Somatic MAP3K3 and PIK3CA mutations in sporadic cerebral and spinal cord cavernous malformations

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Cavernous malformations affecting the CNS occur in ~0.16–0.4% of the general population. The majority (85%) of cavernous malformations are in a sporadic form, but the genetic background of sporadic cavernous malformations remains enigmatic. Of the 81 patients, 73 (90.1%) patients were detected carrying somatic missense variants in two genes: MAP3K3 and PIK3CA by whole-exome sequencing. The mutation spectrum correlated with lesion size (\(P = 0.001\)), anatomical distribution (\(P < 0.001\)), MRI appearance (\(P = 0.004\)) and haemorrhage events (\(P = 0.006\)). PIK3CA mutation was a significant predictor of overt haemorrhage events (\(P = 0.003\), odds ratio = 11.252, 95% confidence interval = 2.275–55.648). Enrichment of endothelial cell population was associated with a higher fractional abundance of the somatic mutations. Overexpression of the MAP3K3 mutation perturbed angiogenesis of endothelial cell models in vitro and zebrafish embryos in vivo. Distinct transcriptional signatures between different genetic subgroups of sporadic cavernous malformations were identified by single cell RNA sequencing and verified by pathological staining. Significant apoptosis in MAP3K3 mutation carriers and overexpression of GDF15 and SERPINA5 in PIK3CA mutation carriers contributed to their phenotype. We identified activating MAP3K3 and PIK3CA somatic mutations in the majority (90.1%) of sporadic cavernous malformations and PIK3CA mutations could confer a higher risk for overt haemorrhage. Our data provide insights into genomic landscapes, propose a mechanistic explanation and underscore the possibility of a molecular classification for sporadic cavernous malformations.

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Introduction

Cavernous malformations (CMs) are among the most prevalent vascular malformations affecting the CNS and occur in ~0.16–0.4% of the general population.1–3 CMs can present incidentally, with seizures or focal neurological deficits most frequently due to haemorrhages.4 Neurosurgical resection remains the only effective treatment, which is limited by lesion accessibility and is associated with non-negligible rates of morbidity and mortality.5,6 CMs tend to fall into two categories: familial (inherited) forms and sporadic forms, with the majority (85%) being sporadic.7,8 The genetic analysis of CMs was mostly based on the inherited form of CMs and was associated with three distinct loci: CCM1 (KRIT1), CCM2 and CCM3 (PDCD10).9–11 Heterozygous loss-of-function of these three mutations was identified in ~90% of familial cases.5 Previous genetic studies on sporadic CMs mainly focused on the known three CCM genes using PCR amplicons, while the detection rate of pathogenic mutations was low.12–14 The genetic background of sporadic CMs remains unclear. Recently, pathogenic somatic mutations have been identified in sporadic arteriovenous malformations, capillary malformations, lymphatic malformations and some types of venous malformations.15–19 We hypothesized that sporadic CMs also arise from somatic mutations in the vasculature of the CNS.

Materials and methods

Study populations

We used whole-exome sequencing (WES) to detect potential pathogenic somatic mutations within the CM lesions and evaluate the possibility of correlating molecular subgroups with clinical characteristics. Patients were eligible for inclusion if they had cerebral or spinal cord CMs with negative family history. The diagnosis of CM was based on the typical appearance of the lesion on MRI and pathological examination of resected samples. Catheter angiography was not used in the evaluation of CMs, unless a differential diagnosis of arteriovenous malformation is being considered. Ninety patients with CMs in the CNS who underwent surgical resection of the lesions at the Beijing Xuanwu Hospital in China between May 2017 and November 2019 were recruited to this study. The study was approved by the ethics committee of Beijing Xuanwu Hospital (no. 2016032) and written informed consent from all patients or their guardians was obtained before surgery.

