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REPORT

Somatic activating *BRAF* variants cause isolated lymphatic malformations

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Summary

Somatic activating variants in *PIK3CA*, the gene that encodes the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K), have been previously detected in ~80% of lymphatic malformations (LMs).[1,2] We report the presence of somatic activating variants in *BRAF* in individuals with LMs that do not possess pathogenic *PIK3CA* variants. The BRAF substitution p.Val600Glu (c.1799T>A), one of the most common driver mutations in cancer, was detected in multiple individuals with LMs. Histology revealed abnormal lymphatic channels with immunopositivity for BRAF^{V600E} in endothelial cells that was otherwise indistinguishable from *PIK3CA*-positive LM. The finding that *BRAF* variants contribute to low-flow LMs increases the complexity of prior models associating low-flow vascular malformations (LM and venous malformations) with mutations in the PI3K-AKT-MTOR and high-flow vascular malformations (arteriovenous malformations) with mutations in the RAS-mitogen-activated protein kinase (MAPK) pathway.[3] In addition, this work highlights the importance of genetic diagnosis prior to initiating medical therapy as more studies examine therapeutics for individuals with vascular malformations.

Disorganized morphogenesis of arteries, veins, capillaries, and lymphatic vessels results in vascular malformations, a relatively common congenital malformation associated with significant morbidity.[4] Vascular malformations are classified into high-flow lesions, which include arteriovenous malformations (AVMs), and low-flow lesions, which include venous malformations (VeMs) and lymphatic malformations (LMs). Individuals with vascular malformations typically have no family history, because most are caused by post-zygotic (mosaic) activating mutations in oncogenes within the phosphatidylinositol 3-kinase (PI3K)-AKT and RAS-mitogen-activated protein kinase (MAPK) pathways.[1–3] Treatments for vascular malformations are primarily invasive and include sclerotherapy, embolization, and open surgical resection,[4] but the identification of specific activating mutations in well-known oncogenic signaling pathways has led to trials examining the efficacy of targeted medical therapies.[5–11]

Previous work has shown that approximately 80% of isolated LMs have somatic pathogenic variants in *PIK3CA*,[1,2,12] the gene that encodes for the catalytic subunit of PI3K, a component of the PI3K-AKT pathway.[13] Although mutations in other genes (including *NRAS*, *KRAS*, *CBL*, *ARAF*, and *EPHB4*) have been identified in complex lymphatic anomalies, such as diffuse lymphangiomatosis and Gorham-Stout disease,[14] *PIK3CA* is the only gene associated with isolated LMs to date. The vast majority (>90%) of LM-associated pathogenic variants occur at one of three locations,[2] referred to as "hotspots": c.1624G>A (p.Glu542Lys), c.1633G>A (p.Glu545Lys), and c.3140A>G (p.His1047Arg), all of which result in PI3K hyperactivation.[15,16] The fraction of DNA molecules that possess the pathogenic *PIK3CA* variant (referred to as the variant allele fraction [VAF]) within LM tissue is typically very low (<10%),[2] and it has been hypothesized that a fraction of LMs without a detected *PIK3CA* variant in fact do carry a *PIK3CA* variant that was "missed" due to low-level mosaicism. It is also possible that additional genes play a role. Here, we report the identification of somatic *BRAF* mutations in LMs without a detected *PIK3CA* variant.

LM tissue from 106 individuals was screened for the three *PIK3CA* (GenBank: NM_006218.4) hotspots (p.Glu542Lys, p.Glu545Lys, and p.His1047Arg) as well as the less common but amplicon-overlapping p.His1047Leu substitution using droplet digital polymerase chain reaction (ddPCR) assays and molecular inversion probes, as previously reported (Supplemental methods).[2] Following this screening, 22 individuals remained without a detected *PIK3CA* variant. Fifteen of these individuals had sufficient DNA (14 lesion-derived and 1 cyst fluid) for further testing, which was sent for high-depth targeted sequencing using a 44-gene panel, referred to as VANseq (vascular anomaly

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Table 1. Somatic variants in LM detected by VANseq and confirmed by ddPCR

