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ORIGINAL ARTICLE

Global gene expression profiling in congenital diaphragmatic hernia (CDH) patients

Kubilay Gürünlüoğlu[1] · Muhammed Dündar[2] · Turgay Unver[3] · Necmettin Akpınar[1] · Ismail Kürşad Gokce[4] · Semra Gürünlüoğlu[5] · Mehmet Demircan[1] · Ahmet Koc[2]

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Abstract

Congenital diaphragmatic hernia (CDH) is an anomaly characterized by a defect in the diaphragm, leading to the passage of intra-abdominal organs into the thoracic cavity. Herein, the presented work analyzes the global gene expression profiles in nine CDH and one healthy newborn. All of the patients had left posterolateral (Bochdalek) diaphragmatic hernia, operated via an abdominal approach, and stomach and bowels in the thorax cavity. Some patients also had additional anomalies. A total of 560 differentially regulated genes were measured. Among them, 11 genes showed significant changes in expression associated with lung tissue, vascular structure development, and vitamin A metabolism, which are typical ontologies related to CDH etiology. Among them, SLC25A24 and RAB3IL1 are involved in angiogenesis, HIF1A and FOXC2-AS1 are related with the alveolus, MAGI2-AS3 is associated with the diaphragm, LHX4 and DHH are linked with the lung, and BRINP1, FZD9, WNT4, and BLOC1S1-RDH5 are involved in retinol. Besides, the expression levels of some previously claimed genes with CDH etiology also showed diverse expression patterns in different patients. All these indicated that CDH is a complex, multigenic anomaly, requiring holistic approaches for its elucidation.

Keywords Congenital diaphragmatic hernia · Global transcriptome · Vitamin A · Multigenic

Introduction

Congenital diaphragmatic hernia (CDH) is an inborn anomaly characterized by the intra-abdominal organs pass into the thoracic cavity (Stolar and Dillon 2006). It is thought to be caused by a developmental defect of the pleuroperitoneal membrane, one of the four parts of the diaphragm (Stolar and Dillon 2006). This anomaly is most often seen on the left side, with a defect on the posterolateral side of the diaphragm. It is thought to be caused by a developmental

✉ Ahmet Koc
ahmet.koc@inonu.edu.tr

Kubilay Gürünlüoğlu
kgurunluoglu@hotmail.com

Muhammed Dündar
muhammed.dundar@inonu.edu.tr

Turgay Unver
turgayunver@gmail.com

Necmettin Akpınar
necmettinakpinarrr@gmail.com

Ismail Kürşad Gokce
ikgokce07@hotmail.com

Semra Gürünlüoğlu
casemra@yahoo.com

Mehmet Demircan
profdemircan@yahoo.com

1 Department of Pediatric Surgery, Faculty of Medicine, Inonu University, Malatya, Turkey

2 Department of Medical Genetics, Faculty of Medicine, Inonu University, Malatya, Turkey

3 Ficus Biotechnology Ostim OSB Mah, Inonu University, 100. Yıl Blv. No:55 Yenimahalle, Malatya, Turkey

4 Department of Pediatrics and Division of Neonatology, Faculty of Medicine, Inonu University, Malatya, Turkey

5 Department of Pathology, Malatya Education and Research Hospital, Malatya, Turkey

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defect of the pleuroperitoneal membrane, one of four parts of the diaphragm (Ameis et al. 2017). Retinoic acid, which is an acidic form of retinol or vitamin A, has been demonstrated to have an important role in the development of the heart, lungs, and diaphragm in the embryonic period (Kling and Schnitzer 2007). Thus, any disorder in vitamin A metabolism is considered to be associated with CDH occurrence (Kling and Schnitzer 2007).

The etiology and pathogenesis of CDH are still not fully understood. There is no study model that explains the etiology of CDH in humans. Studies on this subject are mostly experimental studies. Experimentally, studies trying to explain the etiopathogenesis of CDH have proposed three hypotheses, which are surgical models, pharmacological agent, and nitrofen, and the genetic model (Van Loenhout et al. 2009, Clugston et al. 2006, Unger et al. 2003). In fact, these three models might create a single model, such as complementary puzzle pieces. If these three models were combined into a single model, at the time, it would seem that the initial link in the chain of events was genetic changes that occurred in the early embryonic period (Cannata et al. 2021). It was claimed that nitrofen is one of the most important environmental factors that led to these genetic changes (Van Loenhout et al. 2009, Clugston et al. 2006, Unger et al. 2003). Nitrofen, an agricultural herbicide, is thought to show its harmful effect by disrupting vitamin A metabolism (Greer et al. 2003, 2000). This chemical agent has been prohibited in many countries because of its teratogenic effect on the embryo (Greer et al. 2003, 2000; Mey et al. 2003; Noble et al. 2007). Studies have also demonstrated that nitrofen exposure during pregnancy is related to CDH occurrence (Montalva and Zani 2019, Zhaorigetu et al. 2018). The major contributions regarding the molecular elucidation of this disease have mainly come from the whole-exome sequencing (WES) studies. For example, a trio exome/WES analysis revealed that the *GATA4*, *ZFPM2*, and *GATA6* genes are associated with CDH (Yu et al. 2013, Longoni et al. 2014; Yu et al. 2020). In particular, *GATA6*, *ZFMP2*, *GATA4*, *SYNC*, *NR2F2*, *EYA1*, *CTNNB1*, and *FGfrL1* genes have been demonstrated to show considerable changes in expression in CDH patients (Ameis et al. 2017, Yu et al. 2013, Longoni et al. 2014, Ackerman et al. 2005, Kammoun et al. 2018, You et al. 2005, Paris et al. 2015, Amann et al. 2014).

