Supplementary Appendix

Somatic *GJA4* Mutation in Intracranial Extra-axial Cavernous Hemangiomas

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Supplemental Methods

DNA isolation from frozen and FFPE samples

For frozen extra-axial cavernous hemangiomas specimens and paired peripheral blood samples, commercially available kits (QIAGEN Gentra Puregene and QIAamp DNA Blood Mini Kit) were used following manufacturer's recommendations. For FFPE samples, genomic DNA was extracted by QIAamp GeneRead DNA FFPE kits (Qiagen) following manufacturer's recommendations and the Uracil-N-Glycosilase (UNG) were used to remove artificially induced uracils from the DNA obtained from the FFPE sample.

WES of extra-axial cavernous hemangioma tissue and peripheral blood samples

WES of extra-axial cavernous hemangiomas specimens and paired peripheral blood samples were performed as our previous study[1]. The quality of isolated genomic DNA was verified by: (1) DNA degradation and contamination were monitored on 1% agarose gels; (2) DNA concentration was measured by Qubit® DNA Assay Kit in Qubit® 2.0 Fluorometer (Invitrogen, USA). For library preparation, a total amount of 0.6 μg genomic DNA per sample was used as input material. Sequencing libraries were generated using Agilent SureSelect Human All Exon V6 kit (Agilent Technologies, CA, USA) following manufacturer's recommendations and index codes were added to each sample. Fragmentation was carried out by the hydrodynamic shearing system (Covaris, Massachusetts, USA) to generate 180-280 bp fragments. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, adapter oligonucleotides were ligated. DNA fragments with ligated adapter molecules on both ends were selectively enriched in a PCR reaction. After PCR reaction, libraries were hybridized in liquid phase with the biotin-labeled probes and captured using magnetic beads enriched for the exons of genes. Captured libraries were enriched in a PCR reaction to add index tags to prepare for sequencing. Products were purified using AMPure XP system (Beckman Coulter, Beverly, USA) and quantified using the Agilent high sensitivity DNA assay on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using Hiseq PE Cluster Kit (Illumina) according to the manufacturer's instructions. After cluster generation, the DNA libraries were sequenced on Illumina Hiseq platform and 150 bp paired-end reads were generated. The depth of extra-axial cavernous hemangiomas lesions and blood controls was 300X and 100X, respectively.

Mapping and variant calling

Whole-exome sequencing (WES) with paired-end 150-bp reads was carried out in extra-axial cavernous hemangiomas lesions (300X) and blood controls (100X) to detect somatic mutations in the discovery cohort. Genomic DNA data in the newly sequenced samples was mapped to hg19 human genome by Burrows Wheeler Aligner (bwa mem)[2]. SAMtools and Picard were applied to sort the reads and mark duplicates[3]. The bam files of the extra-axial cavernous hemangiomas lesion and matched blood control were subjected to the SAVI2 protocol for variant discovery and prioritization[4]. The variant list was filtered as previously reported (MUTracer Step 2: 1-6) to eliminate false positives[5]. In total, we identified 34 somatic mutations in 30 genes (Table S2).

Digital Droplet PCR (ddPCR)

To detect the *GJA4* c.121G>T (p.Gly41Cys), template DNA were analyzed on the QX200 Droplet Digital PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The ddPCR probes were synthesized by BIOLIGO, Shanghai. The sequence of the used primers and probes for ddPCR are as follows: forward primer (TCGACCGTGGTGGGTAAGAT), reverse primer (TTGCTCGTCACCCCACACT), fluorescent reference probe (5'-VIC- CTGGCCGGCGAGT) and mutant allele probe (5'-FAM-CCTGACCATgTTCATGGAGTACA). Then, ddPCR was performed as our previous study[5]. A total of 200 ul 40X probe assay containing 60 ul forward primer (100 uM), 60 ul reverse primer (100 uM), 20 ul reference probe (100 uM), 20 ul mutant allele probe (100 uM), and 40 ul DNase/RNase free water was prepared. The 20 μl reaction mix consisted of 10 μl of 2x ddPCR SuperMix for probes (Bio-Rad Laboratories), 0.5 μl of the 40X probe assay, 7.5 μl DNase/RNase free water and 2 μl of 5 ng/μl genomic DNA. Cycling conditions for the reaction were 95°C for 10 min, followed by 40 cycles of 94°C for 30 sec and 60°C for 1 min, then 98°C for 10 minutes, and finally a 16°C hold on a Life Technologies Veriti thermal cycler. All assays were validated by temperature gradient to ensure optimal separation of reference and variant signals. Data were analyzed using QuantaSoft v1.7.4 (Bio-Rad Laboratories). The 300 bp synthetic mutant DNA fragment and 300 bp synthetic wildtype DNA fragment were constructed (by Shanghai Sangon Biological Engineering and Technological Service Company, China) and used as positive control and negative control, respectively. In addition, DNase/RNase free water used as no template control. We also performed ddPCR for normal control tissue (superficial temporal artery) from 7 FFPE individuals who were undergoing standard craniotomy procedure. To reduce false positive rate, we set a cutoff for fractional abundance of 0.5% for *GJA4* c.121G>T mutations with over five positive droplets.

Cell culture and treatment of human umbilical vein endothelial cells (HUVECs)

Primary human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell and cultured in endothelial cell medium (ECM; ScienCell) containing 1% endothelial cell growth supplement (ECGS; ScienCell) and 5% fetal bovine serum (FBS; ScienCell). The cells were cultivated in a humidified atmosphere at 37°C of 5% CO2. Inhibitor of SGK1 (EMD638683, 10μM, Selleck) was dissolved in DMSO and added in the cell medium for 12 h before harvest. Comparison was made to vehicle-treated (i.e., DMSO, 0.1%) controls.

Lentivirus preparation and transduction

The lentivirus encoding wild-type *GJA4* or mutant *GJA4* were designed, constructed, and produced by Beijing SyngenTech Co. A pLV-EF1a-hluc-P2A-Puro-CMV-3flag vector was used. Empty vector was used as control. At the time of infection, plates were incubated with 20 MOI of lentivirus in culture medium. 24 hours after infection, ECs were selected with 2 μg/ml puromycin.

