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Highly Sensitive Blocker Displacement Amplification and Droplet Digital PCR Reveal Low-Level Parental *FOXF1* Somatic Mosaicism in Families with Alveolar Capillary Dysplasia with Misalignment of Pulmonary Veins

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Abstract

Detection of low-level somatic mosaicism [alternate allele fraction (AAF) 10%] in parents of affected individuals with the apparent de novo pathogenic variants enables more accurate estimate of recurrence risk. To date, only a few systematic analyses of low-level parental somatic mosaicism have been performed. Herein, highly sensitive blocker displacement amplification, droplet digital PCR, quantitative PCR, long- range PCR, and array comparative genomic hybridization were applied in families with alveolar capillary dysplasia with misalignment of pulmonary veins. We screened 18 unrelated families with the FOXF1 variant previously determined to be apparent de novo (n = 14), of unknown parental origin (n = 1), or inherited from a parent suspected to be somatic and/or germline mosaic (n = 3). We identified four (22%) families with FOXF1 parental somatic mosaic single-nucleotide variants (n = 3) and copy number variant deletion (n = 1) detected in parental blood samples and an AAF ranging between 0.03% and 19%. In one family, mosaic allele ratio in tissues originating from three germ layers ranged between <0.03% and 0.65%. Because the ratio of parental somatic mosaicism have significant implications for the recurrence risk, this study further implies the importance of a systematic screening of parental samples for low-level and very- low level (AAF 1%) somatic mosaicism using methods that are more sensitive than those routinely applied in diagnostics. (J Mol Diagn 2020, 22: 447e456; *https://doi.org/10.1016/j.jmoldx.2019.12.007*)

Introduction

During the past decade, growing evidence on the importance of somatic mosaicism in etiology of several human genetic conditions, including cancers and neurodevelopmental dis- eases, has been reported.[1e11] However, somatic mosaic variants have been also detected in clinically unremarkable or mildly affected individuals, including parents of subjects with genetic conditions.[12,13] The incidence and ratio of parental somatic mosaicism have important implications for the recurrence risk,[14,15] because both affected and unaffected carriers of the pathogenic mosaic variant can transmit it to their children if it is also present in germline cells.[4,10,11] However, mainly because of technical limitations, only a few systematic studies on the real incidence of somatic mosaicism in parents of affected individuals with apparent *de novo* events have been performed.[5,12,13] Herein, highly sensitive blocker displacement amplification (BDA), droplet digital PCR (ddPCR), quantitative PCR (qPCR), long-range PCR, and customized array comparative genomic hybridization were applied in families with alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV; Mendelian Inheritance in Man number 265380). ACDMPV is a rare neonatal lethal lung developmental disorder, characterized by unique histopathologic features.[16e20] To date, >70 distinct pathogenic or likely pathogenic single-nucleotide variants (SNVs) and 60 copy number variant (CNV) deletions, involving *FOXF1* (forkhead box F1; Mendelian Inheritance in Man number 601089) or its lung-specific enhancer on 16q24.1, have been reported in 80% to 90% of ACDMPV families.[20e30] The vast majority of ACDMPV cases are sporadic, with *de novo FOXF1* variants being detected.[20,31] Only a few ACDMPV families with a pathogenic *FOXF1* variant transmitted from a somatic mosaic or apparent heterozygous healthy parent have been reported.[20,22,31e34] To examine the efficacy of the applied techniques as well as the scale and ratio of parental somatic mosaicism in

families with ACDMPV, 18 families with a known *FOXF1* variant were studied retrospectively.

Materials and Methods

Subjects

The DNA samples studied were from parents of 18 unrelated index ACDMPV patients with a known pathogenic *FOXF1* SNV ($n = 12$), insertion/deletion ($n = 5$), or CNV deletion ($n = 1$), detected during the standard diagnostic procedure. On the basis of PCR and Sanger sequencing, these variants were originally determined to be apparent *de novo* (alternate allele was not detected in the parents; $n = 14$), of unknown parental origin (parents were not tested; $n = 1$), or inherited from a parent suspected to be somatic and/or germline mosaic (alternate allele was present in the parent, but the precise allelic ratio was not determined and/or alternate allele was not detected in the parents, but the family pedigree suggested the presence of germline mosaicism; $n = 3$) (Figure 1).[20,21,31] Only the families in whom both parental and proband DNA samples were available, and for whom it was possible to design the BDA, ddPCR, or qPCR assays, were included in this study after obtaining informed consent. The study protocol was approved by the Institutional Review Board for Human Subject Research at Baylor College of Medicine (Houston, TX; H-8712 and H-28088).

DNA Extraction

Genomic DNA was previously extracted from peripheral blood, saliva, and frozen or formalin-fixed, paraffin- embedded lung tissue using Gentra Purgene Blood Kit (Qiagen, Germantown, MD), prepIT L2P/PT-L2P kit (DNA GenoTek, Ottawa, ON, Canada), and DNeasy Blood and Tissue Kit (Qiagen), respectively, as described.[20,31] DNA from urine, buccal cells, and hair follicles (family 176) was isolated with Gentra Purgene Blood Kit (Qiagen), prepIT•L2P/PT-L2P kit (DNA GenoTek), and QIAamp DNA Investigator Kit (Qiagen), respectively, according to the manufacturer's instructions.