Clinical characteristics

All medical information was extracted from a computerized online database, which contained demographics, clinical, radiological, and treatment-related information. We defined a haemorrhage event as a symptomatic event with radiographic evidence of overt haemorrhage.8 The observational period of haemorrhage was defined as the interval between the initial diagnosis and surgical treatment. The MRI appearance of CMs were classified into three types based on the Zabramski classification.15 Type I was defined as lesions with high signal intensity cores on T1-weighted imaging and high/low signal intensity on T2-weighted imaging. Type II was defined as lesions with reticulated, mixed-signal intensity cores on T1- and T2-weighted imaging (with surrounding dark rim). Type III was defined as lesions with iso-to-low signal intensity on T1-weighted imaging and low signal intensity on T2-weighted imaging. All films were reviewed by independent neuroradiologists. The Karnofsky Performance Scale score was used to provide an objective measure defining neurological functional status of the patients with cerebral or spinal cord CMs. The neurological status of patients with spinal cord CMs were further assessed by the modified McCormick scale.16

Genetic analysis and functional studies

We performed WES on fresh-frozen tissue samples of CMs. Sequencing depth was anticipated to be 200–300 × for tissue samples. We confirmed the results with droplet digital PCR (ddPCR) analysis to observe mitogen-activated protein kinase kinase kinase 3 (MAP3K3) mutation and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (PIK3CA) mutations. We also compared variant frequencies of the somatic mutations in endothelial cells and non-endothelial cells of tissue samples using fluorescence-activated cell sorting (FACS) and ddPCR. The functional studies of PIK3CA on endothelial cells and animal models have been well performed in previous studies on venous malformations.17,18 We explored the functions of the MAP3K3 (p.Ile441Met) mutation in human umbilical vein endothelial cells (HUVECs) and in a zebrafish model.

Statistical analysis

We used Fisher’s exact test or Pearson’s χ² test for categorical variables and Student’s t-test for continuous variables. Logistic regression analysis was used to assess the impact of multiple variables that predicted an overt haemorrhage event. Cumulative rates of haemorrhage during follow-up were illustrated using the Kaplan-Meier method, and the curves were compared by the log-rank test. All analyses were performed under the guidance of a statistician using SPSS software (version 25, IBM Corp., Armonk, New York, USA). All P-values are two-sided, and we defined statistical significance as P < 0.05.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary...
material. Derived data supporting the findings of this study are available from the corresponding author on request.

Results
Baseline characteristics of the patients

Ninety consecutive patients were included in the study. WES was performed in 90 fresh-frozen tissue samples of cerebral or spinal cord CMs and two associated cutaneous CMs (Patients 45 and 75). Among these, nine patients were excluded because of carrying germline non-synonymous mutations of KRIT1 (5/9), CCM2 (3/9) and PDCD10 (1/9) by WES. Samples from 81 sporadic patients were eligible for final analysis, including 36 cerebral CMs and 45 spinal cord CMs. Among the 81 patients, 43 (53.1%) were male. The mean age on admission was 37.9 ± 15.7 years (ranging from 3 to 82 years). The clinical characteristics of the patients are summarized in Supplementary Table 1 and Supplementary Fig. 1.

Observation of MAP3K3 and PIK3CA somatic variants in patients

Of the 81 sporadic patients, WES and ddPCR resulted in the identification of somatic missense variants in MAP3K3 and PIK3CA in 73 (90.1%) patients (Fig. 1 and Supplementary Fig. 2). The finding of ddPCR was consistent with the results of WES (r = 0.882, P < 0.001). Among the 73 patients, 42 (57.5%) were detected as having an MAP3K3 missense variant (RefSeq accession number NM_002401.3, c.1323C > G, p.Ile441Met) with variant frequencies ranging from 0.4% to 22.7%. The same somatic mutation has been identified in verrucous venous malformations with mutant allele frequencies 0.4% to 22.7%. The same somatic mutation has been identified in verrucous venous malformations with mutant allele frequencies 0.4% to 22.7%.

The somatic mutation genotype was associated with haemorrhage.