Nevertheless, studies regarding the molecular basis of CDH are still limited and away from underlying the genetic reason/s of this disease. In this regard, the current work aimed to dissect the global gene expression profiles in CDH patients and have insights into the disease-associated gene/s and related pathways.

Materials and methods

Patient conditions and consent

This work was conducted upon receiving approval from Inonu University Malatya Clinical Research Ethics Committee (No: 2020/62). This was also in accordance with the Helsinki Declaration. The patient consent forms have been signed by the families of infants participating in this study. All patients had left posterolateral (Bochdalek) diaphragmatic hernia and were operated via an abdominal approach. In all patients, the stomach and bowels were in the thorax cavity. Three patients had herniated sacs. They were all drained with chest tubes. The diaphragm is primarily repaired in all patients. No patients involved in this study were given blood and blood products. Seven patients had additional cardiac anomalies, two of whom had major cardiac anomalies. One patient with cardiac anomaly also had an additional genitourinary system anomaly. Two patients had no additional anomalies.

Sample collection

Blood samples were taken from each patient's upper right or left extremities using the intravenous Seldinger technique. The patients were 2–4 days old when blood samples were taken. Three to four milliliters of blood samples was collected from each patient in vacuum tubes (Vacutainer® for genetic analysis; BD-Plymouth, United Kingdom), which contained K2-etilendiamintetraacetic acid. In addition, 4 cc of blood was taken for a control group from a normal child born by cesarean section who did not have any diseases and anomalies admitted for routine follow-up after birth in the neonatal department.

RNA isolation

Total RNA was extracted directly from whole blood samples of patients and control groups using the QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's directions. RNA was then quantified using a flourimeter (Qubit 3.0, Thermo Fisher Sci, Massachusetts, USA) and stored at − 80 °C until analysis.

Library preparation and sequencing

RNA integrity was checked by 2% gel electrophoresis and Agilent 5400 Fragment Analyzer System. RNA concentration was measured by a spectrophotometer. RNA libraries were prepared using the "TruSeq RNA Sample Preparation

v2" (Illumina, San Diego, CA, USA) kit. Sequencing was done by Illumina NovaSeq 6000 platform as paired-end (PE) 2 × 150 bp layout. 20 M reads were produced per sample on average. Briefly, library preparation included (i) mRNA purification and fragmentation, (ii) single-strand cDNA synthesis, (iii) double-strand cDNA synthesis, (iv) end-repair, (v) 3' adenylation, (vi) adaptor ligation, and (vii) DNA fragment enrichments and library quality check.

Bioinformatics analysis

Adapter and contaminant sequences and low-quality reads were removed using the FASTQC tool (www.bioinformatics.babraham.ac.uk/projects/fastqc/). The clean reads were aligned to the reference genome (*Homo sapiens*, assembly GRCh38.p13) with BWA aligner (http://bio-bwa.sourceforge.net/). The quantification of mapped reads was done using a HTSeq Python library (https://htseq.readthedocs.io/en/master/). DEGs were quantified using an edgeR R package (http://bioconductor.org/packages/release/bioc/html/edgeR.html). Gene ontology (GO) and KEGG pathway analysis were performed using the "Functional Annotation" module in the OmicsBox tool (https://www.biobam.com/omicsbox/).

Gene expression validation by RT-qPCR analysis

RNA samples were reverse-transcribed using the "Maxima First Strand cDNA Synthesis Kit" (Thermo Fisher Sci, Massachusetts, USA). The relative expression level of genes was analyzed by the ABI StepOnePlus RT-qPCR instrument. RT-qPCR conditions were as 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 58 °C for 30 s, and followed by a melting curve analysis. As endogenous control, the *GAPDH* gene was used and the relative expression levels were calculated based on the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). The expression levels of five genes such as *HIF1A*, *WNT4*, *RIB3IL1*, *FZD9*, and *BRINP1* were validated in all ten samples (Table 1). The RT-qPCR validation was performed as three technical replicates for each gene.

Table 1 The list of validated genes and endogenous control, and primer sequences

Gene	Amplicon size (bp)	Design	Primer	Sequence
GAPDH	120	Endogenous control	Forward	GCATCTTCTTTTGCGTCG
			Reverse	TGTAAACCATGTAGTTGAGGT
HIF1A	144	Validated gene	Forward	GCCAGATCTCGGCGAAGTAA
			Reverse	CCAGAAGTTTCCTCACACGC
WNT4	155	Validated gene	Forward	TCGTGTACGCCATCTCTTCG
			Reverse	ACCGTAGGCGATGTTGTCAG
RIB3IL1	127	Validated gene	Forward	AGGTGACAGCCTTGAAGACG
			Reverse	GTGCTCTTGTGGCGAGAGT
FZD9	133	Validated gene	Forward	GACCATCGTCATCCTGACCC
			Reverse	AGGAAACTACTGCCCAGCAC
BRINP1	123	Validated gene	Forward	CTCCTGCAACAAGGGCTACA
			Reverse	CGTAGAGGCGTGAGTCCATC