SDS-PAGE and immunoblotting

Whole-cell lysates were prepared using RIPA buffer (Sigma-Aldrich, St. Louis, USA). Protein concentration was determined using a BCA Protein Assay kit (Pierce). 20μg total proteins were loaded on gel and separated by SDS-PAG and transferred to a polyvinylidene difluoride membranes (PVDF) (Merck KgaA, Darmstadt, Germany). Signals were detected using an Immobilon Western

Chemiluminescent HRP Substrate (WBKLS0500, Millipore, MA). GAPDH was used as the loading control. The detailed information of primary antibodies used are as follow: anti-*GJA4* (1:1000, ab181701, Abcam), anti-SGK1 (1:1000, #12103, CST), anti-p-SGK1 (1:1000, #5599, CST), anti-ERK1/2 (1:1000, #4695, CST), anti-p-ERK1/2 (1:1000, #4376, CST), anti-P38 (1:1000, #8690, CST), anti-p-P38 (1:1000, #4511, CST), anti-TK1 (1:1000, ab76495, Abcam), anti-PCNA (1:1000, #13110, CST), Cyclin D1 (1:1000, #55506, CST), anti-Ephrin B2 (1:1000, ab131536, Abcam), anti-VEGF R2 (1:1000, #9698, CST), anti-GAPDH (1:1000, #5174, CST). Goat Anti-Rabbit IgG-HRP (1:5000, M21002, Abmart) was used as second antibody.

Histological staining

For HE staining, tissue samples were fixed in 4% paraformaldehyde overnight at 4 °C, dehydrated in 100% ethanol, and embedded in paraffin. Tissue sections underwent dewaxing and rehydration through xylene and ethanol treatment were subsequently used for staining. Then, HE staining was performed using a HE staining Kit (G1120, Solarbio) according to the manufacturer's instructions. For immunohistochemical staining, extra-axial cavernous hemangiomas tissue sections were incubated with an anti-p-SGK1 (1:50, AF3001; Affinity), anti-PCNA (1:200, ZM-0213, ZSGB-BIO) or anti- Ephrin-B2 (1:100, ab131536; Abcam) primary antibody overnight at 4°C and were then incubated with a biotin-labeled goat anti-rabbit IgG polymer (ZSGB-BIO) at room temperature for 1 hour followed by horseradish peroxidase–labeled streptavidin for 30 minutes. After washing with Tris buffer, sections were stained with 3,3′-Diaminobenzidine, and nuclei were counterstained with hematoxylin. Images were acquired with a Zeiss Axio Scope A1 microscope. For statistical purpose, six randomly selected non-overlapped vision fields from 3 patients in each group were chosen. A positive reaction was indicated by brown color using 3,3′-Diaminobenzidine. Image-Pro Plus was used to calculate the integrated optical density.

AAV preparation

pAAV-ICAM2p-MCS-eGFP-3Flag-SV40 polyA (AAV-control), -*GJA4*-eGFP-3Flag-SV40 polyA (AAV-*GJA4*-wild-type), and -*GJA4* p.G41C-eGFP-3Flag-SV40 polyA (AAV-*GJA4*-mutant) were designed, constructed, and produced by Genechem Co., Ltd, Shanghai, China. A modified AAV serotype 9 vector (A endothelium-targeted heptapeptides SLRSPPS is inserted into AAV9 capsid A589 site) with target gene expression driven by ICAM2 promoter was used[6,7]

Animals and AAV injection

C57BL/6 mice littermates were purchased from SPF Biotechnology Co., Ltd. (Beijing, China). The animals were maintained in a temperature- and humidity-controlled animal care facility with a 12 h light/12 h dark cycle and free access to water and food. Litters were randomly divided into different groups. Investigators were blinded to group allocation when performing all data collection. No animals were excluded for failure to achieve the predicted phenotypes. P1 littermates were administered 10μl of phosphate buffered saline (PBS) containing 3×10^{10} (3×10^{10} genome copies [GC]) AAV-control or 3×10^{10} GC AAV-*GJA4*-wild-type or 3×10^{10} GC AAV-*GJA4*-mutant by retro-orbital venous sinus injection as described previously[8]. For EMD638683 experiments, 2-week-old C57BL/6 mice injected with AAV-

GJA4-mutant received oral gavage with 600mg/kg body weight EMD638683 (Selleck) for a week; control mice received only vehicle.

Immunofluorescence staining

For immunofluorescence staining of GFP, the mice at the post-natal 3 weeks in control group were used and anesthetized with IP injection of sodium pentobarbital. For brain and liver, tissue samples were fixed in 4% PFA overnight at 4 °C, dehydrated in ethanol, and embedded in paraffin. Tissue sections underwent dewaxing and rehydration through xylene and ethanol treatment were subsequently used for staining by Opal Multiplex IHC Assay Kit (PerkinElmer, USA) according to the manufacturer's instructions. Anti-GFP (1:1000, ab290, Abcam) and anti-CD31 (1:200, #77699, CST) were used as primary antibody. For retinal staining, eyes were pre-fixed in 4% paraformaldehyde for an hour and dissected retinas were fixed in 4% paraformaldehyde at 4 °C overnight. Then, retinas were permeabilized in blocking buffer (PBS, 1% of BSA, 0.5% of TritonX-100) at 4 °C overnight. After washes o in Pblec buffer (PBS supplemented with 0.1 mM of MgCl2, 0.1 mM of MnCl2, 0.1 mM of CaCl2, and 1% of Triton X-100), retinas were incubated with Lectin (1:25, MP6313, MKBIO) for 1h at room temperature.

For immunofluorescence staining of brain P-SGK1, the mice at the post-natal 3 weeks in control, *GJA4*-wild-type and *GJA4*-mutant group were used. Brains were fixed in 4% PFA, then dehydrated in ethanol and embedded in paraffin. After dewaxing and rehydration, tissue sections were used for staining by Opal Multiplex IHC Assay Kit (PerkinElmer, USA). Anti-p-SGK1 (1:50, YP0333, Immunoway) and anti-CD31 (1:200, #77699, CST) was used as primary antibody.

