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ARTICLE

Co-existence of 2 clinically significant variants causing disorders of somatic mosaicism

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ABSTRACT

Purpose: Disorders of somatic mosaicism (DoSM) are a heterogeneous group of conditions caused by postzygotic variants in genes within the PI3K/AKT/mTOR and RAS/MAPK signaling pathway. The co-existence of 2 activating variants in this disease group is extremely rare.
Methods: A deep sequencing next-generation sequencing assay for the molecular diagnosis of DoSM was run on 936 individuals with DoSM.
Results: A single pathogenic or likely pathogenic (P/LP) variant was identified in 584 of 617 (94.8%) positive cases; 33 of 617 (5.2%) cases carried 2 P/LP variants. Of these 33 cases, 22 carried 2 P/LP variants in the same gene, including 8 associated with a loss-of-function disease mechanism and 14 associated with a gain-of-function disease mechanism. Eleven cases had P/LP variants in 2 different genes, including *PIKC3A* variants in 7 cases and 4 cases with 2 P/LP variants in non-*PIK3CA* genes.
Conclusion: To our knowledge, this is the largest cohort with the co-existence of 2 P/LP somatic variants causing DoSM. The study of the co-existence of 2 clinically significant variants in DoSM requires unique considerations regarding variant allelic fractions, the combination of variants, affected tissue types, and the severity of the disease. Investigations into this unique cohort may further our understanding of the disease mechanism and potential therapeutic options.

Introduction

Disorders of somatic mosaicism (DoSM) is a heterogeneous group of malformations caused by mosaic genetic alterations. The clinical manifestations of this disease group are highly variable and heterogeneous, depending on the function of the altered genes, allelic diversity, and when and where the alterations occurred during development. These characteristics overlap the features found in different conditions. Therefore, genetic testing plays a significant role in

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making a molecular diagnosis as well as identifying therapeutic targets. Next-generation sequencing (NGS) has become an important method of genetic testing to improve the molecular diagnosis of genetic disorders. Because of the nature of mosaic genetic alterations in DoSM, high-sensitivity NGS testing plays a significant role in increasing the diagnostic yield with its capability of detecting mosaic genetic alterations with relatively low variant allele fractions (VAFs).

The genetic alterations observed in DoSM usually affect the function and regulation of key genes in cell survival and proliferation pathways, mainly in 2 intracellular signaling pathways: the PI3K/AKT/mTOR and RAS/RAF pathways, both of which also play significant roles in the setting of cancer. Several activating variants in the key genes are associated with both congenital malformations and oncogenesis. For example, activating *PIK3CA* variants are associated with both breast cancer and *PIK3CA*-related overgrowth spectrum (PROS), and activating *AKT1* variants are associated with both non–small cell lung cancer and Proteus syndrome.[1] The RAS/RAF pathway is often referred to as the proliferation pathway. Germline variants in its key players are associated with a group of congenital disorders known as RASopathies. Mosaic variants in *RAS* genes (*HRAS*, *KRAS*, and *NRAS*) that result in constitutive RAS signaling are widely observed in cancer, whereas they have been recently described in DoSM.[2]

DoSM are malformation syndromes that share overlapping genetic alterations with cancer.[3] Unlike cancer, which is known to be caused primarily by multiple genetic alterations, most known congenital genetic conditions are caused by single-gene events.[4] There are no reports to date studying the evaluation of possible gene combinations in this unique disease group. Here, we review the 7 years' experience in the Clinical Genetics and Genomics Laboratory at WUSM, during which 936 individuals with DoSM were tested using a high-depth NGS gene panel to identify the individuals with 2 pathogenic or likely pathogenic variants in this cohort.

Materials and Methods

Samples from 936 individuals with DoSM as the indication for testing were submitted to the Clinical Genetics and Genomics Laboratory at Washington University in St. Louis between October 2013 and July 2021. DNA extraction was performed on specimens from affected tissues, including fresh tissues, formalin-fixed paraffin-embedded tissue, cultured fibroblasts, and buccal swab specimens. Target enrichment was performed using oligonucleotide-based targeted capture of exonic regions of up to 37 genes known to underlie DoSM. Sequencing of enriched libraries was performed in multiplex, using the paired-end, ~150-bp configuration with an average unique on-target coverage depth of ~2000×. Peripheral blood was obtained as an unaffected comparator alongside the primary affected tissue for 23 of the 33 individuals with clinically significant findings. In those cases, peripheral blood was submitted to a clinical reference laboratory for confirmation of the variant of interest by Sanger sequencing. Detection of the variant in peripheral blood indicates either a multitissue distribution of a mosaic alteration or that the variant has a constitutional origin, whereas the absence of the variant in peripheral blood is taken as indicating mosaicism. The detailed descriptions of the DNA isolation, library preparation, sequencing, bioinformatics analysis, and variant interpretation have been described in our previous publication.[5] Of note, tissue-profiling NGS assays, such as the assay used for a majority of cases in this study, are not capable of determining whether discrete variants are present in the same cells, or different cells (or cell types), within affected tissues.

Abbreviations

DoSM – Disorders of Somatic Mosaicism
NGS – Next-generation sequencing
P/LP – Pathogenic or likely pathogenic
PROS – *PIK3CA*-related overgrowth spectrum

Results

In the cohort of 936 individuals with DoSM, 617 (65.9%) tested positive, with at least 1 clinically significant, pathogenic or likely pathogenic (P/LP) variant in the submitted specimen. A single clinically significant variant was identified in 584 of 617 (94.8%) cases, whereas 33 of 617 (5.2%) cases carried 2 clinically significant variants in the submitted specimens, including 8 cases with 2 P/LP variants in the same gene associated with a loss-of-function (LOF) disease mechanism (Table 1), 14 cases with 2 P/LP variants in the same gene associated with a gain-of-function (GOF) disease mechanism (Table 2), and 11 cases with P/LP variants in 2 different genes (Tables 3 and 4, Figure 1). All the P/LP variants were classified based on American College of Medical Genetics and Genomics (ACMG) 2015 guideline, and the detailed variant information, including nomenclature and classification, can be found in Supplemental Table 1.