Subject	Age[a]	Sex	Variant	Sample	VANseq reads			ddPCR droplets		
					VAF (%)	Var	WT	VAF[b] (%)	Var	WT
LR18-536	2 years	F	*PIK3CA* p.Asn345Lys	lesion A	1.3	21	1,669	1.1	144	13,401
				lesion B	–	–	–	0.2	23	10,709
				lesion C	–	–	–	NEG	5	12,251
				lesion D	–	–	–	0.2	22	12,569
				lesion E	–	–	–	1.4	151	10,827
				lesion F	–	–	–	0.5	88	23,161
				lesion G	–	–	–	0.9	74	9,877
				skin	–	–	–	0.3	30	9,480
				salivary gland	–	–	–	NEG	0	31,662
LR16-278	2 years	F	*PIK3CA* p.Glu545Lys	lesion	0.7	12	1,709	0.5	48	12,295
LR16-264	3 years	F	*PIK3CA* p.Glu545Lys	lesion	10.6	7	59[c]	4.8	437	9,551
LR17-322	1 year	M	*BRAF* p.Val600Glu	lesion	2.1	34	1,618	1.7	165	10,555
				skin	–	–	–	NEG	0	10,374
LR19-346	5 months	F	*BRAF* p.Val600Glu	lesion, deep	0.6	7	1,143	1.2	69	5,872
				lesion, inferior	–	–	–	0.9	56	6,389
				lesion, superior	–	–	–	NEG	2	9,584
				lesion, no location	–	–	–	3.6	91	2,479
				skin	–	–	–	NEG	0	4,304
				fat	–	–	–	NEG	1	5,340
				muscle	–	–	–	NEG	0	6,129
LR19-443	1 month	M	*BRAF* p.Val600Glu	cyst fluid, A[d]	–	–	–	0.2	29	13,055
				cyst fluid, B[d]	0.3	4	1,458	0.1	10	9,661
				cyst fluid, C[d]	–	–	–	0.3	31	13,292
				cyst fluid pellet	–	–	–	NEG	15	30,798

ddPCR, droplet digital polymerase chain reaction; NEG, no variant detected; VAF, variant allele fraction; Var, variant; WT, wild type.
[a]Age at time of tissue or cyst fluid attainment.
[b]ddPCR VAF calculated using droplet concentrations and only reported for samples in which sample variant concentration was statistically different from WT control variant concentration based on 95% total error confidence intervals.
[c]Lower than typical coverage.
[d]Cell-free DNA was assayed from cyst fluid samples.

sequencing) (see Supplemental methods and Table S1) throughout the rest of this paper.

VANseq identified variants in 6/15 individuals (Table 1). One individual (LR18-536) had a non-hotspot *PIK3CA* variant, c.1035T>A (p.Asn345Lys) that could not have been detected by hotspot allele-specific ddPCR screening. This variant is absent from the Genome Aggregation Database (gnomAD), is predicted to be damaging by several *in silico* tools, and has been previously reported in numerous individuals with cancer as well as one individual with congenital lipomatous overgrowth, vascular malformations, epidermal nevis, spinal/skeletal anomalies/scoliosis (CLOVES) syndrome.[1,17,18] Functional studies have demonstrated that this substitution results in PI3K pathway hyperactivity.[15,16] Although not previously reported in association with isolated LMs, we interpreted this variant as being pathogenic[19] and confirmed the presence of the variant in additional samples from that individual using ddPCR. There was variation in VAF from undetectable to 1.4% within lesion samples (Table 1), as we have previously described.[2]

VANseq detected a hotspot *PIK3CA* variant (p.Glu545Lys) in two individuals (LR16-278 and LR16-264) who had previously been screened for this allele by ddPCR.[2] We reexamined prior data from both cases. LR16-278's prior ddPCR had six variants and 1,055 reference droplets but did not meet our positive criteria, as the 95% confidence interval overlapped with wild-type samples (Supplemental methods). The initial ddPCR run for LR16-264 had one variant and 5,305 reference droplets, but subsequent testing from the original stock DNA dilution was unambiguously positive by VANseq and ddPCR (VAFs of 10.6% and 4.8%, respectively). Although provenance testing was not

