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A recurrent 8 bp frameshifting indel in *FOXF1* defines a novel mutation hotspot associated with alveolar capillary dysplasia with misalignment of pulmonary veins

Justyna A. Karolak[1,2], **Albino Bacolla**[3], **Qian Liu**[1], **Patrick E. Lantz**[4], **John Petty**[5], **Pamela Trapane**[6], **Karin Panzer**[7], **Balagangadhar R. Totapally**[8], **Zhiyv Niu**[1], **Rui Xiao**[9], **Nina G. Xie**[10], **Lucia R. Wu**[10], **Przemyslaw Szafranski**[1], **David Yu Zhang**[10], **Paweł Stankiewicz**[1]

[1]Department of Molecular & Human Genetics, Baylor College of Medicine, Houston, TX 77030 [2]Department of Genetics and Pharmaceutical Microbiology, Poznan University of Medical Sciences, 60-781 Poznan, Poland [3]Departments of Cancer Biology and of Molecular and Cellular Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030 [4]Department of Pathology, Wake Forest School of Medicine, Baptist Medical Center, Winston-Salem, NC 27157 [5]Department of General Surgery, Wake Forest School of Medicine, Baptist Medical Center, Winston-Salem, NC 27157 [6]Department of Pediatrics, Division of Pediatric Genetics, University of Florida College of Medicine-Jacksonville, Jacksonville, FL 32207 [7]Department of Pediatrics, University of Iowa Stead Family Children's Hospital, Iowa City, IA 52242 [8]Department of Pediatrics, Florida International University, Nicklaus Children's Hospital, Miami, FL 33155 [9]Baylor Genetics, Houston, TX 77021 [10]Department of Bioengineering, Rice University, Houston, TX 77030.

Abstract

Alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV) is a rare lethal lung developmental disease. Affected infants manifest with severe respiratory distress and refractory pulmonary hypertension and uniformly die in the first month of life. Heterozygous point mutations or copy-number variant deletions involving *FOXF1* and/or its upstream lung-specific enhancer on 16q24.1 have been identified in the vast majority of ACDMPV patients. We have previously described two unrelated families with a *de novo* pathogenic frameshift variant c. 691_698del (p.Ala231Argfs*61) in the exon 1 of *FOXF1*. Here, we present a third unrelated ACDMPV family with the same *de novo* variant and propose that a direct tandem repeat of eight consecutive nucleotides GCGGCGGC within the ~ 4 kb CpG island in *FOXF1* exon 1 is a novel mutation hotspot causative for ACDMPV.

Correspondence Paweł Stankiewicz; pawels@bcm.edu.

SUPPORTING INFORMATION Additional supporting information may be found online in the Supporting Information section

DATA ACCESSIBILITY The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST DYZ and LRW have a patent pending on Blocker Displacement Amplification. DYZ and LRW are consultants of NuProbe Global. DYZ owns equity of NuProbe Global and Torus Biosystems.

Keywords

recurrent mutation; *FOXF1* haploinsufficiency; tandem repeats; CpG island

1. INTRODUCTION

Alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV, MIM# 265380) is a rare neonatal developmental lung disease, lethal due to severe respiratory distress and refractory pulmonary hypertension (PAH) (Bishop, Stankiewicz, & Steinhorn, 2011). To date, more than 70 distinct ACDMPV-related *FOXF1* heterozygous point mutations and 60 copy-number variant (CNV) deletions involving *FOXF1* and/or its upstream lung-specific enhancer at 16q24.1 have been identified in 80–90% of ACDMPV patients (Abu-El-Haija et al., 2018; Everett, Ataliotis, Chioza, Shaw-Smith, & Chung, 2017; Hayasaka et al., 2018; Ma et al., 2017; Nagano, Yoshikawa, Hosono, Takahashi, & Nakayama, 2016; Pradhan et al., 2019; Sen, Gerychova, et al., 2013; Stankiewicz et al., 2009; Szafranski et al., 2013, 2014, 2016). *FOXF1* (Forkhead box F1, MIM#601089) encodes a transcription factor of the fork-head family, and is regulated by the sonic hedgehog (SHH) signaling pathway during lung development (Fernandes-Silva, Correia-Pinto, & Moura, 2017; Kalinichenko et al., 2001).

