PIK3CA mutation correlates with mTOR pathway expression but not clinical and pathological features in Fibro-adipose vascular anomaly (FAVA)

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Abstract

Background: Fibro-adipose vascular anomaly (FAVA) is a rare and new entity of vascular anomaly. Activating mutations in the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) gene were identified at a frequency of 62.5% in FAVA cases. The PIK3CA mutations excessively activate mammalian target of rapamycin (mTOR) pathway, which promotes angiogenesis and lymphangiogenesis, implying that PIK3CA mutations may act as drivers of FAVAs. This study investigated the correlations between PIK3CA mutational status, clinicopathological features and immunohistochemical expression of the mTOR pathway in a series of FAVA.

Methods: We retrospectively evaluated the clinical and pathological findings of four FAVA cases. We performed next-generation sequencing (NGS) with a custom panel of genes associated with the mTOR pathway and genes responsible for other vascular anomalies; followed by direct sequencing and immunohistochemical analysis of the mTOR pathway.

Results: Two PIK3CA-mutation cases and two PIK3CA-wild-type (wt) cases exhibited similar typical clinical features of FAVA. Histological analysis revealed venous malformation, lymphatic malformation, nerves containing enlarged abnormal vessels and fibrofatty tissue were observed regardless of PIK3CA mutational status. In contrast to clinical and histological findings, the immunohistochemical expression of activated AKT and mTOR that are upstream of the mTOR pathway was detected in abnormal vessels of PIK3CA-mutation cases but not in those of PIK3CA-wt cases. However, activated eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and ribosomal protein S6 kinase 1 (S6K1), both of which are downstream effectors of the mTOR pathway, were expressed in abnormal vessels of both PIK3CA-mutation and PIK3CA-wt cases. Furthermore, targeting NGS did not find any common genetic mutations involved in the mTOR pathway among PIK3CA-wt cases.
Background

Fibro-adipose vascular anomaly (FAVA) is a newly described vascular anomaly [1]. FAVA is extremely rare and occurs most commonly in the muscles of the lower extremities of young patients [1, 2]. Histologically, FAVA is composed of venous malformation (VM), lymphatic malformation (LM), and the presence of fibro-adipose tissues with the atrophic skeletal muscle [1, 3]. A recent study identified somatic and mosaic gain-of-function mutations of the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) gene in a subset of FAVAs (62.5%) [4]. The identified PIK3CA mutations are p.E542K, p.E545K and p.Q546K in the helical domain (encoded within exon 9), and p.H1047R in the kinase domain (encoded within exon 20) [4]. These PIK3CA mutations are termed hotspot mutations, and are present in a subset of VMs and the majority of LMs [4–10]. PIK3CA mutations excessively activate the phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway in the endothelial cells during vascular developments [4–10]. Activation of PI3K results in the phosphorylation of AKT (p-AKT), and p-AKT phosphorylates mTOR. Furthermore, the phosphorylated form of mTOR (p-mTOR) phosphorylates downstream effectors such as eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1), ultimately promoting angiogenesis and lymphangiogenesis [11, 12]. These results highly suggested that PIK3CA mutations may act as drivers of FAVAs through activation of the PI3K/AKT/mTOR pathway. Furthermore, the presence of PIK3CA mutations in VMs and the genotype of PIK3CA mutation in LMs correlate with both clinical severity and histological features [8, 10, 13]. However, little is known regarding the correlations among PIK3CA mutational status, the mTOR pathway activation status and clinicopathological features in FAVA. Here, we report the results of clinical, histological, immunohistochemical, and genetic analyses examining a small series of isolated FAVA cases.

Methods

Four FAVA cases with formalin-fixed paraffin-embedded (FFPE) tissues were retrieved from the pathology files of Osaka University Hospital from 2010 to 2020. A final diagnosis of FAVA was determined by consensus agreement after consideration of clinical, radiologic, and histological findings [1–3]. This study was approved by the Ethical Review Board of the Graduate School of Medicine, Osaka University (IBR No. 17,214).

