Assessment of PI3K/AKT and MAPK/ERK pathways activation in oral lymphatic malformations

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Objective. Lymphatic malformations are characterized by the overgrowth of lymphatic vessels during development. Activation of PI3K/AKT and MAPK/ERK signaling pathways occur in isolated lymphatic malformation and in those associated with syndromes such as CLOVES and Klippel-Trenaunay. We aimed to assess the activation of these pathways in sporadic oral lymphatic malformations.

Study Design. A convenience sample of 14 formalin-fixed paraffin-embedded samples of oral lymphatic malformations underwent immunohistochemical reactions for the phosphorylated forms of AKT1 (pAKT-Ser473) and ERK1/2 (pERK1/2-Thr202/Tyr204), which are markers of PI3K/AKT and MAPK/ERK pathways activation, respectively.

Results. Positive staining for pAKT1 and pERK1/2 was observed in the endothelial cells in all samples of oral lymphatic malformations evaluated.

Conclusions. Our results suggest that activation of PI3K/AKT and MAPK/ERK signaling pathways participates in the pathogenesis of oral lymphatic malformations. (Oral Surg Oral Med Oral Pathol Oral Radiol 2022;133:216–220)

Lymphatic malformations, also known as lymphangiomas, are benign lesions originating from embryonic lymphatic sacs that failed to connect to the drainage system. Lymphatic malformations occur mostly in lymphatic-rich areas, mainly in the head and neck (45%-52%), but also in the axilla and mediastinum.[1-3] Lymphatic malformations are classified into 3 morphologic subtypes: macrocystic, microcystic, and combined. Macrocystic lesions are large and are habitually located below the level of the mylohyoid muscle. By contrast, microcystic lesions are smaller and usually found in the oral cavity. Combined lesions are characterized by the fusion of these 2 subtypes.[3]

Lymphatic malformations most often occur sporadically (isolated), but they can also occur in syndromes such as Klippel-Trenaunay syndrome (OMIM #149000), CLOVES (congenital lipomatous overgrowth, vascular malformations, epidermal nevis, spinal/skeletal anomalies/scoliosis) (OMIM #612918), and CLAPO syndrome (capillary vascular malformation of the lower lip, lymphatic malformations of the head and neck, asymmetry, and partial or generalized overgrowth) (OMIM #613089).[4]

Oral lymphatic malformations usually manifest as a translucid plaque with small thin-walled vesicles resembling frog eggs. These lesions occur more frequently in the dorsum of the tongue, but they can also affect the lips, buccal mucosa, soft palate, and floor of the mouth.[5] The pathogenesis of oral lymphatic malformations is poorly characterized.

Recently, somatic activating *PIK3CA* mutations have been reported in isolated and syndrome-associated lymphatic malformations.[6] *PIK3CA* encodes the PI3K catalytic subunit p100α, and somatic activating mutations lead to the PI3K/AKT pathway signaling activation.[6-9] Notably, the activation of PI3K/AKT and MAPK/ERK signaling pathways play an important role in angiogenesis and were recently reported in lymphatic malformations.[10-14]

The PI3K/AKT and MAPK/ERK are compensatory pathways that mediate cell survival, proliferation, tumor growth, and angiogenesis. Cell surface receptors initiate the signaling processes, then signaling occurs through cytoplasmic kinases and other proteins, culminating with the control of gene expression in the nucleus.[15-17] There are many mechanisms of cross talk

Statement of Clinical Relevance

The activation of PI3K/AKT and MAPK/ERK in oral lymphatic malformation reveals that these pathways play a role in the pathogenesis of these lesions. These results can pave the way for targeted therapies as a step preceding surgical removal in challenging cases.

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between PI3K/AKT and MAPK/ERK signaling pathways.[18] Among these cross talk mechanisms, there are cross-activation and pathways convergence on substrates, which are represented in Figure 1.