Recently, we described a genomic instability hotspot at 16q24.1, involving two evolutionarily young LINE-1 and *Alu* elements located at the edge of the *FOXF1* enhancer, that mediate formation of different-sized CNVs (Szafranski et al., 2018). Here, we define a novel indel mutation hotspot in *FOXF1* causative for ACDMPV.

2. MATERIAL AND METHODS

Ethical statement Patients were recruited and genetic testing was performed as a part of research protocol after obtaining parental consents. The study protocol was approved by the Institutional Review Board for Human Subject Research at Baylor College of Medicine (H-8712).

Clinical descriptions **Patient 69.4** was a Caucasian girl reported by Sen et al., (2013) (pt#6). She was born at 39 weeks to a 28 years old G2P1 mother via vaginal delivery. Her birthweight was 3200 g and Apgar scores were 8/1 and 9/5. She was placed on extracorporeal membrane oxygenation (ECMO) and treated with nitric oxide, Milrinone, and Epoprostenol and died at 28 days of age due to respiratory failure. Histopathological evaluation of a lung biopsy sample revealed the characteristic constellation of changes seen in ACDMPV, including medial hyperplasia, small pulmonary arteries marked with extension of arterial smooth muscle into small alveolar wall vessels, lobular simplification with alveolar enlargement and poor subdivision, deficiency of normally positioned alveolar capillaries, and malposition of pulmonary veins adjacent to small pulmonary arteries. In addition to ACDMPV, there were interstitial changes highly suggestive of pulmonary interstitial glycogenosis with alveolar wall widening by bland mesenchymal cells with abundant clear to bubbly cytoplasm. An increased airway smooth muscle with intralobular extension was also observed.

Patient 187.3, reported by Pradhan et al., (2019), was born at 35 3/7 weeks with a birthweight of 2325 g and Apgar scores 3/1 and 7/5. Pregnancy was complicated by fetal hypoplastic left heart, polyhydramnios, and enlarged fetal stomach. Labor and delivery were complicated by premature prolonged rupture of membranes with meconium stained fluid 55 hours prior to delivery. After delivery child required resuscitation. The child developed apnea and was subsequently intubated. There were persistently hazy lung fields on chest x-ray (CXR). The child received surfactant, antibiotics, and diuretics with no improvement on CXR. Cardiac catheterization was completed to rule out anomalous pulmonary venous return and the child was noted to have severe pulmonary venous desaturations. Lung biopsy was completed and pathology was consistent with ACDMPV. Lung support was withdrawn on day 16 of life.

Patient 188.3 was a girl born through an emergency caesarian section at 38 weeks with a birthweight of 3420 g. Pregnancy was complicated by polyhydramnios. The child had omphalocele, pyloric atresia, and intestinal malrotation which were operated on day 1 of life. The child also found to have severe PAH with structural cardiac anomalies. In spite of intensive medical therapy, she continued to have severe PAH and hypoxemic respiratory failure and was placed on veno-arterial ECMO on day 9 of her life After two weeks, she was off the ECMO support. After less than 48 hours a second run of ECMO was initiated due to worsening PAH and hypoxemic respiratory failure. ECMO support was discontinued on day 36 of life. No lung biopsy or autopsy was performed; ACDMPV diagnosis was based on clinical and genetic findings.

Molecular studies The entire coding region of *FOXF1* was amplified and sequenced in patient 188.3 as described (Sen et al., 2013). To determine whether identified variants in 187.3 and 188.3 arose *de novo*, parental DNA samples were tested using PCR and Sanger sequencing. In family 187.3, the level of somatic mosaicism in the patient's mother was ascertained using the Mutation Surveyor (SoftGenetics, State College, PA).

3. RESULTS

In patient 188.3, Sanger sequencing analysis revealed a deletion of one of two tandemly repeated 8-mers GCGGCGGC (NC_000016.9:g.86544866_86544873del) within a ~ 4 kb CpG island in the exon 1 of *FOXF1* (Figure 1), resulting in a predicted codon frameshift and premature termination and protein truncation, c.691_698del, (p.Ala231Argfs*61). This variant is absent in the ExAc (v1.0), gnomAD (v2.1.1), and dbSNP (build 151) databases and its further investigation by PolyPhen and MutationTaster revealed that it is likely deleterious due to reading frame alteration. NMDEscPredictor (Coban-Akdemir et al., 2018) showed that the c.691_698del variant is expected to be subject to degradation by nonsense-mediated decay and thus to lead to *FOXF1* haploinsufficiency. Interestingly, we have reported the same variant in two unrelated ACDMPV patients 69.4 (pt#6) (Sen et al., 2013) and 187.3 (Pradhan et al., 2019).

