Maternal mutations of *FOXF1* cause Alveolar capillary dysplasia despite not being imprinted

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Abstract.

Alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV) is a rare cause of pulmonary hypertension in newborns. Maternally inherited point mutations in Forkhead Box F1 gene (*FOXF1*), deletions of the gene or its long-range enhancers on the maternal allele are responsible for this neonatal lethal disorder. Here we describe monozygotic twins and one full-term newborn with ACD and gastrointestinal malformations caused by *de novo* mutations of *FOXF1* on the maternal inherited alleles. Since this parental transmission is consistent with genomic imprinting, the parent-of-origin specific monoallelic expression of genes, we have undertaken a detailed analysis of both allelic expression and DNA methylation. *FOXF1* and its neighboring gene *FENDRR* were both biallelically expressed in a wide range of fetal tissues, including lung and intestine. Furthermore detailed methylation screening within the 16q24.1 regions failed to identify regions of allelic methylation, suggesting that disrupted imprinting is not responsible for ACDMPV.

Alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV; MIM# 265380) is a rare lethal lung disorder caused by persistent pulmonary hypertension (Alameh et al. 2002) in which newborns subsequently die of hypoxemic respiratory failure. More than half of patients have associated congenital anomalies: the most frequent being gastrointestinal, genitourinary and cardiovascular malformations (Alameh et al. 2002; Al Hathol et al. 2000). Familial occurrence has been reported in 10% of patients, although the true incidence of this condition is unknown. Heterozygous mutations in the *FOXF1* transcription factor (Stankiewicz et al. 2009) or deletions of distant mesodermal enhancers, encompassing the long non-coding (lnc)RNAs *LINC01081* and *LINC01082*, are present in most patients (Sen et al. 2013; Szafranski et al. 2013; Szafranski et al. 2014; Szafranski et al. 2016a). Interestingly deletions that remove *FOXF1* gene also affect the neighboring transcript *FENDRR* (previously known as *FOXF1-AS1*) a ncRNA transcribed from the opposite DNA strand (Grote and Hermann, 2013). Intriguingly when samples are informative ~90% of *FOXF1* mutations are maternally derived (Sen et al. 2013; Leiden Open Variation Database, LOVD). Furthermore, ~95% of reported pathogenic deletions involving the upstream regulatory region are *de novo* on the maternal allele, consistent with this locus being subject to genomic imprinting (Szafranski et al. 2016a; Decipher database). However to date, no imprinted transcripts have been identified at this locus.

Genomic imprinting involves the marking of the genes during gametogenesis to achieve monoallelic, parent-of-origin expression. Allele-specific methylation of DNA, as well as chromatin structure and histone modifications, is also involved in this process in somatic cells (reviewed in Monk, 2015). Many imprinted genes are organized in clusters with monoallelic expression resulting from the sharing of *cis*-regulating features such as differentially methylated regions (DMRs). Genetic aberrations encompassing imprinted loci

are associated with several of the known imprinting disorders, which have helped characterize the mechanisms leading to these rare diseases. For example uniparental disomies (UPDs) or reciprocal deletions of 15q11.2-13 result in two clinically distinct neurobehavioural disorders, Prader-Willi syndrome (PWS) and Angelman syndrome (AS)(Malcolm et al. 1991; Nicholls et al. 1989). The anticipated mechanisms leading to ACDMPV are proposed to be similar to those resulting in AS, with mutations in an imprinted protein coding gene or deletions of long-range regulatory elements (Buiting et al. 1999).

Here we describe two monozygotic twins and a third case corresponding to a full-term newborn diagnosed with ACD associated with abdominal malformations and mutations of *FOXF1* on the maternal allele (see supporting information for full clinical details). In the first two cases antenatal ultrasound at 20 weeks gestation of a healthy 30 years old mother's first pregnancy showed an abdominal cystic mass in monochorionic diamniotic twins. There was history of consanguinity in the parents, but no neonatal deaths in the family reported. Female twins were born at 36 weeks of gestational age. The first twin (I.1) had a birth weight of 2280g (27th centile). At 24 hours of age, she developed respiratory distress with pulmonary hypertension. On day 5 severe coagulopathy with a grade three intraventricular hemorrhage was observed by cerebral ultrasound. Extracorporeal membrane oxygenation (ECMO) treatment was not considered because of the poor prognosis, and the patient died soon after. The anatomopathology of the lung confirmed the diagnosis of ACD (Supp. Fig S1). Partial annular pancreas and colonic stenosis were present at autopsy; the rest of the examination was unremarkable.

