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ORIGINAL ARTICLE

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High-level gonosomal mosaicism for a pathogenic non-coding CNV deletion of the lung-specific *FOXF1* enhancer in an unaffected mother of an infant with ACDMPV

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Abstract

Background: Alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV) results from haploinsufficiency of the mesenchymal transcription factor *FOXF1* gene. To date, only one case of an ACDMPV-causative CNV deletion inherited from a very-low level somatic mosaic mother has been reported.

Methods: Clinical, histopathological, and molecular studies, including whole genome sequencing, chromosomal microarray analysis, qPCR, and Sanger sequencing, followed by in vitro fertilization (IVF) with preimplantation genetic testing (PGT) were used to study a family with a deceased neonate with ACDMPV.

Results: A pathogenic CNV deletion of the lung-specific *FOXF1* enhancer in the proband was found to be inherited from an unaffected mother, 36% mosaic for this deletion in her peripheral blood cells. The qPCR analyses of saliva, buccal cells, urine, nail, and hair samples revealed 19%, 18%, 15%, 19%, and 27% variant allele fraction, respectively, indicating a high recurrence risk. Grandparental studies revealed that the deletion arose on the mother's paternal chromosome 16. PGT studies revealed 44% embryos with the deletion, reflecting high-level germline mosaicism.

Conclusion: Our data further demonstrate the importance of parental testing in ACDMPV families and reproductive usefulness of IVF with PGT in families with high-level parental gonosomal mosaicism.

KEYWORDS

genetics of lung development, germline mosaicism, parental mosaicism, somatic mosaicism

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1 | INTRODUCTION

Postzygotic formation of single nucleotide variants (SNVs) or copy-number variants (CNVs) results in somatic, germline, or gonosomal mosaicism (Biesecker & Spinner, 2013; Iourov et al., 2010; Lupski, 2013). Somatic mosaicism has been described in a wide variety of genetic disorders with all modes of inheritance and across different tissues (Ansari et al., 2014; Bartnik et al., 2011; Biesecker & Spinner, 2013; Boone et al., 2010; Cao et al., 2019; Conlin et al., 2010; Erickson, 2010; Goriely et al., 2010; King et al., 2015; Lim et al., 2017; Morales et al., 2020; Myers et al., 2018; Poduri et al., 2013; Sano et al., 2020; Serra et al., 2020; Stosser et al., 2018; Xin et al., 2017). Depending on mosaicism levels and tissue distribution, somatic mosaic variants have been found both in affected and unaffected individuals, including parents of patients with genetic disease (Campbell, Yuan, et al., 2014; Huang et al., 2014). Importantly, the level of somatic mosaicism in the parents has been shown to correlate positively with the recurrence risk if the pathogenic variant is present in the parental germline (Campbell, Stewart, et al., 2014; Jonsson et al., 2018; Rahbari et al., 2016).

Alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV, MIM #265380) is a rare neonatal lethal lung developmental disorder, characterized by severe respiratory distress with refractory pulmonary arterial hypertension, from which patients die most often during the first postnatal month (Bishop et al., 2011; Janney et al., 1981; Langston, 1991; Sen et al., 2004; Szafranski et al., 2016). Heterozygous SNVs in the *FOXF1* gene on chromosome 16q24.1 or CNV deletions involving *FOXF1* (MIM# 601089) and/or its distant lung-specific enhancer, located ~286 kb upstream, are found in 80%–90% of patients with ACDMPV. To date, more than 64 distinct pathogenic or likely pathogenic SNVs (missense and nonsense), 29 indels (frameshift) and 70 CNV deletions have been reported (Abu-El-Haija et al., 2018; Everett et al., 2017; Hayasaka et al., 2018; Ma et al., 2017; Nagano et al., 2016; Pradhan et al., 2019; Sen et al., 2013; Stankiewicz et al., 2009; Szafranski et al., 2013, 2014, 2016, 2019, 2022; Yildiz Bolukbasi et al., 2022).