CNV Deletion Analysis

To study CNV deletion in family 176, array comparative genomic hybridization analysis was performed using customized 16q24.1-specific (1 Mb region flanking *FOXF1*) high-resolution 180K microarray (Agilent Technologies, Santa Clara, CA), as described.[20,23] Deletion junction fragment was amplified by long-range PCR with LA *Taq* DNA polymerase (TaKaRa Bio, Madison, WI), followed by Sanger sequencing.

BDA and qPCR Experiments

To determine the alternate allele fraction (AAF) in parental samples, 17 families (Table 1) were tested using BDA or qPCR using the probands' DNA samples as positive controls. BDA principles were described in detail by Wu et al[35] (2017). The workflow of BDA data analysis is shown in Supplemental Figure S1. Primer and blocker sequences (Table 2) were designed according to the previously developed protocol.[35] To prevent unspecific binding of primers to FOXF2, a highly similar genomic sequence to FOXF1, primers used in BDA experiments were not fully complementary to the FOXF2 and thus have much weaker binding energy to FOXF2. Moreover, the short extension time (30 seconds) prevented amplification of longer, potentially nonspecific amplicons (Supplemental Figure S2). Sanger sequencing of the amplified products further confirmed the specificity of all primers.

The patient, mother, and father genomic DNA samples were tested with blocker (ie, standard BDA) and without blocker (ie, forward and reverse primers only). The qPCR assays were performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA) with 400 nmol/L of each primer, 4 mmol/L of blocker, and 10 ng of DNA per well. For GC-rich amplicons, betaine was added to a final concentration of 1 mol/L (Sigma Aldrich, St. Louis, MO) to reduce template secondary structures (Supplemental Table S1). Reactions were performed in the final volume of 10 mL using CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with incubation at 95_C for 180 seconds, followed by 60 cycles of 95_C for 10 seconds and 60_C for 30 seconds. Each qPCR was repeated at least twice. Change in quantification cycle (DCq) values were calculated for each sample using Cq values obtained in both experiments (with and without blocker). First, the DCq_{samp} was calculated for each sample: DCq_{samp} Z (median with blocker Cq) _ (median no-blocker Cq). All calculated Cq and DCq_{samp} values are shown in Supplemental Table S2. A smaller DCq_{samp} indicates a higher likeliness of the sample containing a mutation. Next, PCR products from two replicated qPCR experiments for the parental sample with smaller DCq_{samp} were purified and Sanger sequenced. Because it is extremely unlikely that both parents carry the same pathogenic variant, the PCR product from the other parent was not sequenced. To avoid false positives caused by Taq polymerase errors, a sample was called as positive when the

variant appeared in both of the duplicate Sanger results. If the presence of alternate allele was confirmed by Sanger sequencing (Figure 2), the qPCR Cq values were used to calculate the AAF. AAF was calculated as follows:

$$\mathrm{AAF} = \frac{50\%}{2^{\Delta Cq_{\mathrm{samp,parent}} - \Delta Cq_{\mathrm{samp,patient}}}} \qquad (1)$$

where $DCq_{samp,parent}$ is the DCq_{samp} of the parent sample with a positive result, and $DCq_{samp,patient}$ is the DCq_{samp} of the corresponding patient sample. Sanger chromatopherograms of negative (no mosaicism detected) parental samples are shown in Supplemental Figure S3. In family 91, calibration experiments were performed with the use of series of dilutions (1%, 0.3%, 0.1%, and 0.03%) of wild-type human genomic DNA (NA18537; Coriell Institute for Medical Research, Camden, NJ) and synthetic double-stranded DNA (gBlocks Gene Fragment; Integrated DNA Technologies, Coralville, IA) bearing the c.294C > A variant. To avoid DNA loss during dilution, the 1_ Tris-EDTA buffer with 10 ng/mL carrier RNA and 0.2% Tween 20 was used for dilution of samples. BDA calibration was performed using the 30 ng of each sample. All experiments were performed in triplicate (or six replicate for 0.03% AAF). A mastermix bulk reaction mixture was made and then split into replicates to decrease the variability due to pipetting error. The assay sensitivity of BDA method was determined as 0.03% (Supplemental Figure S4). DNA samples from family 176were analyzed using standard qPCR without the blocker using the same parameters as those in the BDA experiments. The forward and reverse primers were designed upstream and downstream to the approximately 7-kb deletion, respectively. The amplicon length of the variant template was 167 bp, and the amplicon length on the wild-type template was 7303 bp. qPCR was performed using non-long-range Taq polymerase with short (30 seconds) extension time. Thus, amplicons >1000 bp cannot be amplified (Supplemental Figure S3), and only variant template was detected. Duplicated Sanger sequencing was performed to confirm the correct (167- bp) length of the obtained amplicon. For positive samples, AAF was estimated as follows: amplicons >1000 bp cannot be amplified (Supplemental Figure S3), and only variant template was detected. Duplicated Sanger sequencing was performed to confirm the correct (167-bp) length of the obtained amplicon. For positive samples, AAF was estimated as follows:

$$\mathrm{AAF} = \frac{50\%}{2^{Cq_{\mathrm{median,parent}} - Cq_{\mathrm{median,patient}}}} \qquad (2)$$

where $Cq_{median,parent}$ is the median Cq of the parent sample with a positive result, and $Cq_{median,patient}$ is the median Cq of the corresponding patient sample. Herein, the AAF in a patient is assumed to be 50% and the PCR amplification efficiency for the mutant to be two per cycle in the presence of the blocker, so that the Cq difference between parent and patient can be used to infer AAF in the parent. The assumption about PCR amplification efficiency is consistent with the DCq_{samp} in most patient samples (Supplemental Table S2), although some patient samples showed a high DCq_{samp} value, indicating the PCR yield for some mutations was lower than two with the blocker. However, even in the case of the largest DCq_{samp}, the amplification efficiency per cycle was approximately 1.75, so the above equation for AAF estimation can still be used. To further improve AAF quantitation, the PCR amplification efficiency for each different mutation can be calculated on the basis of DCqsamp values.

ddPCR Assays

To further assess the AAF in parental samples, three families were tested using the probe-based ddPCR (Table 1). The *FOXF1* primers and probes specific to alternate or wild-type allele were designed using droplet digital PCR assays tool (Bio-Rad). To ensure the highest specificity between the mutant and wild-type clusters, the ddPCR assays for each variant were validated and optimized with the use of a temperature gradient and the probands' and wild-type DNA samples (positive and negative controls, respectively), as well as a non-template control. The droplets were classified on the basis of the fluorescence amplitude observed in the positive, negative, and non-template controls. In the clean reaction, there should be no mutant-positive droplets in both negative and non-template control wells. In family 105, additional calibration experiments were performed with the use of a series of dilutions of proband's DNA in the control wild-type DNA (50%, 10%, 5%, 1%, 0.1%, and 0.03%). The cutoff sensitivity of this ddPCR assay was determined as 0.1% (Supplemental Figure S5). The ddPCR experiments were performed using QX200 AutoDG Droplet Digital PCR System (Bio-Rad). Each 20- mL PCR contained 10 mL of 2 ddPCR Supermix for probes (Bio-Rad), 1 mL of custom-designed TaqMan probes and primers mix, and 50 to 100 ng of DNA. In addition, 1 U of *Hind*III (family 123) or *Mse*I (families 85, 105, and 138) restriction enzyme (New England Biolabs, Ipswich, MA) was added to each reaction to perform restriction digestion of DNA samples directly in the ddPCR. After emulsification with Automated Droplet Generator (Bio-Rad), a plate containing ddPCR droplets was transferred to the thermocycler. Samples were denatured at 95C for 600 seconds, followed by 40 cycles of 94C for 30 seconds and 54C (families 85, 123, and 138) or 56 C (family 105) for 60 seconds, and final incubation at 98C for 600 seconds. After thermal cycling, droplets were read with the use of the QX200 Droplet Reader, followed by data analysis with Quantasoft version 1.7

Studio (Bio-Rad). Only samples with total droplets count 13,000 were included in calculations. Each parental sample was run in at least eight repeats.

Results

Mosaic *FOXF1* variants in reportedly unaffected parents were identified in 4 of 18 families studied. Two of these variants were initially detected in the maternal samples (families 91 and 176) using routine molecular testing with Sanger sequencing and CNV deletion-specific PCR, as previously described.[20,31] The AAF of studied variants, determined in parental blood samples using BDA, ddPCR, or qPCR, ranged between 0.03% and 19% (Table 1, Figure 2, Figure 3, and Supplemental Tables S2 and S3).

A heterozygous approximately 7-kb CNV deletion involving *FOXF1* was found in the patient from family 176. The same-sized junction fragment of weaker intensity was identified in the apparently healthy mother. The intertissue AAFs ranged from <0.03% to 0.65%; they were determined at 0.2% in saliva, 0.14% in redrawn saliva, 0.04% in blood, <0.03% in urine, and 0.65% in buccal cells using BDA. In the hair follicles, the deletion could not be detected; ddPCR was not performed (Figure 2D, Table 1, and Supplemental Tables S2 and S3). In the maternal sample of family 91, somatic mosaicism for SNV c.294C>A (p.His98Gln) was initially detected by Sanger sequencing.[31] BDA enabled precise measurement of the variant AAF at 19%; ddPCR was not performed (Figure 2C, Table 1, and Supplemental Table S2). The level of maternal somatic mosaicism of SNV c.539C>A (p.Ser180*) in family 85 was estimated by BDA and ddPCR at 1.5% and 1.0%, respectively (Figure 2A, Table 1, and Supplemental Table S2). In family 105, SNV c.316T>C (p.Phe106Leu) was found in the paternal DNA sample. The variant allele was detected using BDA method, and its ratio was estimated at 0.03% (Figure 2B, Table 1, and Supplemental Table S2). This variant was undetectable by ddPCR method. No evidence of parental mosaicism was found in the remaining 14 ACDMPV families, including family 123 with two children manifesting ACDMPV with the *FOXF1* insertion/deletion c.849_850del (p.Ile285Glnfs*9)[20] (Table 1, Figure 3, and Supplemental Table S2).