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Validation of the impact of MAP3K3 mutation on angiogenesis in a zebrafish model

To investigate the loss-of-function of Map3k3 in a zebrafish model, morpholino oligonucleotides (MOs) were designed to knockdown wild-type MAP3K3 in zebrafish embryos. We observed that all of the embryos died within 48 h post-fertilization (hpf) (MO concentration range: 0.05–5 ng), which indicated an indispensable role of Map3k3 in zebrafish embryonic development. Subsequently, to investigate the function of the MAP3K3 (I441M) mutation, we identified that the CD31 + cells had a higher fractional abundance of mutations in PIK3CA (I441M) or PIK3CA (H1047R), which indicated that the somatic mutations mainly occurred in endothelial cells (Fig. 3A and Supplementary Fig. 4).

The somatic mutations mainly occurred in CD31 + cells

To identify the types of cells that have MAP3K3 or PIK3CA mutations in CMs, we used FACS to sort CD31 + cells (endothelial cell) and CD31 – cells from four fresh samples derived from cerebral and spinal cord CMs (Fig. 3A and Supplementary Fig. 4). The ddPCR analysis showed that the CD31 + cells derived from the four samples all had a higher fractional abundance of mutations in MAP3K3 (I441M) and PIK3CA (H1047R), which indicated that the somatic mutations mainly occurred in endothelial cells (Fig. 3A and Supplementary Fig. 4).

MAP3K3 mutation showed an impact on angiogenesis

Cells transfected with MAP3K3 (I441M), as compared with the control, showed significant activation of phosphorylated ERK5, JNK and p58 (Fig. 3B). However, the extracellular signal-regulated kinase 1/2 (ERK1/2) activation was not found. Upregulated KLF2 and KLF4 were reported in previous studies, but no significant difference was observed in our results (Supplementary Fig. 5A and C–H). Overexpression of MAP3K3 (I441M) increased cell proliferation and cell migration (P < 0.001) with VEGFA (20 ng/ml) stimulation compared with the control group (Fig. 3C). On cell tube formation, overexpression of the mutation in HUVECs led to disruption and disorganization of spontaneous vascular tube formation (P < 0.001), with significant reductions of total vascular tube length and total mesh area (Fig. 3D). On cell senescence, overexpression of the MAP3K3 mutation evidently modified endothelial cell morphology and increased the amount of β-galactosidase (a recognized senescence marker) positive cells (Supplementary Fig. 6 and E). The activating functions of mutations in PIK3CA were also performed and overexpression of mutations in PIK3CA also promoted cell proliferation, migration, senescence and disrupted tube formation compared with wild-type (Supplementary Figs 5B and 6).

The size of lesions carrying PIK3CA hotspot activating mutations was significantly larger (P = 0.001; Table 1) than the other two genotypes. The majority (60.0%) of patients with PIK3CA mutations had a Zabramski type I appearance of MRIs. While in patients with MAP3K3 mutations, only 28.6% had a Zabramski type I appearance. In patients with co-occurred mutations, Zabramski type II (53.3%) was the dominant MRI appearance.
injected wild-type and MAP3K3 (I441M) mRNAs into zebrafish embryos. As expected, in the control group injected with PBS, the upper blood vessels were neatly distributed and the density was uniform. However, in the experimental group injected with wild-type or MAP3K3 (I441M) mRNAs, part of the upper blood vessels were absent and the blood vessel density was uneven (Fig. 3E).

Overexpression of the MAP3K3 (I441M) mutation caused more severe phenotypes than wild-type mRNA in zebrafish at the same concentration ($P < 0.001$) (Fig. 3F). The observations of endothelial cell models and zebrafish embryos suggested that the MAP3K3 (I441M) mutation was an activating mutation.

Single cell RNA sequencing revealed distinct transcriptional signatures between MAP3K3 and PIK3CA mutations

In single cell RNA sequencing, 74 326 single cells from nine samples (six CMs samples and three control samples) were profiled and 65 297 cells passed stringent quality control filters for further processing. The six samples of CMs included two CMs with MAP3K3 mutations (one with cerebral CMs, one with associated cutaneous CMs), three cerebral CMs with PIK3CA mutations, and one with cerebral CMs with double mutations. The cutaneous lesion was from Patient 75 with spinal cord CMs at the same spinal metameric segment (Table 3).