Two LOF clinically significant variants in the same gene

We identified 8 cases with 2 clinically significant variants detected in the same gene associated with an LOF disease mechanism, including 3 cases with variants in *RASA1* (OMIM 139150), 2 with variants in *PTEN* (OMIM 601728), 2 with variants in *NF1* (OMIM 613113), and 1 with variants in *TSC1* (OMIM 605284) (Table 1). Germline LOF variants in *RASA1* are associated with an autosomal dominant capillary malformation–arteriovenous malformation (CM-AVM; OMIM 608354). A second somatic clinically significant variant of *RASA1* has been reported in vascular endothelial

Table 1 Eight cases were identified with 2 clinically significant variants in the same gene associated with the loss-of-function disease mechanism

Case ID	Age/ Gender	Clinical Presentation	Tissue for Sequencing	Gene	Variant	Protein Change	Variant Type	VAF (%)	Detected in Blood?	Allele Ratio
1-a	5 mo/F	Capillary malformation and minimal overgrowth	Fresh-tissue biopsy from the left foot, plantar surface with capillary malformation	*RASA1*	NM_002890.3:c.1328_1331dup	p.(Gln444Hisfs*10)	Frameshift	4.00	N	11.5
					NM_002890.3:c.2920del	p.(Asn976Metfs*20)	Frameshift	46.0	Y	
1-b	2 y/F	Capillary malformation, possible CM-AVM	Fresh-tissue biopsy from right foot	*RASA1*	NM_002890.3:c.2603+2dup	NA	Splice site	4.25	N	10.8
					NM_002890.3:c.599del	p.(Lys200Serfs*7)	Frameshift	46.0	Y	
1-c	2 y/M	Capillary malformation and segmental overgrowth	FFPE >80% affected tissue from the AVM region on the back	*RASA1*	NM_002890.3:c.934_938del	p.(Glu312Argfs*14)	Frameshift	2.84	N	1.1
					NM_002890.3:c.2925+1del	NA	Splice site	2.99	N	
1-d	9 y/F	Congenital vascular malformation with predominant venulocapillary component within prominent fibroadipose tissue	FFPE >90% affected tissue	*PTEN*	NM_000314.8:c.388del	p.(Arg130Glufs*4)	Frameshift	2.20	N	20.9
					NM_000314.8:c.723dup	p.(Glu242*)	Nonsense	46.0	Y	
1-e	13 y/F	AVM of skin on the back and FAVA on the left thigh	FFPE >80% affected tissue from the AVM region on the back	*PTEN*	NM_000314.8:c.133del	p.(Val45Tyrfs*9)	Frameshift	2.20	N	21.8
					NM_000314.8:c.569del	p.(Pro190Glnfs*9)	Frameshift	48.0	Y	
1-f	76 y/F	Recent onset eruptive neurofibromas, no other symptoms of neurofibromatosis, history of breast/ ovarian/bladder cancer	FFPE skin punch biopsy from the right upper inner breast	*NF1*	NM_000267.3:c.5439_5440inv	p.(Gln1814*)	Nonsense	11.9	NA	1.1
					NM_000267.3:c.6652C>T	p.(Gln2218*)	Nonsense	13.6	NA	
1-g	6 y/F	Hyperpigmentation, plexiform neurofibroma in the neck, no family history of neurofibromatosis	Fresh tissue from the neck	*NF1*	NM_000267.3:c.4537C>T	p.(Arg1513*)	Nonsense	12.6	N	1.1
					NM_000267.3:c.7846C>T	p.(Arg2616*)	Nonsense	13.3	N	
1-h	2 y/M	Hemihypertrophy and port-wine stain	Fresh tissue from the left forearm	*TSC1*	NM_000368.5:c.901_902del	p.(Gln301Glufs*2)	Frameshift	3.62	N	12.9
					NM_000368.5:c.441del	p.(Lys148Asnfs*19)	Frameshift	47.0	Y	

AVM, arteriovenous malformation; *CM*, capillary malformation; *F*, female; *FAVA*, fibroadipose vascular anomaly; *FFPE*, formalin-fixed paraffin-embedded; *M*, male; *N*, no; *NA*, not applicable; *VAF*, variable allele fraction; *Y*, yes.

Table 2 Fourteen cases were identified with 2 clinically significant variants in the same gene associated with the gain-of-function disease mechanism