Based on the previous analyses using PCR with primers amplifying the proband-specific junction fragment in the blood samples from the parents of patient 69.4, the c.691_698del variant has been determined to be *de novo*. The latter result is consistent with the analyses

using the blocker displacement amplification (BDA) method, whereby a blocker oligonucleotide that overlaps with the primer results in the preferred amplification of the mutated allele (Wu, Chen, Wu, Patel, & Zhang, 2017, manuscript submitted). Unexpectedly, Sanger sequencing of the blood sample of patient 187.3's apparently unaffected mother, revealed the presence of somatic mosaicism. We have estimated the level of maternal somatic mosaicism at 25.08% (Figure S1).

4. DISCUSSION

The local DNA environment, including sequence context and epigenetic modifications, has been shown to mediate a formation of the significant fraction of point mutations and indels in the human genome. Cooper et al. (2011) estimated that ~5% of point mutations causing human genetic diseases could be linked to methylation-mediated deamination of 5-methylcytosine (5mC) within a CpG context. Their hypermutability is a result of DNA replication/repair errors generated during removal and base excision repair of guanine-thymine mismatches, which are produced by spontaneous deamination of 5mC to thymine (Cooper, Mort, Stenson, Ball, & Chuzhanova, 2010). The first evidence of increased mutability at CpG motif related to human disease was identification of the recurrent missense mutations in *F8* in patients with hemophilia A (Youssoufian et al., 1986). To date, the CpG hotspots have been associated with several constitutional recurrent mutations in multiple disease-related genes, e.g. *FGFR3* (MIM# 134934), *NF1* (MIM# 613113), *RB1* (MIM# 614041), *DMD* (MIM# 300377), and *NACC1* (MIM#610672) in patients with achondroplasia (MIM#100800), neurofibromatosis type 1 (MIM# 162200), retinoblastoma (MIM# 180200), Duchenne muscular dystrophy (MIM# 310200), and neurodevelopmental disorder (MIM# 617393), respectively.

The formation of indel hotspots has been associated also with the local nucleotide context (Kondrashov & Rogozin, 2004). Recurring frameshifting indels have been described in repeated sequences in *APC* (MIM# 611731), *FOXG1* (MIM# 164874), *PRRT2* (MIM# 614386), and *RAI1* (MIM# 607642) in patients with familial adenomatous polyposis 1 (MIM# 175100), congenital variant of Rett syndrome (MIM# 312750), episodic kinesigenic dyskinesia 1 (MIM#128200), and Smith-Magenis syndrome (MIM# 182290), respectively.

The presence of repetitive DNA sequences, including short direct or inverted repeats, have been recognized as an important factor predisposing to the formation of indels, likely due to slipped-strand mispairing during DNA replication/repair (Ball et al., 2005; Kondrashov & Rogozin, 2004). Specifically, Kondrashov & Rogozin (2004) showed that the impact of nucleotide periodicity on mutation rate increases with the number and length of repeated segments. For example, homonucleotide tracts (sequences with period equal to one) are more prone for mutations if they are four or more nucleotide long, while if the period is three nucleotides or longer, even two direct repeats in tandem can increase the deletion rate (Kondrashov & Rogozin, 2004). Moreover, it has been shown that the crystal structure for the 8-mer presented here $(GCGGCGGC)_2$, containing the CGG RNA repeats associated with the fragile X disorders, can form a duplex with non-canonical G-G pairing and G-quadruplex DNA secondary structures; a hybrid between DNA (the non-template strand) and

nascent RNA is also possible (Kiliszek, Kierzek, Krzyzosiak, & Rypniewski, 2011). The region around the repeats is also prone to other secondary structures (Figure 1).

