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Research Article

Clinical Relevance of Rapid *FOXF1*-Targeted Sequencing in Patients Suspected of Alveolar Capillary Dysplasia With Misalignment of Pulmonary Veins

Gabriëla G. Edel[a], Janna A. Hol[b], Evelien Slot[a,b], Jan H. von der Thüsen[c], Yolande van Bever[b], Rogier C.J. de Jonge[d], Marianne van Tienhoven[b], Hennie T. Brüggenwirth[b], Annelies de Klein[b], Robbert J. Rottier[a,*]

[a] *Department of Pediatric Surgery, Erasmus MC Sophia Children's Hospital, Rotterdam, The Netherlands;* [b] *Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands;* [c] *Department of Pathology and Clinical Bioinformatics, Erasmus MC, Rotterdam, The Netherlands;* [d] *Pediatric Intensive Care Unit, Department of Pediatrics and Pediatric Surgery, Erasmus MC Sophia Children's Hospital, Rotterdam, The Netherlands*

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ABSTRACT

Alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV) is a lethal congenital lung disorder that presents shortly after birth with respiratory failure and therapy-resistant pulmonary hypertension. It is associated with heterozygous point mutations and genomic deletions that involve the *FOXF1* gene or its upstream regulatory region. Patients are unresponsive to the intensive treatment regimens and suffer unnecessarily because ACDMPV is not always timely recognized and histologic diagnosis is invasive and time consuming. Here, we demonstrate the usefulness of a noninvasive, fast genetic test for *FOXF1* variants that we previously developed to rapidly diagnose ACDMPV and reduce the time of hospitalization.

Introduction

Alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV) is a lethal congenital lung disorder resulting in respiratory distress and therapy-resistant pulmonary hypertension (PH).[1] In 95% of cases, patients are born at term with normal birth weight, but they mostly present with respiratory decline within the first days of life.[1,2] There is, however, also a subset of patients with a delayed presentation that usually has a milder phenotype.[3-5] Patients are treated with pulmonary vasodilatory agents, like nitric oxide, to reduce PH, and they receive cardiorespiratory support, including mechanical ventilation and, often, extracorporeal membrane oxygenation (ECMO). In atypical or late-presenting ACDMPV patients, lung transplantation has so far been the only treatment for prolonged survival, although several lung transplant–free cases have been described as well.[6,7] ACDMPV is characterized by a reduced capillary density, thickening of the alveolar septa, and misalignment of the pulmonary veins.[2] Also, extrapulmonary anomalies are found in 50% to 80% of the patients, for example, in the genitourinary, gastrointestinal, or cardiovascular systems.[2] Definitive diagnosis is based on histologic examination for aforementioned pulmonary malformations. However, open lung biopsy is a high-risk intervention, especially with anticoagulation while on ECMO in an already critically ill child. This may lead to a delay in the decision for a lung biopsy and contributes to the fact that approximately 90% of patients with

* Corresponding author.
E-mail address: r.rottier@erasmusmc.nl (R.J. Rottier).

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ACDMPV are diagnosed postmortem.[2] To minimize suffering for these patients, it is important to diagnose ACDMPV fast and noninvasively.[8]

It has been reported that approximately 40% of ACDMPV cases are associated with genomic alterations in the forkhead box F1 (*FOXF1*) gene or its 60-kilobase pair (kb) upstream enhancer,[2,9] and more recent reports even demonstrate association in 80% to 90% of ACDMPV cases.[10,11] These alterations are either point mutations or insertion-deletion mutations in the *FOXF1* gene or large copy number variations involving the gene itself or the enhancer region.[9,10] FOXF1 is an important transcription factor during development and in the lungs, it is expressed in mesenchymal cells and vascular endothelium.[12-14] Homozygous *Foxf1*$^{-/-}$ mice already die around embryonic day 9.5 because of defective development in the extraembryonic mesoderm,[15] whereas heterozygous *Foxf1*$^{+/-}$ mice have features that resemble ACDMPV.[16,17] However, a new model in which mice are heterozygous for a patient-derived c.155C>T, p.(Ser52Phe) missense mutation in the *FOXF1* gene represents an even more clinically relevant mouse model for ACDMPV.[18] In this model, misalignment of the pulmonary veins as seen in ACDMPV was recapitulated for the first time in the mouse, underscoring the impact of small *FOXF1* variants on phenotypic outcome.

