ARTICLE

Comprehensive targeted next-generation sequencing in patients with slow-flow vascular malformations

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Recent studies have shown that the PI3K signaling pathway plays an important role in the pathogenesis of slow-flow vascular malformations (SFVMs). Analysis of genetic mutations has advanced our understanding of the mechanisms involved in SFVM pathogenesis and may identify new therapeutic targets. We screened for somatic variants in a cohort of patients with SFVMs using targeted next-generation sequencing. Targeted next-generation sequencing of 29 candidate genes associated with vascular anomalies or with the PI3K signaling pathway was performed on affected tissues from patients with SFVMs. Fifty-nine patients with SFVMs (venous malformations n = 21, lymphatic malformations n = 27, lymphatic venous malformations n = 1, and Klippel–Trenaunay syndrome n = 10) were included in the study. TEK and PIK3CA were the most commonly mutated genes in the study. We detected eight TEK pathogenic variants in 10 samples (16.9%) and three PIK3CA pathogenic variants in 28 samples (47.5%). In total, 37 of 59 patients (62.7%) with SFVMs harbored pathogenic variants in these three genes involved in the PI3K signaling pathway. Inhibitors of this pathway may prove useful as molecular targeted therapies for SFVMs.

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INTRODUCTION

Vascular anomalies comprise both malformations and tumors. [1] Vascular malformations have conventionally been classified as slow flow or high flow according to the affected blood flow characteristics. [1] Slow-flow vascular malformations (SFVMs) include venous and lymphatic malformations as well as combined malformations, such as Klippel–Trenaunay syndrome (KTS). [2] SFVMs often cause deformity, pain, chronic anemia, coagulation abnormalities, and functional impairment. [2] Conventional treatments, such as surgery and/or sclerotherapy, are rarely curative, underscoring the urgent need for new therapeutic modalities. [3]

A number of genetic changes have been identified in patients with vascular anomalies, the majority of which occur within two of the major intracellular signaling pathways; namely, the RAS/mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway. [4–6] Recent studies have shown that the PI3K/AKT/mTOR pathway is typically mutated in patients with SFVMs, raising the possibility that targeted therapy could be a useful treatment strategy. [4–6] Indeed, sirolimus, also known as rapamycin, is an allosteric inhibitor of mTOR [7] that has been tested in a monocentric prospective phase II clinical trial for patients with SFVMs that were refractory to standard treatments. [3] Sirolimus was found to be highly effective, resulting in a partial response in all patients, reducing symptoms and increasing quality of life. [3] Genetic analysis has thus advanced our understanding of the mechanisms involved in SFVM pathogenesis and may also suggest new targets for molecular targeted therapies.

In this study, we performed targeted next-generation sequencing (NGS) of 29 candidate genes associated with vascular anomalies or with the PI3K signaling pathway in affected tissue samples from a cohort of patients with SFVMs.

MATERIALS AND METHODS

Patients and tissue samples

Patients diagnosed with SFVMs at six academic hospitals in Japan were enrolled in this study. Sample acquisition and genetic analyses were approved by the institutional review board at each institution, and written informed consent was obtained from all patients or their guardians. Tissue samples were collected during clinically indicated surgical procedures (surgical excision, punch biopsy, and needle biopsy) for therapeutic or diagnostic purposes. Areas of necrosis or hemorrhage on gross inspection

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were removed from the fresh specimens. Tissues were rapidly frozen in liquid nitrogen until analyzed.

DNA extraction and next-generation sequencing

Genomic DNA was extracted from frozen tissues using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) or NucleoSpin® Tissue kit (Macherey-Nagel, Duren, Germany) according to the manufacturers’ protocols. For NGS, primer pairs were designed to amplify the exonic regions of 29 candidate genes associated with vascular anomalies or the PI3K signaling pathway (listed in Table 1) using the Ion AmpliSeq Designer (v7.24, Thermo Fisher Scientific, Waltham, MA, USA). [5, 6, 8–10] A total of 1297 primer pairs with 125 to the manufacturer Kit Plus (Thermo Fisher Scientific) and the indicated primer pairs, according to the manufacturer’s protocol. The libraries were subsequently sequenced on an Ion Proton™ Sequencer (Thermo Fisher Scientific) using the Ion PI™ Chip v3 (Thermo Fisher Scientific) with 15–16 samples per chip.

