursors with a charge state of 2–6 were sampled for MS2. The dynamic exclusion duration was set to 15 s with a 10 ppm tolerance. The instrument was run in top speed mode with 3-s cycles. The raw data was processed using Proteome Discoverer 1.4 (Thermo Fisher Scientific Inc.) in conjunction with MASCOT search engine, version 2.6.0 (Matrix Science Inc., Boston, MA) for protein identification. Peptides and proteins were identified against the human protein database in UniProt (release 2018_11) with a precursor mass tolerance of 10 ppm and fragment ion mass tolerance of 0.8 Da. Fixed modifications were set for the carbamidomethylation of cysteine, whereas variable modifications were set for the oxidation of methionine. Two missed cleavages by trypsin were allowed.

Protein quantification was performed using the Proteome Discoverer (Thermo Fisher Scientific Inc.), which calculates the peak area of precursor ions on the basis of the top three unique peptides for the given protein.

Proteomic analysis and visualization

Proteins abundance with fold changes of ≤0.5 or ≥2 in the CDH group compared to with those in the Control group were analyzed.[13] In addition, the following filters were applied: (i) minimum two matched unique peptides were considered,[14] and ii) score > 35: it is related to the number of peptide sequences that have been identified for a protein.[15] On the basis of these criteria, proteins were detected as differentially expressed proteins (DEPs). Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov, and version 6.8) was used to analyze DEPs. *Kyoto Encyclopedia of Genes and Genomes* (KEGG, https://www.genome.jp/kegg/kegg_ja.html) was used to analyze the enrichment pathways involved in DEPs. Proteomaps were used to show the graphical areas of each protein reflecting the magnitude of the average fold change in DEPs for cases compared with that for controls (https://www.proteomaps.net/). Reactome was used to describe the functions and pathways in which proteins were involved (https://reactome.org/dev/graph-database). Protein interactome map was used to predict protein·protein interaction analyzed using STRING online database (https://string-db.org/). The nodes in the interactome map expressed retrieved data as the results of the neighboring network analysis.

Statistical Analysis

Data sets were tested for normality and then analyzed using SPSS ver. 25 for Windows (IBM Corp., Armonk, NY). Group data were presented as median [range] and compared using Mann-Whitney *U* test. Statistically significant difference was set at a p value of < 0.05.

RESULTS

All patients in the CDH group were treated for PH with high-frequency oscillatory ventilation and inhaled nitric oxide. Two patients required further extracorporeal membrane oxygenation therapy because of severe CDH. One patient died 7 days after birth because of severe PH. Several characteristics of the Control group (n = 4) were matched, including gestational age at birth. Most of the maternal and neonatal clinical characteristics were similar, such as maternal age, birth weight, and umbilical arterial blood pH (Table 1). Only Apgar scores at 1 (5 [5–6] vs 9 [9–9]; $p < 0.001$) and 5 (3.5 [3–8] vs 10. [9–10]; $p = 0.005$) min were the significantly different characteristic between the CDH and Control groups.

The 697 proteins identified via LC–MS/MS were analyzed using the MASCOT program, and 98 of these were found to be DEPs. Of these DEPs, the complement C1q (C1q) subcomponent showed the highest fold change, followed by complement C5 (C5) (Table 2).

In the pathway enrichment analysis, the complement and coagulation cascades showed the highest significance ($p = 2.4 \times 10^{-26}$; Fig. 1A). In addition, enrichment analysis of the Gene Ontology terms for a biological process showed complement activation of the classical pathway as significant ($p = 1.3 \times 10^{-29}$; Fig. 1B). Nine of the 20 pathways, such as complement activation, complement activation classical pathway, and platelet degranulation, were related to the complement and coagulation cascades (Fig. 1B). The Proteomap showed the involvement of complement and coagulation cascades (Figs. 1C–E). In Reactome, several protein interactions were expressed in the immune system in the CDH group (Fig. 1F). Furthermore, the Protein Interactome map showed that the complement and coagulation factors were present in CDH (Fig. 1G). [Figure 1]