In order to capture all possible *FOXF1* variants that are associated with ACDMPV, including copy number variants, when using Sanger sequencing, this needs to be combined with single nucleotide polymorphism (SNP) array, array comparative genomic hybridization, or multiplex ligation-dependent probe amplification, which makes diagnostics cumbersome and expensive. An alternative to simultaneously detect all *FOXF1* variants is whole genome sequencing, which is even more expensive and produces a vast amount of data, making bioinformatics and analysis more complicated.[19] This results in a time-consuming workflow, and the analysis may take days, causing delay in setting the right diagnosis and unnecessary suffering for both patients and parents. Preferably, a genetic test for this specific diagnosis should be fast, reliable, and cost-effective. To meet these requirements, we previously developed a targeted next-generation sequencing (NGS) panel to identify all *FOXF1* variants associated with ACDMPV in one diagnostic test.[20] For this study, the test was used clinically in a prospective manner on patients who were suspected of ACDMPV at the pediatrics department of the Erasmus MC Sophia Children's Hospital, in comparison with traditional testing methods. We show the speed and effectiveness of the test as a diagnostic tool.

Materials and Methods

Sample Collection and DNA Isolation

Blood samples of patients and parents, when applicable, were collected in the Erasmus MC Sophia Children's Hospital. Samples were processed, and DNA was isolated using the Gentra Puregene Blood Kit (Qiagen) as previously described.[20] The study was reviewed and approved by the Daily Board of the Medical Ethics Committee of the Erasmus MC Rotterdam, The Netherlands.

FOXF1 Test With Alveolar Capillary Dysplasia With Misalignment of Pulmonary Veins—targeted Next-Generation Sequencing Panel and Analysis

DNA of patients and parents was collected for genomic analyses. Generation of the ACDMPV-targeted NGS panel has been described previously.[20] In short, the ACDMPV panel consists of 157 amplicons covering the *FOXF1* gene and its regulatory region. A total of 38 overlapping amplicons covered *FOXF1* to detect single nucleotide substitutions and small deletions or duplications within the gene. The other 119 amplicons were used to detect copy number variants by covering selected SNPs, of which the majority were distributed around the *FOXF1* gene and its regulatory region, and the rest were distributed across the other parts of chromosome 16.

Histologic Evaluation and Immunohistochemical Stainings

Histologic examination of the lungs was performed by an experienced thoracic pathologist (J.H.v.d.T). Staining was performed on slides of formalin-fixed and paraffin-embedded lung tissue obtained by biopsy, including hematoxylin-eosin and immunohistochemistry for CD31 (JC70 clone) (Cell Marque, Rocklin) with ready-to-use antibodies on a Benchmark Ultra system, using an Ultraview Dab kit for visualization (all from Ventana Medical Systems).

Results

The ACDMPV-targeted NGS panel was performed on newborns with a severe unexplained lung phenotype and for which the pediatric pulmonologist or clinical geneticist suspected a possible *FOXF1* mutation, including a possible second diagnosis. In a time frame of approximately 1 year, 4 patients were tested with the ACDMPV-targeted NGS panel. Additional diagnostic testing was performed because the ACDMPV-targeted NGS panel was not yet accepted as a definitive diagnostic test. For each patient, the clinical representation, genetic results, and histologic evaluation, if available, are summarized in the Table.

Patient 1

Out of the 4 patients, 1 patient was diagnosed with ACDMPV. This patient (Table, patient 1) was born at term and developed respiratory insufficiency and PH 12 hours after birth. ECMO treatment was initiated 18 hours after birth. He had anal atresia, duodenal stenosis, and an absent gall bladder. Eight days after birth, the ACDMPV-targeted NGS test was performed, which showed a de novo missense variant (NM_001451.3(FOXF1):c.301T>C, p.(Ser101Pro) in the first exon of the *FOXF1* gene. A few days before the ACDMPV-targeted NGS test was performed, exome sequencing (ES) had been requested simultaneously and the ES analysis confirmed the *FOXF1* variant identified by the ACDMPV-targeted NGS panel. However, because the ES analysis had a turnaround time of 17 days, the analysis with the ACDMPV-targeted NGS panel identified this variant earlier, with a turnaround time of just 2 days. The identified missense variant, which has not been previously reported in patients with ACDMPV, was classified as pathogenic on the basis of American College of Medical Genetics and Genomics criteria PS2, PM1, PM2_supporting, PM5, and PP3 (ClinGen Sequence Variant Interpretation Recommendation for PM2—version 1.0; date approved: September 4, 2020).[21] Based on the results of the ACDMPV-targeted NGS panel, a lung biopsy was performed 3 days later, and ACDMPV was confirmed histologically, with clear misalignment of pulmonary veins and disordered capillary networks in alveolar septa (Supplementary Fig. S1), even before the test results