Retinal vascular analysis

The mice at the post-natal 3 weeks in control, *GJA4*-wild-type, *GJA4*-mutant and *GJA4*-mutant with EMD638683 group were used for retinal vascular analysis. Mice were anesthetized with IP injection of sodium pentobarbital. Eyes were pre-fixed in 4% paraformaldehyde and dissected retinas were fixed in 4% paraformaldehyde at 4 °C overnight. Then, retinas were permeabilized in blocking buffer overnight and washed in Pblec buffer. After that, retinas were incubated overnight at 4°C with α-SMA primary antibody (1:400, #19245, CST). Then, retinas were incubated with Goat Anti-Rabbit IgG H&L Alexa Fluor® 594 (1:500, ab150080, Abcam) and Lectin (1:25, DL-1178, Vector) for 2 hours at room temperature. After three washes in PBS, whole retinas were flat-mounted in PBS containing 50% of glycerol, and visualized using a Zeiss LSM 880 confocal microscope, and Zen software. The vessel density was the percentage of area occupied by vessels inside the retinal tissue; The diameter of retinal arteries and vein was averaged from three measurements taken at the proximal, middle, and distal part of the vessel segment.

Supplementary Figures

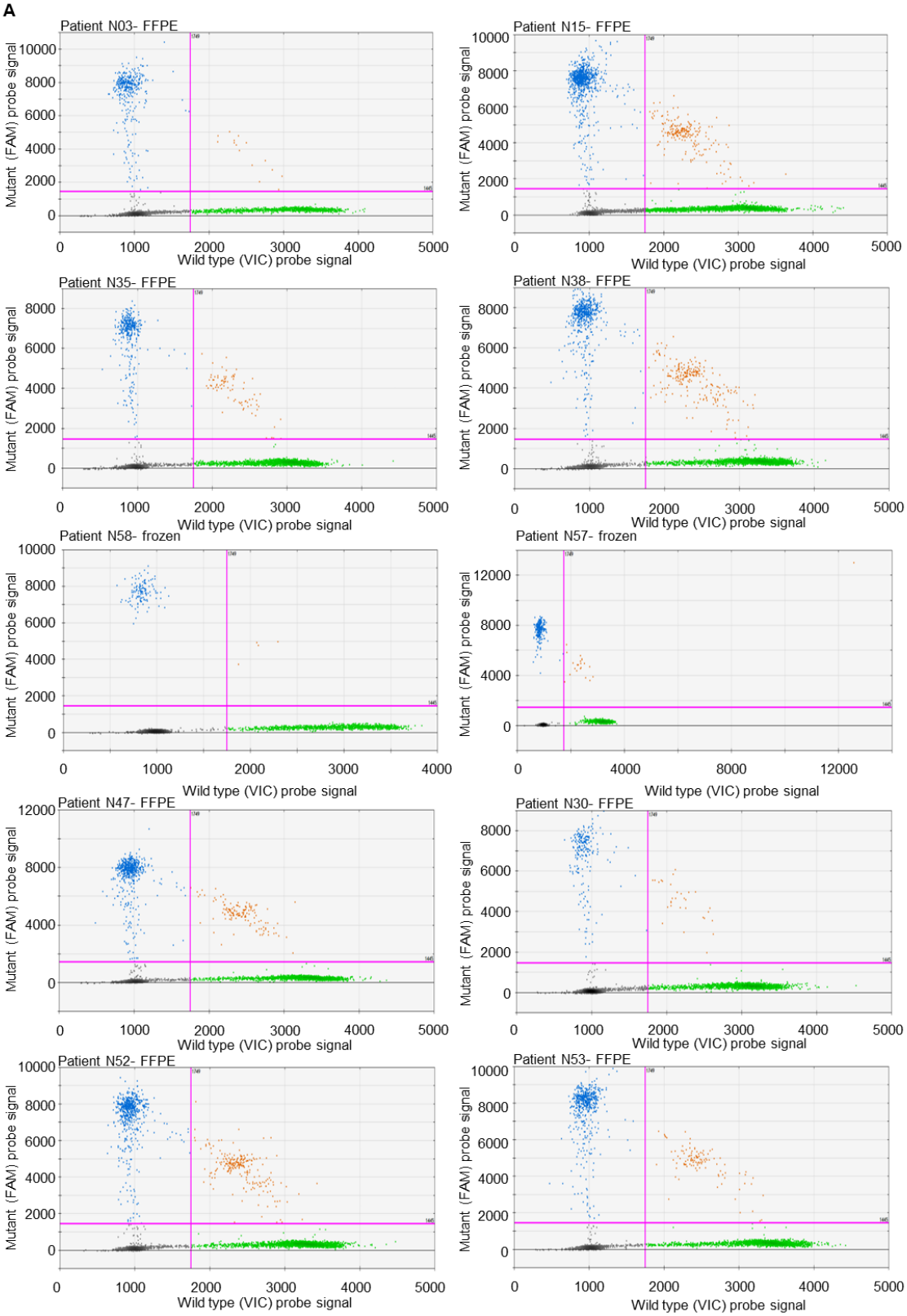
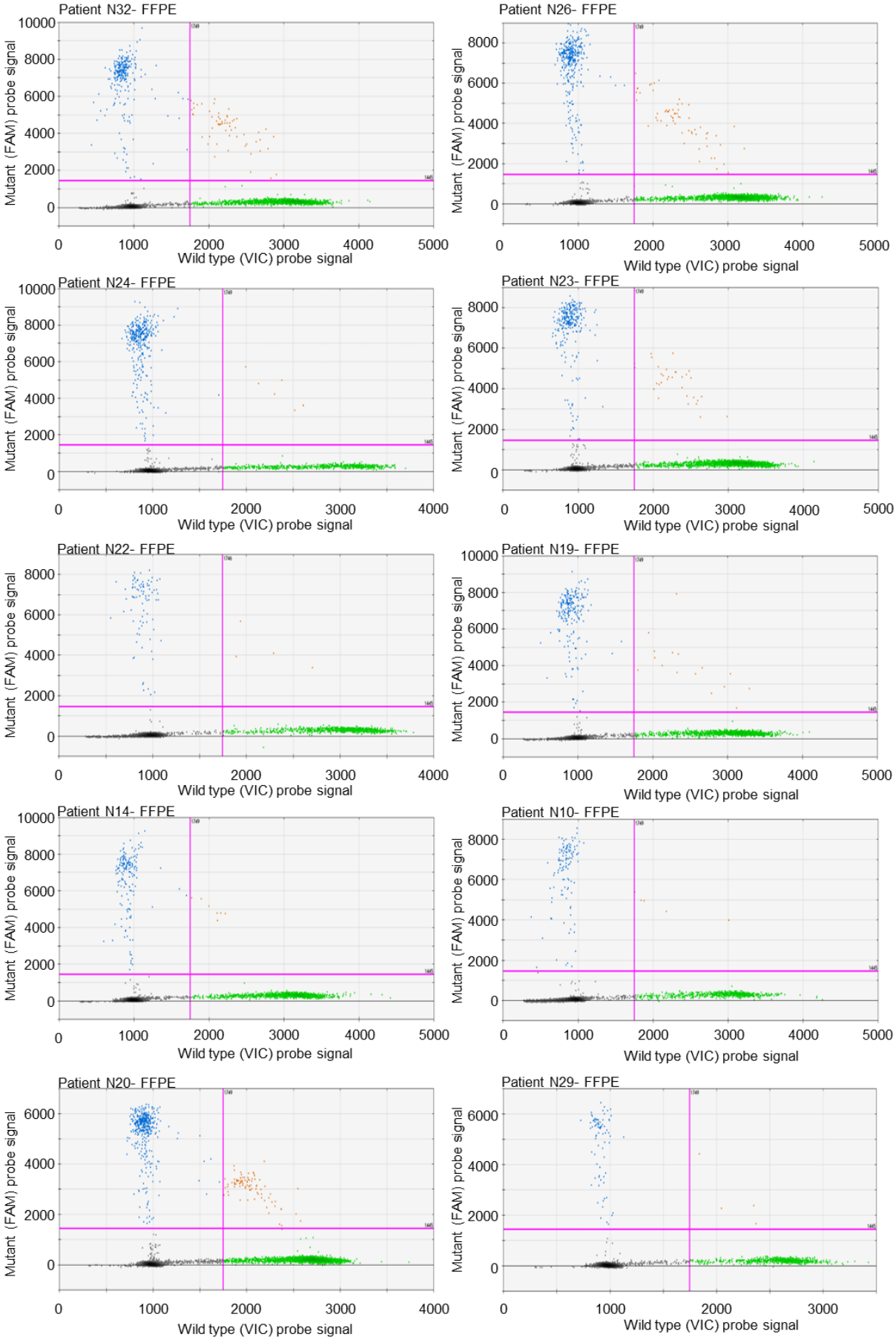


