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Endothelial Cells from Capillary Malformations are Enriched for Somatic *GNAQ* Mutations

Javier A. Couto, BS^{1,*}, Lan Huang, PhD^{2,5,*}, Matthew P. Vivero, BA¹, Nolan Kamitaki⁶, Reid A. Maclellan, MD, MMSc¹, John B. Mulliken, MD^{1,4}, Joyce Bischoff, PhD^{2,6}, Matthew L. Warman, MD^{3,4,6,7}, and Arin K. Greene, MD, MMSc^{1,4}

¹Department of Plastic and Oral Surgery, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115

²Department of Surgery, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115

³Department of Orthopedic Surgery, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115

⁴Department of Vascular Anomalies Center, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115

⁵Department of Vascular Biology Program, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115

⁶Department of Genetics, Harvard Medical School, Boston MA 02115

⁷Howard Hughes Medical Institute, Boston Children's Hospital, Boston MA 02115

Abstract

Background—A somatic mutation in *GNAQ* (c.548G>A;p.R183Q), encoding $G\alpha_q$, has been found in syndromic and sporadic capillary malformation tissue. However, the specific cell type(s) containing the mutation is unknown. The purpose of this study was to determine which cell(s) in capillary malformations have the *GNAQ* mutation.

Methods—Human capillary malformation tissue was obtained from 13 patients during a clinically-indicated procedure. Droplet digital PCR (ddPCR), capable of detecting mutant allelic frequencies as low as 0.1%, was used to quantify the abundance of *GNAQ* mutant cells in capillary malformation tissue. Six specimens were fractionated by fluorescence activated cell sorting (FACS) into hematopoietic, endothelial, perivascular, and stromal cells. The frequency of *GNAQ* mutant cells in these populations was quantified by ddPCR.

Results—Eight capillary malformations contained *GNAQ* p.R183Q mutant cells, 2 lesions had novel *GNAQ* mutations (p.R183L; p.R183G), and 3 capillary malformations did not have a detectable *GNAQ* p.R183 mutation. Mutant allelic frequencies ranged from 2% to 11%. Following

Corresponding Author Arin K. Greene, MD, MMSc, Department of Plastic Surgery and Oral Surgery, Boston Children's Hospital, 300 Longwood Ave., Boston, MA 02115, Phone: 617.355.2306, Fax: 617.738.1657, arin.greene@childrens.harvard.edu.

* indicates co-first authorship

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FACS, the *GNAQ* mutation was found in the endothelial but not the platelet-derived growth factor receptor- β -positive (PDGFR β) cell population; mutant allelic frequencies were 3% to 43%.

Conclusions—Endothelial cells in capillary malformations are enriched for *GNAQ* mutations and are likely responsible for the pathophysiology underlying capillary malformation.

Keywords

capillary malformation; ddPCR; endothelial cell; *GNAQ*; somatic mutation; Sturge-Weber syndrome

INTRODUCTION

Capillary malformation is the most common type of vascular malformation, affecting approximately 0.3% of newborns.¹ This lesion is characterized by enlarged venule-type channels in the papillary dermis.² Capillary malformations are present at birth and can affect any cutaneous site. Lesions may be single, multiple, localized or diffuse. Most capillary malformations are sporadic, but may be syndromic as well (e.g., Sturge-Weber syndrome).³

Recently, a somatic mosaic mutation in *GNAQ* (c.548G>A; p.R183Q), encoding G α_q , was found in sporadic and syndromic capillary malformations.⁴ G α_q is an alpha subunit of heterotrimeric G proteins that activate phospholipase C β .⁵ The arginine (R) residue at position 183 in G α_q resides within the GTP binding pocket. Thus, the p.R183Q missense mutation likely reduces GTPase activity and thereby maintains G α_q in its GTP-bound, active state. *GNAQ* is normally expressed in many cell types including blood, neuronal and vascular cells.⁶

The purpose of this study was to identify which cell type(s) in capillary malformations contain the somatic *GNAQ* mutation. Identifying the mutant cell that drives the capillary malformation phenotype will pinpoint the relevant cellular context in which to study the mutation, may uncover new mechanisms underlying capillary morphogenesis, and might eventually translate to targeted therapies for this vascular malformation.