Interestingly, in contrast to pathogenic SNVs that have been found either on the maternal or paternal chromosome, CNVs encompassing *FOXF1* and/or its distant lung-specific enhancer apparently arise almost exclusively de novo on maternal chromosome 16 ($n = 50$ vs. $n = 5$) (Szafranski et al., 2019, 2022; Yildiz Bolukbasi et al., 2022). We proposed a model of *FOXF1* regulation with the distant lung-specific enhancer acting stronger on the paternal chromosome 16 and suggested that paternal deletions may lead to more severe non-lung anomalies (Szafranski et al., 2016, 2022) unless mitigated by hypermorphic modifier(s), e.g., located within the remaining allele of the enhancer (Szafranski et al., 2019). Although the pLI score of *FOXF1* is 0.96, reflecting low tolerance of *FOXF1* to the loss-of-function, six cases of pathogenic SNVs involving *FOXF1* have been described to be inherited from a nonmosaic parent (Karolak et al., 2020; Luk et al., 2016; Reiter et al., 2016; Szafranski et al., 2016) and only one CNV deletion inherited from the very low-level (0.4% in peripheral blood) mosaic mother (Karolak et al., 2020) has been reported.

Here, we describe clinical, histopathological, and molecular findings and application of in vitro fertilization (IVF) with preimplantation genetic testing (PGT) in a family with a deceased neonate with ACDMPV due to a non-coding CNV deletion of the distant *FOXF1* enhancer inherited from the unaffected mother found to be high-level mosaic for this variant in different tissues.

2 | METHODS

2.1 | Patients and samples

Peripheral blood samples from the deceased neonate proband (ACD209.3) and his parents (ACD209.1 and ACD209.2), as well as urine, buccal, saliva, hair, and nail samples from the proband's mother, and the saliva sample from the proband's grandparents (ACD209.4 and ACD209.5), were collected after obtaining written informed consent approved by IRB at Baylor College of Medicine (protocol H-8712).

2.2 | DNA extraction

Peripheral blood DNA was extracted using Gentra Purgene Blood Kit (Qiagen, Germantown, MD). DNA from urine was extracted 24 hours after collection using the Quick-DNA Urine Kit (Zymo Research, Irvine, CA). The prepIT-L2P (DNA Genotek, Ottawa, Canada) reagent was used to isolate DNA from buccal cells and saliva. The QIAamp DNA Investigator Kit (Qiagen) was used to extract DNA from hair follicles and nail clippings from fingers and toes. All procedures were followed to the manufacturer's protocols.

2.3 | Histopathological analyses

Histopathological evaluation was carried out on formalin-fixed, paraffin-embedded 5-μm sections of lung tissue specimens from infant autopsy stained with hematoxylin and eosin (HE).

2.4 | Rapid whole genome sequencing

Clinical rapid whole genome sequencing (rWGS) of the proband was performed at the Rady Children's Institute for Genomic Medicine in San Diego (CA) (Kingsmore et al., 2022). Sequence via next-generation sequencing (NGS) technology was generated from genomic DNA. PCR-free library preparation was performed prior to rWGS. An average genomic coverage of at least 35x, and/or at least 90% of OMIM genes were achieved 100% of coding base coverage of >10x for each proband. Alignment and variant calling were performed with the Illumina DRAGEN pipeline using the official reference build 37.1 (hg19). CNV calling was performed using a combination of CNV callers. Interpretation of CNVs was focused on variants that overlap or have a boundary that lies within 1 kb of an exon in all coding genes.

2.5 | CNV deletion analyses

Chromosomal microarray analysis (CMA) was performed using high-resolution custom-designed 16q24.1 region-specific 4x180K oligonucleotide microarrays (Agilent Technologies, Santa Clara, CA). CNV deletion junction was amplified using long-range PCR with LA *Taq* polymerase (Takara Bio, Madison, WI) and was Sanger sequenced to map the deletion breakpoints. Sequences were assembled using Sequencher v4.8 (Gene Codes, Ann Arbor, MI). Parental and maternal grandparental DNA samples were tested for the presence of the CNV deletion using junction-specific PCR.