Case ID	Age/ Gender	Clinical Presentation	Tissue for Sequencing	Gene	Variant	Protein Change	Variant Type	VAF (%)	Detected in Blood?	Allele Ratio	Phase[a]
2-a	30 y/M	Arteriovenous malformation of left upper limbs	Fresh tissue	*TEK*	NM_000459.5: c.2690A>G	p.(Tyr897Cys)	Missense	2.60	N	2.54	*In cis*
					NM_000459.5: c.2752C>T	p.(Arg918Cys)	Missense	6.60	N		
2-b	17 y/F	Soft tissue mass left lateral distal thigh, excision, venous malformation	FFPE	*TEK*	NM_000459.5: c.2740C>T	p.(Leu914Phe)	Missense	2.10	NA	1.26	Unknown
					NM_000459.5: c.2545C>T	p.(Arg849Trp)	Missense	2.64	NA		
2-c	11 y/F	Capillary malformation and vascular anomaly with mild overgrowth	Fresh tissue	*TEK*	NM_000459.5: c.2743C>T	p.(Arg915Cys)	Missense	1.44	N	1.41	*In cis*
					NM_000459.5: c.2689T>A	p.(Tyr897Asn)	Missense	2.03	N		
2-d	13 y/F	Vascular malformation, predominantly venous	FFPE biopsied from right shoulder	*TEK*	NM_000459.5: c.3336_3337del	p.(Tyr1113 Cysfs*4)	Frameshift	1.85	NA	19.68	Unknown
					NM_000459.5: c.2545C>T	p.(Arg849Trp)	Missense	36.4	NA		
2-e	5 y/M	Right-sided congenital vascular malformation causing upper and lower extremity asymmetry	FFPE specimen from the skin and soft tissue of the right neck	*TEK*	NM_000459.5: c.3339T>A	p.(Tyr1113*)	Frameshift	16.7	NA	1.06	Unknown
					NM_000459.5: c.2545C>T	p.(Arg849Trp)	Missense	17.7	NA		
2-f	11 mo/M	Venous malformation of the lower lip, vascular birthmarks, seizures, developmental delay	FFPE from the lower lip	*TEK*	NM_000459.5: c.2753G>A	p.(Arg918His)	Missense	3.00	N	14.43	Unknown
					NM_000459.5: c.2545C>T	p.(Arg849Trp)	Missense	43.3	Y		
2-g	10 y/M	Congenital vascular hamartomas, including predominate venous malformation with possible lymphatic components affecting the buttock	FFPE tissue of venous malformation of the buttock	*TEK*	NM_000459.5: c.3339del	p.(Tyr1113*)	Frameshift	11.0	NA	4.42	Unknown
					NM_000459.5: c.2545C>T	p.(Arg849Trp)	Missense	48.6	NA		
2-h	12 y/F	Multifocal vascular malformations, recurrent bleeding from a vascular malformation of the jejunum, segmental bowel resection, possible blue rubber bleb nevus syndrome	FFPE specimen of the jejunal vascular malformation	*TEK*	NM_000459.5: c.2690A>G	p.(Tyr897Cys)	Missense	4.80	N	5.96	*In cis*
					NM_000459.5: c.2753G>A	p.(Arg918His)	Missense	28.6	Y		
2-i	6 y/F	Vascular malformation, possible blue rubber bleb nevus syndrome	FFPE specimen of the right shoulder with vascular malformation	*TEK*	NM_000459.5: c.2752C>T	p.(Arg918Cys)	Missense	13.8	NA	1.06	*In cis*
					NM_000459.5: c.2690A>T	p.(Tyr897Phe)	Missense	14.6	NA		
2-j	3 y/F	Congenital vascular malformation	Fresh frozen tissue specimen from skin biopsy	*TEK*	NM_000459.5: c.3327_3328del	p.(Lys1110 Valfs*7)	Frameshift	1.07	N	1.46	*In cis*
					NM_000459.5:c.3314C>A	p.(Thr1105Asn)	Missense	1.56	N		
2-k	14 y/M	Multiple vascular malformations and telangiectasias	FFPE tissue further on left upper abdomen with venous malformation	*TEK*	NM_000459.5:c.2689T>C	p.(Tyr897His)	Missense	5.90	NA	1.64	*In cis*
					NM_000459.5:c.2753G>A	p.(Arg918His)	Missense	9.70	NA		

(continued)

Table 2 Continued

Case ID	Age/ Gender	Clinical Presentation	Tissue for Sequencing	Gene	Variant	Protein Change	Variant Type	VAF (%)	Detected in Blood?	Allele Ratio	Phase[a]
2-l	12 y/M	Lymphangioma circumscriptum of the left flank, sacral agenesis, left leg length discrepancy, clubfoot, constipation, unequal kidney size, autism, ADHD, generalized anxiety disorder, microcystic lymphatic malformation with macrocystic component. nevus on nape	Fresh tissue specimen of hemangioma of left chest	*PIK3CA*	NM_006218.4:c.1624G>A NM_006218.4:c.1636C>A	p.(Glu542Lys) p.(Gln546Lys)	Missense Missense	1.25 2.06	N N	1.65	Not *in cis*
2-m	31 y/F	Hemangioma of the tongue, with extensive cautery, hemorrhage, fibrosis, and acute inflammation	FFPE sample of the excised lesion	*PIK3CA*	NM_006218.4:c.263G>A NM_006218.4:c.3140A>G	p.(Arg88Gln) p.(His1047Arg)	Missense Missense	2.09 11.5	NA NA	5.52	Unknown
2-n	18 y/F	Arteriovenous malformation	Fresh tissue from the affected skin	*KRAS*	NM_004985.5:c.34G>A NM_004985.5:c.65A>G	p.(Gly12Ser) p.(Gln22Arg)	Missense Missense	1.98 2.16	N N	1.09	Not *in cis*

ADHD, attention-deficit hyperactivity disorder; *F*, female; *FFPE*, formalin-fixed paraffin-embedded; *M*, male; *N*, no; *NA*, not applicable; *NGS*, next-generation sequencing; *VAF*, variant allele fraction; *Y*, yes.

[a]Phase: *in cis* indicates that the 2 variants were observed on the same NGS sequencing read. Not *in cis* indicates that the 2 variants were observed on different NGS sequencing reads. As those are somatic variants, they could be either *in trans* in the same cell or in different cell populations. Unknown indicates that the 2 variants are located too far to be able to observed on the same NGS sequencing read. Phase remains unclear.

