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PII: S0022-3476(22)00208-6

DOI: https://doi.org/10.1016/j.jpeds.2022.03.023

Reference: YMPD 12896

To appear in: *The Journal of Pediatrics*

Received Date: 4 October 2021

Revised Date: 1 March 2022

Accepted Date: 15 March 2022

Please cite this article as: Katz OL, Wild KT, McEldrew D, Kaur M, Raible S, Skraban CM, Zackai EH, Medne L, Izumi K, Fortunato S, Weatherly J, Hartman T, Deppen P, Blair J, Devkota B, Schindler E, Hedrick HL, Peranteau W, Krantz ID, Molecular Mechanisms Contributing to the Etiology of Congenital Diaphragmatic Hernia: A Review and Novel Cases, *The Journal of Pediatrics* (2022), doi: https://doi.org/10.1016/j.jpeds.2022.03.023.

Molecular Mechanisms Contributing to the Etiology of Congenital Diaphragmatic Hernia: A Review and Novel Cases

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Keywords: Congenital diaphragmatic hernia, CDH, diaphragm development, CDH genetics, isolated CDH, complex CDH, syndromic CDH, *STAG2*, *MED14*, trisomy 12p

Abbreviations & Acrynomns: CDH = Congenital Diaphragmatic Hernia, PPF = pleuro-peritoneal fold, ES = Exome Sequencing, LHR = fetal lung area-to-head circumference ratio, CNV = copy number variants, CdLS = Cornelia de Lange Syndrome, DOL = day of life, PKS = Pallister-Killian Syndrome

Funding: Fore Hadley Foundation grant, Agnew Surgical Society.

COI: The authors declare no conflicts of interest.

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Congenital diaphragmatic hernia (CDH) is a relatively common birth defect, affecting approximately one in 2500-3000 live births, with a slight male predominance (1.5:1).[1-17] CDH is characterized by a herniation of the abdominal viscera through a defect in the diaphragm, leading to significant pulmonary hypoplasia and pulmonary hypertension and substantial morbidity and mortality.[6,8,11,18-25,34] Prenatal diagnosis, referrals to high volume tertiary care centers, and treatment advances in the Neonatal Intensive Care Unit (NICU) have significantly improved survival rates to about 70%. When combining spontaneous miscarriages, elective terminations and postnatal demise, overall survival is approximately 50%.[1,3,6,8,11,12,21,24-34]

Despite many lines of evidence supporting a strong genetic contribution to CDH, genetic causes have only been identified in approximately 30% of cases.[5,6,9,25,30] Further identification of genetic etiologies of CDH will likely drive advances in fetal and prenatal management of CDH, and ultimately improve prognosis.

Classification of CDH

Important CDH characteristics that significantly impact pre- and postnatal survival, as well as long-term prognosis, include size, location, presence of the liver above or below the diaphragm, lung volume, and associated abnormalities.[6] Only two of these classification modalities have been reproducibly linked with genetic differences; the association of Trisomy 21 with more rare, centrally-located diaphragm defects, and the presence of associated anomalies in many genetic syndromes.[6,8,25,32,35-38]

Isolated vs. Complex CDH

In approximately 60% of cases, CDH presents without any other major developmental malformations and is referred to as isolated or non-syndromic CDH.[6,8,32,39] Additional associated findings considered part of the CDH sequence include pulmonary hypoplasia, intestinal malrotation, patent ductus arteriosus, patent foramen ovale, left heart hypoplasia, cardiac dextroposition, tricuspid or mitral valve regurgitation, undescended testes, and presence of an accessory spleen.[8,32,37,39,40]

In the remaining roughly 40% of cases, the CDH occurs alongside a variety of other anomalies and is considered complex or syndromic CDH (or CDH+). The terms “complex,” “syndromic,” and “non-isolated” are sometimes used interchangeably.[6,8,32,34,39] The most common malformations co-occurring with CDH include congenital heart defects, urogenital anomalies, musculoskeletal deformities, craniofacial defects, and central nervous system abnormalities.[13,25,32,34,39,41] Overall, a genetic etiology is identified in about 30% of infants with CDH, primarily in infants with complex or syndromic CDH. An underlying genetic etiology is identified in <5% of infants with isolated CDH.[42]

Assessing for the presence or absence of other non-CDH-associated anomalies is critical in evaluating prognosis and guiding management, as well as providing informed counseling about recurrence risk.[6,25,32,33,39,43,44] In a recent study by Shanmugam et al, one-year survival in patients with isolated CDH was 78.7% compared with only 56.2% in patients with CDH + other congenital anomalies but without a recognizable syndrome. The one-year survival of those with recognizable syndromic forms of CDH was 18.2%.[10] CDH-related mortality in some specific syndromes has also been studied. For example, Gupta et al showed that mortality was 76% for patients with CDH and Cornelia de Lange Syndrome (CdLS) and 29% for non-CdLS patients.[45]

CDH Etiology

Multiple lines of evidence suggest a strong genetic contribution to the etiology of CDH, however a genetic etiology is currently found in only ~30% of cases.[5,6,9,25,30,36,37,46] Studies identifying causative genes are complicated by multiple factors including; the highly heterogeneous nature of CDH, incomplete penetrance and variable expressivity of CDH variants, limited pedigrees available for study with vertical transmission of CDH, the significant contribution of *de novo* variants, the possibility of multifactorial (gene/environment) or multigene interactions, the potential contribution of epigenetic and/or non-coding changes, tissue mosaicism of gene variants or aneuploidies, and the current limitations of genomic, bioinformatic, and variant functional prediction analysis tools and techniques.[24,25,39,47-52] It is difficult to extrapolate from many of these studies the

exact prevalence of CDH in a particular diagnosis (due to poor documentation in clinical reports, the embryonic or early fetal demise in many affected individuals with syndromic forms of CDH, and the overall rarity of any individual diagnosis), as well as the exact contribution of a particular pathway, gene or mutation/variant to the prevalence of CDH (as these studies have not been consistently undertaken in large cohorts, again due to the rarity of the diagnosis and specific genetic causes). However, some of these obstacles may be overcome through the work of large-scale consortiums (e.g. the Diaphragmatic Hernia Research and Exploration, Advancing Molecular Science (DHREAMS) consortium).[53]