Discussion

The incidence of somatic mosaicism varies between different diseases, genes, and type of variants. Studies in several human genetic conditions have shown that the rate of parental somatic mosaicism, explaining a familial recurrence of apparently *de novo* mutations, is higher than previously thought. Low-level (AAF 10%) and very- low level (AAF 1%) parental somatic mosaicism for CNV deletions and SNVs associated with genetic disorders have been detected in 4% and 8% of families, respectively, with AAFs ranging between <9% for CNVs and 0.22% to 6.15% for SNVs.[12,36] Rahbari et al[37] (2016) reported 3.8% of mutations in mosaic state in at least 1% of parental blood cells. Mosaic mutations linked to S*CN1A*-related epilepsy have been identified in 5% of parents, whereas 2% to 3% of patients with vascular Ehlers-Danlos syndrome and pre- sumed *de novo* variants in *COL3A1* could have a parent with low-grade mosaicism.[8,9]

Among ACDMPV families supported by the Alveolar Capillary Dysplasia Association (*https://acdassociation. org*), a nonprofit organization dedicated to increasing ACDMPV awareness, there are a few families with two or more affected siblings and no previous family history, suggesting the possibility of parental mosaicism. However, thus far, only five ACDMPV families with mosaic *FOXF1* variants in parents have been reported.[20,32,33] The real incidence of parental somatic or germline mosaicism, including low-level or very-low level mosaicism, in ACDMPV families remains unknown because methods applied for standard molecular di- agnostics, including Sanger sequencing, are not sensitive enough to detect low percentages of a variant allele and often fail to precisely determine the allelic ratio.[38] More- over, mutational screening is usually limited to one type of tissue (ie, blood or saliva). To overcome the technical diagnostic challenges, new methods are now being implemented for more efficient detection of somatic mosaicism, including BDA.

BDA is a relatively new PCR-based allele enrichment technology that preferably amplifies single-base variants, small insertions, and CNV deletions 1000-fold over wild-type allele, allowing for rare allele quantitation with precision similar to ddPCR, which is considered as a gold standard in rare event detection.[35] BDA does not require any chemically modified oligonucleotides or specialized instruments (only standard qPCR or PCR thermocyclers); thus, it is fast and economical for rare allele detection. It is also compatible with downstream sequence analysis methods (Sanger sequencing or next-generation sequencing) to verify the amplicon sequences. BDA's performance is consistent within an 8 C temperature window of the annealing/extension PCR step; therefore, the optimization of temperature is not required.

Herein, 18 ACDMPV families were retrospectively analyzed, with the previously identified pathogenic or likely pathogenic variants determined by conventional molecular techniques to be apparent *de novo* ($n = 14$), of unknown parental origin ($n = 1$), or inherited from a parent suspected to be somatic and/or germline mosaic ($n = 3$). The use of

BDA, ddPCR, and qPCR methods with higher sensitivity allowed us to characterize parental somatic mosaicism of *FOXF1* variants detected in 4 (22%) of 18 tested ACDMPV families.

Among four families with parental mosaicism, two were tested in parallel with the use of two different high-sensitive techniques. In family 85, the mosaic ratios of *FOXF1* variant measured by BDA and ddPCR were comparable (1.5% and 1%, respectively), indicating that both methods can be used to accurately quantitate low-level mosaicism. However, in family 105, BDA turned out to be more efficient for detection of very-low level mosaicism than ddPCR. Using BDA, the level of parental *FOXF1* mosaicism was determined at 0.03%, whereas it remained undetectable by ddPCR. On the basis of calibration experiments performed for c.316T>C variant in *FOXF1*, the sensitivity cutoff for this particular ddPCR assay was determined as 0.1%. Because the amount of DNA available is the limiting factor for sensitivity of rare allele detection using ddPCR, the possibility that use of more DNA in calibration (proband's DNA) and actual (maternal DNA) experiment could increase the limit of detection cannot be ruled out. However, because of insufficient amount of both proband's and parental DNA samples, further experiments could not be performed.

In family 176, the presence of somatic mosaicism in the mother was detected by junction-specific long-range PCR performed in DNA extracted from saliva and was further confirmed and quantitated in saliva and other tissues by qPCR. Although the level of somatic mosaicism was very low (<0.03% to 0.65%), the presence of CNV deletion in tissues originating from all three germinal layers suggests that it still might have occurred during early embryonic development. The observed variation in very-low level mosaic ratios across tested cells could be a result of many different factors, including tissue-specific selection effects.[4,14]

Family 123, in which two siblings had *FOXF1* frameshift variant and died of ACDMPV 6 years apart,[20] originally screened with the use of Sanger sequencing, was now tested with ddPCR. Neither ddPCR nor Sanger screening of *FOXF1* has detected the corresponding pathogenic *FOXF1* variant allele in the maternal blood sample, suggesting that germline maternal mosaicism or extremely low-level maternal somatic mosaicism is the most plausible cause of the unusual recurrence of ACDMPV in this family.