Table 3 Seven cases were identified with 2 clinically significant variants in 2 different genes (*PIK3CA* + *X*)

Case ID	Age/ Gender	Clinical Presentation	Tissue for Sequencing	Gene	Variant	Protein Change	Variant Type	VAF (%)	Detected in Blood?	Allele Ratio
3-a	33 y/F	Clinical diagnosis of Proteus syndrome	FFPE specimen: right thigh - dysplastic melanocytic nevus	*PIK3CA* *BRAF*	NM_006218.4:c.1633G>A NM_004333.6:c.1799T>A	p.(Glu545Lys) p.(Val600Glu)	Missense Missense	0.92 5.10	N N	5.57
3-b	12 y/M	Rapidly involuting congenital hemangioma	Fresh frozen tissue specimen from a right adrenal mass	*PIK3CA* *GNAQ*	NM_006218.4:c.2908G>A NM_002072.5:c.626A>C	p.(Glu970Lys) p.(Gln209Pro)	Missense Missense	35.7 10.3	N N	3.47
3-c	35 y/F	Venous vascular malformation, clinical history of a port-wine stain	FFPE tissue from the brain's right temporal	*PIK3CA* *GNAQ*	NM_006218.4:c.3073A>G NM_002072.5:c.626A>G	p.(Thr1025Ala) p.(Gln209Arg)	Missense Missense	2.23 11.2	NA NA	5.03
3-d	8 mo/M	Capillary malformation, soft tissue hypertrophy, right inner thigh mass, overgrowth, vascular anomaly	Fresh tissue from the right inner thigh	*PIK3CA* *GNAQ*	NM_006218.4:c.2176G>A NM_002072.5:c.626A>C	p.(Glu726Lys) p.(Gln209Pro)	Missense Missense	20.6 2.67	Y, mosaic N	7.72
3-e	4 y/M	Diffuse capillary malformation and overgrowth of the leg, angiokeratoma	FFPE biopsy from the skin of the left posterior thigh	*PIK3CA* *KRAS*	NM_006218.4:c.3140A>G NM_004985.5:c.35G>A	p.(His1047Arg) p.(Gly12Asp)	Missense Missense	2.78 1.77	N N	1.57
3-f	10 mo/M	Congenital malformation syndromes involving early overgrowth/port-wine stain	Fresh tissue specimen from right leg	*PIK3CA* *RASA1*	NM_006218.4:c.1634A>G NM_002890.3:c.2739del	p.(Glu545Gly) p.(Met914Cysfs*17)	Missense Frameshift	6.10 2.08	N N	2.93
3-g	10 y/F	Congenital malformation of the peripheral vascular system	Fresh tissue specimen	*PIK3CA* *TEK*	NM_006218.4:c.2740G>A NM_000459.5: c.3340_3347del	p.(Gly914Arg) p.(Ala1114*)	Missense Frameshift	8.00 1.93	Y, mosaic N	4.15

F, female; *FFPE*, formalin-fixed paraffin-embedded; *M*, male; *N*, no; *NA*, not applicable; *VAF*, variant allele fraction; *Y*, yes.

Table 4 Four cases were identified with 2 clinically significant variants in 2 non-*PIK3CA* genes

Case ID	Age/ Gender	Clinical Presentation	Tissue for Sequencing	Gene	Variant	Protein Change	Variant Type	VAF (%)	Detected in Blood?	Allele Ratio
4-a	16 y/M	Cafe au lait spots, congenital facial asymmetry, capillary malformation involving left scapula and left arm, nevus spilus on back, right arm with junctional nevi and epidermal nevus, scalp and neck with a linear reddish rough alopecic patch of left temporal scalp	Fresh tissue from nevus of right forearm	*BRAF*	NM_004333.6: c.1780G>A	p.(Asp594Asn)	Missense	4.80	N	2.36
				NRAS	NM_002524.5: c.179G>A	p.(Gly60Glu)	Missense	2.03	N	
4-b	16 y/M	Capillary venous lymphatic malformation	FFPE tissue from right dorsal foot	*PIK3R1*	NM_001242466.2:c.601A>G	p.(Asn201Asp)	Missense	13.0	NA	1.29
				MTOR	NM_004958.4: c.7280T>G	p.(Leu2427Arg)	Missense	10.1	NA	
4-c	14 y/F	Clinical diagnosis of Klippel-Trenaunay syndrome, venous malformation, vascular malformation, features of arteriovenous malformation	FFPE specimen from affected left foot	*IDH1*	NM_005896.4: c.394C>T	p.(Arg132Cys)	Missense	9.70	N	1.53
				PIK3R1	NM_001242466.2:c.266_276delinsTTCAAGAAAAAAGTTTCTTGAAA	p.(Tyr89_Gln92delinsPheGlnGluLysSerPheLeuLys)	In-frame delins	6.36	N	
4-d	34 y/F	Arteriovenous malformation of the right upper arm	Fresh tissue from the affected right arm	*RASA1*	NM_002890.3: c.2035C>T	p.(Arg679*)	Missense	16.0	N	4.00
				PDGFRB	NM_002609.4: c.2548G>T	p.(Asp850Tyr)	Missense	4.00	N	

F, female; *FFPE*, formalin-fixed paraffin-embedded; *M*, male; *N*, no; *NA*, not applicable; *VAF*, variant allele frequency; *Y*, yes.

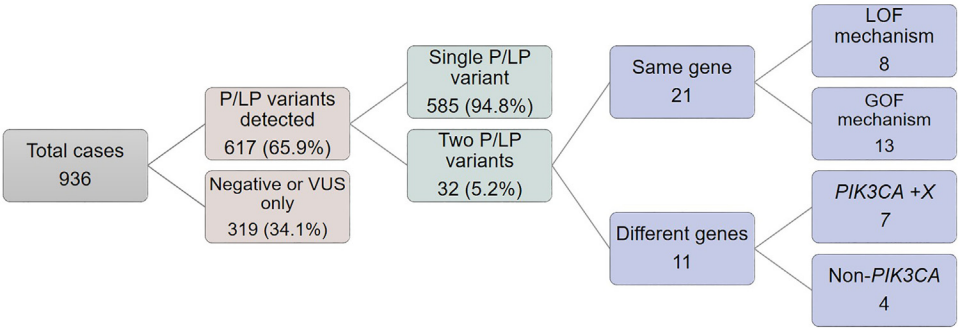

Figure 1 Genetic results summary of all the 936 cases with DoSM. In the cohort of 936 individuals with DoSM, 617 (65.9%) were reported with at least 1 clinically significant variant in the submitted specimen. A single clinically significant variant was identified in 584 of 617 (94.8%) cases, whereas 33 of 617 (5.2%) cases carried 2 clinically significant variants in the submitted specimens. Of the 33 cases with 2 clinically significant variants, 22 cases were identified with 2 clinically significant variants in the same gene, including 8 cases associated with LOF disease mechanism and 14 cases associated with GOF disease mechanism. The remaining 11 cases with 2 clinically significant variants in 2 different genes, including PIKC3A variants observed in 7 cases, and the remaining 4 cases with 2 clinically significant variants in non-*PIK3CA* genes only. DoSM, disorders of somatic mosaicism; GOF, gain of function; LOF, loss of function.