PATIENTS and METHODS

Capillary malformation specimens and cell isolation

This study was approved by the Committee on Clinical Investigation at Boston Children's Hospital. Specimens were collected when removing overgrown skin/subcutaneous tissue to improve the patient's appearance and/or to facilitate the fitting of clothing. The diagnosis of capillary malformation was based on history, physical examination, and histopathology. Six capillary malformations were studied retrospectively (stored flash frozen at -80°C), and seven were studied prospectively. Two patients underwent multiple procedures to remove their capillary malformation; three specimens were obtained from Patient 5 and two from Patient 12. After being removed immediately from the operating room, six specimens were fractionated by fluorescence-activated cell sorting (FACS).

DNA was extracted from each capillary malformation with the QIAamp DNA Mini kit (Qiagen, MD). A portion of the freshly obtained sample was used for cell isolation by FACS. Specimens were washed to remove blood cell contaminants, digested with collagenase, and filtered through a 70µm strainer. Cells were fractionated into 4 populations (hematopoietic, endothelial, perivascular, stromal) using the following antibodies: anti-human CD45 conjugated to allophycocyanin (APC) (eBioscience; San Diego, CA), anti-human platelet derived growth factor receptor beta (PDGFRβ) conjugated to fluorescein isothiocyanate (FITC) (R&D Systems), and a mixture of anti-human CD31 (BD Biosciences Pharmingen; Bedford, MA), anti-vascular endothelial-cadherin (VE-Cadherin) (R&D Systems), anti-CD34 (Miltenyi Biotec; Auburn, CA), anti-VEGFR2 (R&D Systems), all conjugated to phycoerythrin (PE). DNA then was extracted from the different populations of sorted cells.

Fluorescence activated cell sorting method (FACS) and analysis

Sorting of cells from capillary malformation tissue specimens was performed on a Becton Dickinson FACS Aria II machine with the FACS Diva v.6.1.3 program. FlowJo v.10 software was used for data analysis. Isotype-matched IgG control antibodies conjugated to APC, PE and FITC were used to set gates for background staining such that <0.2% of the diploid cells were in the positive fractions. For the triple-fluorescent label cell sorting, compensation was set prior to sorting using single antibody-APC, -PE or FITC staining of cells such the % cells in other two channels was <0.2%. Fluorescence minus one controls were not performed due to the limited number of cells obtained from small capillary malformation specimens available for this study. CD45 is a common leukocyte marker and CD45-negative cells could be readily distinguished from CD45-dim and -positive cells in the capillary malformation-derived cell populations. We used a set of four antibodies against endothelial markers – anti-CD31, anti-CD34, anti-VECadherin and anti-VEGFR2 - to ensure that all endothelial cells in the capillary malformation tissue were detected and sorted; this is based on a newly published technique developed in our laboratory.⁷ Because of the relative abundance of CD31 and VE-cadherin, endothelial fluorescence intensity was strong and therefore the boundary between positive and negative cells was readily identified. Anti-PDGFRβ staining was verified by staining human retinal pericytes as a positive control and human umbilical vein endothelial cells as a negative control (not shown).

Droplet digital PCR (ddPCR)-based mutational analysis

PCR primers and fluorescent probes for detecting wild-type and mutant *GNAQ* alleles were designed using Primer3 and purchased from Integrated DNA Technologies (Coralville, IA).⁸ The wild-type allele probe was 5' HEX labeled with a 3' Iowa Black quencher and the mutant (p.R183Q) probe was 5' FAM labeled with a 3' Iowa Black quencher. Droplet digital PCR was performed as previously described.⁹ We used 30 ng of template DNA, corresponding to ~4000 cells, in each reaction. The mixture was partitioned into ~20,000 droplets using the QX200 Droplet Generator (Bio-Rad), subjected to 40 PCR cycles, and then analyzed using the QX200 Droplet Reader and QuantaSoft software (both from Bio-Rad).

Immunostaining and confocal microscopy

Formalin-fixed, paraffin-embedded tissue sections (5µm) were deparaffinized and either directly stained with hematoxylin and eosin (H&E) or immersed in an antigen retrieval solution (Citrate-EDTA buffer: 10mM Citric Acid, 2mM EDTA, 0.05% Tween-20, pH 6.2;) for 20 minutes. Frozen sections (10µm) fixed in acetone at -20°C for 10 minutes and evaporated at room temperature for 20 minutes. Sections were blocked for 30 minutes in 5% serum and incubated overnight with anti-human VE-Cadherin (1:40, C19, Sigma), anti-human PDGFRβ (1:40, AF385, R&D Systems), or anti-human CD31 (1:40, clone JC70A, Dako). Purified class- and species-matched IgGs (Vector Lab) were used as controls. Sections were incubated with fluorescein or Texas Red conjugated secondary antibody (Vector Laboratories). Slides were stained with the nuclear marker DAPI (Molecular Probe, Eugene, OR). Images were acquired using a Leica TCS sp2 Acousto-Optical Beam Splitter confocal system equipped with DMIRE2 inverted microscope camera (Leica Microsystems, Wetzlar, Germany).