Finally, although the inheritance of CDH is poorly understood, familial recurrence is thought to be as high as 2%, even when an underlying genetic etiology is not identified. In cases of diaphragmatic agenesis, where recurrence suggests an autosomal recessive inheritance pattern, the underlying genetic etiology remains unknown.[54] In a small study of monozygotic twins with CDH, discrepant copy number variants were not a common cause of twin discordancy.[55]

Aneuploidies

The most common aneuploidy syndromes associated with CDH **[Table I]** are trisomy 18, 13, and 21, and Turner syndrome (45,X). Trisomy 9, 22, and X, and mosaic trisomy 2, 8, 9, and 16 have also been reported with CDH, although less frequently.[6,8,25,32,34,36,37,44,56-73] Despite the higher incidence of CDH among individuals with these aneuploidies than in control populations, CDH is a rare feature even in these syndromes, observed in fewer than an estimated 10% of individuals with these cytogenetic results.[6,25,32,36,37,39,56,64,74]

Copy Number Variants

Various copy number variants (CNVs) have also been reported in association with diaphragmatic defects **[Table I]**. Among these, CDH is seen most frequently in del 15q26.1-q26.2, del 8p23.1, Pallister-Killian syndrome (PKS; mosaic tetrasomy 12p), del 1q41-q42, Wolf-Hirschhorn syndrome (del 4p16.3), del 15q24, del 15q25.2, and del 17q12. It is estimated that 10-40% of individuals with each of these CNVs present with CDH.[6,8,25,32,36,37,44,56,68,75-88] For the other listed

CNVs in **Table I**, it is believed that fewer than 10% of patients present with CDH as part of their clinical picture.[6,36,37,89,90]

Patients with chromosomal aberrations – either aneuploidies or CNVs – comprise approximately 13% of CDH cohorts and the majority have complex or syndromic CDHs.[6,8,25,32,34,37,44,57,64,65,69,73,77,86,88,91,92] Smaller deletions (5p15.2, 8p23.1, 15q25.2, 16p11.2, 17q12) or duplications (1q41, 16p11.2, and Xq13.1) of some of these regions have also been found to be rare causes of isolated CDH.[6,37,49,69,76,93,94] Improved high-resolution cytogenetic methodologies have enabled more precise delineation of the minimal critical regions for CDH development, and improved the ability to pinpoint candidate genes within these regions.

Single Gene Disorders

Numerous single gene disorders have also been described with CDH as a recurrent feature **[Table II].** Most of these diagnoses are syndromic, however some individuals may present with an apparently "isolated" CDH (to be discussed in more detail later). By virtue of the multisystem nature of these diagnoses, the genes responsible and the pathways in which they are involved play a role in different aspects of organogenesis and diaphragm development. In these syndromic diagnoses, whether or not an individual presents with CDH may be partially related to the specific nature of the variant, but is also likely affected by variation in other genes or environmental factors, as evidenced by the fact that the same variant in a specific gene can be reported in individuals both with and without CDH. In some of these syndromes – such as Donnai-Barrow syndrome, Cutis Laxa (*LTBP4*-related and *EFEMP2/FBLN4*-related subtypes only), Cardiac-urogenital syndrome, Matthew-Wood syndrome, and Tonne-Kalscheuer syndrome, CDH is considered a core feature and is observed in greater than 40% of individuals.[6,25,37] CDH is also considered one of the six cardinal features of CdLS, with approximatley 30% of CdLS patients having a CDH [Clark et al 2012; Kline et al 2018].[95,96] Moreover, for patients with CdLS, CDH is the leading cause of death in the first 28 days of life, accounting for more than 1/3 of neonatal deaths and the cause of 15-18% of CdLS deaths overall.[96,97] Additional syndromes including microphthalmia with linear skin defects

syndrome, multiple congenital anomalies-hypotonia-seizures syndrome, arterial tortuosity syndrome, Denys-Drash syndrome, and Simpson-Golabi-Behmel syndrome, are associated with diaphragmatic defects in 10-40% of cases. For the remainder of disorders listed in **Table II**, CDH is observed in a significant, but smaller fraction of cases, or the incidence of CDH in patients with the disorder is unknown.[6,25,37] The genes *DISP1* and *MYH10* are listed in **Table II** even though they are not associated with a formally named syndrome. Variants in these genes are thought to contribute to cases of syndromic CDH.[98,99]

Single Gene Variants in Isolated CDH

Finally, a few genes have been implicated in the development of apparently isolated CDH, including *GATA4, ZFPM2/FOG2, NR2F2/COUP-TFII*, *EFNB1, FREM1*, and *HLX* **[Table III]**.[48,52,76,86,100-105] Although all of these genes have been implicated in the development of isolated CDH, variants in these genes have also been seen in patients with complex CDH (particularly those with structural heart defects), those with isolated heart defects (in the absence of CDH), and in unaffected family members of individuals with isolated or complex CDH. Thus, variants within these "isolated CDH genes" show significant variable expressivity and incomplete penetrance. The location and type of the variant within the genes may explain some of the variation in phenotypic presentation, but other genetic or epigenetic modifiers are expected to play an important role.[48,52,76,86,100-105]

Novel, Illustrative Case Reports

STAG2

Proband 1 is a male child born at 39w1d gestational age (singleton pregnancy) to a 33 year old G2P1001 mother. Routine 20-week anatomy ultrasound diagnosed a left-sided CDH; further fetal evaluation at 21w5d demonstrated a left-sided CDH with liver, stomach, spleen, and nondilated bowel loops within the fetal thorax. He was also found to have a small bronchopulmonary sequestration. Fetal echocardiogram demonstrated severe rightward cardiomediastinal shift with mild compression of the cardiac chamber, but normal cardiac structure and function.