cells in CM-AVM, indicating a 2-hit mechanism of disease.[6,7] All 3 individuals with 2 clinically significant variants in *RASA1* were ≤2 years of age and had capillary malformations. The type 2 segmental mosaicism, a combination of an initial germline mutational event and a second somatic event, was observed in 2 of the 3 cases, with the germline variants detected in peripheral blood specimens by Sanger sequencing. The third case contained 2 somatic *RASA1* variants (VAFs: 2.84% and 2.99%). Although the germline variants in *RASA1* are known to cause CM-AVM, *RASA1* somatic alterations have been reported to cause CM-AVM in the literature. Germline LOF variants in *PTEN* are associated with the *PTEN* hamartoma tumor syndrome, a group of disorders that include Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome, *PTEN*-related Proteus syndrome, and *PTEN*-related Proteus-like syndrome.[8-11] Somatic LOF variants in *PTEN* occur in sporadic primary tumors, with the highest frequencies in endometrial carcinoma and glioblastoma.[12] The 2 cases identified in this study with 2 P/LP variants in *PTEN* were associated with vascular anomalies within fibroadipose tissue. Each of the 2 cases contained 1 germline variant (VAFs: 46.0% and 48.0%) and 1 somatic variant (VAFs: 2.20% and 2.20%). Two cases with 2 P/LP variants in *NF1* were observed in a 76-year-old woman and a 6-year-old girl, both of whom had neurofibromas with no other features of neurofibromatosis and no significant family history of neurofibromatosis. Both *NF1* variants in these 2 cases were consistent with somatic origin (VAFs: 11.9% and 13.6% in case ID 1-f; 3.3% and 12.6% in case ID 1-g).

Two GOF clinically significant variants in the same gene

Fourteen cases were identified with 2 clinically significant variants in the same gene with GOF as a disease mechanism, including 11 cases with *TEK* (OMIM 600221) variants, 2 with *PIK3CA* (OMIM 171834) variants, and 1 with *KRAS* (OMIM 190070) variants (Table 2).

A total of 22 *TEK* variants have been identified in those 11 cases. The *TEK* variants' distribution is illustrated in Figure 2, including 17 variants found at 5 mutational hotspots located in the tyrosine kinase (TK) functional domain and 5 variants at 3 mutational hotspots located in the C-terminal tail. Among the 9 unique variants, 6 were missense variants, mainly located in the TK functional domain, whereas 1 nonsense and 2 frameshift variants were located downstream of the TK functional domain in the C-terminal tail. Because the nonsense and frameshift variants were located in the C-terminal tail, they may help reserve protein function instead of causing loss of function.

Among the 11 *TEK* cases, 2 patterns were noted. Pattern #1 was observed in 5 cases with one p.Arg849Trp variant along with another variant either at the 3′ end (case IDs: 2-d, 2-e, and 2-g) or in the middle TK functional domain (case IDs: 2-b and 2-f). The p.Arg849Trp variant was observed as a potential germline variant (either detected by Sanger sequencing in the patient's peripheral blood or assumed based on the VAF) in 3 of the 5 cases. Of note, because the p.Arg849Trp variant and its second hit locate too far away to be observed on the same NGS read, we cannot determine the phase of those 2 variants. Pattern #2 was observed in 5 cases with the co-existence of the p.Tyr897Cys variant and another variant in *cis* at either p.Arg918His (case IDs: 2-a, 2-h, 2-i, and 2-k) or p.Arg915Cys (case ID: 2-c). The remaining case (case ID: 2-j) contains 2 variants in *cis* (p.Lys1110ValfsTer7 and p.Thr1105Asn). Four of the 11 cases contained a potential germline *TEK* variant, either confirmed by Sanger sequencing in the peripheral blood specimen (case IDs: 2-f and 2-h) or assumed based on the VAFs and internal experience (VAFs: 36.4% and 48.6% in case IDs: 2-d and 2-g, respectively). All other variants, with VAFs ranging from 1.07% to 17.7%, are consistent with a somatic origin. Although a total of 9 unique *TEK* variants were identified in this cohort, the 4 germline variants were located only at 2 loci, 3 at p.Arg849Trp (case IDs: 2-d, 2-f, and 2-g) and 1 at p.Arg918His (case ID: 2-h). Although 4 of