RESULTS

Most capillary malformations contain *GNAQ* p.R183Q mutant cells

We detected mutant *GNAQ* alleles in 10 out of 13 capillary malformation patients tested; the frequency ranged from 1.9% to 11.1%, corresponding to a mutant cell frequency of 4% to 22%, assuming the *GNAQ* mutation is heterozygous in these cells (Fig. 1, Table 1). The majority of specimens contained a c.548G>A; p.R183Q mutation (n=8). Two lesions exhibited novel *GNAQ* mutations: c.548G>T; p.R183L and c.547C>G; p.R183G. ddPCR failed to detect a *GNAQ* mutation at this site in 3 specimens.

GNAQ mutations are enriched in capillary malformation endothelial cells

Capillary malformation cells were sorted into hematopoietic and non-hematopoietic cells based on CD45 expression (Fig. 2). The non-hematopoietic, CD45-negative cells were separated based on the expression of endothelial markers (VE-cadherin, CD31, VEGFR2, CD34) and the pericyte/smooth muscle marker PDGFRβ. This produced 4 cell populations: 1) CD45-positive (hematopoietic) cells; 2) endothelial marker-positive cells; 3) PDGFRβ-positive (pericyte/smooth muscle) cells, and 4) triple negative (stromal) cells. The endothelial population showed a mutant allelic frequency of 2.8%–42.9% (Table 2). Mutant cells were not detected in the PDGFRβ-positive/endothelial marker-negative population. The stromal population, which may include fibroblasts and undifferentiated cells, contained mutant cells in 3 out of the 6 specimens tested. Mutant cells were detected in the CD45+ population in one of 6 specimens examined.

Histology and immunostaining for VE-cadherin and PDGFRβ

Capillary malformation tissue sections from Patient 2 stained with H&E show the expected enlarged lumens and disorganized perivascular cells. Sections were stained with anti-VE-cadherin and anti-CD31 (not shown) to examine endothelial integrity and phenotype. The ectatic lumens that are characteristic of capillary malformation were again evident. Sections were also stained with anti-PDGFRβ to verify that the perivascular cells express this

receptor used to isolate the cells by FACS. Cells surrounding the endothelial-lined lumen stained positive for PDGFR β . For both anti-VE-cadherin and anti-PDGFR β , purified class- and species-matched IgGs were used as controls to verify specific immunostaining (not shown).

DISCUSSION

Here we: (1) confirm that *GNAQ* p.R183Q is the most common mutation found in capillary malformation,⁴ (2) identify two novel *GNAQ* mutations (p.R183L and p.R183G) in capillary malformation, and (3) show that *GNAQ* mutations are enriched in the endothelial cell compartment. The identification of two new missense mutations at amino acid position 183 indicates that loss of this arginine residue from the GTP binding pocket of Gq α is deleterious. We did not detect a mutation in 3 patients with lower extremity lesions, possibly because: (1) the *GNAQ* p.R183 mutation was at a frequency too low to detect by our ddPCR assay; (2) a *GNAQ* mutation was present, but affected an amino acid residue not queried by our ddPCR assay; or (3) the lesion was caused by a mutation in a gene other than *GNAQ*.

Capillary malformation arises from anomalous vascular development and is characterized histologically by enlarged capillary lumens, ectatic venular morphology, and disordered perivascular coverage.² Our data indicate that the underlying genetic defect in capillary malformation resides within the endothelial cells. Based on the chaotic appearance of perivascular cells around the vessel in capillary malformations,² we hypothesize that abnormal communication between mutant endothelial cells and wild-type perivascular cells contributes to the pathogenesis of capillary malformation. Our results pinpoint the origin of the mutant Gq α signaling – the endothelial cell, which then may affect other cells in a non-autonomous manner.