Amniocentesis revealed a normal fetal karyotype and microarray. CDH was repaired on day of life (DOL) 24 . He was discharged on DOL 79 without supplemental oxygen or pulmonary hypertension medications. On follow-up at 12 months of age, he was appropriately meeting developmental milestones and feeding entirely by mouth; follow up at 24 months was significant only for mild expressive language delay. Clinical genome sequencing identified a hemizygous (on the X chromosome) missense variant of uncertain significance (VUS) in *STAG2* c.713G>C (p.Ser238Thr) (**Figure**). This variant was also seen in his clinically-unaffected mother but was absent in the maternal grandparents, the proband's father and healthy older sister. Although the *STAG2* variant was classified as a VUS, it is of particular interest due to the overlap of the proband's phenotype with reports of CDH in other individuals with *STAG2* variants.[106-110] STAG2 is a core component of the cohesin complex along with RAD21, SMC1A, and SMC3. The cohesin complex and its many regulatory proteins – NIPBL, ESCO2, HDAC8, DDX11, SGOL1, WAPL, PDS5A, PLK1, AURKB, and ATRX – play critical roles in regulating transcription and chromatin looping/architecture, as well as maintaining genomic stability during DNA replication by mediating sister chromatid cohesion and accurate chromosomal segregation.[96,106-119] Germline variants in the structural or regulatory components of the cohesin complex lead to various multisystem developmental disorders, collectively known as cohesinopathies, which tend to share clinical findings such as distinctive facial features, short stature, developmental delay/intellectual disability, and limb anomalies.[106-111,118,120-127] Recently, a few patients with *STAG2* variants have been reported, all displaying multi-system phenotypes with common features incuding intellectual disability, developmental delay, short stature, microcephaly, dysmorphic facies, cleft palate, thoracic vertebral anomalies, and sensorineural hearing loss. CDH was present in two of these patients who also had a collection of other features.[106-110] Most reported *STAG2* variants have been missense variants, distributed throughout the entire protein, which can be inherited from unaffected female carriers or *de novo*, though one male has been reported with a very severe phenotype and a null variant. Affected females tend to have heterozygous truncating variants or

highly skewed X-inactivation.[106-110] Together, this information supports a highly dosage-sensitive X-linked dominant-like pattern of inheritance where a tiered reduction in gene function produces a stepwise increase in phenotype severity. Accordingly, it is possible that Proband 1's *STAG2* missense variant produces only a very minimal reduction in protein function – enough to disrupt only diaphragm development, resulting in a very mild *STAG2*-related phenotype.

MED14

Proband 2 is a male child born at 35w3d gestational age (singleton pregnancy) to a 30 year old G4P2 mother. Pregnancy was complicated by gestational diabetes and polyhydramnios requiring amnioreduction. Routine 20-week anatomy ultrasound demonstrated a cleft lip and palate and abnormal genitalia. Fetal MRI at 21w5d confirmed these findings and also identified a deviation of the nose and anterior nasal septum, low-set ears, and hypospadias. Repeat fetal MRI at 28w4d also revealed a left-sided posterolateral CDH containing bowel and spleen with an intra-abdominal stomach and liver position. Amniocentesis revealed a normal microarray. CDH was repaired on DOL 3. He was discharged on DOL 18 with a nasogastric tube, but without any pulmonary hypertension medications. Interim history is significant for gastroesophageal reflux, bilateral moderate mixed hearing loss, hypertonia, decreased range of motion of all four extremities, mild cerebral palsy, sacral dimple with possible tethered cord, developmental delay, and transition off of nasogastric tube feeds. Clinical exome sequencing (ES) identified a *de novo* hemizygous VUS in *MED14* (c. 1981-2A>G) (**Figure**). This variant is absent from normal control databases. The position of the variant at the -2 position of the splice acceptor site of intron 15 is predicted to alter splicing, likely resulting in a partial to complete loss-of-function allele. The variant is predicted to be deleterious by multiple *in silico* tools. The MED14 protein is a key subunit of the mediator complex, which interacts with a number of other proteins to facilitate transcriptional initiation by the RNA polymerase II apparatus.[128-134] This complex also participates in other phases of transcription and RNA processing, interacts with cohesin to facilitate chromatin looping, and and is essential to vertebrate embryogenesis, cell differentiation, and stem cell maintenance.[134-137] *MED14* is

expressed in various tissues throughout the body including the developing diaphragm in e14.5 mice, and has been shown to play a role in neural crest cell derived craniofacial development in zebrafish models.[138-140] Defects in neural crest cell migration, differentiation, signaling, and apoptosis have been shown to result in development of cleft palates.[141-143] Although variants in *MED14* have never been annotated with a human phenotype before, its critical role in multiple developmental processes, evidence that the Xp11.4 locus escapes X-inactivation,[144] and a pLI score of 1.0 suggests *MED14* is likely intolerant of heterozygous loss of function variants. Thus, absence of previous human disease-causing variants in *MED14* may be due to embryonic lethality.

Additional members of the mediator complex have been shown to lead to specific genetic syndromes when mutated.[130,134,145,146] The clinical features of Proband 2 including developmental delay, intellectual disability, urogenital anomalies, eyelid abnormalities, hypertonia, low-set ears, and hearing loss, overlap with many differences observed with variants in other mediator complex genes. Although CDH has not been reported in any of these mediator complex-component syndromes, it is a feature of syndromes caused by variants in proteins that interact closely with this complex, such as cohesin complex variants that result in CdLS, which is discussed further below.[95,96,104-110,134,136,147-149]

Mosaic Trisomy 12p

Proband 3 is a male child born at 38w6d gestational age to a 26 year old G1P0 mother. On routine 18-week anatomy ultrasound, he was noted to have a left-sided CDH. Further evaluation with detailed ultrasound and MRI at 21 weeks gestation showed left-sided CDH containing stomach, bowel, spleen, and a very small portion of the left lobe of the liver. No other fetal anomalies were noted, and fetal echocardiogram revealed a structurally and functionally normal fetal heart.
He underwent patch repair of the CDH on DOL 8 and was discharged at 7 weeks of age with a nasogastric tube, but without any pulmonary hypertension medications. He was also found to have an undescended R testicle, trace upper pole central calyceal dilation of R kidney, and gastroesophageal reflux. He was also noted to have a bitemporal sparing pattern of his hair, a

transitional crease of the L hand, and a single palmar crease of the R hand. In the nine months following discharge, he continued to experience significant gastroesophageal reflux and feeding intolerance, was noted to have mildly increased tone in his bilateral upper extremities, and had one hospitalization for fever and cough of unknown etiology. At 11 months of age, developmental milestones were notable for mild gross motor and expressive language delays.