Table
Patient data and genetic testing

Patient no.	Gestational age born (week + day)	Time between birth and onset of respiratory symptoms (type of symptoms)	Additional clinical features	ACDMPV-targeted NGS panel results (time to result; time from birth to result)	Additional genetic tests performed[a] (time to result; requested before/simultaneously/after ACDMPV-targeted NGS panel)	Final diagnosis
1	38 + 2	12 h, start of ECMO after ±18 h - Respiratory insufficiency: yes - Pulmonary hypertension: yes	Anal atresia, duodenal stenosis and absent gall bladder	FOXF1 pathogenic variant identified: c.301T>C,p.(Ser101Pro) (2 d; 10 d)	a) SNP array (14 d; before) b) Trio-ES multiple congenital anomalies (17 d; before)	ACDMPV
2	38 + 2	Directly after birth CPAP and high-flow ventilation, lung hypoplasia - Respiratory insufficiency: yes - Pulmonary hypertension: yes	Giant omphalocele, congenital heart defect (Fallot tetralogy) and lung hypoplasia	No variants (6 d; 5 mo)	a) ES Multiple Congenital Anomaly and Intellectual Disability panel (prenatally) b) SNP array (prenatally) c) XON array (18 d; after) d) Sanger & MLPA FOXF1 (8 d; after) e) ES reanalysis congenital heart diseases (12 d; before) f) ES reanalysis *BMPR2* gene (39 d; after)	Unknown
3	37 + 2	13 min, requiring intubation and start of ECMO within several hours - Respiratory insufficiency: yes - Pulmonary hypertension: yes	Choanal atresia, dysmorphic ears, and aplasia of the nipples	No variants (5 d; 5.5 mo)	a) SNP array (12 d; before) b) Directed analysis *CHD7* gene (14 d; before) c) Directed analysis *KMT2D* gene (14 d; before)	Kabuki syndrome (*KMT2D* mutation)
4	30 + 5	Directly after birth CPAP and Optiflow ventilation, deterioration after 2 wk requiring intubation - Respiratory insufficiency: yes - Pulmonary hypertension: yes	None	No variants (7 d, because of logistics problem; 1 mo)	a) ES interstitial lung diseases (*FOXF1* included) (29 d; simultaneously)	Bronchopulmonary dysplasia

ACDMPV, alveolar capillary dysplasia with misalignment of pulmonary veins; CPAP, continuous positive airway pressure; ECMO, extracorporeal membrane oxygenation; ES, exome sequencing; MLPA, multiplex ligation-dependent probe amplification; NGS, next-generation sequencing; SNP, single nucleotide polymorphism.
[a] In chronological order.

of the ES were available. After ACDMPV diagnosis, palliative treatment was initiated, and the patient died at 3 weeks of age.

Patients 2 to 4

In 3 patients (Table, patients 2-4), a diagnosis of ACDMPV was made less likely using the ACDMPV-targeted NGS panel. This included patients with early-onset neonatal respiratory insufficiency and PH. Additional clinical features included giant omphalocele, congenital heart defect, and lung hypoplasia in patient 2 and choanal atresia, dysmorphic ears, and aplasia of the nipples in patient 3. The ACDMPV-targeted NGS panel did not reveal any *FOXF1*-variants in these patients. The turnaround times were 6 and 5 days for patients 2 and 3, respectively. The turnaround time for the results of patient 4 could have been shorter, but because of a logistics problem, it took 7 days for the final results to become available. In patient 2, the absence of pathogenic *FOXF1* variants was supported by further genetic testing, including SNP array, XON array, Sanger sequencing/multiplex ligation-dependent probe amplification of the *FOXF1* gene, and ES. An alternative genetic diagnosis has not yet been identified. Due to major comorbidity, no lung biopsy has been performed. However, the patient was still alive at the last follow-up at the age of 12 months, making the (histologic) diagnosis of ACDMPV unlikely. In patient 3, an alternative genetic diagnosis explaining the patient's phenotype was identified by ES. This patient was diagnosed with Kabuki syndrome on the basis of a likely pathogenic, de novo variant in *KMT2D*, and the patient was alive at the last follow-up at the age of 9 months. In patient 4, who was born prematurely, the absence of pathogenic *FOXF1* variants was supported by ES analysis of a gene panel for interstitial lung diseases and SNP array. No other genetic explanation was found, and computed tomography imaging of the lungs did not show signs of interstitial lung disease. She improved clinically, and a diagnosis of bronchopulmonary dysplasia was considered most likely. She was alive and well at the last follow-up at the age of 19 months.