### Table 1. Genes analyzed in this study

| Gene      | Related disorders/diseases |
|-----------|----------------------------|
| AGGF1     | KTS                        |
| AKT1      | Proteus syndrome           |
| AKT2      | VM                         |
| AKT3      | VM                         |
| BRAF      | AVM                        |
| EPHB4     | CM-AVM                     |
| GNA11     | CH, CM with bone and/or soft tissue hyperplasia, diffuse CM with overgrowth |
| GNAQ      | CH, CM “port–wine” stain, nonsyndromic CM, CM of Sturge–Weber syndrome |
| HRAS      | AVM                        |
| IDH1      | MS, SCH                    |
| IDH2      | MS, SCH                    |
| IGFR1     | see PIK3CA                 |
| KRAS      | AVM                        |
| MAP2K1    | AVM                        |
| MAP3K3    | Verrucous VM               |
| MTOR      | see PIK3CA                 |
| NF1       | see PIK3CA                 |
| NRAS      | Pyogenic granuloma         |
| PIK3CA    | Common (cystic) lymphatic malformation, common VM, KTS |
| PIK3CB    | see PIK3CA                 |
| PIK3R1    | see PIK3CA                 |
| PIK3R2    | see PIK3CA                 |
| PIK3R3    | see PIK3CA                 |
| PTEN      | Bannayan–Riley–Ruvalcaba syndrome, hamartoma of soft tissue/angiomatosis of soft tissue |
| RASA1     | CM-AVM, Parkes Weber syndrome |
| RICTOR    | see PIK3CA                 |
| RPTOR     | see PIK3CA                 |
| STAMBP    | Microcephaly-capillary malformation syndrome |
| TEK       | Common VM, familial cutaneomucosal VM, blue rubber bleb nevus syndrome |
| AVM       | arteriovenous malformation, CH congenital hemangioma, CM capillary malformation, KTS Klippel–Trenaunay syndrome, MS Maffucci syndrome, SCH spindle cell hemangioma, VM venous malformation |

*Gene located upstream of the PI3K signaling pathway

*Gene located within the PI3K signaling pathway

### Sequencing data analysis and variant annotation

The sequencing data were processed using a standard procedure with Ion Torrent Suite Software, and mutations were called using the Torrent Variant Caller plug-in. Called mutations were annotated with SnpEff [11], SnpSift [12], and ClinVar [13]. To remove error mutations from called sites and to identify somatic variants, mutations were selected to satisfy the following criteria: sequencing depths of 1000 or more, variant allele frequencies (VAFs) of ≥1% and <45%, and base qualities of ≥30. Mutations resulting in peptide alterations were annotated with dbNSFP [14] to identify single nucleotide variants or with ClinVar [13] to find insertion/deletions and were selected as the functional candidates. Candidate mutations were manually reviewed using Integrative Genomics Viewer [15]. Likely if they were excluded if they were reported as benign or likely benign in ClinVar [13] or IntVar [16] and their CADD Phred scores [17] were ≤20, as previously reported. [18] Then, as previously described [19], variants were denoted as pathogenic if one or more of the following criteria was satisfied: (i) the nucleotide sequence change (or affected amino acid residue) had previously been documented to be pathogenic, (ii) the change resulted in a shift of the transcript open reading frame, (iii) the change introduced a premature stop codon, (iv) the change altered the canonical splice-site sequence, and (v) the change was a start- or stop-loss mutation. In addition, new missense variants having a minor allele frequency of <0.01 in the 1000 Genomes Project database [20] and having deleterious effects predicted by at least two of three in silico pathogenicity prediction tools (SIFT [21], PolyPhen-2 [22], and MutationTaster [23]) were considered potentially pathogenic variants. Fisher’s exact test was used to compare frequencies of somatic variants in SFVMs with those in Genome Aggregation Database (gnomAD [24]; version 2.1.1) controls or the Catalog of Somatic Mutations in Cancer (COSMIC [25]; v96) pan-cancer cohort. Values of P < 0.05 were considered to indicate statistical significance. GraphPad Prism 7 software (GraphPad Inc., San Diego, CA, USA) was used for this analysis.

### Cis versus trans evaluation of double somatic activating variants in the TEK gene

PCR amplification was performed of fragments containing double somatic activating variants in TEK from tissue DNA (Patients VM7 and VM9). PCR fragments were subcloned using a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA, USA), and individual clones were sequenced to evaluate whether the variants occurred in cis (clones carrying both variants or neither) or trans (clones carrying one or the other variant alone).