With the exception of standard cell free DNA screening for trisomies 13,18, and 21 and sex chromosome aneuploidy, there was no diagnostic genetic testing done prenatally. Family history was notable for a paternal first cousin with CDH, normal development, and a normal chromosomal microarray. On genetics evaluation on DOL 0, his physical examination was notable for a bilateral temporal sparing pattern of the hairline reminiscent of Pallister-Killian Syndrome (PKS), a common syndromic form of CDH. Clinical chromosomal microarray analysis revealed a mosaic pathologic duplication of a 34.31Mb region within chromosome 12p13.33p11.1 (191,619-34,506,279) with a mosaic fraction of 40-45% in blood (**Figure**).

Proband 3's genetic results are consistent with a diagnosis of mosaic trisomy 12p. Trisomy 12p is closely related to PKS, which results from tetrasomy 12p and is characterized by coarse facial features with temporal alopecia, CDH, structural heart defects, GU anomalies, hearing loss, seizures, skin pigmentary differences, hypotonia, developmental delay and intellectual disability (with speech delay often most prominent), among other features. Although closely related, trisomy 12p is typically less severe than PKS.[150-152] Interestingly, however, CDH has not been reported in trisomy 12p.[151] It is possible that the mosaic fraction of trisomy 12p in the diaphragm is greater than the 40-45% found in the blood; this could explain the simultaneous existence of a severe phenotype in the diaphragm and mild features elsewhere. The finding of tissue mosaicism for a syndromic CDH spectrum disorder in a patient with a virtually isolated CDH suggests that mosaicism for known CDH-causing variants may underlie more cases of isolated CDH and emphasizes the importance of performing genetic testing on relevant tissue types.

Approaches to Gene Discovery for CDH

The search for genes implicated in the development of CDH has been complicated by significant genetic heterogeneity, incomplete penetrance and variable expressivity. Additionally, mechanisms such as epigenetic modification, common variant associations (e.g. complex trait model), modifier genes/variants/environmental influences would necessitate the study of large cohorts to generate statistically significant data and reproducibility (complicated by the relatively rare prevalence of CDH) as well as the need for functional studies to validate proposed candidate genes. Continued construction of large CDH consortia with collaboration between many large volume centers will be critical to perform these needed studies. One such study, known as the DHREAMS (Diaphragmatic Hernia Research and Exploration; Advancing Molecular Science) study has performed ES on over 400 infants with CDH and has identified an excess burden of *de novo* variants that are likely to disrupt or be deleterious to genes highly expressed during diaphragm development [42,46]

Past approaches to gene discovery for CDH have mostly focused on either sequencing genes in recurrently deleted regions or performing ES that focuses analysis on genes previously implicated in CDH. Candidate causative genes for CDH include those that i) have been previously annotated with diaphragmatic defects in mice or humans, ii) have been shown to be expressed in the developing murine diaphragm (septum transversum, PPFs, diaphragmatic musculature, etc.), iii) are known to interact with proteins expressed in the developing murine diaphragm, and/or iv) are components of signaling pathways known to be important for diaphragm development (retinoic signaling pathway, Wnt pathway, Shh signaling pathway, FGF pathway, TGFβ, EMT signaling, etc).[9,24,39,48,52,56,76,84,86,98,101-105,153-157]

For example, recent large-scale studies have utilized ES on both complex and isolated CDH patient cohorts in attempts to identify new candidate genes for diaphragm development.[5,9,30,46] Longoni et al 2014 identified rare and predicted pathogenic variants in the following genes: *CHAT, CTBP2, GLI2, MET, PDGFRA, NEDD4, GLI3, PAX7, RARA, ROBO2, SLIT3, TBX5, EYA2, FGFRL1, BOXB4, ILF3, KIF7, MMP14, MPP2, MYOD1, SIX4, DNASE2, PAX3,* and *ROBO1*.

These genes had previously only been implicated in mouse models of CDH. The majority of variants identified in this study were inherited from an apparently unaffected parent, although more detailed MRI studies to confirm the absence of subclinical diaphragm defects were not performed.[30] In a complex CDH patient cohort, Yu et al 2015 found novel likely gene disrupting or deleterious variants in: *ARFGEF2*, *CDO1*, *CLCN4*, *DLST*, *INHBB*, *LONP1*, *PPAPDC2*, *PRKACB*, *PTPN12*, *SIN3A*, *SLC5A9*, and *TLN1*.[46] Longoni et al also reported additional novel likely gene disrupting or damaging missense variants in isolated and complex CDH patients in *GRB10, HSPD1, ACTG1, ADD1, NAA15, PLCG1, FOXP4, ARL15,* and *GINS3*.[5] Interestingly, the presence of at least one likely damaging *de novo* variant has been shown to be associated with worse CDH-associated clinical outcomes (including higher mortality, worse pulmonary hypertension, and worse neurodevelopmental outcomes at 2 years of age).[42] Of note, some of these large-scale sequencing studies found a greater incidence of likely gene disrupting variants in the CDH cohort, as well as a small, but significant fraction of variants in one or more genes possibly related to diaphragm development.[9,30] Thus, it is possible that a variant in a single gene may not sufficiently interfere with diaphragmatic development, but that the threshold for disruption of normal development is reached with an alteration in a second gene in parallel or interacting pathways. If or how these variants interact during diaphragmatic development has yet to be determined, but may help to explain the low penetrance of CDH in certain syndromes or families with inherited variants. Other genetic, or environmental, modifiers may increase or decrease the likelihood that a given variant disrupts diaphragm development sufficiently to produce a CDH. This has yet to be mechanistically documented for CDH, but increasing evidence supports such mechanisms in congenital heart defects.[158]

Together, these sequencing methods have identified likely causative gene variants in around 11-15% of CDH patients (this percentage may be a slight underestimate due to additional variants of uncertain significance with contradicting functional predictions and lack of parental

sequencing in some studies, etc).[30,46] Even when added to the roughly 13% of CDH patients with an identified chromosomal anomaly, only ~30% of CDH patients receive a genetic diagnosis.[25] Sequencing analysis can also be performed without CDH candidate gene prioritization, which has the potential to unveil additional variants that may be implicated in CDH. Qi et al 2018 took this type of approach, finding many predicted deleterious missense (n=138) and likely gene disrupting (n=57) SNVs.[9] However, the relevance of these variants to CDH is limited by a lack of understanding of these genes' invovlement in diaphragm development.