Table 1 Maternal characteristics and neonate outcomes

	CDH (n = 4)	Control (n = 4)	*p* value*
Maternal age (year)	36.0 [29.9 – 40.3]	31.1 [25.1 – 38.2]	0.34
Parity (Primiparous/Total)	2/4 (50.0%)	2/4 (50.0%)	
Gestational age at birth (week)	37.9 [37.4 – 39.9]	38.4 [38.1 – 38.7]	
Outcomes of neonates			
Sex (Male/Total)	2/4 (50.0%)	2/4 (50.0%)	
Birth weight (g)	3188 [2765 – 3324]	2939 [2880 – 3236]	0.68
Apgar score 1 min	5 [5 – 6]	9 [9 – 9]	< 0.001
Apgar score 5 min	3.5 [3 – 8]	10 [9 – 10]	0.005
Umbilical blood pH	7.34 [7.31 – 7.44]	7.32 [7.29 – 7.34]	0.49

Data is shown as median [range] or number (%).
*Mann-Whitney's *U* test was used.

Table 2 DEPs

Accession	Description	Fold-change	*p* value*
P02746	Complement C1q subcomponent subunit B	41.520	0.114
P01031	Complement C5	23.591	0.200
Q15063	Periostin	20.247	0.029
P60709	Actin, cytoplasmic 1	17.934	0.029
P08603	Complement factor H	13.429	0.057
P0CG38	POTE ankyrin domain family member I	12.254	0.029
P68133	Actin, alpha skeletal muscle	11.338	0.029

Accession	Description	Fold-change	*p* value*
P07996	Thrombospondin-1	10.122	0.057
P09871	Complement C1s subcomponent	9.055	0.057
Q6S8J3	POTE ankyrin domain family member E	8.033	0.057
Q03591	Complement factor H-related protein 1	7.897	0.114
P01871	Immunoglobulin heavy constant mu	7.883	0.057
P00736	Complement C1r subcomponent	6.679	0.114
Q15485	Ficolin-2	6.081	0.343
P49747	Cartilage oligomeric matrix protein	5.024	0.114
P05155	Plasma protease C1 inhibitor	4.506	0.057
P07359	Platelet glycoprotein Ib alpha chain	4.456	0.057
Q14520	Hyaluronan-binding protein 2	4.437	0.200
Q08380	Galectin-3-binding protein	4.332	0.114
P0DOX6	Immunoglobulin mu heavy chain	4.216	0.114
P04180	Phosphatidylcholine-sterol acyltransferase	4.205	0.114
P07358	Complement component C8 beta chain	4.003	0.114
P40197	Platelet glycoprotein V	3.988	0.486
P00734	Prothrombin	3.987	0.029
P02743	Serum amyloid P-component	3.940	1.000
P35443	Thrombospondin-4	3.908	0.200
P36980	Complement factor H-related protein 2	3.875	0.114
P00747	Plasminogen	3.800	0.114
Q06033	Inter-alpha-trypsin inhibitor heavy chain H3	3.747	0.200
A0A0B4J1U7	Immunoglobulin heavy variable 6-1	3.715	0.029
A0A0C4DH67	Immunoglobulin kappa variable 1-8	3.567	0.029
P00742	Coagulation factor X	3.412	0.200
P36955	Pigment epithelium-derived factor	3.295	0.057
A0A075B6S5	Immunoglobulin kappa variable 1-27	3.260	0.029
Q969T7	7-methylguanosine phosphate-specific 5'-nucleotidase	3.177	0.057
Q9Y566	SH3 and multiple ankyrin repeat domains protein 1	3.110	0.686
P01011	Alpha-1-antichymotrypsin	2.986	0.057
P02751	Fibronectin	2.979	0.343
P07225	Vitamin K-dependent protein S	2.836	0.486
P04004	Vitronectin	2.730	0.200
P00748	Coagulation factor XII	2.724	0.114
P10909	Clusterin	2.710	0.057
A0A087WW87	Immunoglobulin kappa variable 2-40	2.701	0.114
A0A075B6P5	Immunoglobulin kappa variable 2-28	2.666	0.114
P0C0L4	Complement C4-A	2.657	0.200
P0C0L5	Complement C4-B	2.657	0.200
P00450	Ceruloplasmin	2.635	0.114
Q04756	Hepatocyte growth factor activator	2.557	1.000
P04275	von Willebrand factor	2.547	1.000
P62937	Peptidyl-prolyl cis-trans isomerase A	2.541	1.000
P29622	Kallistatin	2.521	0.057
O75636	Ficolin-3	2.472	0.343
P01024	Complement C3	2.470	0.114
P15169	Carboxypeptidase N catalytic chain	2.440	0.029
Q96IY4	Carboxypeptidase B2	2.433	1.000