Although the use of BDA and ddPCR allowed us to detect parental mosaicism of *FOXF1* variants in 22% fam- ilies, the real frequency of mosaicism could be still under- recognized because of technical limitations. For example, high GC content of exon 1 of *FOXF1* precluded testing *FOXF1* mutations in some ACDMPV families.

In conclusion, this research proposes that parents of children with ACDMPV who are found negative for *FOXF1* variants by the routine detection techniques (eg, Sanger sequencing or array comparative genomic hybridization) may benefit from reanalyses using more sensitive and quantitative methods, including BDA or ddPCR. These techniques are shown to be efficient tools for the detection of low-level (ddPCR) or even very-low level (BDA) parental somatic mosaicism for both SNVs and CNVs. However, given that in most cases only one type of parental tissue was available for screening, the real frequency of mosaic variants may be underestimated. Data from this study further demonstrate the need for a systematic screening of parental samples for somatic mosaicism, particularly in families in whom more than one affected carrier of the same variant was observed.[12]

Figures

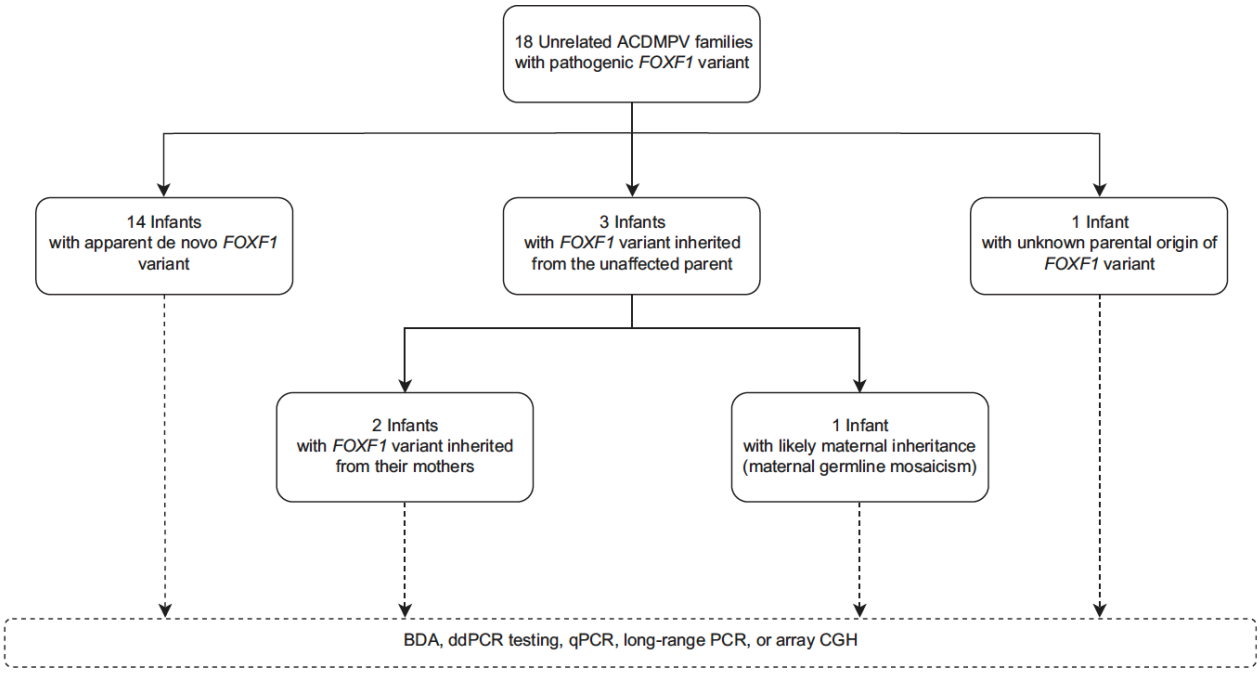

Figure 1 Alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV) families with *FOXF1* variants included in this study. To precisely determine the frequency of variant allele in parental DNA, 18 families with *FOXF1* variants previously indicated to be *de novo*, of unknown parental origin, or inherited were tested using blocker displacement amplification (BDA), droplet digital PCR (ddPCR), quantitative PCR (qPCR), long-range PCR, and array comparative genomic hybridization (array CGH). Flowchart was generated using *https://www.draw.io* (last accessed December 16, 2019). $n = 14$ (*de novo* variants); $n = 1$ (variant of unknown parental origin); $n = 3$ (inherited variants).

Table 1 The List of Studied Families with Distribution and Parental Origin of *FOXF1* Pathogenic Variants