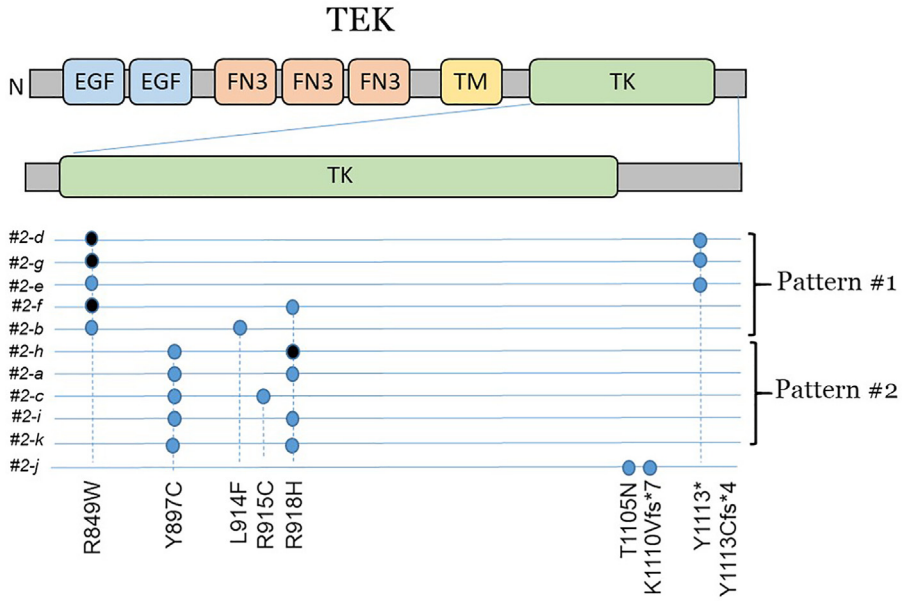

Figure 2 TEK protein structure and variant distribution in the 11 cases with *TEK* 2 clinically significant variants. TEK protein functional domains include EGF, FN3, TM, and TK. A total of 11 cases (case IDs from 2-a to 2-k) were listed with variant distribution in 2 major patterns. Variant distribution pattern #1 was observed in 5 cases with one p.Arg849Trp variant along with another variant either at the 3′ end (case IDs: 2-d, 2-e, and 2-g) or in the middle TK functional domain (case IDs: 2-b and 2-f). Pattern #2 was observed in 5 cases with the co-existence of the p.Tyr897Cys variant and another variant in cis at either p.Arg918His (case IDs: 2-a, 2-h, 2-I, and 2-k) or p.Arg915Cys (case ID: 2-c). EGF, epidermal growth factor-like domain; FN3, fibronectin type-III domain; TK, tyrosine kinase domain; TM, transmembrane domain.

5 cases with pattern #1 were documented to have a venous malformation, because of the lack of comprehensive clinical presentation, the genotype and phenotype correlations among different cases are still unclear.

Two cases (case IDs: 2-l and 2-m) were identified with the co-existence of 2 *PIK3CA* variants, all of which are unique variants consistent with somatic origin (VAFs range from 1.25% to 11.5%). Manual inspection of the variants in case 2-l showed that the 2 *PIK3CA* variants (p.Glu542Lys and p.Glu546Lys) were not present on the same NGS reads, suggesting that the 2 variants either occurred on different alleles (in *trans*) in the same cell population or occurred in 2 different cell populations. The co-existence of 2 activating *PIK3CA* variants is extremely rare in DoSM; it has been reported in a female patient with multiple congenital lipomas, vascular malformations, and an ovarian cyst.[13] The clinical significance of the co-existence of multiple activating *PIK3CA* variants remains unclear.

One case (case ID: 2-n) was identified with the co-existence of 2 somatic *KRAS* variants (p.Gly12Ser and p.Gln22Arg) with similar VAFs (1.98% and 2.16%, respectively). Manual inspection of the variants in case 2-n showed that these 2 *KRAS* variants were present on the same sequencing reads, consistent with the 2 variants occurring in *cis*. Interestingly, the co-existence of p.Gly12Ser and p.Gln22Arg in *cis* has been observed mainly in cancer cases, including a patient with intramuscular hemangioma capillary type (IHCT), which is a fast-flow vascular lesion classified as a tumor, although its phenotype overlaps with arteriovenous malformations (AVMs).[14,15] The current case in our study was referred for genetic testing with clinical indication of AVM, which is often difficult to distinguish from IHCT clinically because they share several features. Along with a previously reported case, the co-existence of *KRAS* p.Gly12Ser and p.Gln22Arg in *cis* has been observed recurrently in patients with the fast-flow vascular anomaly, including AVM and IHCT.[15] The pathophysiologic significance of the co-existence has yet to be described.

Co-existence of 2 clinically significant variants in 2 different genes

PIK3CA plus a second gene

Besides the 22 cases with 2 P/LP variants in the same gene, 11 cases were identified in this study with 2 P/LP variants in 2 different genes on the clinical gene panel (Figure 1). Seven of the 11 cases contain a pathogenic variant in *PIK3CA* (Table 3). Among these 7 cases, 3 cases have an additional pathogenic variant in the gene *GNAQ*, each of the remaining 4 cases has a pathogenic variant in the genes *BRAF*, *KRAS*, *TEK*, or *RASA1*, separately. The *PIK3CA* variants are considered the primary mutational event (with an allele ratio of approximately 3 or higher) in 4 cases (case IDs: 3-b, 3-d, 3-f, and 3-g). Peripheral blood specimens were available from 6 patients in this cohort. Two *PIK3CA* variants were detected by Sanger sequencing in the peripheral blood specimen with notably reduced VAF,