Accession	Description	Fold-change	*p* value*
P06310	Immunoglobulin kappa variable 2-30	2.297	0.200
Q15848	Adiponectin	2.288	0.343
P26927	Hepatocyte growth factor-like protein	2.284	0.114
P07357	Complement component C8 alpha chain	2.263	0.486
A0A075B6I9	Immunoglobulin lambda variable 7-46	2.256	0.343
P04196	Histidine-rich glycoprotein	2.249	0.114
P02042	Hemoglobin subunit delta	2.228	0.029
P04278	Sex hormone-binding globulin	2.220	0.057
O75420	PERQ amino acid-rich with GYF domain-containing protein 1	2.209	0.114
P48740	Mannan-binding lectin serine protease 1	2.209	0.486
P05109	Protein S100-A8	2.189	0.200
P19823	Inter-alpha-trypsin inhibitor heavy chain H2	2.142	0.343
P02656	Apolipoprotein C-III	2.138	0.200
A0A0B4J1Y8	Immunoglobulin lambda variable 9-49	2.059	0.200
P05452	Tetranectin	2.051	0.114
P02760	Protein AMBP	2.018	0.114
A0A075B6S6	Immunoglobulin kappa variable 2D-30	2.018	0.114
P01703	Immunoglobulin lambda variable 1-40	0.490	0.343
P22570	NADPH:adrenodoxin oxidoreductase, mitochondrial	0.489	0.486
Q12805	EGF-containing fibulin-like extracellular matrix protein 1	0.460	0.886
A0A075B6J9	Immunoglobulin lambda variable 2-18	0.453	0.486
P43251	Biotinidas	0.429	0.343
P04114	Apolipoprotein B-100	0.423	1.000
P22105	Tenascin-X	0.405	0.200
P00739	Haptoglobin-related protein	0.390	0.343
P04406	Glyceraldehyde-3-phosphate dehydrogenase	0.367	1.000
P35527	Keratin, type I cytoskeletal 9	0.365	0.445
P01876	Ig alpha-1 chain C region	0.357	0.043
P01817	Immunoglobulin heavy variable 2-5	0.320	0.343
P01034	Cystatin-C	0.320	0.200
P80108	Phosphatidylinositol-glycan-specific phospholipase D	0.317	0.886
P46199	Translation initiation factor IF-2, mitochondrial	0.300	0.200
Q6EMK4	Vasorin	0.298	0.114
Q9NZP8	Complement C1r subcomponent-like protein	0.284	0.200
P12259	Coagulation factor V	0.273	0.886
P13671	Complement component C6	0.270	0.200
P05543	Thyroxine-binding globulin	0.210	0.114
P27361	Mitogen-activated protein kinase 3	0.204	0.057
P35542	Serum amyloid A-4 protein	0.157	0.057
Q96QT4	Transient receptor potential cation channel subfamily M member 7	0.123	0.114
P12036	Neurofilament heavy polypeptide	0.106	0.486
Q86YZ3	Hornerin	0.023	0.200
P33151	Cadherin-5	0.011	1.000

*Mann-Whitney's *U* test was used.