The second twin (I.2) had a birth weight of 2180g (19th centile) with abdominal X-ray revealing dilated bowel loops. On day 2 of life she developed severe hypoxemia, with episodes of bradycardia and hypotension, requiring progressively increasing ventilatory and inotropic support with no response despite treatment with inhaled nitric oxide (iNO). On the

4th day of life she was admitted to neonatal intensive care due to severe pulmonary hypertension, thus ECMO was initiated. On 7th day of life, the sister's pathology report confirmed ACD, so a lung biopsy was performed. This also showed features of ACD. In the light of the poor prognosis associated with ACD, ECMO was withdrawn and the infant died. The autopsy confirmed ACD, with partial annular pancreas and colonic stenosis.

The third ACD patient was a full-term male neonate and was the first pregnancy of a healthy 32 years old female who has subsequently had an unaffected daughter. During the second trimester polyhydramnios was detected, raising the suspicion of a congenital abdominal malformation. Delivery occurred by cesarian-section at 38 weeks gestation with a birth weight of 2900g (24th centile). Ultrasound and radiologic studies showed bowel obstruction. He was taken to theatres at 8 hours of life where an annular pancreas, duodenal stenosis and intestinal malrotation were found. The second day of life, the oxygen requirements increased, with clinical signs of severe pulmonary hypertension refractory to escalating ventilatory and hemodynamic support as well as iNO. After 72 hours of life the patient presented a progressive respiratory deterioration with increasing oxygenation index and was cannulated for veno-venous ECMO. Within the following 5 days there was no decrease in pulmonary hypertension and ACD was suspected. A pulmonary biopsy was performed that showed histologic features of ACD. Given the poor prognosis of the disease, the decision was made to withdraw care and the patient died. All clinical and genetic details of these cases have been submitted to Leiden Open Variation Database (www.lovd.nl/).

To determine whether mutations in *FOXF1* were responsible for these cases we performed genetic analysis by PCR and Sanger sequencing (see supporting information for materials and methods). This identified a heterozygous nonsense mutation c.810G>A in RefSeq transcript NM_001451.2 (p.W270*) in the twin cases with no evidence of the mutation in the parental samples consistent with the mutation arising *de novo*. Interestingly,

this is a recurrent heterozygous mutation in ACDMPV and has previous been observed by Sen and colleagues (Sen et al. 2013). To ascertain whether the mutation was on the maternal chromosome we performed genotype analysis of single nucleotide polymorphisms (SNPs) within the *FOXF1* region. This revealed that SNP rs2078304:C>G within intron 1 was informative, being heterozygous in the cases 1 and 2 but homozygous in the mother. Cloning of long-range PCR products incorporating both the mutation and SNP in the amplicon revealed that the mutation was on the maternal allele (Figure 1A, B). Similar mutation screening of *FOXF1* in case 3 detected a *de novo* heterozygous one base pair deletion (c.105delG) (p.A36*). Using a similar strategy as described above, we identified that the polymorphism rs6145927: -/GGAGGGCTGCCT was informative, revealing that the mutation had also arisen on the maternal allele (Figure 1C).

To better understand the molecular mechanisms associated with this disease, we determined the allelic expression of *FOXF1* in fetal and adult tissues and fully characterized the methylation profile throughout the 16q24.1 interval. To assess if *FOXF1* and the neighboring transcript *FENDRR* are subject to genomic imprinting, we identified transcribed SNPs that would allow for allelic discrimination. Imprinting analysis was performed in fetal and adult lung, intestine and term placenta samples since qRT-PCR revealed these were the most abundant for *FOXF1* (Figure 1D; Supp. Fig S2; Supp. Table S1). Allele-specific RT-PCR followed by Sanger sequencing revealed that both genes are biallelically expressed in multiple fetal tissues (Figure 1E, G) and adult lung biopsies (Supp. Fig S2), with results quantified by pyrosequencing (Figure 1F, H). Unfortunately no heterozygous SNPs were identified in our tissue cohort for the lncRNAs, *LINC01081* and *LINC01082*.