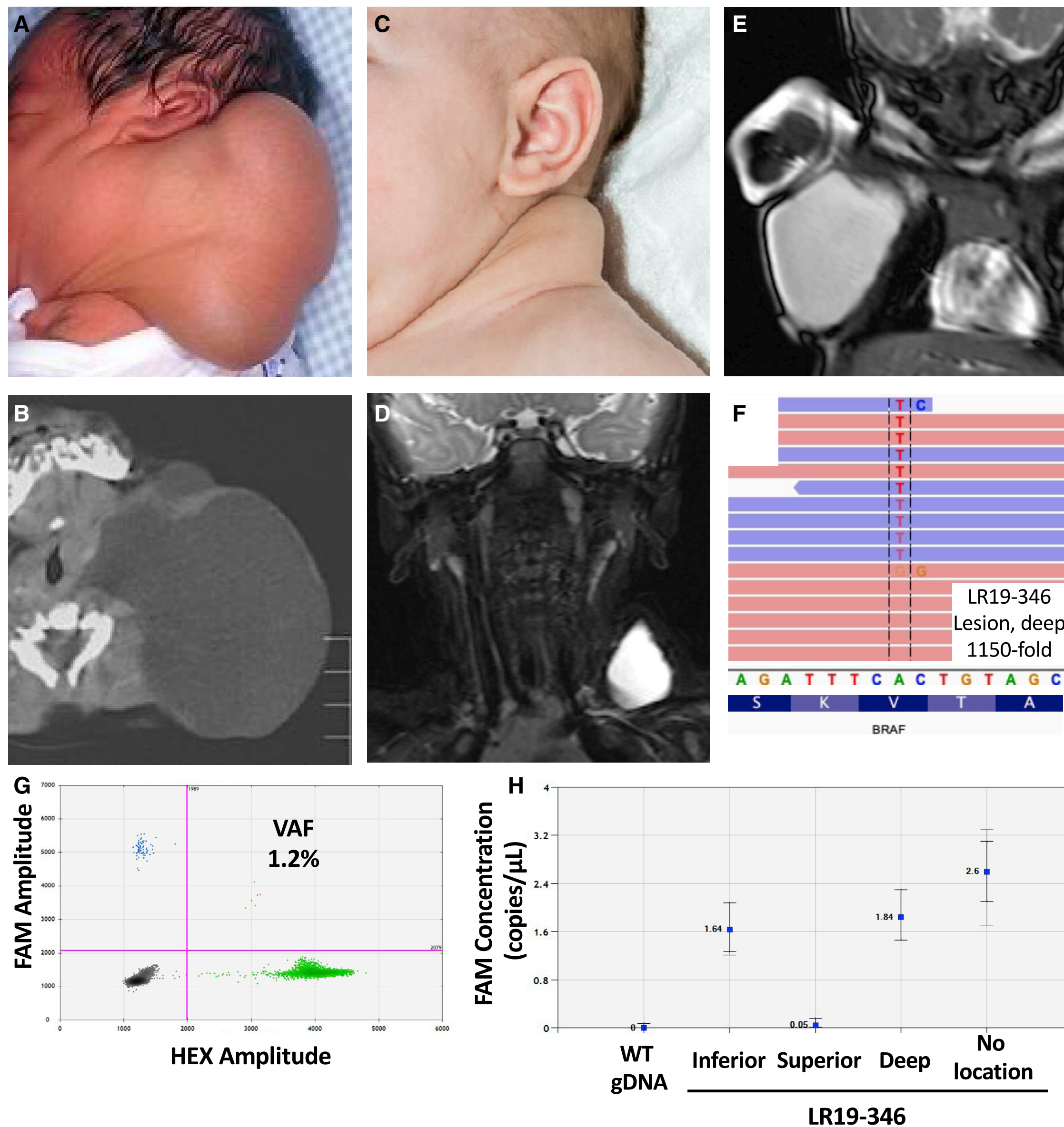

Figure 1. Clinical features of *BRAF*-mutated LM and confirmation of genetic diagnosis
(A–G) Clinical photos of LR17-322 (A) and LR19-346 (C), showing posterior neck LMs. Corresponding computed tomography (CT) (LR17–322; B) and MRI (LR19-346, D; LR19-443, E) images demonstrate macrocystic lesions with minimal septations of the posterior lateral neck and axilla. Integrated Genomics Viewer image for LR19-346 demonstrates somatic BRAF p.Val600Glu variant (F), confirmed on droplet digital PCR (G).
(H) Variant concentration image from Quantasoft shows variability in mutation prevalence between samples (H).
Note: (A) and (B) were previously published prior to identification of this individual's genetic variant.[43]

possible to prove it, we suspect this resulted from a sample swap during the original screening. Poor sample quality could also be a factor, as LR16-265 had lower than typical coverage on VANseq (Table 1). These examples highlight difficulties in using tiered screening assays, which increase the likelihood of sample swaps, and also demonstrate consideration for repeat testing when the diagnostic pre-test probability is high.[20] Previous publications from our lab and others have highlighted the utility of repeat testing when the diagnostic pre-test probability is high.[2,21] We are confident that the pathogenic variant has now been identified for both of these individuals.