The entire population was classified into three cell types, including endothelial cells, mural cells and other cells based on their respective molecular features: VWF, CLDN5, CDH5 (endothelial cells) and PDGFRB, ACTA2, PDLIM3 (mural cells) (Fig. 4A and B). Other cells’ were identified as mast cells, astrocytes, plasmacytoid dendritic cells (pDCs), B cells, natural killer (NK) cells, dendritic (DC) cells, oligodendrocytes, microglia, macrophages/monocytes, epithelial cells and T cells by interrogating the expression patterns of known marker genes (Fig. 4A and Supplementary Fig. 7). Interestingly, all three CM samples with PIK3CA mutations had minimal counts of endothelial cells and ratio to the total number of sample cells (Fig. 4C). Most differentially expressed genes (DEGs) in endothelial cells were consistent in different
Table 1 Clinical characteristics and genotype of 73 sporadic CMs patients with somatic mutations

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall Genotype</th>
<th>MAP3K3 mutation</th>
<th>PIK3CA mutation</th>
<th>Co-occurred mutations</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, n (%)</td>
<td>73</td>
<td>28 (38.4)</td>
<td>30 (41.1)</td>
<td>15 (20.5)</td>
<td></td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>31 (42.5)</td>
<td>13 (46.4)</td>
<td>14 (46.7)</td>
<td>4 (26.7)</td>
<td>0.366</td>
</tr>
<tr>
<td>Mean age at onset ± SD (range), years</td>
<td>36.3 ± 14.4 (8–75)</td>
<td>33.6 ± 14.6 (8–64)</td>
<td>38.1 ± 13.1 (10–58)</td>
<td>37.9 ± 16.8 (9–75)</td>
<td>0.423</td>
</tr>
<tr>
<td>Duration of symptoms ± SE, months</td>
<td>22.4 ± 4.4</td>
<td>26.3 ± 8.0</td>
<td>14.7 ± 4.9</td>
<td>30.5 ± 12.1</td>
<td>0.303</td>
</tr>
<tr>
<td>Locations of presenting or largest CMs (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Supratentorial lobar and cerebellum</td>
<td>18 (24.7)</td>
<td>3 (10.7)</td>
<td>11 (36.7)</td>
<td>4 (26.7)</td>
<td></td>
</tr>
<tr>
<td>Supratentorial deep (basal ganglia/thalamus) and brainstem</td>
<td>14 (19.2)</td>
<td>3 (10.7)</td>
<td>11 (36.7)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Spinal cord</td>
<td>41 (56.2)</td>
<td>22 (78.6)</td>
<td>8 (26.7)</td>
<td>11 (73.3)</td>
<td></td>
</tr>
<tr>
<td>Mean size of CMs ± SD, mm</td>
<td>14.0 ± 8.3</td>
<td>11.1 ± 7.7</td>
<td>17.9 ± 8.7</td>
<td>11.9 ± 5.5</td>
<td>0.001</td>
</tr>
<tr>
<td>MRI appearance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.004</td>
</tr>
<tr>
<td>Type I</td>
<td>33 (45.2)</td>
<td>8 (28.6)</td>
<td>18 (60.0)</td>
<td>7 (46.7)</td>
<td></td>
</tr>
<tr>
<td>Type II</td>
<td>27 (37.0)</td>
<td>10 (35.7)</td>
<td>9 (30.0)</td>
<td>8 (53.3)</td>
<td></td>
</tr>
<tr>
<td>Type III</td>
<td>13 (17.8)</td>
<td>10 (35.7)</td>
<td>3 (10.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Overt haemorrhage presentation (%)</td>
<td>45 (61.6)</td>
<td>11 (39.3)</td>
<td>24 (80.0)</td>
<td>10 (66.7)</td>
<td>0.006</td>
</tr>
<tr>
<td>Mean KPS score ± SD</td>
<td>75.5 ± 16.0</td>
<td>79.6 ± 14.0</td>
<td>74.7 ± 16.3</td>
<td>69.3 ± 17.5</td>
<td>0.076</td>
</tr>
</tbody>
</table>

KPS = Karnofsky Performance Scale.