Next-generation sequencing (NGS)

Genomic DNA was extracted from FFPE tissue using the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Two pathologists (Y.H. and K.H.) selected FFPE blocks with greater than 50% abnormal tissue content in all cases. The gene panel was designed by SureDesign (https://earray.chem.agilent.com/suredesign) to cover a whole exon of 14 genes associated with the mTOR pathway signaling or responsible for other vascular anomalies (PIK3CA, TEK, GNA11, GNAQ, AKTI, PTEN, mTOR, CCM, BRAF, MAP3K3, KRAS, NRAS, HRS, RASA1). On average 70 ng of the extracted DNA was fragmented by SureSelect Fragmentation Enzyme (Agilent Technologies, Inc. Santa Clara, CA, USA) to 150–200 bp. Sequence libraries were prepared with a custom SureSelect Low Input Target Enrichment System (Agilent Technologies, Inc. Santa Clara, CA, USA) according to the manufacturer’s instructions and sequenced with the Illumina MiSeq (Illumina, San Diego, CA, USA). SureCall ver4.0 (https://www.agilent.com/en/download-software-surecall) was used for variant calling. DNA in introns or non-coding DNA were excluded. To confirm PIK3CA gene mutations, polymerase chain reaction (PCR) assays and direct sequencing were performed using the following primers: PIK3CA-Exon9 Forward, CAGCTCAAAG CAATTTCTAC; PIK3CA-Exon9 Reverse, CACTTACC TGTTGACCTCAT; PIK3CA-Exon20 Forward, AACTGA GCAAGAGGCTTTGG; PIK3CA-Exon20 Reverse, TGTTGGAGAGATCCAAATCCA. A mixture of 5% PIK3CA-mutant DNA against a background of 95% wild-type (wt) DNA was used as a positive control.

Histological and immunohistochemical staining

Resected tissue samples were fixed with 10% formalin, routinely embedded in paraffin, cut into 4 μm thick serial sections, and used for H&E and immunohistochemical staining. Immunohistochemical staining was performed using a Roche Ventana BenchMark GX autostainer (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer’s instructions. Primary

Conclusions: There was no significant association between the presence of PIK3CA mutations and the clinicopathological features of FAVA, suggesting that the PIK3CA gene is not necessarily involved in the onset of FAVA. FAVAs lacking PIK3CA mutations may be caused by other gene mutations that activate 4EBP1 and S6K1.

Keywords: Fibro-adipose vascular anomaly, FAVA, PIK3CA, mTOR, Vascular anomaly, Lymphatic malformation, Venous malformation, Sirolimus
antibodies against p-AKT (#4060, 1:100; Cell Signaling Technology, Danvers, MA, USA), p-mTOR (clone 49F9, 1:100; Cell Signaling Technology), p-S6K1 (#9204, 1:100; Cell Signaling Technology), p-4EBP1 (clone 236B4, 1:500; Cell Signaling Technology), S100 (polyclonal, Ventana Medical Systems), CD31 (clone JC70A, 1:200, Dako), CD34 (clone QBEnd10, 1:200, Dako), D2-40 (760-4395, Ventana Medical System), and PROX1 (ab199359, 1:500; Abcam, Cambridge, UK) were used. Samples were considered positive when at least 10% of the endothelial cells of abnormal vessels exhibited a signal for the targeted protein.

Results
Clinical data and molecular genetic findings
The four patients included four men, and they ranged in age from 3 to 15 years (median, 12.5 years). Two patients presented at birth (cases 1 and 3). The presenting symptoms were pain (4/4 cases), swelling (3/4 cases), and functional restriction (2/4 cases). The preoperative clinical diagnosis was vascular anomalies, including infantile hemangioma, vascular malformation, and FAVA. The lesions were located within and in the vicinity of the thigh muscles, and knee (Fig. 1 A-D). Heterogenous PIK3CA hotspot mutation (p.H1047R) was identified in two cases (cases 1 and 2) (Fig. 1E), while none of the PIK3CA mutations were detected in other two cases (cases 3 and 4) (Fig. 1 F). We also found mutations in TEK in 2 cases, GNA11 in 1 case, AKT1 in 1 case, PTEN in 2 cases and HRAS in 1 case. The clinical characteristics and the results of the genetic analysis are summarized in Table 1.