Imprinted domains are associated with regions of allelic methylation inherited from the germline (Court et al. 2014) which is stable in somatic tissues irrespective of the expression levels making it an ideal epigenetic signature for screening novel imprinted loci.

Since CpG island promoters associated with imprinted transcripts are often differentially methylated we performed allelic bisulphite PCR on the bidirectional promoter interval associated with *FOXF1* and *FENDRR*. We observe that this region is robustly unmethylated in all tissues analyzed (Figure 2; Supp. Fig S3). In an attempt to identify novel imprinted DMRs we performed a bioinformatic screen of methyl-seq datasets from tissues associated with the ACD phenotype. We identified various non-overlapping, single-copy regions in lung, pancreas and intestine with partial methylation profiles consistent with allelic methylation (for selection criteria see materials and methods section in the supporting information) (Figure 2A). However when assessing the methylation profiles of these regions in methyl-seq datasets from sperm, oocyte and pre-implantation embryos none were consistent with imprinting. Interestingly, ~250kb of the *FOXF1*-lncRNA interval is methylated in sperm and less methylated in oocyte, however this subject to pre-implantation reprograming being largely unmethylated in blastocysts (Figure 2B).

Using standard allelic bisulfite PCR, we assessed regions with partial methylation observed in at least two target tissues in more detail. In all candidate regions analyzed the methylation was mosaically present on both alleles in lung, intestine, pancreas and placenta-derived DNA (Figure 2C; Supp. Fig S3). This includes 6 of the10 CpGs within the proposed DMRs (region 1 in Fig. 2C) mapping within the critical region (Szafranski et al. 2013; Szafranski et al. 2016b).

FOXF1 is a transcription factor expressed in the lung mesenchyme and is essential for normal development. Consistent with previous northern blot analysis (Pierrou et al. 1994), our qRT-PCR expression profiling revealed restricted *FOXF1* expression pattern with highest expression in lung, intestine and pancreas, the target tissues for ACDMPV. Heterozygous point mutations in *FOXF1* and heterozygous deletions encompassing the transcript or upstream enhancers (Szafranski et al. 2013) are the main causes of ACDMPV. Clinical

diagnosis is confirmed by observing misalignment of the pulmonary veins in post-mortem lung biopsies (Janney et al. 1981) although many ACDMPV infants present with extra-pulmonary anomalies, including defects in the gastrointestinal, cardiovacular and genitourinary systems. Consistent with our observations mutations in the coding sequence of *FOXF1* are associated with ACD and intestinal malrotation (Stankiewicz et al. 2009), which are explained by the essential role of *FOXF1* in the formation of the dorsal mesentery during fetal development. Stankiewicz *et al*. also described three cases that presented with ACD, annular pancreas and intestinal stenosis and derived from a mutation in *FOXF1* (Stankiewicz et al. 2009) similar to the three cases we describe.

To date ~200 cases of ACDMPV have been reported in literature, with 52 listed in the LOVD database. The majority of these cases are associated with maternally acquired genetic aberrations (Sen et al. 2013; Dharmadhikari et al. 2015; Szafranski et al. 2016a) suggesting genomic imprinting may be involved in this disorder. Maternal segregation of a missense *FOXF1* mutations in a familial case of ACDMPV (Sen et al. 2013) also suggests that the gene could be maternally expressed. Allele-specific expression analysis for *FOXF1* failed to identify evidence of maternal expression and detailed methylation screening did not find any evidence for allelic methylation, a key epigenetic feature associated with imprinted loci. This is despite previous reports of modest maternal methylation within the newly defined critical region defined by minimal deletion overlap (Szafranski et al. 2016b). However it must be noted that the methylation experiments performed by Szafranski and co-workers were not performed using SNPs and the methylation profile was inferred from patient samples carrying deletions. Our results are consistent with numerous genome-wide methylation screens for DMRs in which no parent-of-origin methylation profiles were reported within the 16q24.1 interval (Court et al. 2014; Sanchez-Delgado et al. 2016). This lack of direct evidence for imprinting is supported by a familial case in which the *FOXF1* mutation, a