### RESULTS

#### Patient characteristics and variant identification

Fifty-nine Japanese patients with SFVMs (31 males and 28 females) were included in the study. The patient characteristics are shown in Table 2. Their ages ranged from 0 to 72 years with a median age of 7.5 years. Of the 59 subjects, 21 were diagnosed with venous malformation (VM), 27 with lymphatic malformation (LM), 1 with lymphatic venous malformation (LVM), and 10 with KTS. The diagnosis of VM, LM, LVM, and KTS was based on clinical presentation and radiologic findings and was confirmed by histopathologic examination. Affected tissue samples from all 59 subjects were analyzable. Tissue acquisition methods consisted of surgical excision (n = 46), punch biopsy (n = 12), and needle biopsy (n = 1). Pathogenic variants were identified in tissues from 37 patients (62.7%) (Table 3). Genes encoding the endothelial receptor tyrosine kinase TIE2 (TEK) and the PI3K catalytic subunit (PIK3CA) were the most commonly mutated genes in the study. Eight pathogenic variants in TEK and three in PIK3CA were identified in 10 samples (16.9%) and 28 samples (47.5%), respectively (Fig. 1). Single variants of unknown significance were detected in genes encoding RAS p21 protein activator 1 (RASA1 in c.1772G > A; p.Arg591His) in one patient with LM, MAPK kinase kinase 3 (MAP3K3 in c.1416 C > G; p.Ile472Met) in one in VM, and Ehrin type-B receptor 4 (EPHB4 in c.52 G > A; p.Glu18Lys) and mTOR (MTOR in c.5490_5501del; p.Ala1831_Thr1834-del) in one patient with KTS each. The frequency of variants identified in patients with SFVMs is shown in Table 4. TEK (c.921 C > G; p.Tyr307*) and PIK3CA (c.1633G > A; p.Glu544Lys), and

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PIK3CA (c.3140 A > G; p.His1047Arg) showed a significant difference in frequency between SFVMs and gnomAD. TEK (c.2743 C > T; p.Arg915Cys), TEK (c.2752 C > T; p.Arg918Cys), TEK (c.2753 G > T; p.Arg918Leu), PIK3CA (c.1633G > A; p.Glu545Lys), PIK3CA (c.1624G > A; p.Glu542Lys), and PIK3CA (c.3140 A > G; p.His1047Arg) showed a significant difference in frequency between SFVMs and COSMIC. PIK3CA (c.3140 A > G; p.His1047Arg) and TEK (c.2690 A > G; p.Tyr897Cys, c.2689 T > C; p.Tyr897His, c.2740 C > T; p.Leu914Phe, c.2743 C > T; p.Arg915Cys, c.2752 C > T; p.Arg918Cys, c.2753 G > T; p.Arg918Leu, and c.3295 C > T; p.Arg1099*) were not detected in gnomAD. TEK (c.2740 C > T; p.Leu914Phe), TEK (c.3295 C > T; p.Arg1099*), TEK (c.2690 A > G; p.Tyr897Cys), TEK (c.2689 T > C; p.Tyr897His), and TEK (c.921 C > G; p.Tyr307*) were not detected in COSMIC.

Genetic variants associated with VM

Of the 21 patients with VMs, 14 (66.7%) harbored pathogenic genetic variants (Table 3). The maximum diameter of the lesions ranged from 1.0 to 15.0 cm. Six of the 14 patients showed evidence of multiple lesions. Pathogenic variants in TEK were identified in 10 patients, of whom 6 harbored variants in the mutational hotspot Leu914. [5] Although the TEK nonsense variant (c.921 C > G; p.Tyr307*) identified in patient VM9 was shown to result in a premature stop codon, the VAF of this variant (4.0%) was lower than that of other activating variants (8.6% and 9.0%) observed in the same patient, suggesting that c.921 C > G; p.Tyr307* is not a major cause of SFVM in this patient. Two of the 14 patients with VMs possessed double somatic activating variants in TEK (c.2690 A > G; p.Tyr897Cys + c.2752 C > T; p.Arg918Cys, and c.2689 T > C; p.Tyr897His + c.2753 G > T; p.Arg918Leu). TA cloning analysis showed that the double somatic activating variants were in cis in these patients (Fig. 2).