Table 2 The experimental design and sequencing statistics of samples

Sample name	Experimental design	Platform/layout	Raw reads	Q20 (%)
Control	Control group	Illumina Novaseq, Paired-end (PE)	18,724,944	96.88
Patient 1	Test group	Illumina Novaseq, Paired-end (PE)	21,376,204	96.94
Patient 2	Test group	Illumina Novaseq, Paired-end (PE)	15,531,679	96.75
Patient 3	Test group	Illumina Novaseq, Paired-end (PE)	21,922,735	97.07
Patient 4	Test group	Illumina Novaseq, Paired-end (PE)	22,463,625	97.11
Patient 5	Test group	Illumina Novaseq, Paired-end (PE)	22,260,369	96.91
Patient 6	Test group	Illumina Novaseq, Paired-end (PE)	21,782,614	97.09
Patient 7	Test group	Illumina Novaseq, Paired-end (PE)	21,732,216	97.04
Patient 8	Test group	Illumina Novaseq, Paired-end (PE)	21,519,190	97.17
Patient 9	Test group	Illumina Novaseq, Paired-end (PE)	20,114,591	97.02

Table 3 Reference genome mapping statistics

Sample name	Mapped reads (count)	Mapped reads (percentage)	Average mapped length (base)
Control	37,225,247	99.40%	149.66
Patient 1	42,482,069	99.37%	149.67
Patient 2	30,846,347	99.30%	149.64
Patient 3	43,576,182	99.39%	149.68
Patient 4	44,694,711	99.48%	149.68
Patient 5	44,251,622	99.40%	149.66
Patient 6	43,287,920	99.36%	149.67
Patient 7	43,202,993	99.40%	149.67
Patient 8	42,793,600	99.43%	149.69
Patient 9	39,965,469	99.34%	149.67

Results

Global gene expression profiling

The global gene expression profiles of nine CDH patients and one healthy individual as a control group were revealed using the Illumina platform. An average of 21 M paired-end reads per sample was sequenced with a Q20 score of > 96% (Table 2). More than 99% of the reads were mapped to the reference genome (Table 3). A total of 560 genes, with 292 up- and 268 downregulated were found to be differentially expressed based on the $-1 < \log2FC > 1$ threshold (Table 4; refer to Suppl. File 1 for expression of all genes).

RNA-seq profiles in all groups were validated by RT-qPCR analysis using five selected genes, *WNT4*, *HIF1A*, *RAB3IL1*, *BRINP1*, and *FZD9* (Fig. 1). These genes were selected based on their expression profiles and ontology (GO) terms related to CDH disease.

Differentially expressed genes (DEGs) annotation

To attribute functional roles, differentially expressed 560 genes were annotated with gene ontology (GO) terms such as molecular function (MF), biological process (BP), and cellular component (CC). Molecular function-related terms were mainly associated with "binding," "transporter activity," "catalytic activity," "molecular function regulator," and "structural molecular activity" (Fig. 2). Besides, the terms with biological processes involved "regulation of biological process," "multicellular organismal process," "biological regulation," "metabolic process," "developmental process," "biological adhesion," "developmental process," and "growth" (Fig. 3). Cellular component associated terms were mainly with "protein-containing complex" and "cellular anatomical entity" (Fig. 4).

The number of studies on congenital diaphragmatic herniation (CDH) has been limited but our current knowledge postulates that multiple genetic mutations are the main culprits behind this disease. From DEGs reported in this work, in particular, 11 genes were associated with lung tissue, vascular structure development, and vitamin A metabolism (Table 5).

Expression status of genes with claimed CDH etiology

In this work, we also investigated the expression levels of genes that have been previously claimed to have

Table 4 The differential gene expression (DEGs) analysis statistics

Comparison groups	Total DEGs number (probability > 0.9)	Upregulated gene number (M > 0*)	Downregulated gene number (M < 0*)
Patient 1 vs control	1878	980	898
Patient 2 vs control	2470	1304	1166
Patient 3 vs control	1298	766	532
Patient 4 vs control	2336	1340	996
Patient 5 vs control	1614	942	672
Patient 6 vs control	820	465	355
Patient 7 vs control	2884	1558	1326
Patient 8 vs control	1590	958	632
Patient 9 vs control	702	419	283
Comparison groups	Total DEGs number (FDR < 0.05)	Upregulated gene number (logFC > 1)	Downregulated gene number (logFC < − 1)
All patients vs control	560	292	268