Family ID	DNA variant	Protein variant	Parental origin of *FOXF1* pathogenic variants				
			Sanger sequencing (original detection method)	BDA or qPCR	% Of variant allele detected in parent (type of tissue)	ddPCR	% Of variant allele detected in parent (type of tissue)
2	c.225C>A	p.Tyr75*	*De novo*	*De novo*	0.0	N/A	N/A
46	c.1031_1032del	p.Phe344Cysfs*66	*De novo*	*De novo*	0.0	N/A	N/A
48	c.1138T>C	p.*380Argext*73	*De novo*	*De novo*	0.0	N/A	N/A
55	c.145C>T	p.Pro49Ser	*De novo*	*De novo*	0.0	N/A	N/A
56	c.89C>A	p.Ser30*	*De novo*	*De novo*	0.0	N/A	N/A
61	c.872_879del	p.Ser291*	*De novo*	*De novo*	0.0	N/A	N/A
66	c.899_903dup	p.Gly302Cysfs*79	*De novo*	*De novo*	0.0	N/A	N/A
67	c.191C>A	p.Ser64*	*De novo*	*De novo*	0.0	N/A	N/A
69	c.691_698del	p.Ala231Argfs*61	*De novo*	*De novo*	0.0	N/A	N/A
76	c.1139G>C	p.*380Serext*73	*De novo*	*De novo*	0.0	N/A	N/A
83	c.221T>A	p.Ile74Asn	*De novo*	*De novo*	0.0	N/A	N/A
85	**c.539C>A**	**p.Ser180***	***De novo***	**Maternal**	**1.5 (Blood)**	**Maternal**	**1.0 (Blood)**
91	**c.294C>A**	**p.His98Gln**	**Maternal**	**Maternal**	**19.0 (Blood)**	**N/A**	**N/A**
101	c.862C>T	p.Gln288*	*De novo*	*De novo*	0.0	N/A	N/A
105	**c.316T>C**	**p.Phe106Leu**	**Unknown**	**Paternal**	**0.03 (Blood)**	**Mosaicism not confirmed**	**N/A**
123	c.849_850del	p.Ile285Glnfs*9	Probably maternal	N/A	N/A	Probably maternal germline mosaicism	0.0 (Blood)
176	**7-kb CNV deletion involving *FOXF1* chr16:86,542,131-86,549,266(hg19)**		**Maternal**	**Maternal**	**0.2 (Saliva) 0.14 (Redrawn saliva) 0.04 (Blood) <0.03 (Urine) 0.65 (Buccal)**	**N/A**	**N/A**
182	c.145C>G	p.Pro49Ala	*De novo*	*De novo*	0.0	N/A	N/A

Families with parental somatic mosaicism determined by BDA, ddPCR, or qPCR are in bold. The array comparative genomic hybridization data were deposited in the dbVar database (*https://www.ncbi.nlm.nih.gov/dbvar*, accession number nstd178). The sequence variant data were submitted to the ClinVar database (*https://www.ncbi.nlm.nih.gov/clinvar*, submission ID SUB6388016, accessions numbers SCV001055831 to SCV001055847).

BDA, blocker displacement amplification; chr, chromosome; ddPCR, droplet digital PCR; ID, identifier; N/A, not available (not tested); qPCR, quantitative PCR.

Table 2 BDA and qPCR Oligonucleotide Sequences

Family ID	Name	Nucleotide sequence
2	Forward primer	5′-GCCTGACGCTGAGCGAGATCTA-3′
	Reverse primer	5′-AAGGCCCTTGGGTAGCTTGATG-3′
	Blocker	5′-AGCGAGATCTACCAGTTCCTGCAGAGCCaaaa-3′
46	Forward primer	5′-CCCAGCATGTGTGACCGAAA-3′
	Reverse primer	5′-GCAGCCTCACATCACGCAA-3′
	Blocker	5′-TGACCGAAAGGAGTTTGTCTTCTCTTTCAACaaat-3′
48	Forward primer	5′-TCACCTACCAAGACATCAAGCC-3′
	Reverse primer	5′-CGACGGTTATACCTCGAGAAGAAAG-3′
	Blocker	5′-ATCAAGCCTTGCGTGATGTGAGGCaaaa-3′
55	Forward primer	5′-CCGGCGCCCGGAGAA-3′
	Reverse primer	5′-GGTAGGAGCCCCGGAAGAAG-3′
	Blocker	5′-CGGAGAAGCCGCCCTATTCCTACAaaaa-3′
56	Forward primer	5′-CGGCCATGGACCCCGC-3′
	Reverse primer	5′-GCGCTTGGTGGGTGAACTCT-3′
	Blocker	5′-CCCGCGTCGTCCGGCCCGaaat-3′
61	Forward primer	5′-GGCGCCTCTTATATCAAGCAGCA-3′
	Reverse primer	5′-GGCGTTGTGGCTGTTCTGGT-3′
	Blocker	5′-AAGCAGCAGCCCCTGTCCCCCTaatt-3′
66	Forward primer	5′-CCTGTAACCCCGCGGCCA-3′
	Reverse primer	5′-CGGCCTCCCCACTCACCTT-3′
	Blocker	5′-CGGCCAACCCCCTGTCCGGCAaaaa-3′
67	Forward primer	5′-CGCGCTCATCGTCATGGC-3′
	Reverse primer	5′-GGTAGGAGCCCCGGAAGAA-3′
	Blocker	5′-GTCATGGCCATCCAGAGTTCACCCACatta-3′
69	Forward primer	5′-ACATGGGCGGCTGCGG-3′
	Reverse primer	5′-CGCCGAGCCCGAGTAGAC-3′
	Blocker	5′-CTGCGGCGGCGCGGCGaaaa-3′
76	Forward primer	5′-GTCACCTACCAAGACATCAAGCCT-3′
	Reverse primer	5′-GACGGTTATACCTCGAGAAGAAAGCA-3′
	Blocker	5′-ATCAAGCCTTGCGTGATGTGAGGCTGaaaa-3′
83	Forward primer	5′-ACCCACCAAGCGCCTGAC-3′
	Reverse primer	5′-AAGGCCCTTGGGTAGCTTGATG-3′
	Blocker	5′-GCCTGACGCTGAGCGAGATCTACCAaaaa-3′
85	Forward primer	5′-GGGCTCGGCCGGCG-3′
	Reverse primer	5′-CGTTGGAAGGCAGGTGGGG-3′
	Blocker	5′-CGGCGGCCTCTCGTGCCCGaaat-3′
91	Forward primer	5′-AGGGCTGGAAGAACTCCGT-3′
	Reverse primer	5′-CTCCTCGAACATGAACTCGCT-3′
	Blocker	5′-AACTCCGTGCGCCACAACCTCTaaaa-3′
101	Forward primer	5′-CAACAGCGGCGCCTCTTATATCA
	Reverse primer	5′-GGCGTTGTGGCTGTTCTGGT-3′
	Blocker	5′-GCCTCTTATATCAAGCAGCAGCCCCTGTaaaa-3′
105	Forward primer	5′-CCTCTCGCTCAACGAGTGC-3′
	Reverse primer	5′-TGGCATTTCCTTCGGAAGCC-3′
	Blocker	5′-CGAGTGCTTCATCAAGCTACCCAAGGaaaa-3′
176	Forward primer	5′-GCCTCTCGCCCCAGCTC-3′
	Reverse primer	5′-CGCAGTTGGGTTTCTCCTAATCA-3′
	Blocker	None
182	Forward primer	5′-CCGGCGCCCGGAGAAG-3′
	Reverse primer	5′-CTGGTAGGAGCCCCGGAAGAA-3′
	Blocker	5′-CGGAGAAGCCGCCCTATTCCTACATCGaaaa-3′