Table 2 Univariate and multivariate analysis for risk factors of overt haemorrhage in 73 sporadic patients with somatic mutations

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients, n</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>73</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Male</td>
<td>42</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Female</td>
<td>31</td>
<td>0.607 (0.234–1.578)</td>
<td>0.306</td>
</tr>
<tr>
<td>Age, per each additional year</td>
<td>73</td>
<td>0.991 (0.958–1.024)</td>
<td>0.575</td>
</tr>
<tr>
<td>Locations</td>
<td>73</td>
<td>0.271</td>
<td>0.987 (0.950–1.026)</td>
</tr>
<tr>
<td>Supratentorial and cerebellum</td>
<td>18</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Thalamus and brainstem</td>
<td>14</td>
<td>3.667 (0.758–17.728)</td>
<td>0.106</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>41</td>
<td>1.563 (0.511–4.774)</td>
<td>0.434</td>
</tr>
<tr>
<td>Size, per 0.1 cm increase</td>
<td>73</td>
<td>1.073 (1.001–1.150)</td>
<td>0.048</td>
</tr>
<tr>
<td>Genotype</td>
<td>73</td>
<td>0.008</td>
<td>1.050 (0.968–1.139)</td>
</tr>
<tr>
<td>MAP3K3 mutations</td>
<td>28</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>PIK3CA mutations</td>
<td>30</td>
<td>6.182 (1.913–19.974)</td>
<td>0.002</td>
</tr>
<tr>
<td>Co-occurred mutations</td>
<td>15</td>
<td>3.091 (0.830–11.506)</td>
<td>0.092</td>
</tr>
</tbody>
</table>

Comparison groups, which indicated similar general genetic alterations in the sporadic CMs with either MAP3K3 or PIK3CA or both mutations (Fig. 4D). The number of DEGs in different comparisons is shown in Supplementary Fig. 8A. Furthermore, Gene Ontology (GO) analysis of DEGs in three mutation groups versus the control group showed consistency of important pathways such as angiogenesis, blood vessel morphogenesis and blood vessel development (Supplementary Fig. 8B). GO analysis of DEGs between subgroups of MAP3K3 and PIK3CA mutations showed the apoptotic signalling pathway was high ranking (Fig. 4E), which was confirmed by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) immunostaining of eight samples of sporadic CMs with somatic mutations and three control samples of normal brain tissue. Strong positive staining of apoptosis was observed in endothelial cells of CM samples with MAP3K3 mutations (n = 4), and negative staining was observed in the other two genetic subgroups (three patients with PIK3CA mutations, one patient with double mutations) and control samples (n = 3), shown in the representative graphs (Fig. 4F). In DEGs between three genetic subgroups and the control group, GDF15 and SERPINA5 were notably overexpressed in samples with PIK3CA mutations (Fig. 4G), which was consistent with the results following grouping by genotypes (Fig. 4H). These results were also verified by immunofluorescence staining (Fig. 4i).

Discussion

To our knowledge, this is the first study that has demonstrated high prevalence of pathological somatic activating MAP3K3 and PIK3CA mutations in the majority of sporadic CMs in CNS and illustrated the correlations of genotypes with phenotypes. The somatic mutations of MAP3K3 and PIK3CA mainly occurred in endothelial cells. The MAP3K3 mutations activated the phosphorylated ERK5, JNK and p38 pathway in endothelial cells and caused vascular malformations in zebrafish. Distinct transcriptional signatures between different genetic subgroups were identified. Significant apoptosis in MAP3K3 mutation carriers and overexpression of GDF15 and SERPINA5 in PIK3CA mutation carriers contributed to their phenotype.