Another fraction of currently unknown CDH etiologies may be due to tissue mosaicism. Mosaicism is a feature of the syndromic CDH disorder Pallister-Killian syndrome (PKS) and has, albiet inconsistently, been shown to be a contributor to other forms of CDH as well.[98,159-163] An individual may have a pathogenic variant in a CDH-causing gene in a subset of cells including the diaphragm, but the variant may be absent in blood lymphocytes. Tissue mosaicism may also explain why some patients may have a variant in a syndromic CDH gene but present with an isolated or complex (but non-syndromic) CDH. In order to assess for the possibility of mosaicism, sequencing studies will need to be performed on multiple tissue types including diaphragm tissue when available, not just DNA derived from lymphocytes.[163,164]

Non-coding DNA variants may also play a significant role in CDH etiology by altering the expression of nearby genes, particularly those that are critical to diaphragm development. Sequence alterations within promoters or upstream regulatory elements (enhancer regions) are a potential mechanism for the development of isolated CDH due to their tissue and developmental stage specificity. Whereas a coding change in an important developmental gene may result in a syndromic form of CDH or even be incompatible with life (as it would be anticipated to disrupt the morphogenesis of any tissue in which it is expressed and necessary for normal development), a variant in a diaphragm-specific regulatory element of the same gene would interfere with the expression of that gene specifically in the developing diaphragm. Non-coding sequence changes might also impact chromatin looping, methylation patterns, and histone modifications/nucleosome

positioning, which could also impact nearby gene expression. Assays addressing these mechanisms are becoming important for investigating the causes of CDH as well.[165-168]. In order to assess for the presence of non-coding DNA changes, genome sequencing (GS) would need to be undertaken, ideally on patient trios and coupled with bioinformatic analyses that capitalize on regulatory element data such as is available in the ENCODE database (https://www.encodeproject.org) and other sources.

RNA sequencing of diaphragmatic tissue may also yield insight into new candidate genes and pathways important in diaphragm development. Gene expression may be altered for a variety of reasons – from the aforementioned non-coding DNA variants, epigenetic changes, or structural coding variants of genes affecting transcription of downstream genes. Analysis of diaphragmatic transcriptomes can also provide information about the expression of non-protein-coding genes such as long non-coding RNAs and micro RNAs that serve to regulate the expression of other genes and may also be important in CDH formation when disrupted. Interestingly, this is a proposed mechanism of CDH formation in patients with PKS (as well as in mosaic trisomy 12p discussed above); it has been hypothesized that extra copies of miR-200c (transcribed from the chromosome 12p-defined PKS critical region) lead to increased degradation of its target *ZFPM2* transcripts and therefore reduced ZFPM2 protein levels which is associated with CDH.[169] Therfore in PKS, if levels of cells with the isochromsome 12p achieve a critical level in the developing diaphragm, it is postulated that the resultant levels of miR-200c may be elevated enough to significantly reduce transcritpional levels of the *ZFPM2* gene and result in a CDH.

Insights gleaned from studying syndromic forms of CDH can be leveraged to target novel and complex molecular etiologies of isolated CDH. For example, CdLS, one of the most common syndromic forms of CDH, is a multisystem developmental diagnosis caused by disruption of Cohesin structural and regulatory elements (*NIPBL*, *SMC1A*, *SMC3*, *RAD21*, and *HDAC8*). As a master regulator of gene expression, cohesin facilitates long-range regulatory element and promoter interactions throughout the genome via chromatin looping and topologically associated

domain (TAD) formation.[170-173] Two of the novel cases presented here, involving cohesin member (STAG2) and cohesin-interacting complexes (Med14 as part of the Mediator complex), highlight the importance of enhancer-promoter interactions and how disruption of downstream targeted developmental gene expression can lead to CDH (**Figure**). Thus, syndromic forms of CDH, caused by global regulators of developmental transcriptional regulation, can allow for the downstream mapping of interactional partners (e.g. regulatory elements with promoters). These disrupted downstream genes can point towards additional new candidate genes and regulatory elements that drive the tissue- and temporal-specific expression of those genes in the developing diaphragm. Although evidence supporting non-genic and mosaic contributions to the etiology of structural birth defects is compelling,[174,175]studies looking at these mechanisms in CDH have been limited but encouraging.[93,164] Additional studies are critical, but are complicated by the need for large cohorts (preferably trios) and samples from the tissues of interest (diaphragm), as well as appropriate cellular and animal developmental models to test the functional consequences of identified variants.

Importance of Genetic Answers

Idenitifying an underlying genetic etiology of CDH helps to alleviate feelings of parental guilt facilitates more accurate counseling about prognosis, as well as recurrence risks, both for the family in future pregnancies, and eventual transmission risk for the patient (something that will be of particular importance as more and more children with CDH are surviving to adulthood and reproducing), improves understanding of the pathophysiology of CDH and possible genotype-phenotype correlations and leads to opportunities for developing novel therapeutic approaches, which will likely be gene and variant specific. This is important in the era of fetal interventions such as gene editing; the ability to target aberrant genes during development to facilitate proper diaphragm formation could have far reaching benefits on a fetus found to have CDH at an early point in pregnancy.

Acknowledgements

We would like to thank the Fore Hadley Foundation and the Agnew Surgical Society of Penn Medicine for mentorship and support. We would also like to thank the Congenital Diaphragmatic Hernia Frontier Program and the families and patients with CDH at The Children's Hospital of Philadelphia who have graciously provided samples for research.