Figure S1 continued



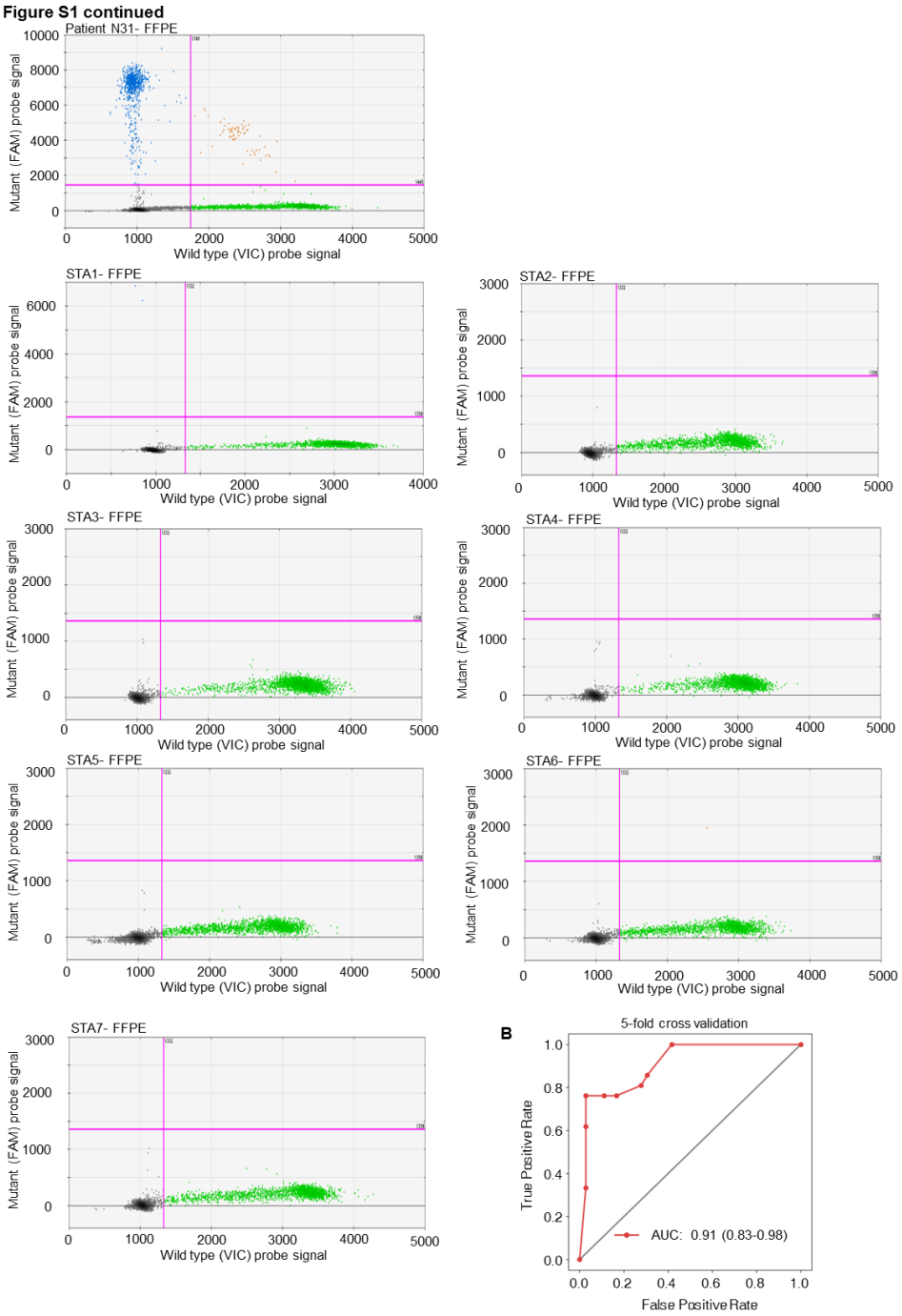


Figure S1. Amplitude scatter plots of *GJA4* c.121G>T, p.G41C ddPCRs. A, Amplitude scatter plots of *GJA4* c.121G>T, p.G41C ddPCRs of mutant samples and superficial temporal artery (STA). Dots represent single well data. Blue: the droplet encloses at least one copy of mutant template. Green: the droplet encloses at least one copy of wild-type template. Orange: the droplet encloses at least one copy of wild-type and one copy of mutant template. Black: the droplet encloses no target molecular; x axis: the signal intensities of HEX probe for the reference allele; y axis: the signal intensities of FAM probe for the mutant allele. **B,** Receiving operation characteristic (ROC) curve showing the cross-validation performance of the decision tree model.

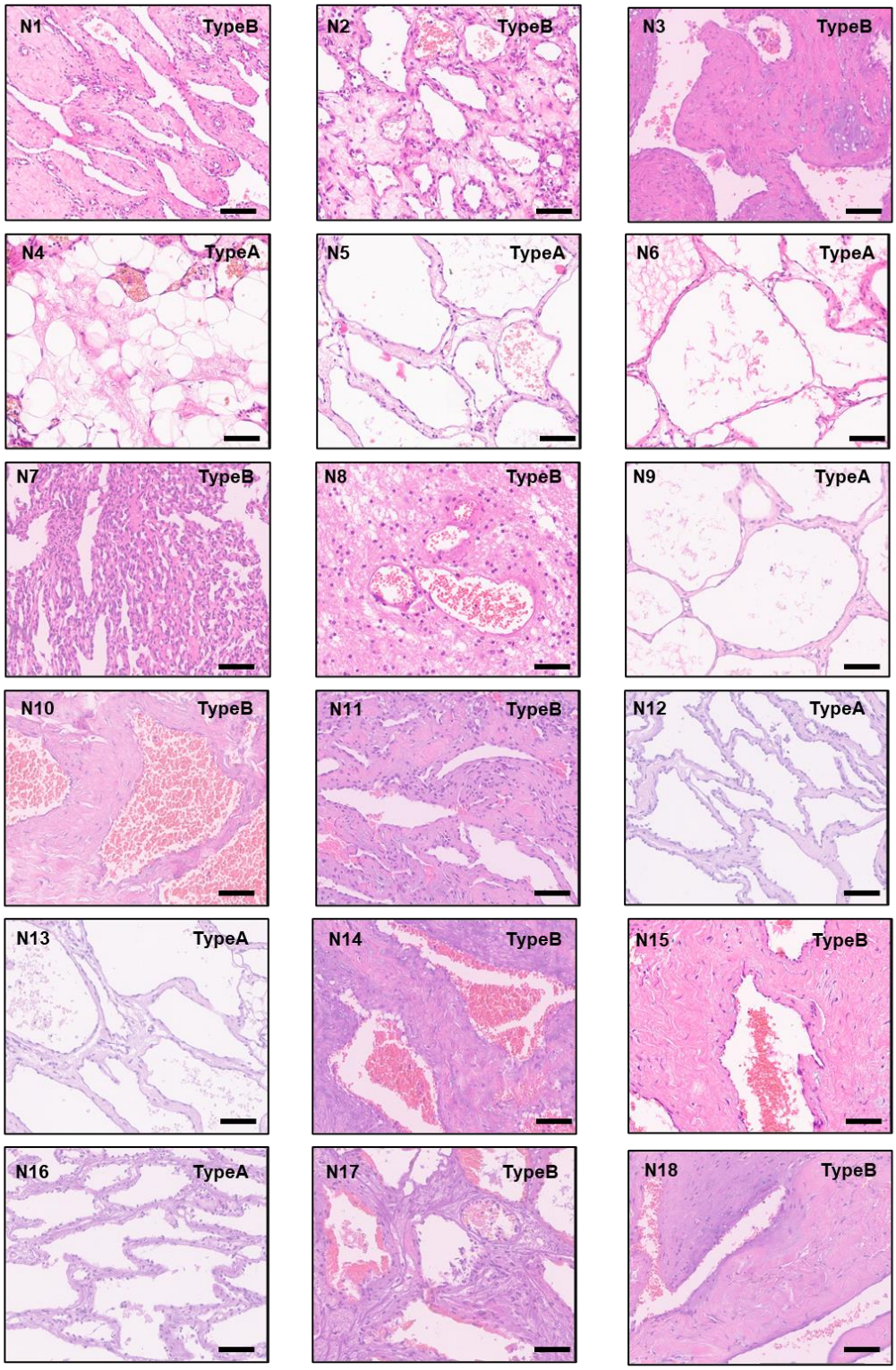

N1 TypeB
N2 TypeB
N3 TypeB
N4 TypeA
N5 TypeA
N6 TypeA
N7 TypeB
N8 TypeB
N9 TypeA
N10 TypeB
N11 TypeB
N12 TypeA
N13 TypeA
N14 TypeB
N15 TypeB
N16 TypeA
N17 TypeB
N18 TypeB