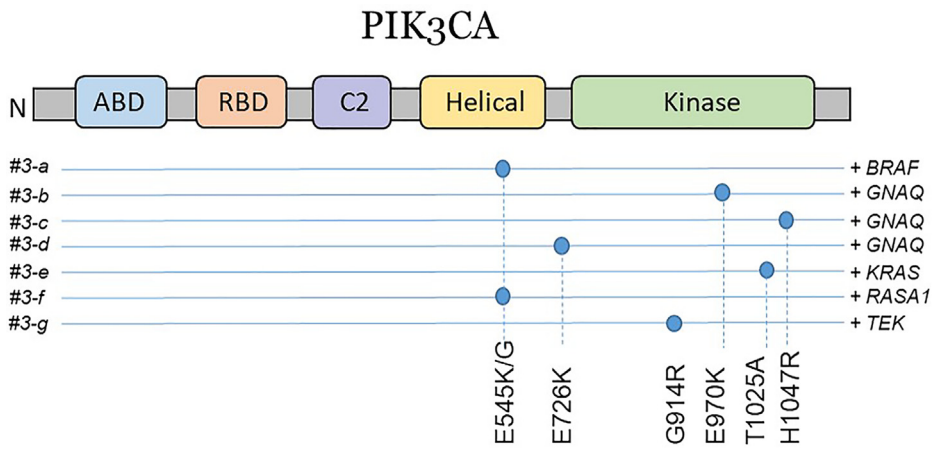

Figure 3 PIK3CA protein structure and variant distribution in the 7 cases with clinically significant variants in *PIK3CA* and another gene. PIK3CA protein functional domains include ABD, C2, Helical, Kinase, and RBD. A total of 7 cases (case IDs from 3-a to 3-g) were listed with 5 partner genes (*BRAF*, *GNAQ*, *KRAS*, *TEK*, and *RASA1*). Each of the 7 cases has a unique *PIK3CA* variant: 3 harboring the PI3K helical domain, and 4 harboring the PI3/4-kinase domain. ABD, adapter binding domain; C2, calcium-dependent phospholipid-binding domain; Helical, PI3K helical domain; Kinase, PI3/4-kinase domain; RBD, Ras binding domain.

suggesting multitissue mosaicism. The presence of *PIK3CA* variants in multiple tissues may indicate that these *PIK3CA* alterations occurred at a relatively earlier stage during development than the lower-VAF mutation. Based on the limited clinical information available, at least 4 cases (case IDs: 3-b, 3-d, 3-f, and 3-g) have associated congenital malformations, which are consistent with the observation of early-stage *PIK3CA* alterations in this cohort of patients. Of note, when compared with the cases with single clinically significant *PIK3CA* variants, there was no obvious preference for the location of the *PIK3CA* variants seen in these 7 cases in the cases with 2 clinically significant variants (Figure 3). Interestingly, none of the second mutational events are in the genes in the PI3K/AKT pathway, which may suggest the intolerance of a secondary clinically significant variant in the PI3K/AKT pathway along with a pathogenic *PIK3CA* variant.

Two clinically significant variants in non-*PIK3CA* genes

The remaining 4 cases contain 2 clinically significant variants in non-*PIK3CA* genes. (Table 4) In a 16-year-old male with multiple skin and vascular findings (case ID: 4-a), activating variants in *BRAF* and *NRAS* were identified as consistent with somatic origin. *PIK3R1* variants were identified in 2 cases with multiple vascular malformations (case IDs: 4-b and 4-c), along with additional pathogenic variants in *MTOR* in case 4-b and *IDH1* in case 4-c. In case 4-d, P/LP variants were identified in genes *RASA1* and *PDGFRB*. Because *RASA1* is known to have a second affected allele to cause the disease, the *PDGFRB* alteration may be responsible for the patient's vascular finding.

Unlike the cases with pathogenic *PIK3CA* variants, most cases have 2 pathogenic variants in 2 genes within the same pathway, including the PI3K/AKT pathway. It may suggest that 2 pathogenic non-*PIK3CA* variants are more tolerated than 2 pathogenic *PIK3CA* variants. The age at the time of testing ranged from 14 to 34 years among these 4 patients. Peripheral blood specimens were available from 3 patients (case IDs: 4-a, 4-c, and 4-d) in this cohort. None of the pathogenic non-*PIK3CA* variants was detected by Sanger sequencing in the peripheral blood specimen.

Discussion

The coexistence of 2 clinically significant variants in the same tissue in patients with DoSM is uncommon, and the study of this topic is just emerging. In 2019, a group from The Netherlands reported a cohort of patients with coexistence of 2 clinically significant variants, including 6 cases with *PIK3CA* variants, 9 cases with *PTEN*2, and 1 case with *RASA1*, in a cohort of patients with vascular malformations.[16] Here, we report an additional 33 individuals with 2 clinically significant variants in the patients with DoSM. There are several considerations to examine, such as whether the 2 mutational events occur at the same time in development or whether they occur in the same cell population. The allelic ratio of the 2 variants can help to determine whether the variants are simultaneous events or successive events. For example, if 2 variants have an allelic ratio close to 1 (approximately the same allelic fraction), the 2 variants could potentially occur as simultaneous events; on the other hand, if the 2 variants have an allelic ratio much greater than 1 (the VAFs are quite different from each other), it suggests the 2 variants occurred at successive events at different time points during the development. However, it is difficult to determine whether 2 variants are in the same cell using short read sequencing unless they are

close enough to each other in the same gene. Given the complex considerations and genetic heterogeneity, we discuss these cases based on the disease mechanism and affected genes.

Two LOF clinically significant variants in the same gene

Some of the genes associated with DoSM are also known as tumor suppressor genes. Co-occurring LOF clinically significant variants were identified in 8 cases, involving 4 different genes (*RASA1*, *PTEN*, *NF1*, and *TSC1*) in the current study. All 4 of these genes are characterized as tumor suppressor genes.[17-21] Similar to the oncogenesis mechanism of tumor suppressor genes in cancer, those genes also require both alleles to be inactivated to cause disease manifestations. This theory is called the 2-hit hypothesis, also known as the Knudson hypothesis, first formulated by Dr Alfred G. Knudson in 1971.[19,22] According to the Knudson hypothesis, there are 2 patterns of the 2-hits to explain the nonhereditary (sporadic) and hereditary (constitutional predisposition) forms of diseases. In the nonhereditary pattern, both alleles are affected by somatic alterations; in the hereditary pattern, one allele is affected by germline alteration (heterozygous), and the second allele is affected by a somatic mutational event. This pattern is recognized as type 2 segmental mosaicism.[23] Among the 8 cases in our study, 5 cases (case IDs: 1-a, 1-b, 1-d, 1-e, and 1-h) are consistent with constitutional predisposition; the remaining 3 cases (case IDs: 1-c, 1-f, 1-d, and 1-g) are consistent with sporadic diseases. Of note, some patients were referred to the DoSM NGS panel after a negative constitutional genetic evaluation, such as by germline NGS panel or exome sequencing. Because of such patient population bias, the genetic contribution of the constitutional changes may be underestimated. Therefore, a precise evaluation of the genetic patterns (constitutional vs sporadic) in this disease type remains challenging.