Capillary malformations progress over time: the stain becomes darker, the skin thickens, pyogenic granulomas develop, and tissue underneath the lesion becomes overgrown.³ Some patients have minimal soft-tissue and osseous hypertrophy, while others experience significant overgrowth. We did not observe a correlation between disease severity and *GNAQ* mutant allele frequency in capillary malformation tissue, likely because the specimens contained a heterogeneous amount of different cell populations. In contrast, we did note an association between disease severity and endothelial cell mutant allele frequency in the 5 specimens that underwent FACS (i.e., patients with worse lesions had a higher mutant allele frequency). If this observation proves to be true, a biopsy potentially could be performed in childhood to predict the natural history of the lesion. For example, if the endothelial cell mutant allele frequency is high, then the patient would be expected to have greater progression of the lesion and a less favorable prognosis.

Treatment of capillary malformation requires pulsed-dye laser to lighten the lesion and resection to remove overgrown tissues; redarkening and recurrence of overgrowth are common.^{3,10} Pharmacotherapy for capillary malformation does not exist. Further study to understand the precise mechanism by which altered Gq α signaling in endothelial cells contributes to the pathophysiology of capillary malformation may lead to improved treatments. Topical, intralesional, or systemic drug therapy might be developed to improve

capillary malformation, prevent its progression, and/or eliminate recurrence following laser and excision.

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Figure 1.

(Above) Examples of patients with capillary malformation included in the study cohort.

(Below) Droplet digital PCR (ddPCR) shows *GNAQ* p.R183Q in resected tissue. Left upper quadrant illustrates droplets containing mutant alleles. Right upper quadrant shows droplets with mutant and wild-type alleles. Left lower quadrant exhibits empty droplets. Right lower quadrant illustrates droplets with wild-type alleles.

Figure 2.

Isolation of cell types from human capillary malformation tissue. (A) (Left) FACS of a freshly obtained capillary malformation specimen (Patient 12a) shows anti-CD45 binding (y-axis) versus side scatter (x-axis). Boxes illustrate gates for CD45-positive cells (upper box) and CD45-negative cells (lower box). (Right) Further analysis of the CD45-negative gated cells for PDGFR β (y-axis) versus endothelial markers (x-axis). FITC+ (magenta) indicates PDGFR-positive/endothelial marker negative cells. PE+ (green) identifies endothelial marker-positive cells. DN (orange) are cells that are negative for all antibodies. (B) (Left) H&E-stained capillary malformation tissue section from Patient 2 shows an enlarged capillary malformation vessel. The perivascular cells appear dispersed and non-aligned. (Center) Anti-VE-cadherin (green) and DAPI (blue nuclear marker) illustrates that the endothelium expresses the marker used for FACS sorting. (Right) Anti-PDGFR β (green) and DAPI show that perivascular cells are positive for the PDGFR β antibody used for FACS sorting. Scale bar, 100 μ m.

Table 1***GNAQ* Mutational Analysis in Capillary Malformation Specimens**

Patient	Age	Sex	Location	Mutant Allelic Frequency (%)
1	9	M	Leg	n.d.
2	12	M	Face	7.4
3	13	F	Face	7.0
4	14	F	Leg	n.d.
5a	15	M	Face	5.3
5b	16	M	Face	6.4
5c	18	M	Face	6.7
6	17	F	Leg	n.d.
7*	18	M	Face	2.1
8	23	F	Leg	4.1 [†]
9	23	M	Face	2.5
10*	28	M	Face	11.1
11*	39	F	Face	5.2 [‡]
12a*	72	F	Face	7.1
12b*	73	F	Face	-
13	79	M	Face	1.9

n.d. = mutation not detected

(-) = not performed

Gray shaded area indicates multiple CM specimens from the same patient.

* Sturge-Weber syndrome

[†] p.R183L

[‡] p.R183G

Table 2***GNAQ* Mutational Analysis in Capillary Malformation Cell Populations**

Patient	Mutant Allelic Frequency (%)			
	Endothelial Cells	PDGFR β + Cells	Stromal Cells	CD45+ Cells
5c	11.3	-	0.4	0
10 *	17.1	0	4.6	-
11 *	7.6 [†]	0	11.2 [†]	0
12a *	41.8	0	2.2	0.3
12b *	42.9	0	0	0
13	2.8	0	0	0

(-) = not performed

Gray shaded area indicates multiple CM specimens from the same patient.

* Sturge-Weber syndrome

[†] p.R183G