Five patients with VMs harbored pathogenic variants in PIK3CA, all of which were hotspot variants: one c.1624G > A; p.Glu542Lys, three c.1633G > A; p.Glu545Lys, and one c.3140 A > G; p.His1047Arg. [26] One patient had a pathogenic variant in both TEK (c. 2740 C > T; p.Leu914Phe) and PIK3CA (c.1633G > A; p.Glu545Lys). Allele frequencies of pathogenic variants in patients with VMs ranged from 3.7% to 13.6%, with 64.3% and 35.7% of individuals having VAFs of <10% and <5%, respectively.

Genetic variants associated with LM

Of the 27 patients with LMs, 14 (51.9%) harbored pathogenic genetic variants (Table 3). The maximum diameter of the lesions ranged from 1.0 to 15.0 cm. One patient showed evidence of multiple lesions. Pathogenic variants in PIK3CA were identified in 14 patients with LMs, all of which were hotspot mutations: five c.1624G > A; p.Glu542Lys, four c.1633G > A; p.Glu545Lys, and five c.3140 A > G; p.His1047Arg. [6] One patient had a RASA1 c.1772G > A; p.Arg591His variant. To our knowledge, this variant has not previously been reported in patients with vascular anomalies. RASA1 c.1772G > A; p.Arg591His is listed in COSMIC [25] as a mutation in angiosarcoma and stomach cancer, although it is indicated to be of uncertain significance in ClinVar [13] and InterVar [15]. In silico analyses predicted this variant to be tolerated by SIFT [21], probably damaging by PolyPhen-2 [22], and disease causing by MutationTaster [23], and was considered as potentially pathogenic. Allele frequencies of pathogenic variants in patients with LMs ranged from 3.5% to 10.3%, with 92.9% and 57.1% of individuals having VAFs of <10% and <5%, respectively.

Genetic variants associated with KTS

Eight of the 10 patients with KTS harbored pathogenic variants in PIK3CA (Table 3). All of these were hotspot variants and consisted of two c.1624G > A; p.Glu542Lys, five c.1633G > A; p.Glu545Lys, and one c.3140 A > G; p.His1047Arg. Allele frequencies of pathogenic variants in patients with KTS ranged from 6.0% to 17.7%, with 75% and 0% of individuals having VAFs of <10% and <5%, respectively.

DISCUSSION

We report the results of NGS of 29 candidate genes in affected tissues from 59 patients with SFVMs. Consistent with the results of previous studies [4–6], we identified three somatic PIK3CA variants (c.1624G > A; p.Glu542Lys, c.1633G > A; p.Glu545Lys, and c.3140 A > G; p.His1047Arg) in patients with VM, LM, LVM, and KTS, and seven somatic activating TEK variants (c.2690 A > G; p.Tyr897Cys, c.2689 T > C; p.Tyr897His, c.2740 C > T; p.Leu914Phe, c.2743 C > T; p.Arg915Cys, c.2752 C > T; p.Arg918Cys, c.2753 G > T; p.Arg918Leu, c.3295 C > T; p.Arg1099*) in patients with VM. VMs are painful and deforming lesions caused by dilated vascular channels. [27] Somatic activating variants in TEK have previously been identified in about 60% of VMs. [27] More recently, somatic activating variants in PIK3CA have been identified in about 25% of VM cases. [26] In patients with wild-type TEK and PIK3CA genes, the VMs are likely caused by infrequent variants in other genes connected to the PI3K/AKT/mTOR and RAS/MAPK pathways, as suggested by Castel et al. [8] TEK and PIK3CA variants drive constitutive activation of the PI3K/AKT/mTOR pathway, resulting in increased proliferation and survival of endothelial cells, which could account for the increased

Table 2. Clinicopathological features of patients with SFVM subtypes

| Gender, n (%) | Total (n = 59) | VM (n = 21) | LM (n = 27) | LVM (n = 1) | KTS (n = 10) |
|---------------|---------------|------------|------------|------------|-------------|
| Male          | 31 (52.5)     | 8 (38.1)   | 18 (66.7)  | 1 (100)    | 4 (40)      |
| Female        | 28 (47.5)     | 13 (61.9)  | 9 (33.3)   | 0 (0)      | 6 (60)      |
| Age, median (range), years | 7.5 (0–72)* | 12 (0–56)* | 4 (0–72)* | 66 | 5 (1–19) |

Surgical procedure, n (%)

| Procedure          | Total (n = 59) | VM (n = 21) | LM (n = 27) | LVM (n = 1) | KTS (n = 10) |
|--------------------|---------------|------------|------------|------------|-------------|
| Surgical excision  | 46 (78)       | 18 (85.7)  | 24 (88.9)  | 4 (40)     |             |
| Punch biopsy       | 12 (20.3)     | 3 (14.3)   | 3 (11.1)   | 6 (60)     |             |
| Needle biopsy      | 1 (1.7)       | 1 (100)    |            |            |             |

KTS Klippel–Trenaunay syndrome, LM lymphatic malformation, LVM lymphatic venous malformation, SFVM slow-flow vascular malformation, VM venous malformation.