The molecular pathogenesis of oral lymphatic malformations remains unclear. Therefore, the present study aimed to assess the activation of PI3K/AKT and MAPK/ERK signaling pathways in oral lymphatic malformations via the evaluation of the immunoexpression of the phosphorylated forms of ERK1/2 (pERK1/2) and AKT1 (pAKT1), markers of these pathways activation.[15,16]

MATERIALS AND METHODS

Study design and ethical approval

This retrospective and cross-sectional study evaluated a convenience sample of 14 formalin-fixed paraffin-embedded tissue specimens of sporadic oral lymphatic malformations. The specimens were obtained from the oral pathology service of Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Brazil. This study was approved by the Research Ethics Committee (protocol no. 30531120.4.0000.5149) and was accomplished in accordance with the Declaration of Helsinki.

Patients and samples

The following data of the patients with oral lymphatic malformations were obtained from the files: sex, age, and anatomic location. The histopathologic diagnosis was confirmed by 2 oral pathologists (C.C.G. and R.S.G.) by reviewing the sections stained with hematoxylin and eosin (H&E) retrieved from the files. Diagnostic criteria included the presence of dilated thin-walled lymphatic channels, with empty lumina or containing proteinaceous fluid, lymphocytes, and some erythrocytes.[19]

Immunohistochemistry

To assess PI3K/AKT and MAPK/ERK pathways activation, 14 cases of oral lymphatic malformations were subjected to immunohistochemistry for pAKT1 and pERK1/2, following standard protocols.[20,21] Briefly, tissues were sectioned (3 μm) and placed on silanized slides (StarFrost, Knittel Glass, Germany). The slides were subsequently

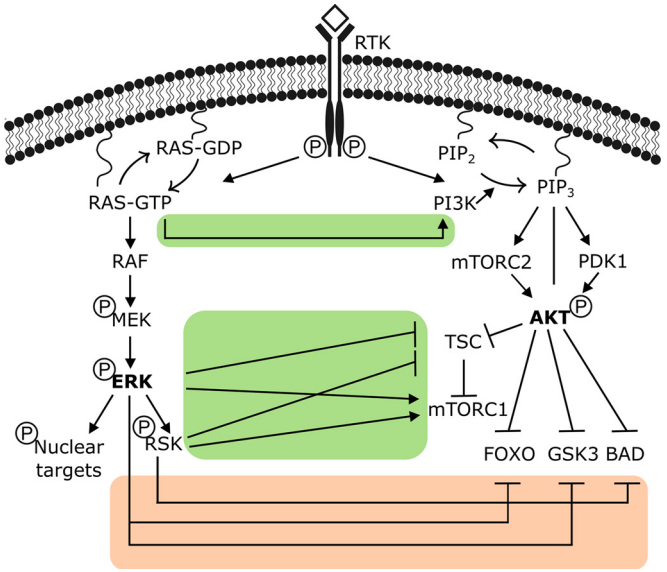

Fig. 1. Cross talk between MAPK/ERK and PI3K/AKT pathways. Interactions between external signals (e.g., growth factors) and receptor tyrosine kinases (RTKs) can activate both pathways. In MAPK/ERK, the activated RAS-GTP interacts and activates RAF, which phosphorylates and activates MEK, which activates ERK by phosphorylation. ERK then phosphorylates a variety of nuclear and cytoplasmic target proteins. In PI3K/AKT, the external signal stimulates the phosphorylation of PIP_2 by PI3K, converting it into PIP_3. AKT is recruited to the plasma membrane by binding to PIP_3 and is then activated owing to phosphorylation by PDK1 and mTORC2 that also bind PIP_3. AKT then inhibits TSC leading to activation of mTORC1, because TSC inhibits mTORC1. AKT leads to cell proliferation and cell survival by inhibiting FOXO, GSK3, and BAD, because these proteins inhibit cell proliferation or induce cell death. Therefore, both MAPK/ERK and PI3K/AKT pathways play important roles in cell survival, proliferation, and growth. Such pathways interact through both cross-activation (green rectangles) and pathway convergence (red rectangles). Cross-activation includes activation of PI3K by RAS and inhibition of TSC by ERK and RSK, which also activate mTORC1. In these cases, members of MAPK/ERK pathway regulate upstream components of the PI3K/AKT pathway, increasing its activity. In addition, both MAPK/ERK and PI3K/AKT can act directly on the same downstream targets, such as FOXO, GSK3, and BAD, negatively regulating their functions and characterizing pathway convergence. Although we have focused on interactions through which both pathways act in the same direction, there are other mechanisms of pathway cross talk, such as cross-inhibition and negative feedback loop.[18]