VANseq identified a pathogenic *BRAF* variant in 3 of the 15 LMs without a detected *PIK3CA* variant (LR17-322, LR19-346, and LR19-443). All three possessed the same variant (GenBank: NM_004333.6:c.1799T>A, resulting in p.Val600Glu), which was confirmed by ddPCR in multiple independent tissues, when available (Table 1; Figures 1F–1H). Three additional LMs without a detected *PIK3CA* variant (LR17-134, LR17-319, and LR18-572) possessed three or more reads supporting the *BRAF* p.Val600Glu substitution but were not confirmed by ddPCR so were not classified as being *BRAF* positive (Table S2). The presence of three or four alternate base calls out of 1,200–1,500 reads

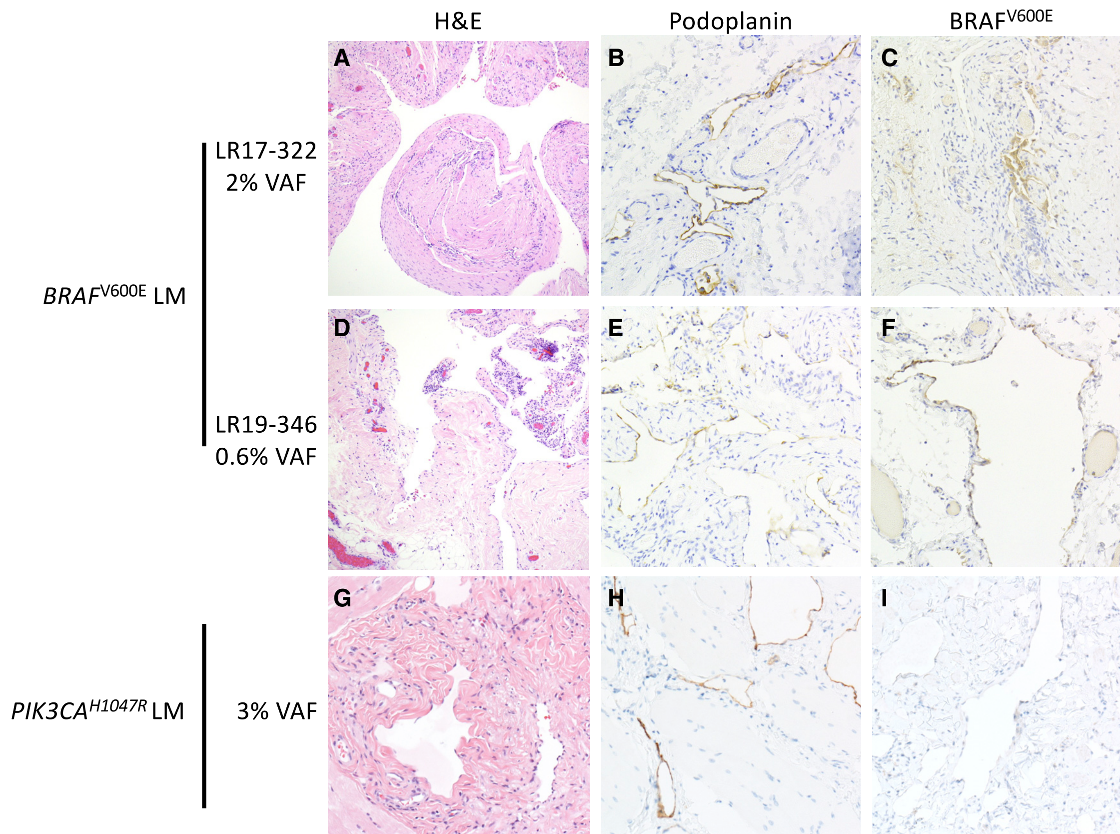

Figure 2. Histology and immunohistochemistry of *PIK3CA* and *BRAF* mutated LMs
LM tissue from two individuals with BRAF p.Val600Glu substitutions (A–F) and one individual with PIK3CA p.His1047Arg substitution. (G–I). H&E stains (A), (D), and (G) show dilated cystic channels with bland, flattened epithelium. (B), (E), and (H) show presence of podoplanin (a.k.a. D2-40) immunoreactivity in endothelial cells. Panels on the right show BRAF p.Val600Glu immunoreactivity (VE1 staining) in endothelial cells in BRAF mutant LM (C and F), but not in PIK3CA mutant LM (I).

is comparable to the inherent error rate of next-generation sequencing (NGS).[22] The discrepancy between the VANseq and ddPCR results for these three samples reflects the lower error rate of ddPCR and highlights the challenges in accurate detection of variants with extremely low allele frequency.