*0 refers to children less than 1 year of age.
| Patient | SFVM type | Gender | Age (years) | Site      | Maximum diameter (cm) | Number of lesions | Specimen type      | Gene       | Variant                        | VAF (%) |
|---------|-----------|--------|-------------|-----------|----------------------|------------------|-------------------|------------|--------------------------------|---------|
| VM1     | VM        | Male   | 2           | Femur     | 4.5                  | Single           | Excision          | TEK        | c.2740 C > T; p.Leu914Phe     | 9.4     |
| VM2     | VM        | Male   | 12          | Neck      | 1.0                  | Multiple         | Excision          | TEK        | c.2740 C > T; p.Leu914Phe     | 10.3    |
| VM3     | VM        | Male   | 16          | Back      | 10.0                 | Single           | Punch biopsy      | TEK        | c.2740 C > T; p.Leu914Phe     | 9.1     |
| VM4     | VM        | Male   | 54          | Lip       | 4.0                  | Multiple         | Excision          | TEK        | c.2740 C > T; p.Leu914Phe     | 4.2     |
| VM5     | VM        | Female | 43          | Neck      | 4.0                  | Multiple         | Excision          | TEK        | c.2743 C > T; p.Arg915Cys     | 12.6    |
| VM6     | VM        | Female | 8           | Pelvis    | 5.0                  | Multiple         | Excision          | TEK        | c.3295 C > T; p.Arg1099*      | 3.7     |
| VM7     | VM        | Female | 2           | Leg       | 4.0                  | Single           | Excision          | TEK        | c.2690 A > G; p.Tyr897Cys     | 4.1     |
| VM8     | VM        | Female | 12          | Leg       | 1.0                  | Single           | Punch biopsy      | TEK        | c.2740 C > T; p.Leu914Phe     | 13.6    |
| VM9     | VM        | Male   | 5           | Head      | 3.0                  | Single           | Excision          | TEK        | c.2689 T > C; p.Tyr897His     | 8.6     |
| VM10    | VM        | Male   | 50          | Arm       | 5.0                  | Single           | Excision          | TEK        | c.1633G > A; p.Glu545Lys      | 3.8     |
| VM11    | VM        | Female | 17          | Clavicle  | 4.0                  | Single           | Excision          | PK3CA      | c.1624G > A; p.Glu542Lys      | 5.3     |
| VM12    | VM        | Female | 20          | Femur     | 4.0                  | Multiple         | Excision          | PK3CA      | c.1633G > A; p.Glu545Lys      | 11.8    |
| VM13    | VM        | Male   | 5           | Axilla    | 15.0                 | Multiple         | Excision          | PK3CA      | c.1633G > A; p.Glu545Lys      | 13.0    |
| VM14    | VM        | Female | 11          | Femur     | 12.8                 | Single           | Excision          | PK3CA      | c.3140 A > G; p.His1047Arg    | 9.2     |
| LM1     | LM        | Female | 1           | Buttock   | 15.0                 | Single           | Excision          | PK3CA      | c.1624G > A; p.Glu542Lys      | 10.3    |
| LM2     | LM        | Male   | 1           | Auricle   | 3.0                  | Single           | Excision          | PK3CA      | c.1624G > A; p.Glu542Lys      | 7.9     |
| LM3     | LM        | Male   | 0           | Neck      | 15.0                 | Single           | Excision          | PK3CA      | c.1624G > A; p.Glu542Lys      | 4.6     |
| LM4     | LM        | Female | 2           | Neck      | 10.0                 | Single           | Excision          | PK3CA      | c.1624G > A; p.Glu542Lys      | 4.6     |
| LM5     | LM        | Male   | 8           | Wrist     | 2.0                  | Multiple         | Excision          | PK3CA      | c.1624G > A; p.Glu542Lys      | 3.5     |
| LM6     | LM        | Female | 1           | Leg       | 5.0                  | Single           | Excision          | PK3CA      | c.1633G > A; p.Glu545Lys      | 4.2     |
| LM7     | LM        | Female | 19          | Mesentery | 5.0                  | Single           | Excision          | PK3CA      | c.1633G > A; p.Glu545Lys      | 6.7     |
| LM8     | LM        | Male   | 5           | Cheek     | 8.0                  | Single           | Excision          | PK3CA      | c.1633G > A; p.Glu545Lys      | 3.8     |
| LM9     | LM        | Male   | 1           | Cheek     | 10.0                 | Single           | Excision          | PK3CA      | c.1633G > A; p.Glu545Lys      | 4.0     |
| LM10    | LM        | Male   | 6           | Tongue    | 1.0                  | Single           | Excision          | PK3CA      | c.3140 A > G; p.His1047Arg    | 8.4     |
| LM11    | LM        | Female | 0           | Neck      | 2.0                  | Single           | Excision          | PK3CA      | c.3140 A > G; p.His1047Arg    | 4.1     |
| LM12    | LM        | Male   | 16          | Cheek     | 5.0                  | Single           | Excision          | PK3CA      | c.3140 A > G; p.His1047Arg    | 4.2     |
| LM13    | LM        | Male   | 5           | Neck      | 10.0                 | Single           | Excision          | PK3CA      | c.3140 A > G; p.His1047Arg    | 8.8     |
| LM14    | LM        | Female | 1           | Finger    | 2.0                  | Single           | Excision          | PK3CA      | c.3140 A > G; p.His1047Arg    | 6.2     |
| VM15    | LM        | Male   | 66          | Waist     | 23.0                 | Single           | Needle biopsy     | PK3CA      | c.1624G > A; p.Glu542Lys      | 17.7    |
| KT1     | KTS       | Female | 17          | Buttock   | –                    | –                | Punch biopsy      | PK3CA      | c.1624G > A; p.Glu542Lys      | 6.0     |
| KT2     | KTS       | Female | 5           | Leg       | –                    | –                | Excision          | PK3CA      | c.1624G > A; p.Glu542Lys      | 8.9     |
| KT3     | KTS       | Female | 19          | Abdomen   | –                    | –                | Punch biopsy      | PK3CA      | c.1633G > A; p.Glu545Lys      | 7.4     |
| KT4     | KTS       | Male   | 4           | Femur     | –                    | –                | Punch biopsy      | PK3CA      | c.1633G > A; p.Glu545Lys      | 7.8     |
| KT5     | KTS       | Male   | 1           | Leg       | –                    | –                | Punch biopsy      | PK3CA      | c.1633G > A; p.Glu545Lys      | 17.7    |
| KT6     | KTS       | Female | 1           | Leg       | –                    | –                | Excision          | PK3CA      | c.1633G > A; p.Glu545Lys      | 7.4     |
| KT7     | KTS       | Female | 4           | Abdomen   | –                    | –                | Excision          | PK3CA      | c.1633G > A; p.Glu545Lys      | 8.1     |
| KT8     | KTS       | Female | 1           | Leg       | –                    | –                | Excision          | PK3CA      | c.3140 A > G; p.His1047Arg    | 10.5    |