Primers and blockers are standard desalted DNA oligonucleotides with no chemical modification. Four lowercase bases at the -3′ of the blocker do not match the template, thus preventing the blocker oligonucleotide from being extended by the polymerase.

BDA, blocker displacement amplification; ID, identifier; qPCR, quantitative PCR.

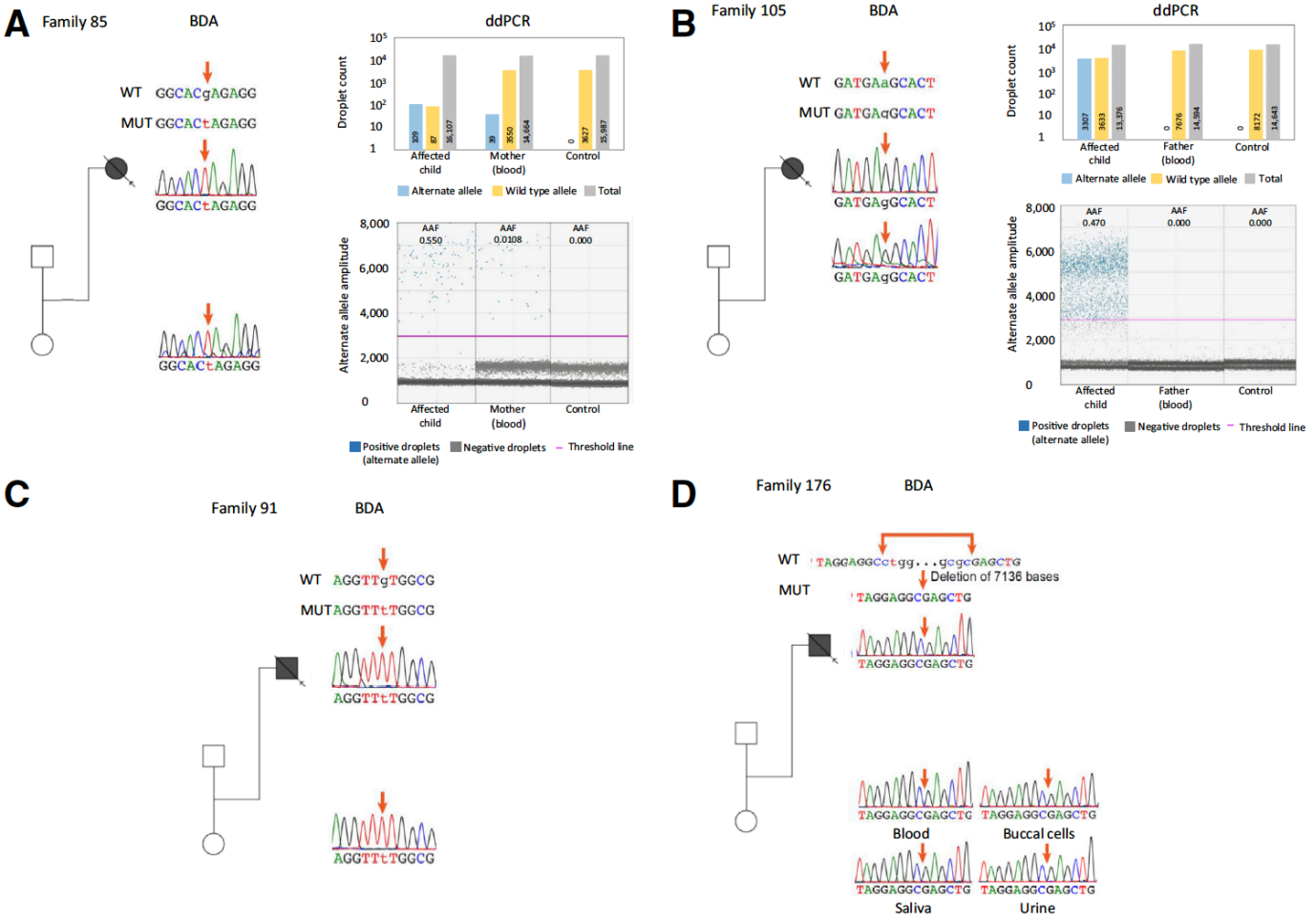

Figure 2 Four families with detected *FOXF1* somatic mosaicism. **A** and **B:** Pedigrees of families 85 (**A**) and 105 (**B**) with chromatopherograms of quantitative PCR (qPCR) Sanger sequencing [blocker displacement amplification (BDA)] and absolute quantification of the allele abundance [droplet digital PCR (ddPCR)]. Chromatopherograms display presence of alternate allele in maternal (**A**) and paternal (**B**) samples. The **upper** ddPCR plots show the examples of droplet counts positive for the alternate (**blue bars**) and wild-type (WT; **yellow bars**) allele and the total droplet counts (**gray bars**) in single reactions. The number of droplets is presented in a logarithmic scale. The **lower** ddPCR plots present alternate allele fraction (AAF) and example of the one-dimensional fluorescence amplitude plot of droplets for alternate allele detection. The positive droplets (blue **dots**), containing the alternate allele, exhibit increased fluorescence compared with negative droplets (**dark gray dots**). The threshold line dividing positive and negative droplets is presented (**pink lines**). **C:** Pedigree