We did not find any somatic non-synonymous mutations of the three CCM genes (KRIT1, CCM2, PDCD10) in the 81 patients with sporadic CMs. According to previous studies, several somatic mutations in the three CCM genes were found in sporadic CMs,
Figure 2 Haemorrhage events of CMs in brain and spinal cord. (A) MRI obtained in patients with CMs in lobes, brainstem and spinal cord with/without overt haemorrhage. (B) Kaplan-Meier analysis illustrating subsequent haemorrhage risks of sporadic CMs in different genotypes. The log-rank test indicated that haemorrhagic events during the follow-up period were more frequent in patients with PIK3CA mutations than with MAP3K3 mutations ($P = 0.012$) and the haemorrhagic risk during the follow-up period of double mutations was in-between; however, the $P$-value for difference between the three genotypes did not achieve statistical significance, though a trend was observed ($P = 0.051$).
Figure 3 Cell locations of MAP3K3 and PIK3CA mutations and phenotypes of endothelial cells and zebrafish expressing activating Map3k3. (A) The FACS plots of flow sorting of four samples of sporadic CMs and the fractional abundance of MAP3K3 and PIK3CA variants in CD31+ and CD31- cells derived from four fresh tissue samples and in the whole tissue sample before sorting. (B) Downstream effectors of MAP3K3. Plasmids encoding MAP3K3 (I441M) and wild-type MAP3K3 (Control) with FLAG-tag were transfected into HUVECs. Significantly increased phosphorylation of extracellular signal regulated kinase 5 (ERK5) was observed with MAP3K3 (I441M). Increased phosphorylation of p38 and Jun N-terminal kinase (JNK) were also seen with MAP3K3 (I441M). While no change in phosphorylation of ERK1/2 was observed with the MAP3K3 (I441M). (C) Increased cell proliferation in mutant MAP3K3 (I441M) compared with wild-type via 5-ethyl-2'-deoxyuridine (EDU) staining 48 h after transfection and VEGFA (20 ng/ml) treatment for 12 h. Green indicates EDU and blue indicates nucleus [4,6-diamidino-2-phenylindole dihydrochloride (DAPI)]. (D) Visible disruption of endothelial vascular tube formation in cells transfected with mutant MAP3K3 (I441M) compared with wild-type (Control). (E) The vascular deficits of zebrafish injected with wild-type or mutant MAP3K3 (I441M) mRNA and control (PBS) at 48 h post-fertilization (hpf). The cardiovascular system was marked with EGFP (kdrl). Errors show the locations of absent blood vessels and uneven blood vessel density. (F) The quantification of zebrafish with vascular phenotypes (one-way ANOVA test). The number in the box above the column represents the number of patients in each group.
while the literature quantity was much fewer and the sample size was smaller than those of the familial CMs.12–14 Regarding the 300 x depth of WES in our study, the possibility of finding somatic mutations in these genes in our study was potentially higher than that in some previous studies. We observed the discrepancy of the somatic mutations in the three CCM genes between our study and previous studies on sporadic CMs, which might be due to genetic background from different populations.

Recently, in a cohort of 31 sporadic patients with CMs, five (16.1%) affected individuals were identified carrying heterozygous germline mutations in RNF213.28 However, the majority of sporadic CMs present with single lesions and are non-familial, which indicated an underlying aetiology of somatic rather than germline mutations. Thus, we used WES to detect somatic mutations of low frequency within the CM lesions. Paraffin-fixed samples are prone to singleton changes and other PCR-introduced errors. To avoid bias associated with sample quality and sequencing process, fresh-frozen tissue was obtained during surgical resections and used in WES. Our results identified that 90.1% of sporadic CMs carried pathogenic somatic missense variants in MAP3K3 and/or PIK3CA. We also found cases with concomitant mutations of both MAP3K3 and PIK3CA. Similar co-occurrence of pathogenic mutations in different genes has previously been detected in other types of vascular malformations and cancer.29,30 In these sporadic CMs with double mutations, the allele frequencies are relatively similar. These results raised an intriguing question regarding whether the mutations occurred in the same cell. Similar abundance is not convincing evidence and the mutations are located far from the 3’ end of the transcripts, rendering them not detectable by the single cell RNA sequencing with short reads. Further studies should be carried out to clarify this.