KTS Klippel–Trenaunay syndrome, LM lymphatic malformation, LVM lymphatic venous malformation, SFVM slow-flow vascular malformation, VAF variant allele frequency, VM venous malformation
accumulation of endothelial cells observed in VMs. [8] In two patients with VMs, we detected double somatic activating variants in \textit{TEK}. The ratio of single/double \textit{TEK} variants in our study (80%/20%) was similar to that (85.7%/14.3%) reported by Limaye et al. [28] Double somatic activating variants in \textit{TEK} found in our study were present in \textit{cis}. Although there have been several reports of VMs with double \textit{TEK} variants on the same allele [28–30], the clinical implications of this are unclear. Double \textit{TEK} variants show a stronger phosphorylation of TIE2 compared with single variants [31]; however, no correlation between disease severity, the number or location of lesions, or the TIE2 activation state has been observed. [28, 32] \textit{TEK} and \textit{PIK3CA} variants are typically mutually exclusive but both occur in some patients with VMs [8, 33, 34], as was the case for one patient with hotspot variants of both \textit{TEK} and \textit{PIK3CA} in the present study. It is likely that this can be explained by the presence of two related events in the same cells, because the VAFs of both variants were similar in our case. Another possibility is that variants in \textit{TEK} and \textit{PIK3CA} do not co-exist in the same cell. Further studies are needed to clarify the roles of simultaneous pathogenic variants in \textit{TEK} and \textit{PIK3CA} in the development of VM.