Histological and immunohistochemical findings
The PIK3CA-mutation cases (cases 1 and 2) (Fig. 2 A-D) and PIK3CA-wt cases (cases 3 and 4) (Fig. 2E-H) possessed similar histology. All four cases exhibited abnormal vessels surrounded by dense fibrous tissue and adipose tissue with atrophic skeletal muscle (Fig. 2 A, E). The abnormal vessels were composed of VM and LM (Fig. 2B, F). The majority of the LM components possessed vascular clusters consisting of thin-walled back-to-back blood-filled sacs (Fig. 2 C, G). The lymphatic phenotype was supported by endothelial D2-40 and/or Prox1 immunopositivity in consistent with our previous study [3]. In one PIK3CA-mutant case (case 2) and one PIK3CA-wt case (case 3), some nerves contained enlarged vessels (Fig. 2D, H). The endothelial cells of these vessels within nerves were positive for CD31 (marker for endothelial cells) and CD34 (marker for blood vessels), negative to weakly positive for PROX1 (marker for lymphatic vessels), and negative for D2-40 (marker for lymphatic vessels) (Fig. 2I). That is, the vessels within nerves had the vein-like characteristics. The other findings included the observation of organized thrombi within abnormal veins in two cases (cases 2 and 4) and lymphocytic aggregates surrounding abnormal vessels in three cases (cases 2-4). The histological findings of all cases are summarized in Table 2.
We next examined the mTOR pathway activation status in abnormal vessels using immunohistochemical staining. The expression of p-AKT and p-mTOR, both of which are upstream of the mTOR pathway, was detected in abnormal vessels of the PIK3CA-mutation cases (cases 1 and 2) (Fig. 3 A, B) but not in those of the PIK3CA-wt cases (cases 3 and 4) (Fig. 3E, F). The expression of p-4EBP1 and p-S6K1, both of which are downstream effectors of the mTOR pathway, was detected in abnormal vessels of both PIK3CA-mutation and PIK3CA-wt cases (Fig. 3 C, D, G, H). The one PIK3CA-wt case (case 4) dose not express p-4EBP1 expression. In normal tissues, including the surrounding skeletal muscle and normal vessels, p-S6K1 exhibited sporadic expression, while p-AKT, p-mTOR, and p-4EBP1 were not expressed at detectable levels. The immunohistochemical results are summarized in Table 3.

Discussion

FAVA is a new entity of vascular anomaly and is exceedingly rare. Alomari et al. (2014) [1] provided a proposed definition of the clinical and histological characteristics of FAVA. Subsequently, PIK3CA mutations were reported in a subset of FAVAs (5/8 cases) [4]. However, the correlations between specific mutations and clinicopathological features remain unclear. The current study is the first reported series of clinical, histological, immunohistochemical, and genetic analyses examining FAVA cases.

According to a clinical series of FAVA, FAVA arises in young patients (median age, 12-17 years) [1, 2]. Common symptoms include pain (100%), functional restriction (81-100%), and swelling (36.5%) [1, 2]. The most common location of the lesion is the lower extremities (94.7%) [2]. Our current study determined that the median age of PIK3CA-mutation patients was 9 years and that of PIK3CA-wt patients was 12.5 years. Both PIK3CA-mutation and PIK3CA-wt patients presented with pain, swelling, and functional restriction. All four lesions were located in the lower extremities. Based on the above findings, our cases exhibited typical clinical features regardless of PIK3CA mutational status (Table 1) [1, 2]. Similarly, both PIK3CA-mutation and PIK3CA-wt cases exhibited the typical histological features of FAVA (Table 2). Histologically, VM, LM, fibrous tissue, and adipose tissue were observed in all cases. Nerves containing enlarged vessels, a condition that is unusual in other vascular anomalies, were also present regardless of PIK3CA mutational status (Fig. 2D, H). Thus, our observations indicated that there was no significant association between the presence of PIK3CA mutations and the clinicopathological features of FAVA.

Immunohistochemical analysis showed that p-AKT and p-mTOR that act upstream of the mTOR pathway were detected in abnormal vessels of PIK3CA-mutation cases, but not in those of PIK3CA-wt cases (Table 3). On the other hand, p-4EBP1 and p-S6K1, downstream of the mTOR pathway, were detected in abnormal vessels of both PIK3CA-mutation and PIK3CA-wt cases (Table 3). One interpretation of this discrepancy was that 4EBP1 and S6K1 were activated in mTOR-independent manner. In fact, phosphorylation of 4EBP1 and S6K1 is subject to mTOR-independent several kinas and feedback loops [14, 15]. Somatic mutations in PIK3CA occur frequently in cancers other than LMs and other PIK3CA-related overgrowth spectrums [4–6, 8]. In cancers, a small number of studies have demonstrated a positive correlation between PIK3CA mutational status and upstream activation of the mTOR pathway [16–18]. p-AKT and p-mTOR were immunohistochemically expressed more frequently in PIK3CA-mutation cases than in PIK3CA-wt cases, while the immunohistochemical expression of p-4EBP1 and p-S6K1 was not correlated with the presence of PIK3CA mutation [16–18]. These results were consistent with the relationship between the mutation and immunohistochemical expression in our FAVA cases.