deparaffinized in xylene (Merck KGaA, Darmstadt, Germany) and hydrated in descending grades of ethanol (Merck). For antigen retrieval, citrate buffer (pH 6.0) solution (pAKT1) and TRIS-EDTA buffer solution (pH 8.0) (pERK1/2), heated to 90°C in a water bath for 30 minutes, were used. Endogenous peroxidase was blocked with hydrogen peroxide/methanol in 1:1 proportion. The slides were then incubated with the primary antibodies: rabbit monoclonal anti-pAKT1 (1:200, s473; Abcam, EP2109Y) and rabbit monoclonal anti-pERK1/2 (1:100; Thr202/Tyr204, Cell Signaling Technology #4376) overnight at 4°C. The detection system used was EnVision (DakoCytomation, Carpinteria, CA, USA). The reactions were revealed with diamonibenzidine tetrahydrochloride (DAB; Novocastra, Newcastle, UK) and counter-stained with Mayer's hematoxylin. Negative controls were obtained by the omission of the primary antibody. The epithelium of oral mucosa from each case served as internal controls for the immunohistochemical reactions.

Results interpretation

Three observers (I.P.G., R.S.G., and C.C.G.) evaluated the results on a light microscope. The entire tissue sections were evaluated, and nuclear and cytoplasmic staining were considered positive reactions, regardless of staining intensity. Slides were scanned using a Pannoramic MIDI Digital Slide Scanner (3DHISTECH, Budapest, Hungary), and the images were visualized and captured using Case Viewer v.2.3 (3DHISTECH, Budapest, Hungary).

RESULTS

Sample characterization

The mean age of the patients with oral lymphatic malformations was 19 years (ranging from 6 to 72), the male to female ratio was 1:1, and the tongue was the most affected location. Available clinical information is shown in Table I.

Microscopically, all cases had lymphatic vessels with marked dilation. The endothelial lining is thin, and the dilated spaces contain proteinaceous fluid and lymphocytes. Surrounding connective tissue stroma consisted of loose fibrotic tissue with a few inflammatory cells (Figure 2).

Immunohistochemistry

The immunohistochemistry reactions for pERK1/2 and pAKT1 of all samples included in the study showed the same staining pattern, and positive staining was observed in endothelial cells. pERK1/2 showed nuclear immunoexpression, whereas pAKT1 immunostaining was mainly observed in the cytoplasm. Also, nuclear immunoexpression of pAKT1 was detected in focal areas. As positive internal controls, pERK1/2 and pAKT1 immunostaining were also observed in epithelial cells of the lining mucosa in all cases (Figure 2).

Table I. Clinical data of the 13 cases of oral lymphatic malformations included in the study

Sample No.	*Age (years)*	*Sex*	*Location*
1	11	M	Buccal mucosa
2	26	M	Alveolar ridge
3	34	F	Buccal mucosa
4	35	F	Tongue (ventral side)
5	6	M	Tongue dorsum
6	23	F	Tongue dorsum
7	7	M	Tongue dorsum
8	10	F	Tongue dorsum
9	8	M	Soft palate
10	12	F	Tongue (ventral side)
11	72	F	Right lateral tongue
12	8	M	Palatoglossal arch
13	8	M	Palatoglossal arch
14	7	F	Tongue dorsum

The mean age of the patients with oral lymphatic malformations was 19 years (ranging from 6 to 72), the male to female ratio was 1:1, and the tongue was the most affected location.
F, female; *M*, male.