2.6 | PCR and sanger sequencing

FOXF1 (NG_016273.1) and the ~7 kb core interval (chr16:86,218,225-86,225,319, hg38) of the lung-specific enhancer essential for lung development were Sanger sequenced for the presence of SNPs that might have acted as phenotype modifiers in the proband and his mother. SNPs in the grandparental samples were analyzed using Sanger sequencing to determine the origin of the CNV deletion.

2.7 | qPCR analysis

The qPCR primers (5′-GGACAACTTCCAAGTGCTTTC-3′ and 5′-ACTGATGGGTCTTGACTCTTTATCC-3′) were designed to amplify the deletion junction fragment of 200–400 bp. The *GAPDH* gene was used as the internal reference. qPCR was performed in triplicates in 20 μl reaction with 10 μl of PowerUp SYBR™ Green Master Mix (ThermoFisher Scientific, Waltham, MA), 0.25 μM of each forward and reverse primer, and 50 ng of blood DNA. DNA from mother's saliva, buccal cells, urine, hair, and nail samples were also tested. The qPCR reactions were carried out by CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and the quantitation cycle (Cq) value for each reaction was read by Bio-Rad CFX Maestro software (Bio-Rad). Relative quantification of the deletion junction fragments (ΔCq) in the proband, the mosaic parent, and an unrelated wild-type control was calculated by comparing it to the internal reference gene. By measuring the fold-change differences between the mother's and the proband's samples (ΔΔCq), the relative level of mosaicism in the mother's samples were determined ($2^{-\Delta\Delta Cq}$) (Liu et al., 2020).

2.8 | Pulmonary and heart studies

Pulmonary function test (PFT) by standard spirometry and plethysmography, measuring lung volumes, flows, and diffusion capacity, oximetry and echocardiogram studies were performed in the proband's mother.

2.9 | IVF and PGT

Chromosomal testing was done at Colorado Center for Reproductive Medicine (CCRM). The normal embryos were tested for CNV deletion of the *FOXF1* lung-specific enhancer at Reproductive Genetic Innovations LLC (RGI) in Northbrook, IL.

3 | RESULTS

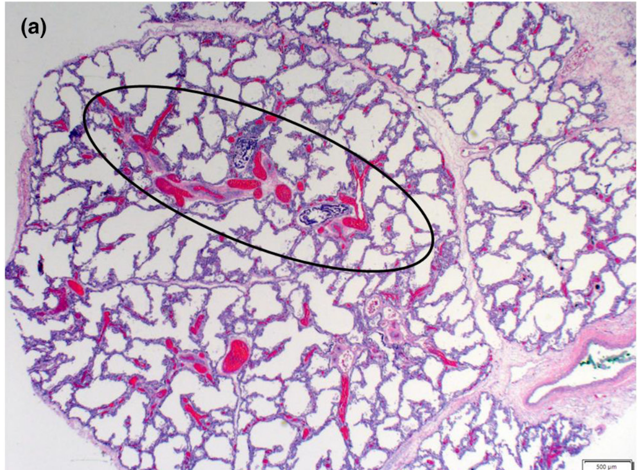
3.1 | Clinical findings

The decedent is a 54-day-old term appropriately grown male infant born via vaginal delivery following a pregnancy complicated by late maternal fever, which led to evaluations for potential neonatal sepsis. Shortly after birth, he developed hypoxemic respiratory failure and pulmonary hypertension. He was intubated and transferred from a community hospital to the neonatal intensive care unit at Children's Hospital Colorado (CHCO), where disease progression with severe persistent pulmonary hypertension required the initiation of extracorporeal membrane oxygenation (ECMO) therapy for 18 days. Investigation as to the etiology of severe pulmonary hypertension was pursued, following separation from ECMO and extubation. Despite treatment with inhaled nitric oxide (iNO), bosentan and remodulin (continuous IV infusion), his PH