Discussion

In this study, we prospectively tested 4 patients suspected of having ACDMPV for *FOXF1* variants with the ACDMPV-targeted NGS panel to quickly provide ACDMPV diagnosis.[20] One patient was diagnosed with ACDMPV and carried a novel pathogenic *FOXF1* variant, a de novo *FOXF1* missense mutation (c.301T>C, p.(Ser101Pro)), which has not been previously published.

The ACDMPV-targeted NGS panel did not yield false positive or false negative results, as confirmed by other genetic analyses, which included the *FOXF1* gene. If a *FOXF1* variant is found, the panel prevents incidental findings in the rest of the genome, is minimally invasive, and prevents unnecessary suffering for the parents and patients. Additionally, the ACDMPV-targeted NGS panel reduces financial costs. The costs for the ACDMPV panel are approximately 2.5 times lower than those for standard ES. Due to the fast turnaround time of the ACDMPV-targeted NGS panel, treatment costs, such as ECMO, can be reduced by several days up until several weeks compared with current diagnostic testing when ACDMPV is diagnosed. Three out of 4 tested patients had no *FOXF1* variants in our study, of which 2 patients were subsequently diagnosed with a different disorder. In the third patient, the prolonged survival makes a diagnosis of ACDMPV unlikely. Nevertheless, a negative test does not

automatically exclude the diagnosis of ACDMPV since it has been previously reported that approximately 30% of ACDMPV cases have no identifiable genetic alterations in the *FOXF1* locus.[10,20] In some cases, this may be explained by hypermethylation of the first exon of *FOXF1* as demonstrated in a recent study,[22] which cannot be identified with the ACDMPV-targeted NGS panel. Therefore, in patients with negative test results and persistent clinical suspicion of ACDMPV, a lung biopsy needs to be performed for histologic evaluation to definitively confirm or rule out ACDMPV diagnosis. Furthermore, as demonstrated by patients 2 and 3, additional genetic testing may be warranted to identify genetic disorders with (initially) similar clinical presentation. Because of other congenital malformations along with respiratory failure and PH, ES analysis for genes involved in multiple congenital anomalies will usually be requested simultaneously. In this study, the ACDMPV-targeted NGS panel was, in most cases, requested after requesting other tests, and the analyses have been performed simultaneously (Table). However, results from the ACDMPV-targeted NGS panel were available sooner because of its fast turnaround time. Based on the success of our ACDMPV-targeted NGS panel, it is favorable to expand the panel with genes and loci that are associated with other congenital lung disorders that show comparable clinical features to omit the need for simultaneous ES analysis. Some examples are *FGF10*, *TBX4*, and *FGFR2*, which are associated with acinar dysplasia and congenital alveolar dysplasia.[23] Inclusion of these genes will contribute to optimal cost and time efficiency.

FOXF1 has 3 different protein domains: a DNA-binding domain and 2 activation domains, which is a region where several other *FOXF1* variants in ACDMPV have been found and which is important for FOXF1 function to regulate downstream molecular pathways.[18,24] At the same amino acid position, the c.302C>T; p.(Ser101Leu) missense variant has been previously reported,[24] emphasizing the functional importance of the Serine residue at position 101.

FOXF1 is expressed during development in multiple organ systems, such as the respiratory, gastrointestinal, and genitourinary tract,[15] and approximately 50% to 80% of patients with ACDMPV have extrapulmonary malformations.[2,10,25] Symptoms are sometimes hard to distinguish from those of other disorders and even more complicated, ACDMPV has been described in patients in addition to other disorders, such as Down syndrome.[26,27] Furthermore, in a retrospective study of newborn patients that died because of idiopathic persistent PH of the newborn and that did not recover on ECMO, 6 out of 7 cases were diagnosed with ACDMPV.[28] These studies show that ACDMPV is most likely to be underdiagnosed or misdiagnosed. Increased awareness and more frequent application of the ACDMPV-targeted NGS panel in a clinical setting can provide a rapid ACDMPV diagnosis and cessation of unnecessary treatment. Moreover, the identification of more patients with ACDMPV will lead to a better scientific understanding of this rare disease.

Author Contributions

Experiments were performed and data were analyzed by E.S., J.H.v.d.T, M.v.T, and H.T.B. R.C.J.d.J. provided samples. G.G.E. wrote the manuscript, and J.A.H., A.d.K., and R.J.R. edited the manuscript. All authors critically commented on the manuscript and had final approval of the submitted and published versions.

Data Availability

The data sets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics Approval and Consent to Participate

The study was reviewed and approved by the Daily Board of the Medical Ethics Committee Erasmus MC Rotterdam, The Netherlands (MEC-2017-302).

Supplementary Material

The online version contains supplementary material available at https://doi.org/10.1016/j.labinv.2023.100233

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