DISCUSSION

Intracellular signaling pathways, such as PI3K/AKT and MAPK/ERK, are often activated by mutations, causing endothelial cell dysfunction. Mutations in such pathways have been reported in lymphatic malformations.[6,8,22] There is no significant correlation between specific *PIK3CA* mutations, the macro- or microcystic phenotype, or clinical severity in lymphatic malformations.[6] Although the activation of PI3K/AKT and MAPK/ERK signaling pathways has recently been reported in lymphatic malformations,[10-14] the involvement of these signaling pathways specifically in oral lymphatic malformations has not been addressed. Therefore, we assessed the activation of MAPK/ERK and PI3K/AKT signaling pathways in oral lymphatic malformations by immunohistochemistry for the phosphorylated forms of ERK1/2 and AKT1. The immunohistochemistry staining for both pERK1/2 and pAKT1 showed positivity of lymphatic endothelial cells in all samples.

The activation of PI3K/AKT and MAPK/ERK observed in oral lymphatic malformations in the present study was similar to that observed in isolated and syndrome-associated lymphatic malformations harboring *PIK3CA* mutations.[12-14] In previous studies, lymphatic endothelial cells from lymphatic malformations demonstrated increased proliferation and expression of pAKT and pERK compared with normal human dermal lymphatic cells, depicting their highly activated angiogenic state.[12-14] PI3K signal transduction via the AKT and mammalian target of rapamycin (mTOR) kinases has been well documented in

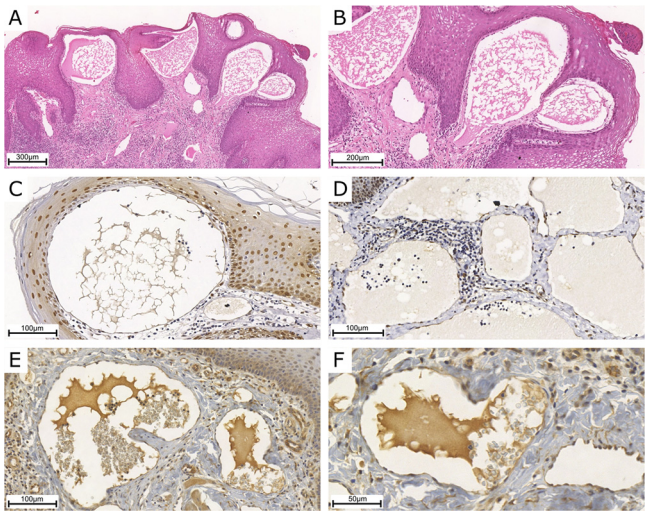

Fig. 2. Histopathologic features and immunohistochemical expression of pERK1/2 and pAKT1 in oral lymphatic malformations samples. **(A, B)** Oral lymphatic malformations are characterized by numerous single endothelial-lined vessels of variable sizes from the subepithelial region to the depth of tissue with lymphatic spaces containing proteinaceous material and occasional inflammatory cells, such as lymphocytes; hematoxylin and eosin staining. **(A)** Original magnification 50×; scale bar 300 μm. **(B)** Original magnification 100×; scale bar 200 μm. **(C, D)** Lymphatic endothelial cells showed marked nuclear pERK1 immunoexpression (original magnification 200×; scale bar 100 μm). **(E, F)** Positive cytoplasmic immunoexpression of pAKT1 was observed in the lymphatic endothelial cells. Positive nuclear immunoexpression was also detected in focal areas. **(E)** Original magnification 200×; scale bar 100 μm. **(F)** Original magnification 400×; scale bar 50 μm. **(C, E)** Oral mucosa immunoexpression served as the positive internal control for the reactions.

tumor growth process and angiogenesis[23] and for lymphatic development in mice.[24] ERK phosphorylation is also a marker for angiogenic endothelial cells.[14] MAPK/ERK and PI3K/AKT signaling pathways are closely related to each other owing to activation by similar extracellular signals and the cross talk between them.[18] Interactions between these pathways are shown in Figure 1**,** and such cross talk is in agreement with the simultaneous activation of MAPK/ERK and PI3K/AKT in our samples.