of family 91 with chromatopherograms of qPCR Sanger sequencing (BDA) showing presence of alternate allele in maternal sample. **D:** Pedigree of family 176 with chromatopherograms of Sanger sequencing showing presence of alternate variant in maternal blood, saliva, buccal cells, and urine. The **red arrows** indicate the analyzed variant of interest. MUT, mutation.

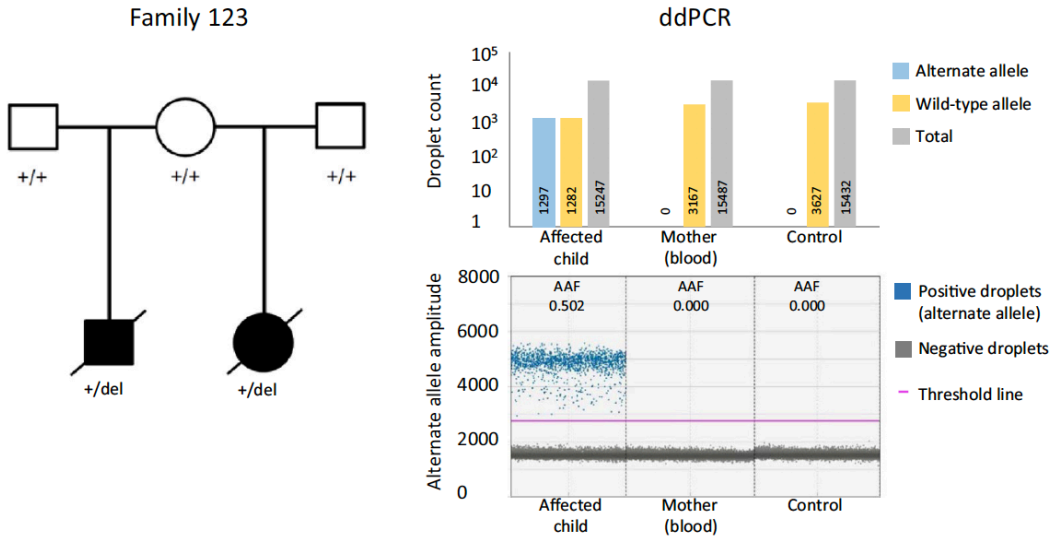

Figure 3 Pedigree of family 123 suspected for maternal germline mosaicism with absolute quantification of the allele abundance by droplet digital PCR (ddPCR) assay showing no evidence of the alternate allele in the maternal blood sample. The **upper** ddPCR plot shows the example of droplet counts positive for the alternate (**blue bars**) and wild-type (**yellow bars**) allele and the total droplet counts (**gray bars**) in single reaction. The number of droplets is presented in a logarithmic scale. The **lower** ddPCR plot presents alternate allele fraction (AAF) and example of the one-dimensional fluorescence amplitude plot of droplets for alternate allele detection. The positive droplets (**blue dots**), containing the alternate allele, exhibit increased fluorescence compared with negative droplets (**dark gray dots**). The threshold line dividing positive and negative droplets is presented (**pink line**).

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Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.jmoldx.2019.12.007*.

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