We propose that similar pre-or postzygotic DNA replication/repair errors predispose to the formation of the recurrent frameshifting indel c.691_698del within the GCGGCGGC tandem repeat in the CpG island of *FOXF1* exon 1, leading to the lethal ACDMPV phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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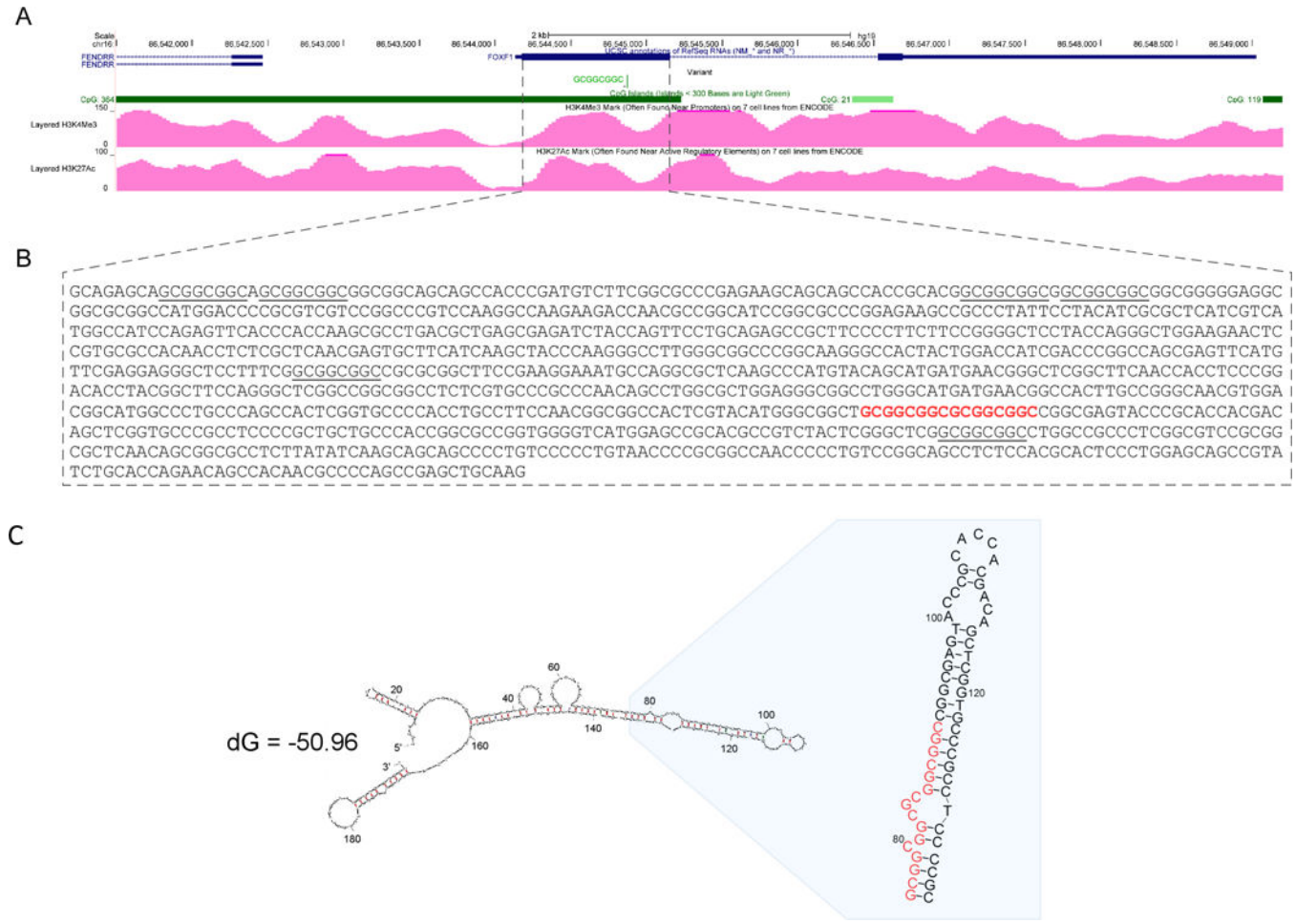

Figure 1. Schematic representation of the *FOXF1* region with exon 1 sequence.

A) The described mutation identified in three unrelated patients with ACDMPV is shown below the gene track. The CpG islands are marked in green. H3KMe3 and H3K27Ac marks in the fetal lungs are shown in pink. **B**) The nucleotide sequence of exon 1 of *FOXF1.* The 8-bp GCGGCGGC repeats are underlined. The 8-bp GCGGCGGC tandem repeat sequence with one motif deleted in three unrelated ACDMPV patients is in red. **C**) Predicted hairpin structure (only the folding of the most stable conformer is shown) of 200 bp sequences around the 8-bp GCGGCGGC repeat assessed using the mfold software (Zuker, 2003).