remained at near systemic levels. Serial echocardiograms demonstrated no improvement in the severity of pulmonary hypertension despite maximal therapy. Several failed attempts with other therapies, including sildenafil, failed to improve his clinical course with increasing episodes of pulmonary hypertensive crises. Due to the high suspicion for a severe or lethal developmental lung disease, rWGS studies were performed, but these were reported as negative for any known disorder, including ACDMPV. With an anticipated high risk of mortality, unresponsive to therapy, the parents elected to withdraw life-sustaining support. Palliative care arrangements were subsequently made and support was withdrawn.

3.2 | Histopathological analysis

HE sections showed global lobular immaturity characterized by enlarged and simplified alveoli and mildly thickened interstitium. The pulmonary vasculature appeared diffusely congested (Figure 1a). The bronchovascular bundles contained many congested and dilated bronchial microvessel and extensively dilated bronchial veins, some with visible connections to pulmonary veins. Pulmonary arteries showed moderately muscularized walls. Many capillaries were dilated and located within the middle portion of the interstitium lacking visible connection to type 1 pneumocytes (Figure 1b). Lymphangiectasia was focally present. Black ink (injected at autopsy into main pulmonary veins) highlighted the extensively dilated bronchial veins, pulmonary veins, and bronchial microvessels, while green ink (injected into main pulmonary arteries) highlighted the pulmonary arteries. The presence of green ink in the bronchial arteries and bronchial microvessels and bronchial veins suggested recruited intrapulmonary bronchopulmonary anastomoses (not shown). The histologic features were diagnostic of ACDMPV characterized by a rich network of recruited bronchial vasculature. The combination of histologic features and differential ink injection suggested open intrapulmonary bronchopulmonary anastomoses as previously reported (Galambos et al., 2014, 2015; Norvik et al., 2020).



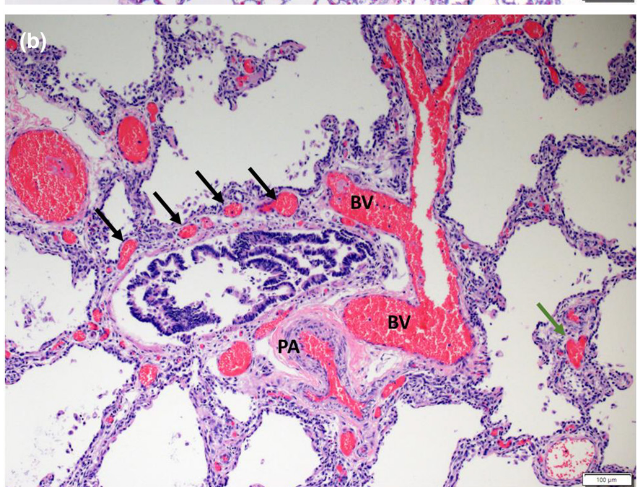


FIGURE 1 (a) Low-power examination of the lung shows global immaturity with diffusely enlarged alveoli and mildly thick interstitium. Markedly dilated pulmonary vessels surrounding the bronchoarterial bundles (circle) are clearly visible. (b) At higher power view disordered circulatory elements including thickened pulmonary artery (PA) and dilated dysplastic capillaries located in the middle of the interstitium lacking connections with type 1 pneumocytes (green arrow) are seen. Surrounding and within the bronchoarterial bundle, markedly dilated and congested bronchial vasculature, including thin-walled bronchial veins (BV) and microvessels (black arrows) are noted.