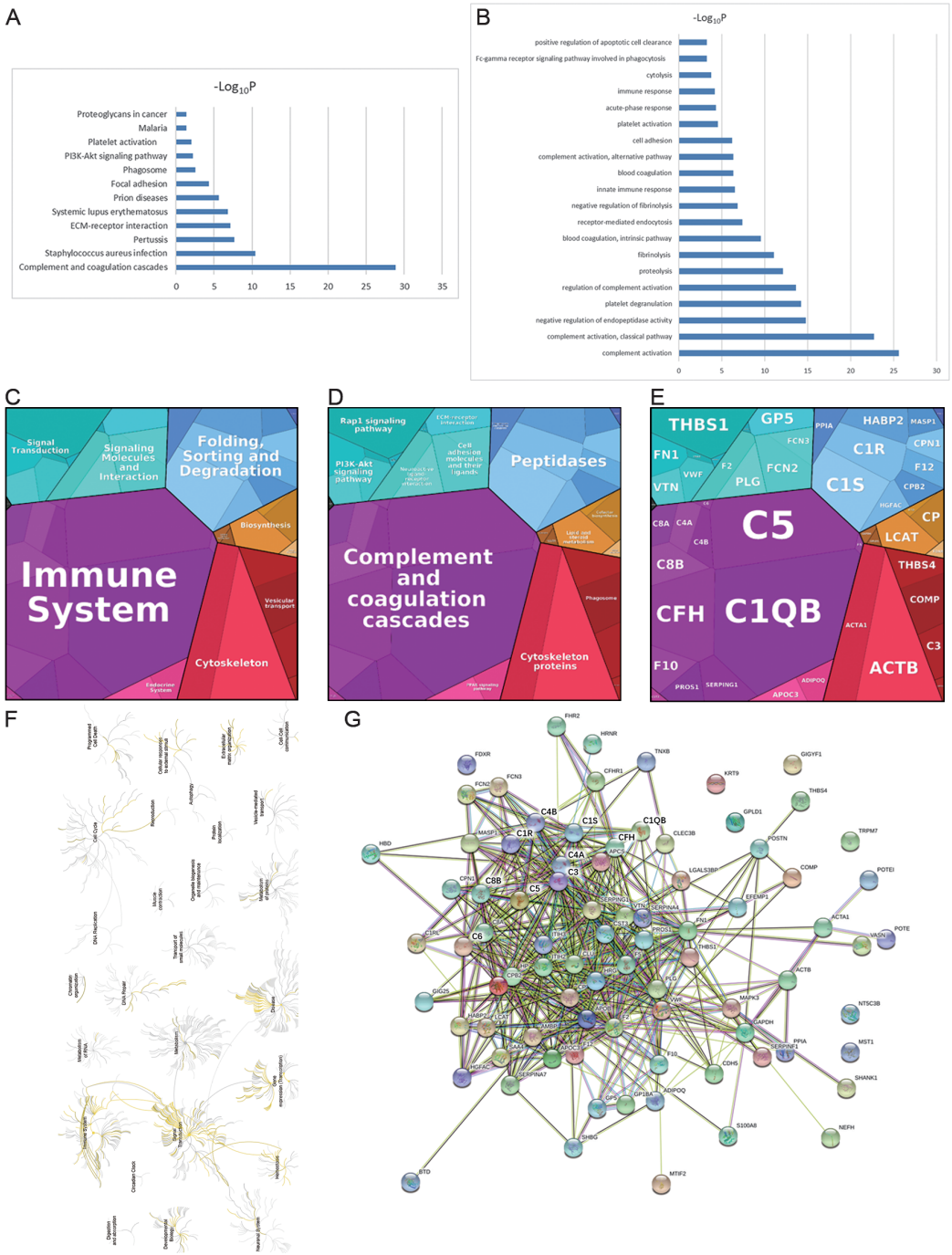

Fig. 1 Proteomic analysis of umbilical cord of neonates with congenital diaphragmatic hernia (CDH) and controls A. Pathway enrichment analysis. B. Gene Ontology enrichment analysis for biological process. Top 20 proteins are shown. C–E. Proteomaps. Low (C), middle (D), and high levels (E) of Gene Ontology annotations. Each colored area reflects the magnitude of the average fold change with respect to protein expression for cases compared with that for controls. F. Reactome map. It shows that DEPs are enriched in the immune system in patients with CDH. G. Protein Interactome map. The nodes contain several complement factors including C1QB, C3, C4A, C4B and C5.

DISCUSSION

This is the first study to describe the proteomic profiling of umbilical cord serum in isolated left-sided CDH, using LC–MS/MS analysis. In the present study, the complement and coagulation cascades, such as C1q subcomponent and C5, were enriched in the umbilical cord serum of CDH cases. To the best of our knowledge, there are only two studies on proteomic profiling of human CDH, these studies have used with amniotic fluid[16] and exhaled breath condensate.[17] In addition, such investigations have been performed using the lung tissue[18] and tracheal fluid[19] of animal models. However, inconsistent results have been obtained possibly because of different tissues or different species. In the amniotic fluid of human CDH cases, no specific protein spot was revealed.[16] Conversely, a number of proteins in the exhaled breath condensates were suggested as biomarkers of CDH.[17] In animal CDH models, myosin light chain was absent in rat lung tissue,[18] and several pathways, including those involved in cell proliferation, were reported to be altered in the ovine tracheal fluid.[19] These factors are considered to be associated with pulmonary hypoplasia in CDH. The present study revealed a new finding that the complement and coagulation cascades were enriched in the umbilical cord serum of CDH cases. Moreover, complement factors, including C1q subcomponent and C5, were identified to be DEPs. Involvement of the complement factors in CDH remains to be investigated. In a previous report with ovine CDH model, complement and coagulation cascades were shown as enriched pathways in CDH lung.