*Statistical analysis result is important;*M*−value (M) is the log2 fold−change

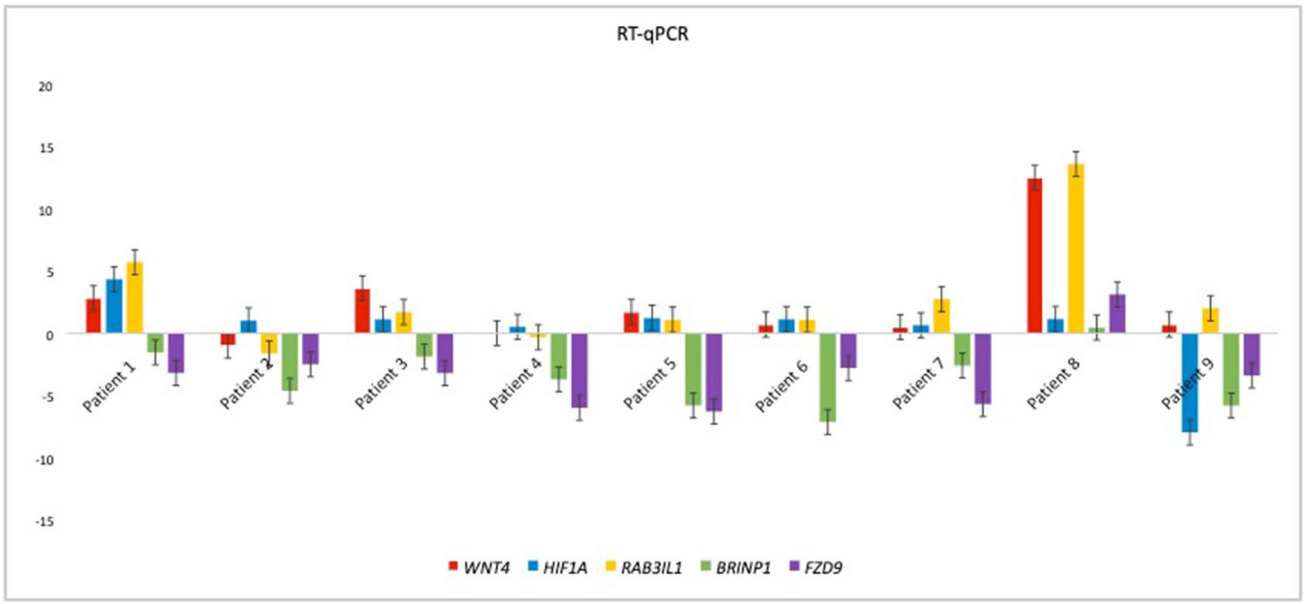

Fig. 1 The expression profiles of five validated genes by RT-qPCR in all CDH patients

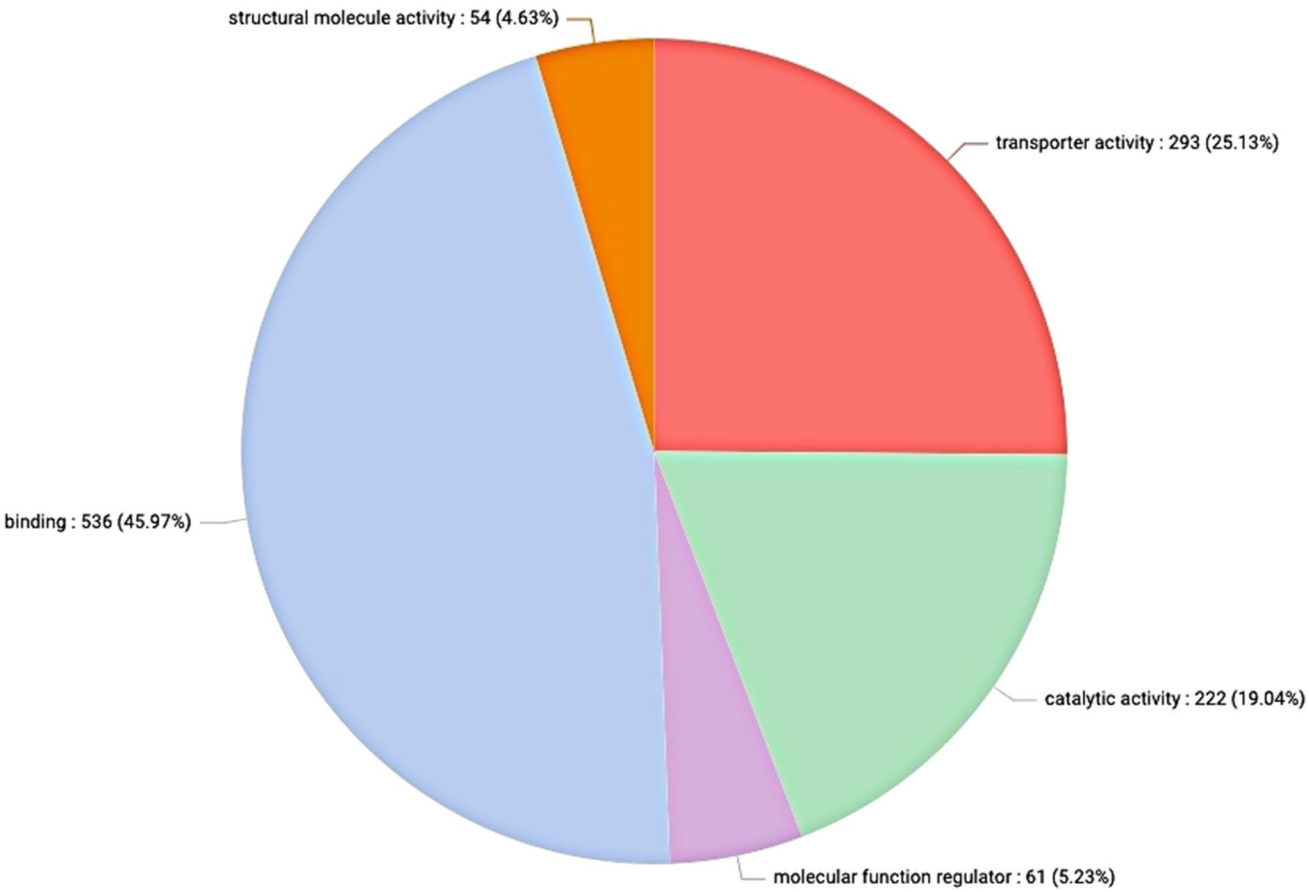

Fig. 2 The pie chart representation of the molecular function (MF) related ontologies in DEGs