The \textit{TEK} nonsense mutation c.921 C $>$ G: p.Tyr307*, identified in one VM patient in the present study, has previously been associated with primary congenital glaucoma. [35] This variant is

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**Table 4.** Comparing frequencies of somatic variants in SFVMs with those in gnomAD or COSMIC

| Variant | SFVMs | gnomAD version 2.1.1 | COSMIC v96 |
|---------|-------|----------------------|------------|
|         | Allele frequency | Population frequency | Allele frequency | Population frequency | Allele frequency | Population frequency |
| TEK p.Leu914Phe | 0.0508 | 0.1016 | Absent | – | Absent | – |
| TEK p.Arg915Cys | 0.0085 | 0.0169 | Absent | – | 4.65e-5 | 0.0041 |
| TEK p.Arg1099* | 0.0085 | 0.0169 | Absent | – | Absent | – |
| TEK p.Tyr897Cys | 0.0085 | 0.0169 | Absent | – | Absent | – |
| TEK p.Arg918Cys | 0.0085 | 0.0169 | Absent | – | 2.33e-5 | 0.0027 |
| TEK p.Tyr897His | 0.0085 | 0.0169 | Absent | – | Absent | – |
| TEK p.Arg918Leu | 0.0085 | 0.0169 | Absent | – | 2.33e-5 | 0.0027 |
| TEK p.Tyr307* | 0.0085 | 0.0169 | 1.99e-5 | 0.0028 | Absent | – |
| PIK3CA p.Glu545Lys | 0.1017 | 0.2034 | 4.03e-6 | <0.0001 | 0.0192 | <0.0001 |
| PIK3CA p.Glu542Lys | 0.0763 | 0.1525 | Absent | – | 0.0118 | <0.0001 |
| PIK3CA p.His1047Arg | 0.0593 | 0.1186 | 4.03e-6 | <0.0001 | 0.0237 | 0.0005 |

SFVMs slow-flow vascular malformations

aP value of Fisher’s exact test comparing allele frequencies of somatic variants in SFVMs with those in gnomAD version 2.1.1

bP value of Fisher’s exact test comparing population frequencies of somatic variants in SFVMs with those in COSMIC v96

Values of $P < 0.05$ were considered to indicate statistical significance.
located in the ectodomain of TEK and is reportedly loss of function. [35] This differs strikingly from TEK variants linked to hereditary and sporadic VMs [27], which are located solely in the intracellular domain and result in enhanced kinase activity. [36] Interestingly, Limaye et al. identified a somatic second hit in TEK, a loss-of-function deletion, in a VM lesion from a patient carrying the TEK R849W variant in the germline. [28] This would suggest that loss-of-function variants in TEK might potentiate the development of VMs [32] and thus also implicates the nonsense variant detected in our study might be associated with development of VMs. Further investigation will be needed to clarify the pathogenicity of this variant.

LMs are characterized by the presence of abnormal lymphatic vessels with progressive cystic dilation. [37] Upregulation of the PI3K/AKT/mTOR pathway may be a causal factor in the development of the abnormal lymphatic vessels. [38] Previous studies performed on LM specimens have identified somatic activating variants in the PIK3CA gene. [38] For example, Blesinger et al. showed that activating PIK3CA variants in patients with LM were specifically localized in lymphatic endothelial cells. [39] Using deep targeted sequencing methods with a cohort of 64 patients, Zenner et al. reported PIK3CA variants in LM tissues from 68.8% of the patients with a VAF of <5%. [40] In our study, 57.1% (8/14) of the individuals with LMs had a maximum VAF of <5%. These data highlight the need for optimization of DNA sequencing methods to enable detection of very low VAFs in LM tissues.

One of our patients harbored a variant in RASA1, which encodes p120-RasGAP protein that inhibits activity of RAS protein. [41] Variants in this gene have been reported to be associated with capillary malformation-arteriovenous malformation and Parkes Weber syndrome, a congenital vascular malformation consisting of capillary malformation, VM, LM, and arteriovenous malformation. [41, 42] Most of the RASA1 mutations responsible for capillary malformation-arteriovenous malformation and Parkes Weber syndrome are loss of function mutations and may lead to activation of RAS and increase downstream signaling via MAPK and PI3K/AKT/mTOR pathways. [41] Although the RASA1 missense variant (c.1772G > A; p.Arg591His) identified in our LM patient is classified as a variant of uncertain significance, it is potentially pathogenic according to two prediction tools. Further genetic and functional studies are needed to determine whether the variant is indeed pathogenic.