Both 4EBP1 and S6K1 are involved in the development of abnormal vessels in VMs and LMs by promoting protein synthesis and cell growth [5–9, 11, 12]. The activation of 4EBP1 and S6K1 may play a key role in the pathogenesis of abnormal vessels in FAVA lesions;
Fig. 2 Histology and immunohistochemical analysis of FAVA. A-H Representative histological findings in PIK3CA-mutation case (A-D) and PIK3CA-wild type (wt) case (E-H). Vascular malformation and adipose and dense fibrous tissue (A, E, lower magnification and B, F, higher magnification). Clusters of vascular channels with thin-walled back-to-back blood-filled sacs (C, G). Nerve containing enlarged vessels (D, H arrows). I Serial sections of a nerve containing enlarged vessels (H) stained for S100, CD31, CD34, PROX1, and D2-40 (arrows indicated abnormal vessels in nerve).

Table 2 Summary of histological findings

| Case | VM | LM | Nerve containing enlarged abnormal vessels | Organized thrombi | Lymphocytic aggregates |
|------|----|----|------------------------------------------|------------------|-----------------------|
| 1    | +  | +  | -                                        | -                | -                     |
| 2    | +  | +  | +                                        | +                | +                     |
| 3    | +  | +  | +                                        | -                | +                     |
| 4    | +  | +  | -                                        | +                | +                     |

VM: venous malformation, LM: lymphatic malformation
however it was unclear what signaling pathways were involved in their activation. Since the identification of PIK3CA mutations in FAVA by Luks et al. [4], further mutational analyses of FAVA have not been performed. Our targeting NGS failed to identify common gene mutations associated with mTOR pathway among PIK3CA-wt cases, although TEK mutation (p.R842H within exon 13) was detected in one PIK3CA-wt case. Somatic gain-of-function mutations in TEK gene that encodes the endothelial tyrosine-protein kinase receptor TIE-2 occurs approximately half of sporadic VMs and in a subset of LMs [8, 19, 20]. TEK hotspot mutations are detected exclusively in exon 17 and are present within the first tyrosine kinase and kinase insert domains of the receptor [8, 19, 20]. TEK hotspot mutations result in a constantly active PI3K/AKT signaling pathway involving angiogenesis [8, 19, 20]. On the other hand, AKT phosphorylates many downstream molecules involved in the regulation of cellular functions. Therefore, little is known about the association with TEK mutations and activation of mTOR downstream effectors in VMs. The identified TEK p.R842H (c.2525G>A) mutation in current study is reported in the COSMIC (Catalogue of Somatic Mutations in Cancer) database, however the function of this mutation is not investigated. Considering that activated AKT was not detected in PIK3CA-wt cases, the TEK p.R842H mutation may not activate AKT in FAVA. Approximately 25% of VMs lacked both TEK and PIK3CA mutations [8, 10], and the responsible genetic aberrations remain unclear. Thus, FAVA lacking PIK3CA mutations may be caused by undiscovered mutations that activate 4EBP1 and S6K1.

**Conclusions**

In this study, we reported the results of clinical, histological, immunohistochemical, and genetic analyses examining a small series of isolated FAVA. There was no significant association between the presence of PIK3CA mutations and the clinical and histological features of FAVA, suggesting that the PIK3CA gene may be not necessarily involved in the onset of FAVA. FAVA lacking PIK3CA mutations may be caused by other mutations that activate 4EBP1 and S6K1.