All three individuals with BRAF p.Val600Glu substitutions had macrocystic LMs diagnosed at birth (Figure 1). LR17-322 had a large, macrocystic lesion of the posterior neck, de Serres stage 1, that resolved spontaneously over the first few months of life (Figures 1A and 1B). Surgery was performed at 1 year of age to remove remaining LM and redundant skin. LR19-346's LM was also isolated to the neck, de Serres stage 1, and was resolving with just observation until an upper respiratory infection induced swelling and the decision was made to remove it surgically (Figures 1C and 1D). LR19-443 had a large macrocystic LM of the axilla that was treated with sclerotherapy at 1 month of age (Figure 1E). This individual did not have surgery, but cell free DNA (cfDNA) from aspirated cyst fluid was available for genetic diagnosis. All individuals did well after intervention with no evidence of recurrence and no further procedures or therapy.

Histopathological examination of tissues from two *BRAF* p.Val600Glu containing LMs showed numerous dilated cystic channels with bland, flattened epithelium that was immunopositive for podoplanin, a marker of lymphatic endothelial cells (Figure 2).[23] There were no distinguishing histopathological features between *BRAF* and *PIK3CA* mutant LMs. The extremely low VAFs of the BRAF p.Val600Glu substitutions (0.3%–2%) indicate that most cells within the malformation do not possess the BRAF substitution.[2] We hypothesized that *BRAF* mutant cells would be primarily located within the lymphatic endothelial cells, as has previously been shown in LMs with *PIK3CA* mutations.[24–27] To test this, we used a BRAF p.Val600Glu-specific monoclonal antibody (VE1).[28] BRAF p.Val600Glu immunostaining was present in cyst-lining endothelial cells in LR17-322 and LR19-346, but not in other cells within the lesion (Figure 2). We detected no BRAF p.Val600Glu staining in two other LM samples bearing p.Glu545Lys and p.His1047Arg PIK3CA substitutions (Figure 2; data not shown), demonstrating specificity. These results both confirm the presence of the BRAF substitutions within these lesions and demonstrate their localization to lymphatic endothelial cells.

When these results are combined with our previous reports,[2,29] a more complete picture of allelic and locus heterogeneity within isolated LMs appears (Table 2). *PIK3CA* variants were found in 88% of the 101 individuals in our

Table 2. The genetic spectrum of LM, including *BRAF* p.Val600Glu

	PIK3CA					*BRAF*	
	p.His1047Arg	p.Glu545Lys	p.Glu542Lys	Other	Total	p.Val600Glu	NEG[a]
Zenner et al.[2]	22	18	18	6	64	–	–
Zenner et al.[29]	7	9	5	0	21	–	–
Current study	1	2	0	1	4	3	9
Total, n = 101[b]	30	29	23	7	89 (88.1%)	3 (3.0%)	9 (8.9%)

[a]All negative samples for the first two studies were included in this study if adequate sample was available for VANseq testing.
[b]Total includes only individuals with detected mutations or sufficient DNA to undergo VANseq testing.

cohort, 92% of which occurred at one of the three *PIK3CA* hotspots. *BRAF* p.Val600Glu variants were found in 3% of individuals with isolated LMs—a small proportion but a clinically important finding, as responses to targeted drug therapies may differ. For example, some BRAF inhibitors produce paradoxical activation of the MAPK pathway and corresponding cellular proliferation in tumors possessing oncogenic mutations in RAS or upstream receptors.[30,31] The application of VANseq to our cohort of 101 individuals with isolated LMs brought our overall diagnostic rate from ~80% to over 90%, and currently, only 9/101 individuals with adequate DNA now remain without a genetic diagnosis.[2]