Figure Legend:

Figure: Representation of the way in which the three novel implicated genetic mechanisms resulting in congenital diaphragmatic hernias (CDHs) confluence on regulating developmental gene expression. STAG2, a known component of the cohesin protein complex has been shown to regulate developmental gene expression through long-range regulator-promoter interactions and topologically-associated domain (TAD) establishment and maintenance. MED14, a key member of the Mediatior complex which interacts with a number of proteins to facilitate transcriptional initiation by the RNA polymerase II apparatus and interacts with cohesin to facilitate chromatin looping. Copy number variations (duplications and triplications) involving chromosome 12p, encompassing a critical region on 12p13.31, are hypothesized to alter downstream targets by overexpressing microRNAs that target these genes critically downregulating them in the relevant developing tissues (e.g. the critical CDH gene *ZFPM2*).

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Table 2. Copy Number Variants Associated with Syndromic/Complex CDH†

Chromosomal Locus	Candidate genes in region	Clinical Features
dup 1q25-q31.2		Cleft palate, dysmorphic facial features, CNS abnormality, renal anomalies[176]
dup 1q41*	*HLX*	Isolated[94]
del 1q41-q42 (OMIM 612530)	*DISP1*, *HLX*	Cleft palate, dysmorphic facial features, club feet, seizures, ID/DD[36,83,98,104]
del 1q44	*ZNF672*, *ZNF692*, *PGBD2*	Colobomas[94]
Trisomy 2 (mosaic)	Full chromosomal aneuploidy	Dysmorphic facial features, cleft lip/palate, heart defects[69,177]
del 2q37 (OMIM 600430)	*DIS3L2*	Autistic behavior, ID/DD, dysmorphic facial features, short hands/feet, heart defects, CNS malformation, renal anomalies, tracheal anomalies, GI tract atresia/stenosis[178]
del 3q22	*RBP1*, *RBP2*	Eyelid malformations typical of BPES[56,179]
del 4p16.3 (Wolf-Hirschhorn syndrome) (OMIM 194190)	*FGFR1*, *ZNF595*, *ZNF718*	Typical facial features, ID/DD, seizures, growth deficiency[36,94,180-183]
del/dup 4q31		del: cleft palate, heart defects, skeletal anomalies[184] dup: Cranial anomalies, heart defects, dysmorphic facial features, wide-spaced nipples, DD[185]
del 5p15.2*	*LINCO1194*	Isolated[94]
del 6q25.3-qter	*ARID1B*	Dysmorphic facial features, CNS malformations, heart defects, skeletal anomalies, ID[186,187]
Trisomy 8 (mosaic)	Full chromosomal aneuploidy	Skeletal anomalies, ID, DD, dysmorphic facial features, corpus callosum agenesis[64,188,189]
dup 8p21-p23.1		CNS anomalies, hyperextendable joints, ID, cleft palate, heart defects, dysmorphic facial features[190]
del 8p23.1* (OMIM 222400)	*GATA4*, *SOX7, NEIL2*	Cardiovascular malformations, mild dysmorphic facial features, ID, neuropsychiatric findings[47,93,191,192]
del 8q23.1 (OMIM 610187)	*ZFPM2*/*FOG2*	Cardiovascular malformations, mild dysmorphic facial features, ID[37,86,193]
Trisomy 9 (complete or mosaic)	Full chromosomal aneuploidy	Spina bifida, facial dysmorphism, limb deformities, GU anomalies, cardiac defects, CNS malformations[58,63,66,72,73]
del 11p13 (WAGR Syndrome) (OMIM 194072)	*WT1*	Wilms tumor, genitourinary anomalies, eye anomalies, ID[44,194,195]
+der (22) t(11;22)(q23.2;q11.2)[ɬ]	*ROBO3*, *ROBO4*	Cleft palate, genitourinary anomalies, heart defects, craniofacial anomalies, ID[196,197]
Mosaic tetrasomy 12p (Pallister-Killian syndrome) (OMIM 601803)	miR-200c	CNS anomalies, shortened limbs, coarse facial features, seizures, congenital heart defects, skin pigmentation differences, sensorineural hearing loss, DD/ID[87,151]
Trisomy 13	Full chromosomal aneuploidy	Cleft lip/palate, dysmorphic facial features, congenital heart defects, polydactyly[198,199]
dup 14q32		Dysmorphic facial features, heart defects[37,200]
del 15q24 (OMIM 613406)		Dysmorphic facial features, digital anomalies, genital abnormalities, CNS malformations, DD/ID[201]
del 15q25.2* (OMIM 614294)	*HGDFRP3*, *BNC1*, *BTBD1*, *HOMER2*	Cardiovascular anomalies, cryptorchidism, short stature, cognitive deficits, possibly Diamond-Blackfan anemia[76,85,89]
del 15q26.1-q26.2 (OMIM 142340)	*NR2F2*/*COUP-TFII*, *IGF1R*, *ARRDC4*	Characteristic craniofacial anomalies, cardiovascular malformations, limb anomalies, growth deficiency[202,203]
Trisomy 16 (mosaic)	Full chromosomal aneuploidy	Dysmorphic facial features, skeletal anomalies, tracheal-esophageal fistula, duodenal atresia[59,62]
del/dup 16p11.2*/* (OMIM 611913; 614671)	*TBX6*, *MESP2* *TP53TG3E*, *TP53TG3B*, *TP53TG3F*, *TP53TG3C*	del: Dysmorphic facial features, structural brain abnormalities, neuropsychiatric issues[76,86,204] dup: facial dysmorphology, cardiac defects, digital anomalies, GU malformation[94]
del 17q12* (OMIM 614527)	*LHX1*, *PIGW*, *FZD2*	Renal anomalies, MODY, psychiatric abnormalities, DD/ID, hearing loss[86,88,205,206]
Trisomy 18	Full chromosomal aneuploidy	Heart defects, spina bifida, dysmorphic facial features, hydrocephalus, omphalocele, arthrogryposis[57,60,199]
del 18q22.1	*DSEL*	Unilateral micropthalmia[207]