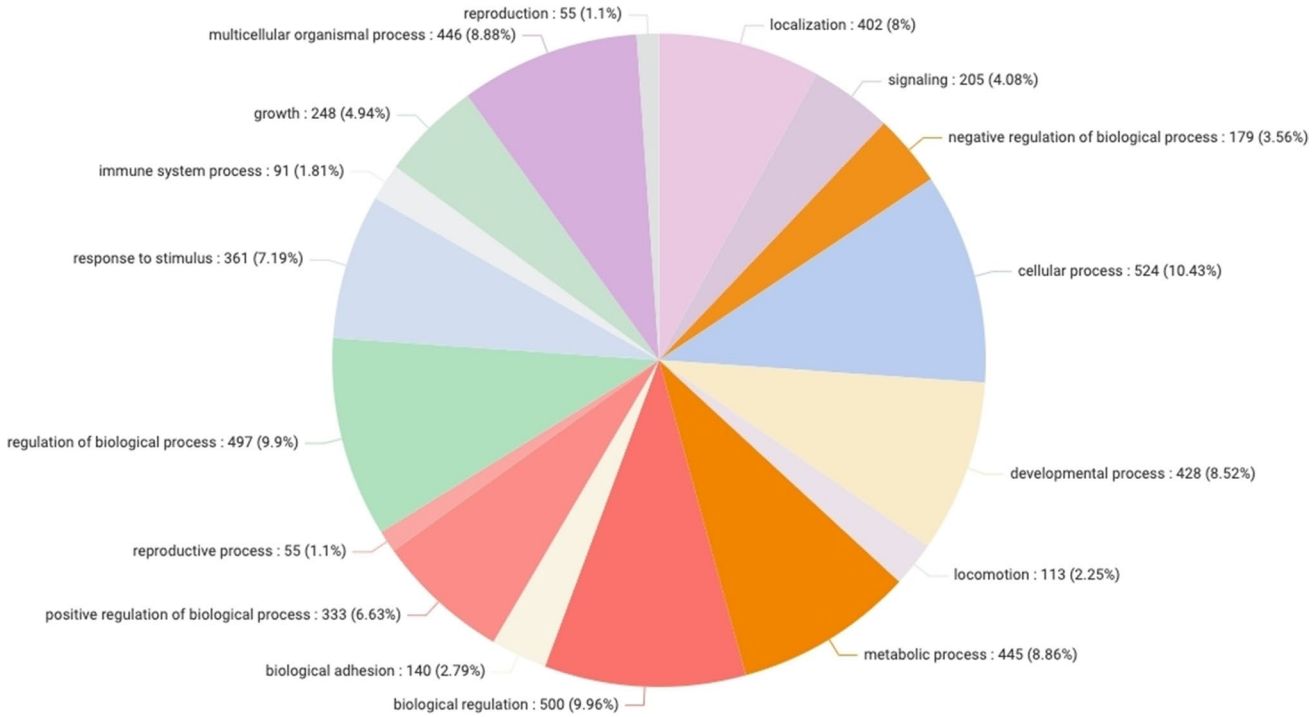

Fig. 3 The pie chart representation of the biological process (BP) related ontologies in DEGs