Figure S2 continued

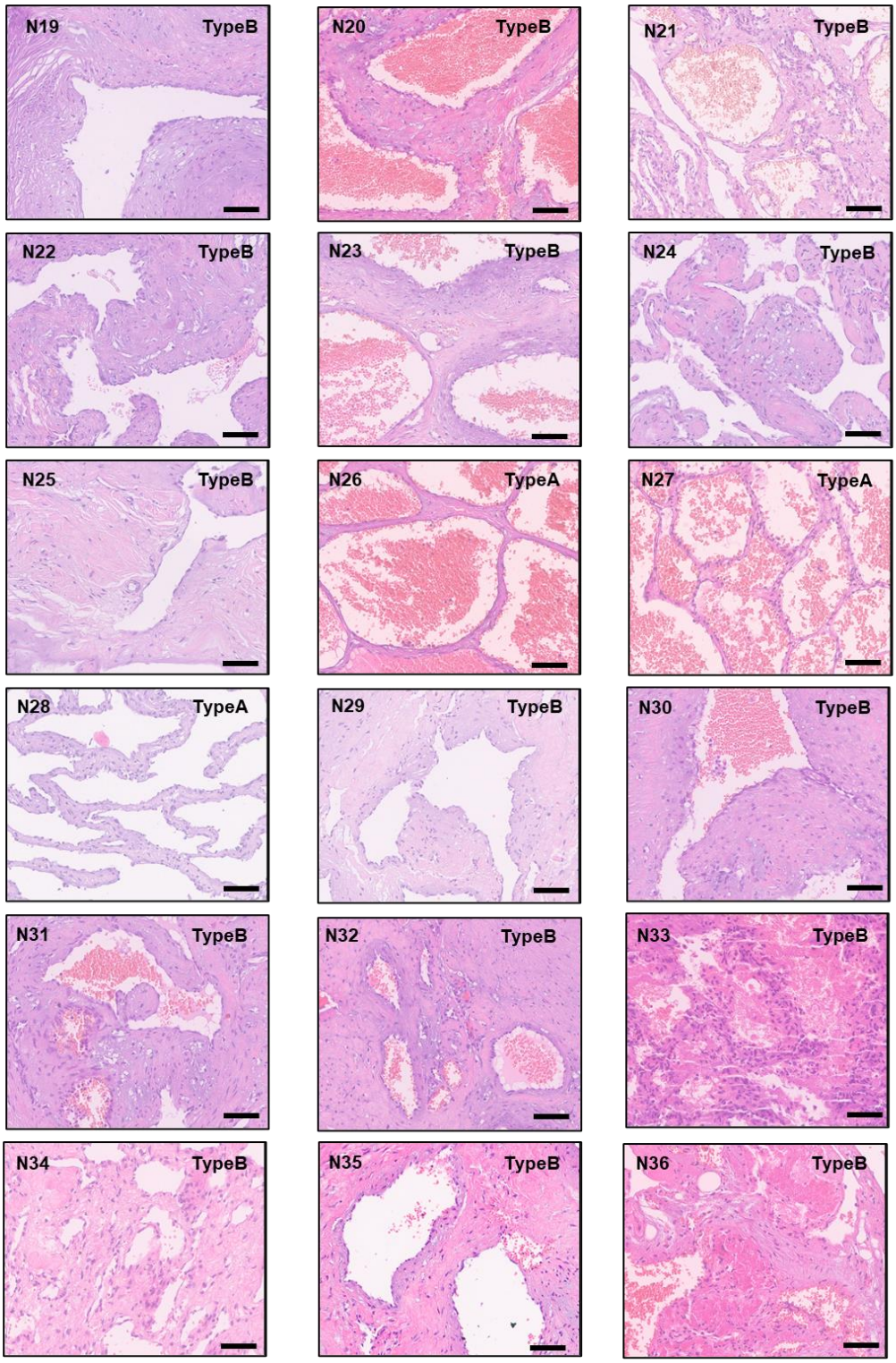
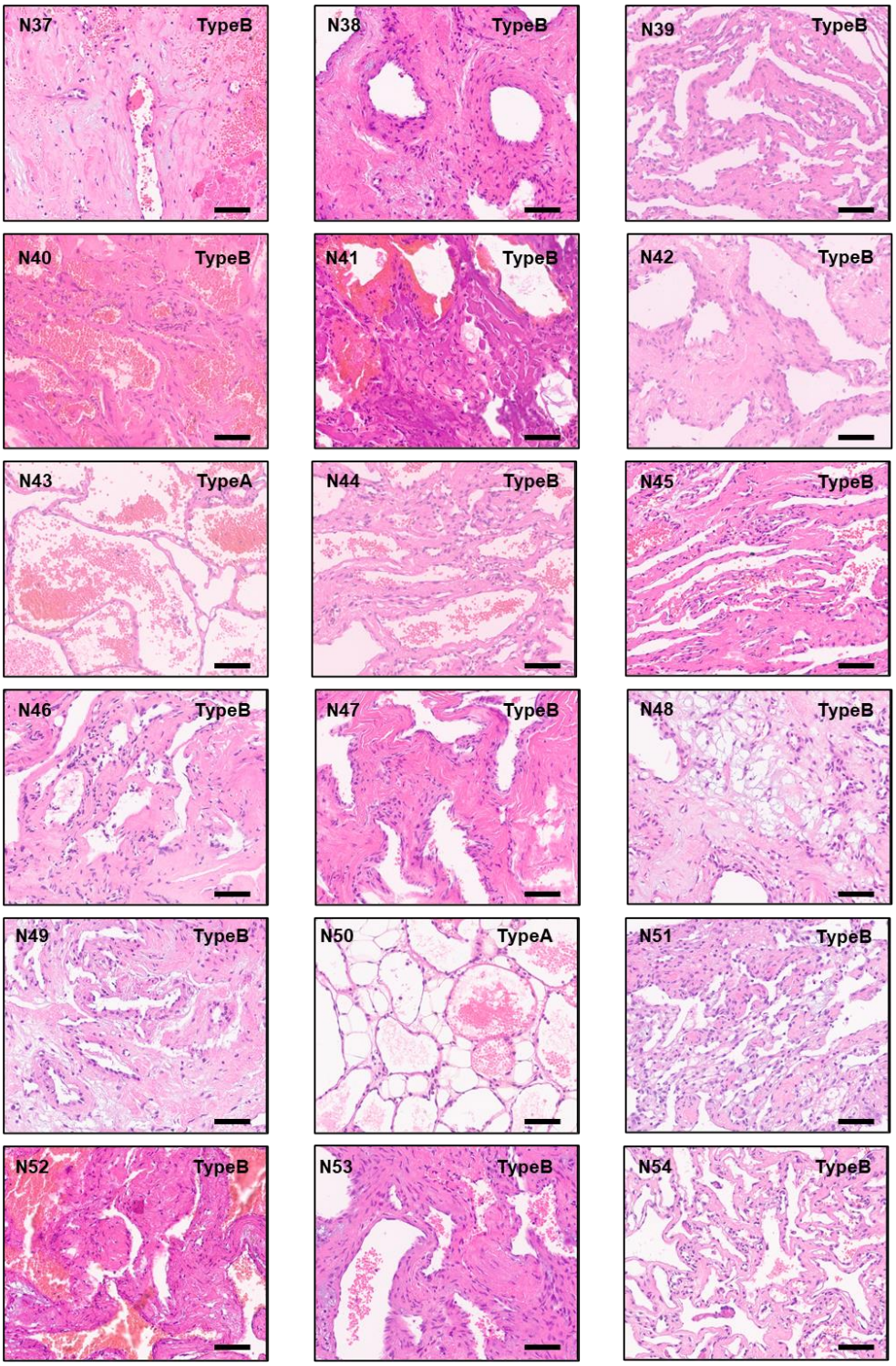