Trisomy 21 (Down syndrome) (OMIM 190685)	Full chromosomal aneuploidy	Characteristic facial features, heart defects, ID, GI anomalies, hypotonia[61,208-210]
Trisomy 22	Full chromosomal aneuploidy	Ambiguous genitalia, GU anomalies, ascites, edema, pleural effusion, dysmorphic facial features[66,211,212]
del 22q11 (OMIM 611867)	*TBX1*, *CRKL*	Cardiovascular malformations, craniofacial anomalies & mild facial dysmorphology, hypocalcemia, absent/hypoplastic thymus, DD/ID[70,90]
Monosomy X (Turner syndrome;(45,X))	Full chromosomal aneuploidy	Heart defects, short stature, premature ovarian failure, prominent nuchal folds, lymphedema, shield chest[34,65,213-216]
Trisomy X (47,XXX)	Full chromosomal aneuploidy	ID, DD, hypotonia, behavioral/emotional difficulty[34,65]
del Xp22-pter	*HCCS*	Features consistent with Microphthalmia with linear skin defects[36,217,218]
dup Xq13.1*	*EFNB1*	Hypertelorism, epicanthal folds, bifid nasal tip[69,219]

†in ≥ 2 patients reported in the literature

* CNVs also implicated in isolated CDH

ᵠ or other unbalanced translocations resulting in dup 11q23.2 (e.g. +der (12) t(11;12)(q23.2;q24.3) or +der (13) t(11;13)(q23.2;q12.3))

CNS = central nervous system. ID = intellectual disability. DD = developmental delay. BPES = blepharophimosis, ptosis, epicanthus inversus syndrome. MODY = maturity onset diabetes of the young.

Table 2. Genes Associated with Syndromic/Complex CDH†

Inheritance Pattern	Syndrome*	Gene(s)**	Chromosomal Locus	Clinical Features
AR	Donnai-Barrow syndrome (OMIM 222448)	*LRP2* (OMIM 600073)	2q31.1	DD, agenesis of corpus callosum, SNHL[32]
AR AR/AD	Matthew-Wood Syndrome (OMIM 601186; 615524)	*STRA6* (OMIM 610745) *RARB* (OMIM 180220)	15q24.1 3p24.2	Anophthalmia, congenital heart defects, ID, progressive motor impairment[220-223]
AR	Cutis Laxa types IB & IC (OMIM 614437; 613177)	*EFEMP2/FBLN4* (OMIM 604633) *LTBP4* (OMIM 604710)	11q13.1 19q13.2	Craniofacial anomalies, bronchopulmonary dysplasia, GI & GU malformations, skin laxity, growth delay[224-226]
AR	Spondylocostal dysostosis (OMIM 277300)	*DLL3* (OMIM 602768)	19q13.2	Multiple vertebral and rib abnormalities[227]
AR	Multiple congenital anomalies-hypotonia-seizures syndrome (OMIM 614080)	*PIGN* (OMIM 606097)	18q21.33	Cardiovascular anomalies, GU dysplasia, cleft palate, dysmorphic facial features, brain anomalies[228]
AR	Arterial tortuosity syndrome (OMIM 208050)	*SLC2A10* (OMIM 606145)	20q13.12	Arterial tortuosity, connective tissue defects[229,230]
AR/het-het interaction w/ FREM2	Fraser syndrome (OMIM 219000; 617666)	*FRAS1* (OMIM 607830) *FREM2* (OMIM 608945)	4q21.21 13q13.3	Cryptophthalmos, syndactyly, renal defects, airway defects, GU anomalies[89,231]
AR	Multiple pterygium syndrome (OMIM 53290)	*CHRNG* (OMIM 100730)	2q31.1	Arthoggyrposis, multiple pterygia, fetal akinesia[232]
AR	Perlman Syndrome (OMIM 267000)	*DIS3L2* (OMIM 614184)	2q37.1	Facial dysmorphology, Wilms tumor, nephroblastomatosis, macrosomia, renal dysplasia[233-235]
AD	Denys-Drash syndrome (OMIM 194080)	*WT1* (OMIM 607102)	11p13	Congenital nephropathy, Wilm's tumor, ambiguous or female external genitalia in individuals w/ a 46,XY karyotype[236]
AD	Meacham syndrome (OMIM 608978)	*WT1* (OMIM 607102)	11p13	Ambiguous or female external genitalia in individuals w/ a 46,XY karyotype, abnormal internal female genitalia, congenital heart defects[237,238]
AD	Frasier syndrome (OMIM 136680)	*WT1* (OMIM 607102)	11p13	Male pseudohermaphroditism, streak gonads +/-, gonadoblastoma, nephrotic syndrome[239]
AD	Cornelia de Lange syndrome (OMIM 122470; 614701)	*NIPBL* (OMIM 608667) *RAD21* (OMIM 606462)	5p13.2 8q24.11	Dysmorphic facial features, hirsutism, limb anomalies, GERD, GU anomalies, congenital heart defects, SNHL, DD, ID, behavioral problems, short stature[147-149]
AD	Marfan syndrome (OMIM 154700)	*FBN1* (OMIM 134797)	15q21.1	Typical presentation: ascending aortic dilation, valvular regurgitation, scoliosis, ocular manifestations, pectus carinatum or excavatum, joint laxity, arachnodactyly[240]
AD	Saethre-Chotzen syndrome (OMIM 101400)	*TWIST1* (OMIM 601622)	7p21.1	Craniosynostosis, syndactyly, facial asymmetry[241]
AD	Kabuki syndrome (OMIM 147920)	*KMT2D* (OMIM 602113)	12q13.12	Dysmorphic facial features, ID, skeletal anomalies, cardiac defects, persistence of fetal finger pads[242,243]
AD	Microphthalmia, syndromic (OMIM 607932)	*BMP4* (OMIM 112262)	14q22.2	Ocular anomalies, dysmorphic facial features[244]
AD	Tuberous sclerosis type 2 (OMIM 613254)	*TSC2* (OMIM 191092)	16p13.3	ID, multi-system hamartomas, seizures, hypopigmented patches[245]
AD	Apert syndrome (OMIM 101200)	*FGFR2* (OMIM 176943)	10q26.13	Craniosynostosis, syndactyly, dysmorphic facies, cleft palate, ID[246]