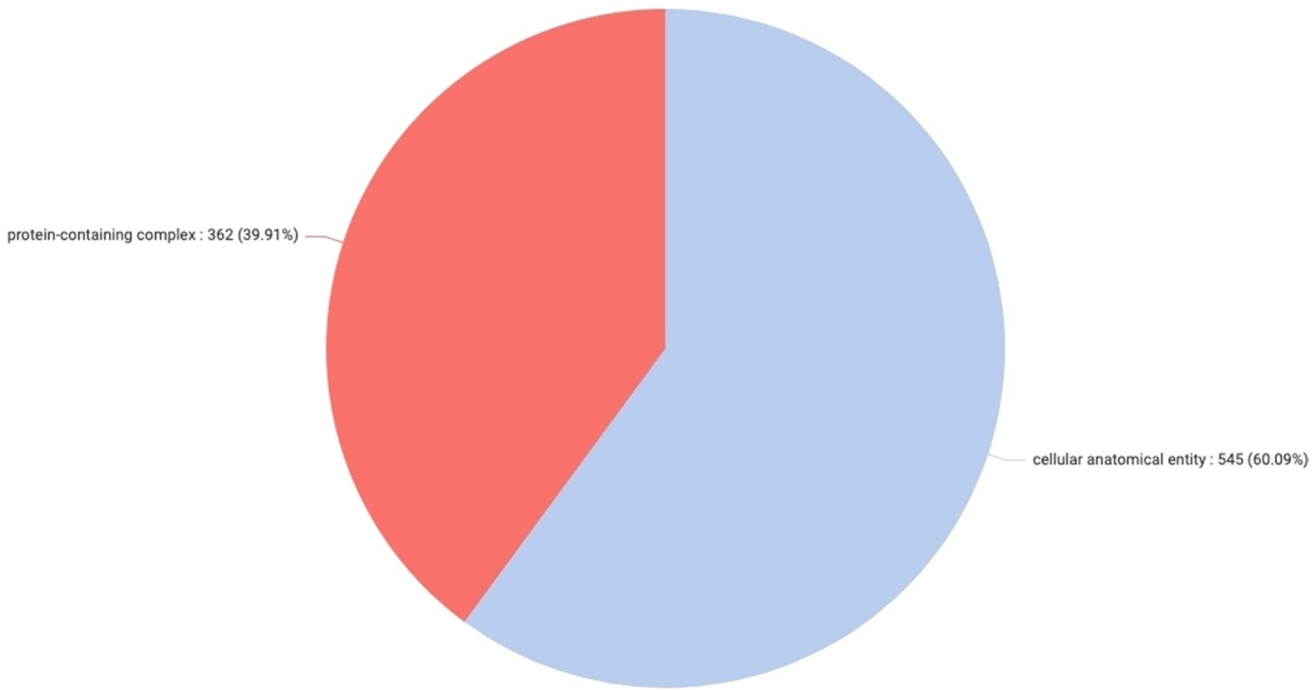

Fig. 4 The pie chart representation of the cellular component (CC) related ontologies in DEGs

Table 5 List of differentially expressed genes involved in CDH-related metabolic processes

Genes with the highest expression level changes	Associated gene ontology terms	Average expression level changes in the transcriptome of patient compared to control
SLC25A24	Angiogenesis	3.82
RAB3IL1	Angiogenesis	6.22
HIF1A	Angiogenesis, alveolus	4.19
FOXC2-AS1	Angiogenesis, alveolus, respiratory	−2.06
MAGI2-AS3	Diaphragm	4.09
LHX4	Lung	3.7
DHH	Lung, retinal	−2.06
BRINP1	Retinoic acid	−2.86
FZD9	Retinoic acid	−3.35
WNT4	Retinoic acid, angiogenesis	3.76
BLOC1S1-RDH5	Retinol	2.98

an association with CDH etiology (Table 6). We found that *GATA6* only showed a considerable increase in two patients (Table 6). We found that *FOG2* (ZFPM2) expression increased significantly in all patients (Table 6). In this work, we found a decrease in *SYNC* gene expression in eight patients. One of the striking results of this study is that we found that desmin expression decreased in all patients (Table 6).

In this study, in all patients, both *ALDH1*and *RALDH2* expressions were found to be decreased. We found that the expression of *ALDH1*, *RALDH2*, *LRATD2*, and *RDH11* genes decreased in all groups, while *RARB* only decreased in seven patients. Besides, *RARA* showed increased activity in all groups, while the expression of *ELN* and *RDH5* genes increased in the majority. In this work, we found that *BRINP1* expression significantly decreased in all CDH patients.

The expression levels of five genes such as *HIF1A*, *WNT4*, *RIB3IL1*, *FZD9*, and *BRINP1* were validated in all ten samples (Table 1). The validation was performed as three replicates for each gene.

Discussion

To list the gene expression pattern in CDH patients compared to a healthy infant, a transcriptome-wide analysis was performed. Differentiation in gene expression led to investigate transcripts and molecular pathways related to the CDH disease. Briefly, *SLC25A24* and *RAB3IL1* genes are associated with gene ontology term of angiogenesis; *HIF1A* and *FOXC2-AS1* with terms angiogenesis, alveolus, and respiratory; *MAGI2-AS3* with term diaphragmatic, *LHX4* and *DHH* with term lung; and *BRINP1*, *FZD9*, *WNT4*, and *BLOC1S1-RDH5* with terms retinoic acid and angiogenesis. Similarly, Dalmer et al. reported that CDH is ontologically associated with "retinol binding" and "retinoic acid binding" activities in relation to molecular function, and "diaphragm development" and "carnial ganglion development" in terms of biological processes (Dalmer and Clugston 2019).

In addition, the CDH-related and other functional roles of these 11 genes have been also mentioned in various studies. For example, the W*NT4* gene is vital for lung development during the embryonic period (Caprioli et al. 2015). In this work, we found that *WNT4*, which was not previously associated with CDH but is vital in lung development, was one of the genes with increased expression in all patients. Another gene, *SLC25A24* has been specifically associated with angiogenesis with upregulated activity in all patients. It has been claimed that this gene is associated with the regulation of the body's fat mass and adipogenesis (Urano et al. 2015). *RAB3IL1* was another gene whose expression increased significantly in all patients herein and it was associated with the term angiogenesis. It was reported to be a gene from prognostic indicators of hepatocellular carcinoma (Ni et al. 2020). Another gene, *HIF1A* was also found with increased activity in all patients with angiogenesis and alveolus functions. *HIF1A* acts as an endogenous feedback function in alveolar-epithelial glucose metabolism to reduce inflammation in acute lung injury and protect the lung (Eckle et al. 2013).