Figure S2 continued



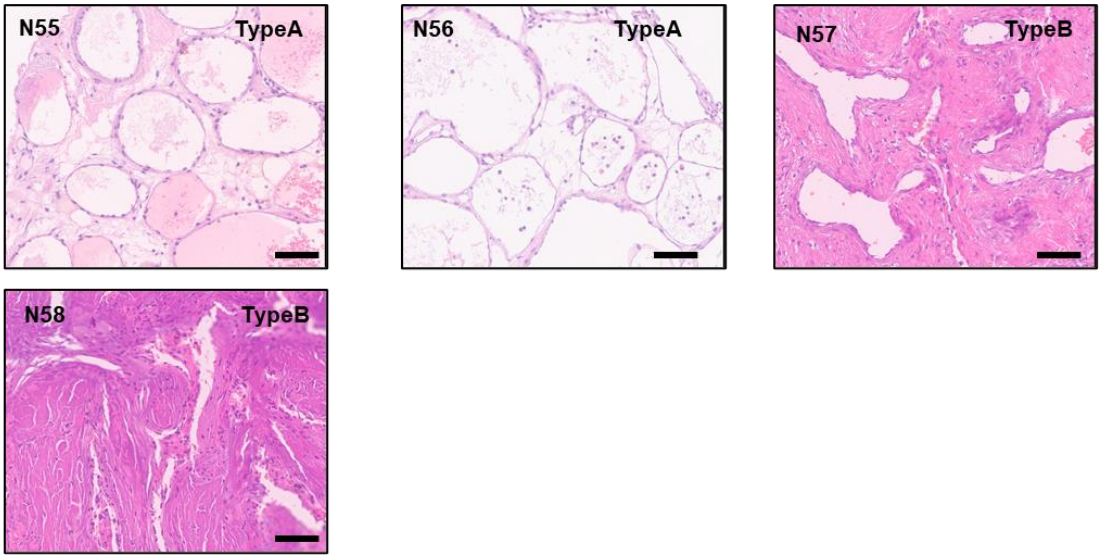
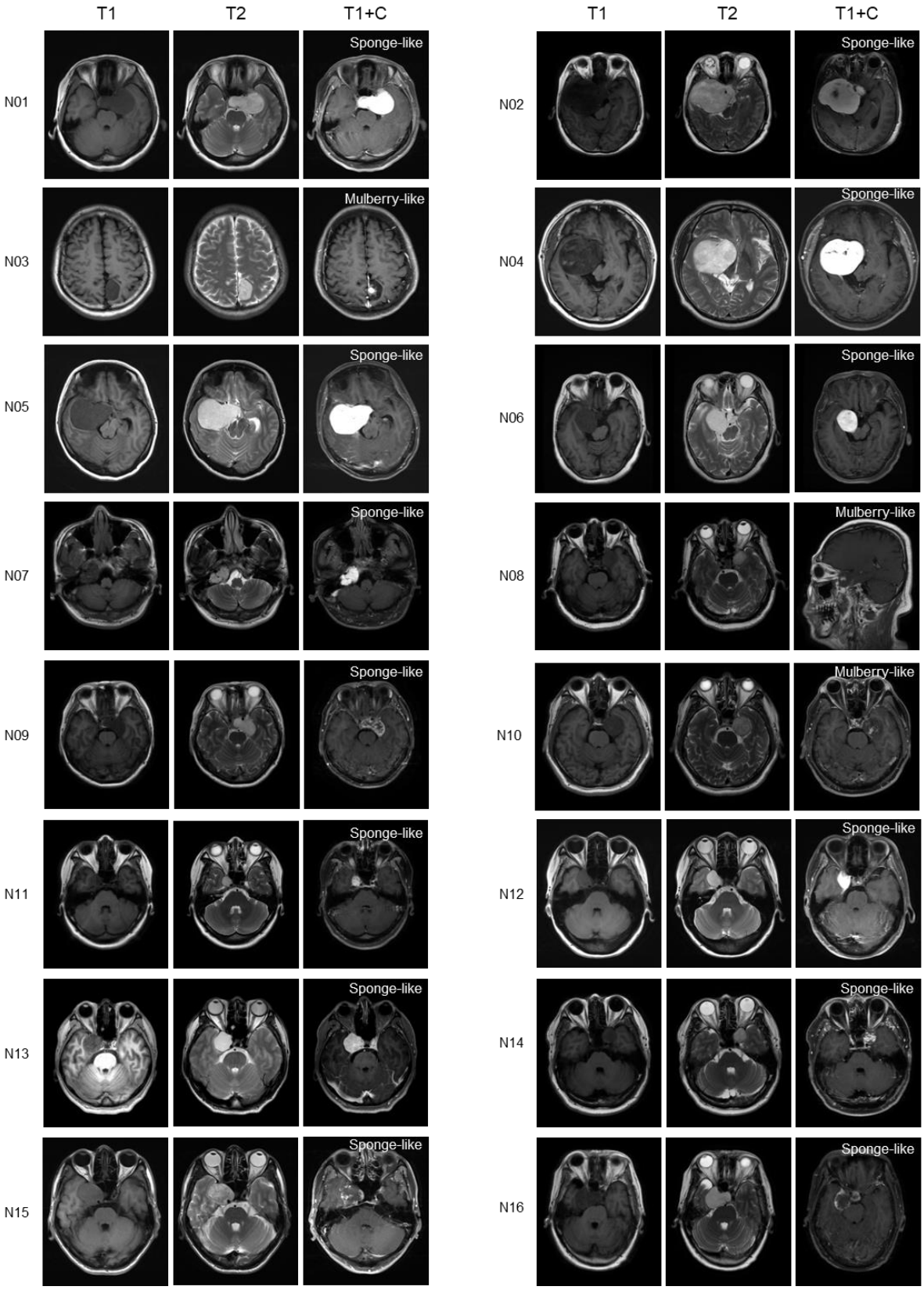