3.3 | Molecular and computational analyses

Clinical rWGS analyses in the proband showed no pathogenic variant. CMA revealed a heterozygous ~91 kb CNV deletion mapping upstream to the *FOXF1* lung-specific enhancer at 16q24.1. Using junction-specific PCR and Sanger sequencing analyses, we mapped the proximal deletion breakpoint within the repetitive LTR element LTR16C (chr16:86,177,468-86,177,904) at chr16:86,177,823. The distal breakpoint mapped within the LINE element L1PA3 (chr16:86,266,902-86,272,916) at chr16:86,268,849, located within the previously reported LINE/*Alu* genomic instability hotspot (Szafranski et al., 2018). At the breakpoint junction, there was a 1 bp (C) microhomology. The same deletion junction-specific fragment of weaker intensity was also detected in the proband's mother's blood, suggesting somatic mosaicism. The variant allele fraction (VAF) in the proband's mother's blood, sample measured using qPCR, was ~18%, corresponding to ~36% of the mutant cell fraction. The VAFs in the remaining maternal tissues were determined at 19% in saliva, 18% in buccal cells,

15% in urine, 19% in nail samples, and 27% in hair follicles (Figure 2).

Sanger sequencing of the ~7 kb core interval (chr16:86,252,422-86,258,902; hg19) within the ~60 kb distant *FOXF1* lung-specific enhancer region mapping upstream to *FOXF1* in the proband and his mother (ACD209.2), for the presence of genetic phenotype modifiers, did not reveal any candidate variant. No evidence of low-level mosaicism in the proband's maternal grandparental saliva samples was revealed, confirming somatic mosaicism in the proband's mother. Analyses of the informative polymorphic markers showed that the deletion arose on the grandpaternal chromosome (data not shown).

3.4 | Pulmonary and heart studies

Standard PFT, including spirometry and plethysmography in the proband's mother revealed normal lung function, including lung volumes, airway resistance, and diffusing capacity, except for a reduced peak inspiratory flow measured at 3.53 L/s (54.5% of the reference value). Her oximetry and echocardiogram studies were completely normal with no evidence of pulmonary hypertension, left or right ventricular hypertrophy, or myocardial dysfunction.

3.5 | IVF and PGT

Out of the 42 collected eggs, 40 were mature, and 36 were subjected for IVF. Out of the 18 embryos tested for the *FOXF1* enhancer deletion, including two mosaic, 10 chromosomally normal, and six chromosomally inconclusive, eight embryos (44%) were found positive.

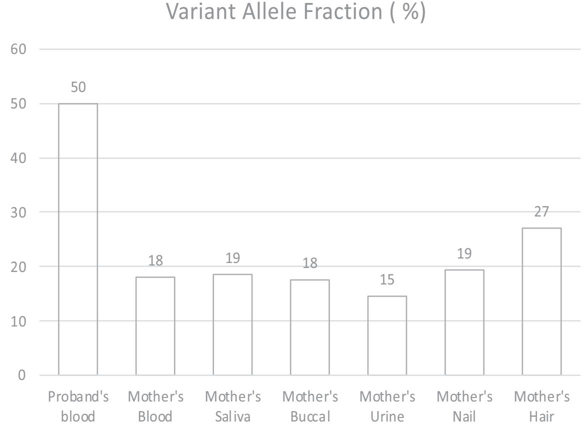


FIGURE 2 Variant allele fraction measured by qPCR in the mother's blood, saliva, buccal swab, urine, hair, and nail tissues compared to the proband's blood tissue