BRAF is one of the most frequently mutated genes in cancer with a predilection for melanoma, thyroid cancer, colon cancer, and non-small cell lung cancer. p.Val600Glu is the most common oncogenic BRAF substitution, accounting for >90% of *BRAF* mutations.[32] Non-mosaic constitutional missense and in-frame deletions in *BRAF* have been reported in RASopathies (e.g., Cardiofaciocutaneous syndrome, Noonan syndrome, and Noonan syndrome with multiple lentigines),[33] but the p.Val600Glu substitution has never been reported in these diseases. This is likely due to the fact that the BRAF p.Val600Glu substitution is not compatible with embryonic survival except in the mosaic state (i.e., the Happle hypothesis).[34] This conclusion is supported by the embryonic lethality seen in constitutional expression of *BRAF* p.Val600Glu in mouse embryos.[35] Somatic *BRAF* p.Val600Glu variants have previously been reported to cause AVMs, though activating mutations in *KRAS* and *MAP2K1* are more common causes.[3,36,37] The precise mechanisms by which somatic BRAF p.Val600Glu substitutions cause LMs in some cases and AVMs in others likely has to do with the timing and location of the post-zygotic mutation. Additional studies are needed to examine this further. Although activating mutations in oncogenes raise concern for an increased risk of cancer, *PIK3CA*-related overgrowth syndromes have a low risk[18] and *BRAF* p.Val600Glu variants are detected in >80% of benign melanocytic nevi, indicating that the single mutation is insufficient to produce melanoma.[38]

All three individuals with *BRAF* p.Val600Glu substitutions in our study had similar clinical phenotypes—large, macrocystic lesions of the neck or body that resolved spontaneously or were treated very early in life. Under the surgical staging system for LMs (de Serres staging), these three individuals would be classified as having stage 1 lesions (unilateral and below the hyoid).[39] Stage 1 lesions make up only ~31% of total LMs in recent studies,[2,39,40] suggesting that the LMs with *BRAF* mutations may represent a milder phenotype than LMs with *PIK3CA* mutations. Although our cohort of LMs with *BRAF* mutations (n = 3) is too small for genotype-phenotype correlations, we speculate that there may be enrichment for *BRAF* mutations in individuals with milder, non-surgical LMs, as genetic diagnosis in most LMs to date has required surgically resected tissue. Additional studies of more LMs with *BRAF* variants, perhaps using non-invasive diagnostic methods, such as cyst-fluid-based cfDNA,[29] will be needed to provide a more balanced view of the genetic spectrum among LMs. The presence of pathogenic *BRAF* variants within the cyst fluid of macrocystic LMs is consistent with our previous study identifying pathogenic *PIK3CA* variants within this compartment.[29] Further studies are needed to assess the relative yield of cyst fluid versus tissue as a diagnostic analyte.

Endothelial cells play a key role in the pathogenesis of vascular malformations, and isolation of endothelial cells from these lesions enriches the detection of somatic variants.[24–27] Prior work in AVMs has shown *KRAS*-mutation-specific staining of endothelial cells,[41] but this has not previously been possible for LMs, as there is no *PIK3CA*-mutant-specific antibody. The presence of $BRAF^{V600E}$ staining in lymphatic endothelial cells within the lesions supports the hypothesis that cell-non-autonomous effects, such as signaling to or recruitment of wild-type cells to the lesion, contribute to the formation of LMs. Cell-non-autonomous effects have been previously suggested to cause cartilage overgrowth in AVMs, but additional studies will be needed to examine this further.[42]

In conclusion, we demonstrate that a somatic activating pathogenic *BRAF* variant (c.1799T>A, [p.Val600Glu]) is present in 3% of our cohort of individuals with isolated lymphatic malformations. Screening isolated LMs for the three PIK3CA hotspots is an efficient and cost-effective approach but will potentially miss clinically important non-hotspot *PIK3CA* and *BRAF* variation. Our use of

VANseq, a high-depth, full-gene sequencing panel, increased the positivity rate for our cohort of LM from ~80% to >90%. In addition, our results suggest the need for studies to examine the efficacy of BRAF inhibition in the treatment of lymphatic malformations.

Data and code availability

The published article includes all data generated or analyzed during this study.

Supplemental information

Supplemental information can be found online at https://doi.org/10.1016/j.xhgg.2022.100101.

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Declaration of interests

R.A.B. is a co-founder of EigenHealth, Inc; a consultant to SpiWay, LLC; and holds a financial interest of ownership equity with Wavely Diagnostics, Inc. The remaining authors declare no competing interests.

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Web resources

Catalogue of Somatic Mutations in Cancer, https://cancer.sanger.ac.uk/cosmic.

Seattle Children's Hospital Lab Test Catalogue, Vascular Anomaly Sequencing Panel (VANSeq) https://seattlechildrenslab.testcatalog.org/show/LAB1920-1.

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