Previous literature has mostly focused on the genetic background of CCMs in a familial form. The concept of Knudson’s two-hit mechanism might play an important role in familial CCM on the basis of the autosomal dominant pattern of inheritance of CCM and the presence of multiple lesions, which have been supported by mouse model of CCM.13,14,31–33 Of the 90 consecutive patients included in the present study, WES identified nine patients carrying germline non-synonymous mutations of KRT15/CCM1 (5/9), MGC4607/CCM2 (3/9) and PDGDC10/CCM3 (1/9). One patient was detected carrying a germline mutation of KRT15 in germline mutant allele and a somatic mutation of KRT15 in the wild-type allele of the gene. That is, the CCM tissue showed a somatic mutation in the CCM gene that harbours the germline mutation, the somatic mutation then inactivates the remaining wild-type allele. The finding of this patient with biallelic somatic and germline mutations of KRT15 indicated a two-hit mechanism of familial CCM pathogenesis.

Patients with sporadic CMs are heterogeneous, for whom predicting future haemorrhagic and neurological risk remains a challenge.34 Previous studies have found that prior haemorrhage event and the location of CMs are valuable in predicting haemorrhage.35,36 We found that in sporadic CMs, the mutation pattern was associated with distinct clinical characteristics. The results were consistent with transcriptomic analysis demonstrating that samples with different genetic mutations had distinct transcriptional signatures. Previous studies have reported that the apoptosis resistance of endothelial cells in pulmonary arterial hypertension promoted vascular obstruction.37 In our study, transcriptomic analysis as well as immunostaining demonstrated that endothelial cells from samples with PIK3CA mutations had a low apoptosis rate, indicating that CM lesions with PIK3CA mutations might also be susceptible to vascular obstruction. The minimal counts of endothelial cells and apoptosis resistance reflect severe dysfunctions of endothelial cells and impairment of vascular structures in CMs with PIK3CA mutations. Moreover, the expression of SERPINA5 and GDF15 in pathological level may functionally correlate with the haemorrhagic characteristics of CMs and may represent a biomarker for haemorrhagic risk and predictor of genotypes in CMs. SERPINA5 (protein C inhibitor, plasminogen activator inhibitor-3) is a secreted, extracellular clade A serpin, which has been related to anticoagulation.38 The expression of growth differentiation factor 15 (GDF15) is increased during endothelial dysfunction, which is in response to a vascular stress and activated by oncogenic PIK3CA.24,19,39 The high prevalence of MAP3K3 and PIK3CA in sporadic CMs and the distinctive clinical characteristics in different genetic subgroups may lead to a new molecular classification of sporadic CMs.

Somatic mutations in the PI3K-AKT-mTOR and RAS-MAPK pathways have been identified in some types of vascular malformations.19,40–45 Activating mutations of the MAP3K3 and PIK3CA are accompanied by dysregulation of the MAPK-ERK pathway and PI3K-AKT-MTOR pathway.17,18,46,47 Previous studies have shown that MEK5 is a CCM2 binding partner and CMs arose from the loss of an adaptor complex (CCM complex) and gain of MEK5. MEK5-ERK5 signalling in brain endothelial cell.48 CCM2 is osmosensing scaffold for MEK5 and modulates the MEK5-dependent p38 activation.49 The p38 pathway contributes to the abnormal development of vessels in CMs. Based on the functional studies of the present study, we can infer that MAPK pathway activation plays an important role in both sporadic and familial CMs. These findings may alter the concept of management of these CMs by introducing the possibility of targeted medical therapy in the context of surgical intervention are not always possible.