Oral lymphatic malformations may not regress or disappear spontaneously.[25] However, complications such as extrusion of the tongue, obstruction of upper airways, and difficulties in chewing and speech can occur depending on their location.[5] Although the most conventional treatments are sclerotherapy for macrocystic lymphatic malformations and surgical resection for microcystic lymphatic malformations, the lesions often recur after treatment.[13,26] Additionally, depending on the location of the lesions, surgical treatment may be challenging.[5]

Activating mutations in components of PI3K/AKT and MAPK/ERK pathways occur in several human cancers, and targeted molecular inhibitors of these pathways have been developed to improve the treatment of these tumors.[27,28] After the discovery of the pathogenic involvement of these same pathways in vascular malformations, targeted therapies also started to emerge in this context.[22] Sirolimus (mTOR inhibitor) is the most well studied drug in lymphatic malformations.[29,30] In addition, inhibitors of *PIK3CA* (Ly294002, Wortmannin) and of mTOR (Rapamycin) have been tested in vitro and show encouraging results in lymphatic endothelial cells from lymphatic malformations.[13,14] The activation of both PI3K/AKT and MAPK/ERK in oral lymphatic malformation observed in the present study reveals that these pathways play a role in the pathogenesis of these oral lesions. The results of this pilot study can be the basis for further studies investigating molecular pathogenesis of oral lymphatic malformations and targeted therapies as a step preceding surgical removal in challenging cases.

CONCLUSIONS

In conclusion, we report activation of PI3K/AKT and MAPK/ERK signaling pathways in endothelial cells of oral lymphatic malformations. Our results suggest that the activation of such signaling pathways plays a role in the pathogenesis of oral lymphatic malformations.