FOXC2-AS1 gene is associated with alveolus, angiogenesis, and respiratory functions, and showed decreased activity in all patients. It was reported that increased *FOXC2-AS1* activity inhibited apoptosis by increasing and regulating proliferation in the vascular flat muscle (Wang et al. 2020). Another gene, *MAGI2-AS3* was associated with the diaphragm ontologically with increased expression in all patients. This gene was reported to be downregulated in non-small cell lung carcinoma (NSCLC) (Hao and Yang 2019). Excessive expression of *MAGI2-AS3* has been claimed to suppress the proliferative and

Table 6 The expression levels of genes related with CDH disease

Gene name	Chr/location	Patient 1 vs control	Patient 2 vs control	Patient 3 vs control	Patient 4 vs control	Patient 5 vs control	Patient 6 vs control	Patient 7 vs control	Patient 8 vs control	Patient 9 vs control
TBX6	Chr16/30085793–30,091,927	−0.603	−0.939	−1.861	−0.801	0.385	0.672	0.001	−0.138	0.593
ARRDC4	Chr15/**97960703–97,973,833**	1.369	0.414	1.696	0.283	0.428	0.864	1.295	0.559	−0.434
IGF1R	Chr15/98648539–98,964,530	1.607	1.813	0.731	1.454	1.349	0.807	2.008	1.122	1.007
PBX3	Chr9/125747372–125,967,377	0.247	0.807	0.546	0.505	0.186	0.434	0.405	0.253	0.169
RUNX1	Chr21/34787801–35,049,334	0.666	0.597	0.459	0.553	0.821	0.790	1.167	0.399	0.169
TGIF1	Chr18/3412009–3,459,978	−0.475	−0.271	−0.533	0.118	−0.342	−0.522	−0.783	0.482	0.133
ZFHX4	Chr8/76681219–76,867,285	1.380	1.596	−1.062	−1.062	−1.062	−1.062	1.689	−0.138	−0.102
FBN1	Chr15/48408313–48,645,709	−1.686	−3.328	−2.095	−1.698	−0.87	−0.659	2.443	−0.002	1.025
FREM1	Chr9/14734666–14,911,653	−1.789	−0.573	−2.647	2.793	0.515	0.164	2.426	1.977	−1.687
DES	Chr2/219418377–219,426,734	−2.188	−4.496	−0.557	−2.129	−0.802	−0.827	−0.081	−2.476	−0.882
PAX3	Chr2/222199887–222,298,998	1.795	−1.062	0.996	2.056	−1.062	−1.062	−1.062	0.861	1.482
MET	Chr7/116672196–116,798,386	−2.062	1.818	−2.062	−0.528	−1.484	−2.062	−0.895	−0.138	−2.06
ZFPM2	Chr8/105318438–105,804,539	2.442	2.073	3.380	3.855	1.577	1.811	2.751	2.509	2.544
DISP1	Chr1/222814514–223,005,995	0.752	0.678	0.493	0.810	1.370	0.362	0.186	1.282	−0.190
NEIL2	Chr8/11769710–11,787,345	2.458	2.513	−0.588	1.208	0.737	0.164	−0.480	0.598	−1.687
MEF2A	Chr15/99565417–99,716,488	1.91	1.160	1.407	1.830	1.417	1.272	2.7207	1.776	0.694
GATA4	Chr8/11676935–11,760,002	0	0	0	2.533	0	0	0	0	0
GATA6	Chr18/22169589–22,202,528	1.857	0	1.058	2.533	.577	.811	0	0.924	0.959
FOXF2	Chr6/1389576–1,395,603	0	0	0	0	0	0	0	0	0

invasive capabilities of *NSCLC* via the axis miRNA-23a-3p/PTEN [21]. Herein found another gene, *LHX4* was associated with increased activity in all patients. This gene is a LIM-transcription factor necessary for the development of the spinal cord and pituitary gland (Dong et al. 2019). A mouse with homozygous *LHX4*-null mutation was reported to have died after childbirth due to a lung developmental defect (Perron et al. 2003). On the other hand, *DHH* expression was found to be decreased in all patients with lung and retinal associations. Although this gene has not been previously associated with lung development and diaphragmatic hernia, it has been claimed that it plays an important role in the differentiation of the fetal retinal epithelium (Perron et al. 2003). *FZD8* and *FZD9* genes are members of the *WNT* gene family, the orchestra that manages embryogenesis (Katoh 2008). Both *FZD8* and *FZD9* expression were significantly decreased in all patients. *BLOC1S1-RDH5* gene was associated with retinol with increased activity in this work. It is worth noting that there has been no report so far referencing this gene. *MEF2A* expression has been claimed to play an important role in all periods of fetal cardiac development (Lida et al. 1999). In this work, we found that *MEF2A* expression increased in all patients but only seven patients had cardiac anomalies.