Taken together, we identified activating MAP3K3 and PIK3CA somatic mutations in the majority (90.1%) of sporadic CMs of the

| Table 3 Details of donors, tissue region, mutation type, and cell numbers of single cell analysis |
|-----------------|----------------|----------------|-----------------|-----------------|----------------|----------------|
| Sample          | Patient        | Donor numbers | Sample type     | Somatic mutations | Group          | Cell numbers  |
| Sample 1        | Patient 28     | 1             | Brainstem CMs   | MAP3K3 I441M      | Lesion         | 7579          |
| Sample 2        | Patient 31     | 1             | Brainstem CMs   | PIK3CA C420R      | Lesion         | 12414         |
| Sample 3        | Patient 32     | 2             | Temporal lobe CMs | MAP3K3 I441M + PIK3CA E545K | Lesion         | 6743          |
| Sample 4        | Patient 32     |               | Control temporal lobe |                  |                | 7568          |
| Sample 5        | Patient 35     | 1             | Brainstem CMs   | PIK3CA H1047R     | Lesion         | 2919          |
| Sample 6        | Patient 36     | 1             | Thalamus CMs    | PIK3CA H1047R     | Lesion         | 6096          |
| Sample 7        | Patient 75     | 2             | Associated cutaneous CMs | MAP3K3 I441M      | Lesion         | 9057          |
| Sample 8        | Patient 75     |               | Control skin    |                  | Control        | 4065          |
| Sample 9        | Control        | 1             | Control temporal lobe |                | Control        | 8856          |
Figure 4 Single cell RNA sequence revealed distinct transcriptional signatures between MAP3K3 and PIK3CA mutations. (A) UMAP (uniform manifold approximation and projection) clustering of 65,297 cells isolated from nine samples (six CMs samples and three control samples). Cells were marked by tissue sources (left) or cell types (right). (B) Violin plots of endothelial cell and mural cell marker genes expressions identified by differential expression analysis. (C) Bar graphs depicting the counts of endothelial cells and mural cells from nine samples. Right: The proportion of endothelial cells, mural cells and other cells. (D) Binary plots indicating with bars whether a gene (column) is a differentially expressed gene (DEG) in a given comparison group (rows) or not (n = 1621 DEGs). Right: DEG counts for each comparison group. (E) Selected top categories from GO enrichment analysis of DEGs to compare the differences of biological functions between MAP3K3 and PIK3CA mutation groups. (F) TUNEL immunostaining of CM tissues with MAP3K3 mutations (Patient 43), PIK3CA mutations (Patient 11), double mutations of MAP3K3 and PIK3CA (Patient 66), and one control tissue of temporal lobe. Overlay of DAPI (blue), CD31 (red) and apoptotic nucleus (green) are shown. The quantification of apoptotic endothelial cells from MAP3K3 mutation groups were much higher than other groups (P < 0.001). The number in the box above the column represents the number of patients in each group. MAP3K3 mutation groups included Patients 13, 43, 45 and 57. PIK3CA mutation groups included Patients 11, 22 and 40. The double mutation group included Patient 66. (G) Heat maps of the expression of related DEGs from different subgroups compared with the control group. (H) Heat maps of GDF15 and SERPINA5 expressions in three genetic subgroups and the control group. (I) Immunostaining of CM tissues with MAP3K3 (Patient 5), PIK3CA (Patient 45) mutations and control tissue for GDF-15 and Serpin A5. MAP3K3 mutation groups included Patients 43, 46 and 68. PIK3CA mutation groups included Patients 5, 11 and 52. Consistent with the results of single cell RNA sequencing, both were positive in patients of PIK3CA mutation and negative in other samples. Single-channel images of DAPI (blue), CD31 (red), GDF-15 (green) or Serpin A5 (green) and overlay of them are all shown.
CNS. The mutations spectrum may correlate with the phenotype of sporadic CMs and PIK3CA mutations may confer a higher risk for overt haemorrhage. The functional studies and single cell transcriptomic analysis provide a blueprint for interrogating the cellular and molecular basis of sporadic CMs.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at Brain online.

References