AD	CHARGE syndrome (OMIM 214800)	*CHD7* (OMIM 608892)	8q12.2	Ocular coloboma, heart defects, choanal atresia, genital anomalies, ear abnormalities, DD[247]
AD	Cardiac-urogenital syndrome (OMIM 618280)	*MYRF* (OMIM 608329)	11q12.2	Congenital heart defects, GU anomalies[9,248,249]
AD	Coffin-Siris syndrome (OMIM 614608; 135900; 614609; 616938; 618027)	*SMARCB1* (OMIM 601607) *ARID1B* (OMIM 614556) *SMARCA4* (OMIM 603254) *SMARCE1* (OMIM 603111) *DPF2* (OMIM 601671)	22q11.23 6q25.3 19p13.2 17q21.2 11q13.1	Hypoplastic 5th finger/toe and nail, coarse facial features, DD, ID, feeding difficulties[250-253]
AD	Ehlers-Danlos syndrome, vascular type (OMIM 130050)	*COL3A1* (OMIM 120180)	2q32.2	Multiple pulmonary artery stenoses, systemic artery tortuosity, may have little to none skin hyperextensibility[254,255]
AD	White-Sutton syndrome (OMIM 616364)	*POGZ* (OMIM 614787)	1q21.3	DD, ID, autism, dysmorphic facial features, structural brain abnormalities[256,257]
AD	Pancreatic agenesis and congenital heart defects (OMIM 600001)	*GATA6* (OMIM 601656)	18q11.2	Pancreatic agenesis, heart defects, neurocognitive abnormalities[46,51,258-260]
AD		*DISP1* (OMIM 607502)	1q41	Heart defects, cleft lip & palate, tethered cord, axial musculoskeletal anomalies, hypotonia[98]
AD		*MYH10* (OMIM 160776)	17p13.1	Kidney defects, musculoskeletal anomalies, hypotonia, microcephaly, structural brain abnormalities, DD/ID[99]
AD XL	Opitz G/BBB syndrome (OMIM 145410;300000)	*SPECC1L* (OMIM 614140) *MID1* (OMIM 300552	22q11.23 Xp22.2	Cleft lip/palate, tracheoesophageal anomalies, congenital heart defects, anogenital malformations, dysmorphic facial features[261,262]
XL	Craniofrontonasal syndrome (OMIM 304110)	*EFNB1* (OMIM 300035)	Xq13.1	Craniosynostosis, hypertelorism, facial asymmetry, syndactyly, (het. females more severely affected)[263-265]
XL	Simpson-Golabi-Behmel syndrome type I (OMIM 312870)	*GPC3* (OMIM 300037)	Xq26.2	Overgrowth syndrome, coarse facial features, cleft palate, skeletal anomalies, supernumerary nipples, ID[266-269]
XL	Focal dermal hypoplasia (Goltz Syndrome) (OMIM 305600)	*PORCN* (OMIM 300651)	Xp11.23	Microphthalmia, patchy skin atrophy with fat herniation, facial asymmetry, dental anomalies[270,271]
XL	Tonne-Kalscheuer syndrome (OMIM 300978)	*RLIM* (OMIM 300379)	Xq13.2	ID, behavioral disorders, GU anomalies[272]
XL	Microphthalmia with linear skin defects syndrome (OMIM 309801; 300887)	*HCCS* (OMIM 300056) *COX7B* (OMIM 300885)	Xp22.2 Xq21.1	Microphthalmia, linear skin defects, DD, congenital heart defects, agenesis of corpus callosum, GU malformations, short stature[273,274]
XL	Mullegama-Klein-Martinez syndrome (OMIM 301022)	*STAG2* (OMIM 300826)	Xq25	ID, DD, cleft palate, short stature, microcephaly, dysmorphic facial features, thoracic vertebral anomalies, SNHL[53,106-108,110]
XL	X-linked Myotubular (centronuclear) myopathy (OMIM 310400)	*MTM1* (OMIM 300415)	Xq28	Hypotonia requiring mechanical ventilation, scoliosis, contractures, ophthalmoplegia, areflexia[275-277]
YL	Swyer syndrome 46,XY sex reversal (OMIM 400044)	*SRY* (OMIM 480000)	Yp11.2	Female external and internal genitalia, bilateral streak gonads[278,279]
Imprinting/ Epigenetic	Beckwith-Wiedemann syndrome (OMIM 130650)	Imprinting defects (IC1-LoM, IC2-GoM) or	11p15.5	Macroglossia, abdominal wall defects, asymmetric overgrowth, organomegaly[280]

AD		uniparental disomy of 11p15.5 or *CDKN1C* mutation (OMIM 600856)	11p15.4	

†in ≥ 2 patients reported in the literature
* syndromes associated with CDH (may not be all the syndromes associated with a given gene)
**genes associated with CDH (may not be all the genes for a given syndrome)
AR – autosomal recessive, AD – autosomal dominant, XL – X-linked , YL – Y-linked
GI – gastrointestinal, GU – genitourinary, SNHL – sensorineural hearing loss, DD – developmental delay, ID – intellectual disability, GERD = gastroesophageal reflux disease,
IC = imprinting center. LoM = loss of methylation. GoM = gain of methylation.

Table 3. Isolated CDH Genes and Candidate Genes

Gene	Chromosomal Locus	Other Associations
GATA4 (OMIM 600567)	8p23.1	Congenital heart defects, subclinical diaphragm defects[55]
ZFPM2/FOG2 (OMIM 603693)	8q23.1	Congenital heart defects, diaphragm eventrations[86,101,103,281,282]
NR2F2/COUP-TFII (OMIM 107773)	15q26.2	Congenital heart defects[48,283]
EFNB1 (OMIM 300035)	Xq13.1	Craniofrontonasal syndrome[105]
FREM1 (OMIM 608944)	9p22.3	BNAR syndrome,[284] MOTA syndrome,[285,286] CAKUT[100]
HLX (OMIM 142995)	1q41	Asplenia, short bowel[102,104]