c.90_96del, was inherited from the father (Szafranski et al. 2016a) and a individual case in which a c.C231A mutation was also paternally transmissed (Reiter et al. 2016). Furthermore a 4.1 kb *de novo* enhancer deletion also arose on the paternal allele (Szafranski et al. 2016a). Additional evidence supporting a lack of imprinted expression of *FOXF1* comes from the recent description of four cases of paternal uniparental disomy for chromosome (UPD(16)pat) none of which presented with features consistent with ACDMPV (Soehn et al. 2016; Donovan et al. 2016). Similarly, no consistent imprinting phenotype has been reported for the reciprocial UPD16mat (Soehn et al. 2016; Donovan et al. 2016; Scheuvens et al. 2016), despite conditional overexpression of *Foxf1* having lethal lung hypoplasia and vascular defects in mice (Dharmadhikari et al. 2016). This strongly suggests that the *FOXF1* gene is highly dosage-sensitive but not imprinted as all imprinting syndromes associated with UPD have classical phenotypes (reviewed in Lapunzina and Monk, 2011).

It remains possible that the *FOXF1* is maternally expressed in very restricted cells within the lung. Recent work using trangenic lacZ-reporter mice revealed that the evolutionarily conserved *Foxf1*long-range enhancers demonstrate tissue-specific activity in the proximal and distal pulmonary blood vessels (Seo et al. 2016), which may regulate allelic expression in these discrete cell populations. However it remains to be seen if non-canonical tissue-specific imprinting occurs at this locus or if other principals can explain the parent-of-origin effects observed for ACDMPV. Gender effects on human mutations rates have previously been reported at non-imprinted loci and are associated with the differences in male and female gametogensesis. This gives rise to distinct mutational signatures in offspring with increased parental age (Goldmann et al. 2016). However there is no obvious association with mothers age that could explain the phenomenon observed for *FOXF1* suggesting another, yet to be identified, mechanism is responsible.

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Figure legends.

Figure 1. Identifying maternal *FOXF1* mutations and analysis of allelic expression. (A) Map of the *FOXF1* gene showing the positions of the mutations and SNPs used to ascertain parental inheritance. (B) The c.810G>A mutation in case 1 is on the same DNA strand as the C allele of the heterozygous rs2078304 SNP, consistent with a *de novo* mutation on the maternal allele. (C) The parental origin of the c.105delG mutation in case 3 was determined using the heterozygous in/del polymorphisms rs6145927, which revealed that the mutation

was on the del allele consistent with a *de novo* mutation on the maternal allele. The

nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the

reference sequence, with the initiation codon as codon 1. (D) Quantitative RT-PCR showing

the expression profile of *FOXF1*. (E) Sequence traces of RT-PCR products for *FOXF1* in

fetal tissues samples heterozygous for SNPs rs7185244:C>T located in the 3'UTR and (G)

rs43140:G>T in exon 3 of *FENDRR*. The numbers under the electropherograms (F, H)

represent the quantified allelic expression by pyrosequencing.

Figure 2. Methylation profiling of the *FOXF1* interval on chromosome 16.

(A) The methyl-seq data reveals several partially methylated domains in multiple target

tissues and that the *FOXF1/FENDRR* promoter CpG island is devoid of methylation in all

tissues. The commonly deleted enhancer interval and the region of minimal deletion overlap

is highlighted in yellow. (B) The same ~ 350 kb interval in sperm, oocytes and blastocysts

methyl-seq datasets. The vertical black lines in the methyl-seq tracks represent the mean

methylation value for individual CpG dinucleotides. A green box highlights the position of

the CpG islands and transcripts are highlighted in blue type.

(C) Bisulphite PCR and subcloning was used for confirmation. Each circle represents a single

CpG dinucleotide on a DNA strand. (•) Methylated cytosine, (o) unmethylated cytosine. Each

row corresponds to an individual cloned sequence. If heterozygous for a SNP the genotype is

indicated. For clarity only methylation profiles for lung and pancreas are shown.