**Table 3** Immunohistochemical expression of mTOR pathway in abnormal vessels

| Case | p-AKT | p-mTOR | p-4EBP1 | p-S6K1 |
|------|-------|--------|---------|--------|
| 1    | +     | +      | +       | +      |
| 2    | +     | +      | +       | +      |
| 3    | -     | -      | +       | +      |
| 4    | -     | -      | -       | +      |

Staining intensity (-; no expression / +; positive)
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References
1. Alomari AI, Spencer SA, Arnold RW, et al. Fibro-adipose vascular anomaly: clinical-radiologic-pathologic features of a newly delineated disorder of the extremity. J Pediatr Orthop. 2014;34:109–17.
2. Amanneh M, Shakh R. Clinical and imaging features in fibro-adipose vascular anomaly (FAVA). Pediatr Radiol. 2020;50:383–87.
3. Horii Y, Hirose K, Aramaki-Hattori N, et al. Fibro-adipose vascular anomaly (FAVA): three case reports with an emphasis on the mammalian target of rapamycin (mTOR) pathway. Diagn Pathol. 2020;15:98.
4. Lukas VL, Kamitaki N, Vivero MP, et al. Lymphatic and other vascular malformative/overgrowth disorders are caused by somatic mutations in PIK3CA. J Pediatr. 2015;166:1048-54.e1-5.
5. Madisen RR, Vanhaesebroeck B, Semiple RK. Cancer-Associated PIK3CA Mutations in Overgrowth Disorders. Trends Mol Med. 2018;24:856–70.
6. Keppler-Noreuil KM, Rios JL, Parker VE, et al. PIK3CA-related overgrowth spectrum (PROS): diagnostic and testing eligibility criteria, differential diagnosis, and evaluation. Am J Med Genet A. 2015;167A:287–95.
7. Blesinger H, Kaulfuß S, Aung T, et al. PIK3CA mutations specifically localized to lymphatic endothelial cells of lymphatic malformations. PLoS One. 2018;13:e0200343.
8. Castillo SD, Baselga E, Graupera M. PIK3CA mutations in vascular malformations. Curr Opin Hematol. 2019;26:70–78.
9. Pont Cal, Carmona FJ, Grego-Bessa J, et al. Somatic PIK3CA mutations as a driver of spordadic venous malformations. Sci Transl Med. 2016;8:332ra42.
10. Limaye N, Kangas J, Mendola A, et al. Somatic Activating PIK3CA Mutations Cause Venous Malformation. Am J Hum Genet. 2015;97:914–21.
11. Whalen SG, Gingras AC, Amankwa L, et al. Phosphorylation of eIF-4E on serine 209 by protein kinase C is inhibited by the translational repressors, 4E-binding proteins. J Biol Chem. 1996;271:11831–7.
12. Dennis PB, Pullen N, Kozma SC, et al. The principal rapamycin-sensitive PI(3)K(p70(S6K)) phosphorylation sites, T-229 and T-389, are differentially regulated by rapamycin-insensitive kinase kinases. Mol Cell Biol. 1996;16:2424–51.
13. Zenner K, Cheng CV, Jensen DM, et al. Genotype correlates with clinical severity in PIK3CA-associated lymphatic malformations. JCO Insight. 2019;4:e129884.
14. Qin X, Jiang B, Zhang Y. 4E-BP1, a multifactor regulated multifunctional protein. Cell Cycle. 2016;15:781–86.
15. Arif A, Ia J, Willard B, Li X, Fox PL. Phosphorylation of S6K1 directs a kinase phospho-code that determines substrate selection. Mol Cell. 2019;73:446–57.
16. Maruyama N, Miyoshi Y, Taguchi T, et al. Clinicopathologic analysis of breast cancers with PIK3CA mutations in Japanese women. Clin Cancer Res. 2007;13(2 Pt 1):408–14.
17. Azim HA, Kassem L, Teilleux L, et al. Analysis of PI3K/mTOR Pathway Biomarkers and Their Prognostic Value in Women with Hormone Receptor-Positive, HER2-Negative Early Breast Cancer. Transl Oncol. 2016;9:114–23.
18. Wang J, Zhu X, Xu X, et al. PIK3CA mutations and downstream effector p-mTOR expression: implication for prognostic factors and therapeutic targets in triple negative breast cancer. Int J Clin Exp Pathol. 2017;10:7682–91.
19. Limaye N, Wouters V, Uebelhoer M, et al. Somatic mutations in angioipetin receptor gene TEK cause solitary and multiple sporadic venous malformations. Nat Genet. 2009;41:118–24.
20. Ye C, Pan L, Huang Y, Ye R, Han A, Li S, Li X, Wang S. Somatic mutations in exon 17 of the TEK gene in vascular tumors and vascular malformations. J Vasc Surg. 2011;54:1760–8.

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