LVMs composed of combined lymphatic and venous elements are present at birth and develop due to errors in venolymphatic development. [43] Two hypotheses have been proposed for the pathogenesis of LVMs. One suggests that the condition results from malformation of lymphatic vascular pathways, while the second considers that it represents a tumor that grows by cellular (mainly endothelial) hyperplasia. [43] A somatic variant of PIK3CA was identified in affected tissues from a LVM patient in the present study; however, the cell type harboring the variant is unknown. Further research is needed to elucidate the cellular and molecular pathways driving LVM pathogenesis.

KTS is a syndrome involving capillary and venous malformations as well as limb overgrowth with or without LM. [44] KTS is caused by a mutation in primitive limb-forming cells that are destined to become blood and lymphatic vessels, fat, and bones. [38] In most cases of KTS analyzed to date, the cause is mosaic activating variants of PIK3CA. [38] In the present study, we identified pathogenic PIK3CA variants in 8 of the 10 patients with KTS.
In recent years, sirolimus has emerged as a new medical treatment option for SFVMs through inhibiting the PI3K/AKT/mTOR signaling pathway. [3] Notably, sirolimus has demonstrated substantial clinical benefit, as reflected by a decrease in the size of most lesions and an improvement in quality of life. [45] However, long-term sirolimus treatment may cause significant side effects due to immunosuppression, and the clinical studies performed thus far suggest that it does not always reduce the volume of existing SFVM lesions. [46] Direct targeting of chronically activated TIE2 and/or PIK3CA kinases using specific inhibitors may provide the best clinical response for patients with SFVMs. [46]

There are several possible reasons why genetic alterations were not detected in 21 of the 59 patients in the present study. For example, the pathogenic alterations could be located in genes other than the 29 investigated here, or they could be located in other gene regions, such as deep intronic regions, not included in our targeted NGS approach. Alternatively, the VAFs may have been below the detection limit, the changes may have been epigenetic alterations or large deletions that were undetected using the current sequencing methodology, or sampling errors may have occurred. Moreover, only four pathogenic variants in two patients (Patients VM7 and VM9) among all pathogenic variants identified by NGS in this study were verified by Sanger sequencing (see Fig. 2). However, recent studies have proven that NGS is a reliable tool for discovering somatic variants in vascular anomalies. [4–6, 30] In addition, this study identified previously reported causative genes and gene variants for patients with vascular anomalies, and the identified low-frequency pathogenic variants were in line with somatic mosaicism in vascular anomalies. [4–6]

We did not examine DNA isolated from the blood of patients enrolled in this study, so cannot exclude the existence of pathogenic germline variants. Germline variants in PIK3CA are associated with a phenotype characterized by overgrowth, severe macrocephaly, mild intellectual disability, and few dysmorphic features. [47] Germline variants in TEK are associated with a phenotype characterized by small, multifocal bluish cutaneous and/or mucosal VMs. [36] However, no patients enrolled in this study appeared to have these features, and they were not clinically suspected of having germline variants in PIK3CA and TEK. In addition, based on the very low VAFs in our PIK3CA- or TEK-positive cases (all 18% or lower), none of the identified variants was likely to be a germline variant.

In conclusion, pathogenic variants in genes involved in the PI3K signaling pathway were predominant among the 29 genes and 59 samples examined here. Inhibitors of this pathway may therefore have utility as molecular targeted treatments for SFVMs.

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AUTHOR CONTRIBUTIONS

AN, AF, SY, SS, AK, FS, NA, KK, KS, and MO conceived and designed the study. KS performed the next-generation sequencing and AN, KK, KS, HO, YA, and MO interpreted the data. AN and MO wrote the manuscript. All authors read and approved the final manuscript.

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COMPETING INTERESTS

Some of the authors declared Financial and Non-Financial Relationships and Activities, and Conflicts of Interest regarding this manuscript as indicated in the supplementary materials.

ETHICS APPROVAL

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and Good Clinical Practice guidelines and approved by the ethics committee or institutional review board of each institution.

ADDITIONAL INFORMATION

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