[19] Moreover, complement pathway activation is known to contribute to PH.[20] In pediatric PH, evaluation of the complement factors C3 and C4 is recommended.[5] In the lung tissues of rat PH models, the complement and coagulation cascades were enriched[21] and C1q expression was increased.[22] Furthermore, C1q was reported to cause hypertensive arterial remodeling and smooth muscle hyperplasia via β-catenin signaling.[23] It has been demonstrated that the complement pathway has a pathological role in the development of PH and is a possible therapeutic target for PH.[24] These findings suggest that the complement pathway might also be involved in the pathophysiology of PH in CDH. In this study, all CDH cases developed PH and half of them exhibited severe PH. In the present study, umbilical serum was used for analyses. Given the fact that serum contains several components from pulmonary vessels, we propose that umbilical serum could be superior to bronchioalveolar secretions in amniotic fluid and exhaled breath condensate in predicting the subsequent PH at birth. Whereas, results from the latter may be affected by the fetus maturity and treatment after birth.

This study has some limitations. First, samples from a single facility were analyzed. Second, the circulating proteins might not reflect the exact processes occurring in the lungs or pulmonary arterial walls. Third, we could not investigate the maternal serum samples. However, the effect of maternal background could be ignored because the participants did not have any other complications. Therefore, further studies with a larger sample size and analyses based on CDH severity are necessary.

CONCLUSION

This is the first study on CDH with proteomic profiling of umbilical cord serum using LC–MS/MS analysis. The complement and coagulation cascades may be associated with the pathophysiology of PH in CDH. This finding may help in the development of new therapeutic strategies, although further investigations are required.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest to disclose regarding this manuscript.

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