4 | DISCUSSION

Genetic counseling on recurrence risk in a family with detected parental mosaicism is often challenging and imprecise due to the uncertainty about the level of mosaicism in germline. In addition, recurrence risk for apparent de novo variants depends on the variant's parental origin. Although the mothers are the parent-of-origin in ~20% of inherited variants, the risk of recurrence for apparent de novo variants is 10-fold higher than when inherited from the fathers (Campbell, Yuan, et al., 2014; Jonsson et al., 2018), likely because of the presence of self-replication phase during spermatogenesis (Campbell, Yuan, et al., 2014). Variants detected in parental blood have been found to positively correlate with a recurrence risk substantially higher than those confined to the germline. Rahbari et al. showed that when mosaicism for the variant is present in more than 1% of parental blood cells, recurrence risk increases to 24% and to 50% when mosaicism is found in greater than 6% of parental blood cells (Rahbari et al., 2016). Recently, paternal germline mosaicism was studied in the families with children with autism spectrum disorders with de novo mutations (Breuss et al., 2020). Using genome sequencing of paternal blood and sperm, the causative variant was detected only in 2.5% of the fathers' blood or sperm, allowing classification of apparent de novo variants into low-risk or high-risk for recurrence (Breuss et al., 2020). The maternal germline mosaicism can be estimated indirectly, e.g., in IVF studies.

A number of sensitive quantitative molecular methods have been applied to measure the levels of somatic mosaicism, e.g., qPCR, droplet digital PCR (ddPCR), multiple independent primer PCR sequencing, amplicon-based NGS, and blocker displacement amplification (BDA) (Wu et al., 2017). ddPCR, amplicon-based NGS, and BDA are more sensitive and precise methods whereas qPCR is less precise but more cost effective. Using qPCR in the proband's mother samples, we have measured the deletion-containing cell fraction across peripheral blood, saliva, buccal cells, urine, hair follicles, and nails ranging between 30% to 54%. High-level mosaicism for the CNV deletion in tissues originating from all three germ layers suggests that it occurred during early embryonic development, most likely in the second postzygotic division.

In the family studied here, identification of high-level somatic mosaicism in the mother allowed for a more refined assessment of the potential disease recurrence risk for the identified deletion and the family was offered IVF and PGD for the future planned pregnancy. Subsequent IVF and PGD studies revealed 44% of germline mosaicism reflecting a high fraction of the deleted allele in the ovaries, and further supporting previous finding that the recurrence risk increases to 24% when mosaicism for the

variant is present in more than 1% of parental blood cells and to 50% when mosaicism is found in greater than 6% of parental blood cells (Rahbari et al., 2016).

The results of PFT, oximetry, and echocardiogram studies in the proband's mother further confirmed normal structure and function of her lungs and heart, and indicated that the estimated ~85% residual amount of the FOXF1 protein was likely sufficient for physiological development. The identified decreased peak inspiratory flow (PIF) value that measures the maximal flow achieved by an individual during an inspiratory maneuver (Clark & Hollingworth, 1993) was reduced almost by half; however, we interpret it as likely not linked to a disease. PIF has been found to vary in normal population depending on the muscle strength and its lower values have been consistently linked to a female gender (Ghosh et al., 2019; Silva et al., 2021). Significantly lower average PIFs have been also reported in patients with chronic obstructive pulmonary disease and asthma.

Our results further confirm the notion that quantitative testing of parental DNA samples for the mosaicism for the causative apparent de novo variants is essential to accurately evaluate the recurrence risk (Zemet et al., 2022). They also demonstrate the need for more extensive genetic testing in patients with histopathologically diagnosed ACDMPV, or with high level of suspicion for this diagnosis, or even in the setting of severe refractory persistent pulmonary hypertension of the newborn when routine clinical rWGS, CMA, or other genetic studies are negative.

AUTHOR CONTRIBUTIONS

EYB executed the experiments; EYB, PSz, JAK, TG, and PS analyzed and interpreted the data; NW and CG performed histopathological evaluations; SHA and JPK provided the clinical data; NW, CG, and JPK interpreted and described clinical findings; EYB, JAK, and PS wrote the manuscript. All authors reviewed and discussed the manuscript during preparation and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ETHICAL COMPLIANCE

Informed consent for genomic analysis and participation in study protocols was obtained from parents, and all research was conducted in accordance with the Declaration of Helsinki. The study research protocols were approved by the Institutional Review Board for Human Subject Research at Baylor College of Medicine (H-8712). Informed consent was obtained from all participants prior to genetic testing.

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