On the other hand, Yu et al. performed a genetic analysis in two families with CDH and congenital heart defects discerning that the *GATA6* gene bears a de novo mutation in both cases (Yu et al. 2014). They stated that the mutation associated with pancreatic agenesis is also related to CDH (Yu et al. 2014). Herein, we found that expression of *GATA6* considerably upregulated in two CDH patients. Ackerman et al. reported that CDH and bilateral pulmonary hypoplasia occurred as a result of mutations in the *FOG2* (*ZFPM2*) gene resulting in N-etHyl-N-nitrosourea (Ackerman et al. 2005). We also indicated that *FOG2* (*ZFPM2*) expression is significantly increased in all patients. Ameis et al. (2017) suggested that *ZFPM2* and *GATA4* genes are associated with abnormal lung development and congenital diaphragmatic hernia (Ameis et al. 2017). In a syndrome known as Nance-Horan syndrome, CDH and eye anomalies such as cataracts, anophthalmia, and microphthalmia have been found coexistent. Kammoun et al. demonstrated mutations in *SYNC* and *ASXL3* genes in a patient with Nance-Horan syndrome (Kammoun et al. 2018). In this work, we measured a downregulation of *SYNC* gene expression in eight CDH patients. In another work, it was claimed that there is an important relationship between CDH disease and the *NR2F2* gene (You et al. 2005). Herein work, *NR2F6* gene expression was found to be decreased significantly in all patients. Desmin (*DES*) expression is vital for the development of diaphragmatic during the fetal period. An experimental study has shown that CDH develops as a result of suppressed Desmin expression due to nitrofen (Takashi et al. 2016). One of the interesting results of this study is that desmin expression is detected as downregulated in all of the CDH patients. Beck et al. have reported mutations in the *FBN1* gene in CDH patients (Beck et al. 2015). In this study, seven CDH patients showed decreased *FBN1* expression while two had increased transcription level. Kardon et al. claimed that *ZFPM2* and *NR2F2* genes were associated with CDH (Kardon et al. 2017). It was reported that *ZFPM2* and *EYA1* genes are mutated in CDH patients (Longoni et al. 2014). Here, we detected that expression of *ZFPM2* is enhanced in all CDH patients and *EYA1* gene expression is decreased in eight CDH patients. Brady et al. found mutations in the *ZFPM2* gene in two CDH twins (Brady et al. 2014). Paris et al. has reported that Catenin Beta 1 gene mutation was associated with a posterior diaphragmatic hernia (Paris et al. 2015). We observed suppression of *CTNNB1* expression in six CDH patients, while a slight increase in three CDH patients. The previous finding on the *FGfrL1* gene claimed that this gene plays a crucial role in the embryological development of the diaphragmatic muscles and kidneys in animals (Amann et al. 2014). Consistently, we found that *FGfrL1* expression is decreased in seven CDH patients.

The retinoic acid pathway has been the most known pathway associated with CDH disease. In this pathway, the retinol, also known as vitamin A, is transported from circulation to target tissues and oxidized with retinaldehyde dehydrogenase *RALDH 1–2* and converted into retinoic acid. *RALDH 2* is thereby critical during this process. The transmission of the signal generated by retinoic acid by binding to the cell is by receptors of the same origin (Harrison 2005). These receptors are known as retinoic acid receptor A (*RAR*A), *RARB*, and *RARD*. Retinoic acid provides the development of fetal lungs and diaphragmatics in the embryo (Harrison 2005). Besides, Gallot et al. claimed that the retionid-signal pathway might deteriorate with emerging CDH defect that vitamin A metabolism plays an important role in the embryological development of lung tissue. Additionally, *ALDH1* and *RALDH2* expression should both increase during normal lung and alveoli development that only *ALDH1* expression should increase in the development of the alveolar septum (Gallot et al. 2005). Notably, we detected downregulation of *ALDH1* and *RALDH2* expressions in all CDH patients.

The diaphragmatic development and the development of lung tissue are associated with each other, and vitamin A is also known to play an important role in the development of these two tissues embryologically (Harrison 2005, Gallot et al. 2005; Hind et al. 2002; Dirami et al. 2004; Duester et al. 2003). Referring to the cadaver genetic studies, there has been a close relationship between vitamin A metabolism disorder and nitrofen, which is a substance used as an herbicide in agriculture and banned due to its teratogenic effect (Greer et al. 2000; Mey et al. 2003; Noble et al. 2007). *ALDH1*, *RALDH2*, *RARB*,

RARES1, *RARA*, *ELN*, *TP53*, *LRATD2*, *RDH2*, *RDH11*, and *RDH12* were among the genes associated with retinol and retinoic acid metabolism (Takashi et al. 2016, Harrison 2005, Gallot et al. 2005, Hind et al. 2002, Dirami et al. 2004, Duester et al. 2003, Chen et al. 2003). Herein, we found that the expression of *ALDH1*, *RALDH2*, *LRATD2*, and *RDH11* genes are downregulated in all CDH patients, while *RARB* decreased in seven CDH patients. Similarly, *RARA* showed an increased expression level in all CDH patients, while the expression of *ELN* and *RDH5* genes were upregulated in most of the patients.

Moreover, retinoic acid is associated with *BRINP1* gene ontology. The presence of retinoic acid and increased expression level of *BRINP1* is required for embryonic neural stem cell development (Terashima et al. 2010). In consistent with previous findings, we also found that *BRINP1* expression is significantly decreased in all CDH patients.

Conclusion

The global gene expression profiles of nine diaphragmatic hernia patients and one healthy newborn were examined with the next-generation sequencing approach. A total of 560 DEGs were observed and attributed with various CDH-related ontology terms. The expression levels of some previously reported genes as well as not-so-far-mentioned genes were found to be differentially regulated. All indicated that CDH is a complex anomaly and therefore regards holistic approaches for its elucidation. We assume that nitrofen might be an important factor due to changes in the retinoic acid pathway in the occurrence of CDH disease and its negative effect on this pathway. We aim to reach enlightening information on this subject, with further studies in the future.

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Data availability The generated RNA-seq datasets were uploaded into the NCBI SRA database under the Bioproject ID: PRJNA768307.

Declarations

Competing interests The authors declare no competing interests.

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