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REFERENCES

1. Wassef M, Blei F, Adams D, et al. Vascular anomalies classification: Recommendations from the international society for the study of vascular anomalies. *Pediatrics*. 2015;136:e203-e214.
2. Elluru RG, Balakrishnan K, Padua HM. Lymphatic malformations: diagnosis and management. *Semin Pediatr Surg*. 2014;23:178-185.
3. Steiner JE, Drolet BA. Classification of vascular anomalies: an update. *Semin Intervent Radiol*. 2017;34:225-232.
4. International Society for the Study of Vascular Anomalies. Classification of vascular anomalies. Available at: www.issva.org/classification. Accessed April 30, 2021.
5. Kolay SK, Parwani R, Wanjari S, Singhal P. Oral lymphangiomas—clinical and histopathological relations: an immunohistochemically analyzed case series of varied clinical presentations. *J Oral Maxillofac Pathol*. 2018;22(1):S108-S111. suppl.
6. Luks VL, Kamitaki N, Vivero MP, et al. Lymphatic and other vascular malformative/overgrowth disorders are caused by somatic mutations in PIK3CA. *J Pediatr*. 2015;166:1048-1054.
7. Kurek KC, Luks VL, Ayturk UM, et al. Somatic mosaic activating mutations in PIK3CA cause CLOVES syndrome. *Am J Hum Genet*. 2012;90:1108-1115.
8. Rodriguez-Laguna L, Agra N, Ibañez K, et al. Somatic activating mutations in PIK3CA cause generalized lymphatic anomaly. *J Exp Med*. 2019;216:407-418.
9. Rodriguez-Laguna L, Ibañez K, Gordo G, et al. CLAPO syndrome: identification of somatic activating PIK3CA mutations and delineation of the natural history and phenotype. *Genet Med*. 2018;20:882-889.
10. Kim T, Tafoya E, Chelliah MP, et al. Alterations of the MEK/ERK, BMP, and Wnt/β-catenin pathways detected in the blood of individuals with lymphatic malformations. *PLoS One*. 2019;14:e0213872.
11. Glaser K, Dickie P, Neilson D, Osborn A, Dickie BH. Linkage of metabolic defects to activated PIK3CA alleles in endothelial cells derived from lymphatic malformation. *Lymphat Res Biol*. 2018;16:43-55.
12. Osborn AJ, Dickie P, Neilson DE, et al. Activating PIK3CA alleles and lymphangiogenic phenotype of lymphatic endothelial cells isolated from lymphatic malformations. *Hum Mol Genet*. 2015;24:926-938.
13. Boscolo E, Coma S, Luks VL, et al. AKT hyper-phosphorylation associated with PI3K mutations in lymphatic endothelial cells from a patient with lymphatic malformation. *Angiogenesis*. 2015;18:151-162.
14. Blesinger H, Kaulfu S, Aung T, et al. PIK3CA mutations are specifically localized to lymphatic endothelial cells of lymphatic malformations. *PLoS One*. 2018;13:e0200343.
15. Vanhaesebroeck B, Stephens L, Hawkins P. PI3K signalling: the path to discovery and understanding. *Nat Rev Mol Cell Biol*. 2012;13:195-203.
16. Lavoie H, Gagnon J, Therrien M. ERK signalling: a master regulator of cell behaviour, life and fate. *Nat Rev Mol Cell Biol*. 2020;21:607-632.
17. Ersahin T, Tuncbag N. Cetin-Atalay R. The PI3K/AKT/mTOR interactive pathway. *Mol Biosyst*. 2015;11:1946-1954.
18. Mendoza MC, Er EE, Blenis J. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends Biochem Sci*. 2011;36:320-328.
19. El-Naggar AK, Chan JKC, Grandis JR, Takata T, Slootweg PJ. World Health Organization Classification of Head and Neck Tumours. 4th ed Lyon, France: IARC; 2017.
20. Guimarães LM, Gomes IP, Pereira TDSF, et al. KRAS mutations in brown tumor of the jaws in hyperparathyroidism. *J Oral Pathol Med*. 2020;49:796-802.
21. Moraes JK, Wagner VP, Fonseca FP, et al. Activation of BDNF/TrkB/Akt pathway is associated with aggressiveness and unfavorable survival in oral squamous cell carcinoma. *Oral Dis*. 2019;25:1925-1936.
22. Nguyen HL, Boon LM, Vikkula M. Vascular anomalies caused by abnormal signaling within endothelial cells: targets for novel therapies. *Semin Intervent Radiol*. 2017;34:233-238.
23. Jiang BH, Liu LZ. PI3K/PTEN signaling in tumorigenesis and angiogenesis. *Biochim Biophys Acta*. 2008;1784:150-158.
24. Zhou F, Chang Z, Zhang L, et al. Akt/protein kinase B is required for lymphatic network formation, remodeling, and valve development. *Am J Pathol*. 2010;177:2124-2133.
25. Van Damme A, Seront E, Dekeuleneer V, Boon LM, Vikkula M. New and emerging targeted therapies for vascular malformations. *Am J Clin Dermatol*. 2020;21:657-668.
26. Zhang B, Ma L. Updated classification and therapy of vascular malformations in pediatric patients. *Pediatr Investig*. 2018;2:119-123.
27. Yang J, Nie J, Ma X, Wei Y, Peng Y, Wei X. Targeting PI3K in cancer: mechanisms and advances in clinical trials. *Mol Cancer*. 2019;18:26.
28. Samatar AA, Poulikakos PI. Targeting RAS-ERK signalling in cancer: promises and challenges. *Nat Rev Drug Discov*. 2014;13:928-942.
29. Adams DM, 3rd Trenor CC, Hammill AM, et al. Efficacy and safety of sirolimus in the treatment of complicated vascular anomalies. *Pediatrics*. 2016;137:e20153257.
30. Yesil S, Tanyildiz HG, Bozkurt C, Cakmakci E, Sahin G. Single-center experience with sirolimus therapy for vascular malformations. *Pediatr Hematol Oncol*. 2016;33:219-225.