TEK

TEK encodes the dimeric endothelial TK receptor Tie2 (or Tek), which regulates embryonic vascular development and endothelial cell functions such as proliferation, survival, and migration.[24,25] *TEK* GOF variants have been associated with the autosomal dominant germline disorder, multiple cutaneous and mucosal venous malformations, and somatic disorders, such as blue rubber bleb nevus syndrome, multifocal venous malformation, and venous malformation.[26] The combination of 2 *TEK* variants, either germline and somatic or both somatic, has been reported in patients with vascular malformations.[27,28] In vitro functional studies showed that the p.Tyr897His variant, together with the second variant *in cis* in the *TEK* gene, caused ligand-independent hyperphosphorylation. It also suggested that double *TEK* clinically significant variants had stronger effects than a constituent of a single *TEK* clinically significant variant. In previously published studies, there was no correlation between clinical phenotype and level of hyperphosphorylation.[29] Additional observations have shown that 2 *TEK* variants *in cis* are more typically associated with sporadic disorders.[27] Although the combination of 2 *TEK* variants has been reported, a clear genotype-phenotype correlation is still lacking.

PIK3CA

PIK3CA encodes the catalytic subunit of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) protein complex. PROS disorders represent clinically recognizable conditions, typically discerned at birth or during early childhood, and are associated with cutaneous, vascular, skeletal, and cerebral anomalies, as well as focal or segmental overgrowth of the body.[30,31] *PIK3CA* variants associated with PROS overlap those reported as oncogenic variants found in multiple tumor types (COSMIC and cBioPortal Databases).[32,33] A large number of PI3K/AKT pathway inhibitors are currently under clinical study, primarily in a cancer setting, but also in the setting of PROS. The co-existence of 2 clinically significant variants in *PIK3CA* and *GNAQ* has been seen in 3 cases in this study. A similar combination of variants has been reported in 2 patients in the literature. In 2018, 1 patient presenting with megalencephaly-capillary malformation was reported to carry both a *PIK3CA* p.Glu45Lys variant and a *GNAQ* p.Gln209His variant detected in DNA extracted from a congenital hemangioma.[3] The other patient was a 58-year-old woman presenting with hepatic small vessel neoplasm. Both *GNAQ* p.Gln209His and *PIK3CA* p.His1047Arg variants were detected in an autopsy performed after a pulmonary embolism.[34] The co-existence of 2 activating *PIK3CA* variants has been reported in at least 1 patient with lipoma, capillary malformation, and an ovarian cyst.[13] To date, no genotype-phenotype correlation has been established regarding specific variants, or combination of variants, and severity of the disease.

Challenges and limitations

The genotype-phenotype correlation is challenging for DoSM. Similar to the cancer paradigm, the somatic clinically significant variants for this disease group can occur in different tissues at different time points. The same variants may lead to different phenotypes depending on the mutational burden and the timing of the somatic alteration (acquired vs congenital, early developmental stage vs late stage). Therefore, the phenotype is not only dictated by the specific post-zygotic somatic variants but also influenced by the VAF, the tissue distribution of variants, modifying constitutional variants, and potentially additional acquired somatic variants.

The limitations of this study include an inability to fully review the phenotype-genotype correlation for every case. An effort to correlate phenotype with genotype was made. However, the clinical information is limited to what has been provided by the ordering clinician as the indication for

testing. The genetic testing laboratory usually does not have access to detailed clinical presentations for every case, especially those ordered by external clinicians. Another limitation is that the age of each patient, based on the date of sample collection, may not be consistent with the date of initial clinical diagnosis or evaluation. Third, although constitutional genetic alterations can be detected by this assay, the genetic contribution of the constitutional changes may be underestimated because of patient population bias. Some of the patients with similar clinical presentations may undergo prior genetic testing focusing on constitutional findings. The patients who receive positive constitutional results may not be referred for genetic testing focusing on somatic alterations. Additionally, more subtle presentations may not be appreciated or referred for additional testing. Therefore, it is challenging to determine the clinical significance of each variant, as the VAF and disease onset are variable from case to case.

Another important limitation of this study is the inability to determine the clonal distribution of discrete variants based on all tissue-profiling analyses using NGS technology. Bioinformatics tools are imperfect in their ascertainment of VAF, although clustering of variants present within statistically similar VAF has been widely applied in studies describing the oligoclonality of cancer. This limitation could conceivably be addressed by single-cell omics analysis, and it is a logical next step in understanding the presence and mechanism(s) of cooperativity between cases with multiple pathogenic and likely pathogenic variants in the mix.

Despite the limitations, this study includes the largest cohort of cases with the co-existence of 2 clinically significant variants in DoSM. To date, no study has looked specifically at this unique group of cases. The observation and description of these cases with 2 clinically significant variants, including both clinical presentations and genetic results, will shed light on a better understanding of this unique group of diseases. In addition, this study highlights the importance of broader sequencing in DoSM, which may affect prognosis and treatment responses in those cases. The emerging evidence of the co-existence of multiple clinically significant variants in DoSM in this study also guides future research direction to better understand the genetic mechanism of DoSM.

Data Availability

All relevant data are reported in the article and Figures 1-3 and Supplemental Table 1.

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Ethics Declaration

This single IRB (sIRB) that approved this study was the Washington University School of Medicine sIRB, #: 202103225. These data were collected during clinical testing, and no separate consent was needed for the deidentified accumulated data from the clinical analyses. The work was conducted in a manner consistent with the Belmont Report and the Declaration of Helsinki.

Conflict of Interest

The authors declare no conflicts of interest.

Additional Information

The online version of this article (https://doi.org/10.1016/j.gimo.2023.100807) contains supplementary material, which is available to authorized users.

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