Figure S2. The HE staining of 58 individuals in this study. 58 individuals were categorized into Type A or Type B according to the pathological features. The individual number and classification results are given in the upper left and upper right of the panel, respectively. Scale bar: 100μm.


T1
T2
T1+C
N01
Sponge-like
N02
Sponge-like
N03
Mulberry-like
N04
Sponge-like
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N06
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N07
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Mulberry-like
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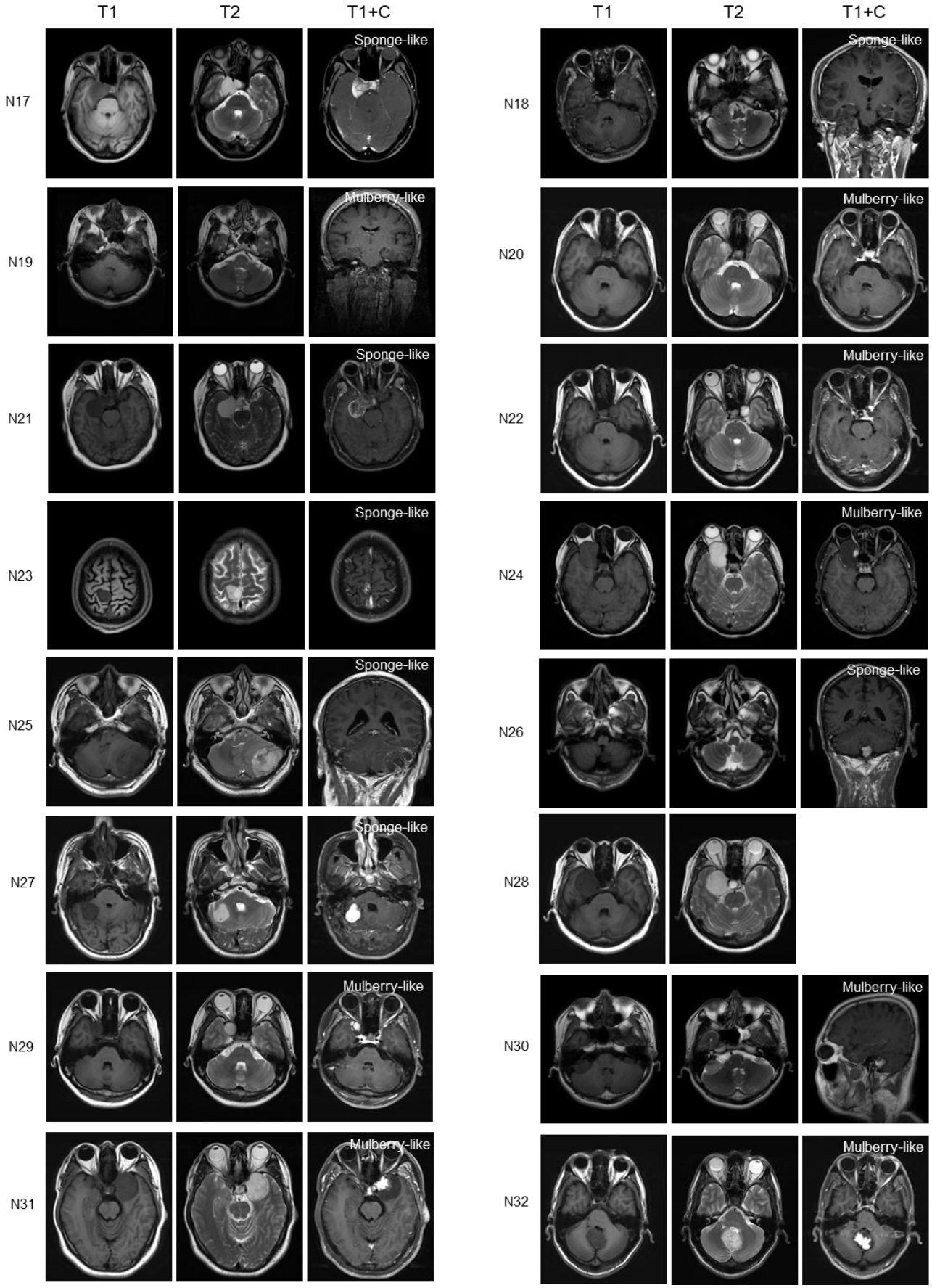
Figure S3 continued



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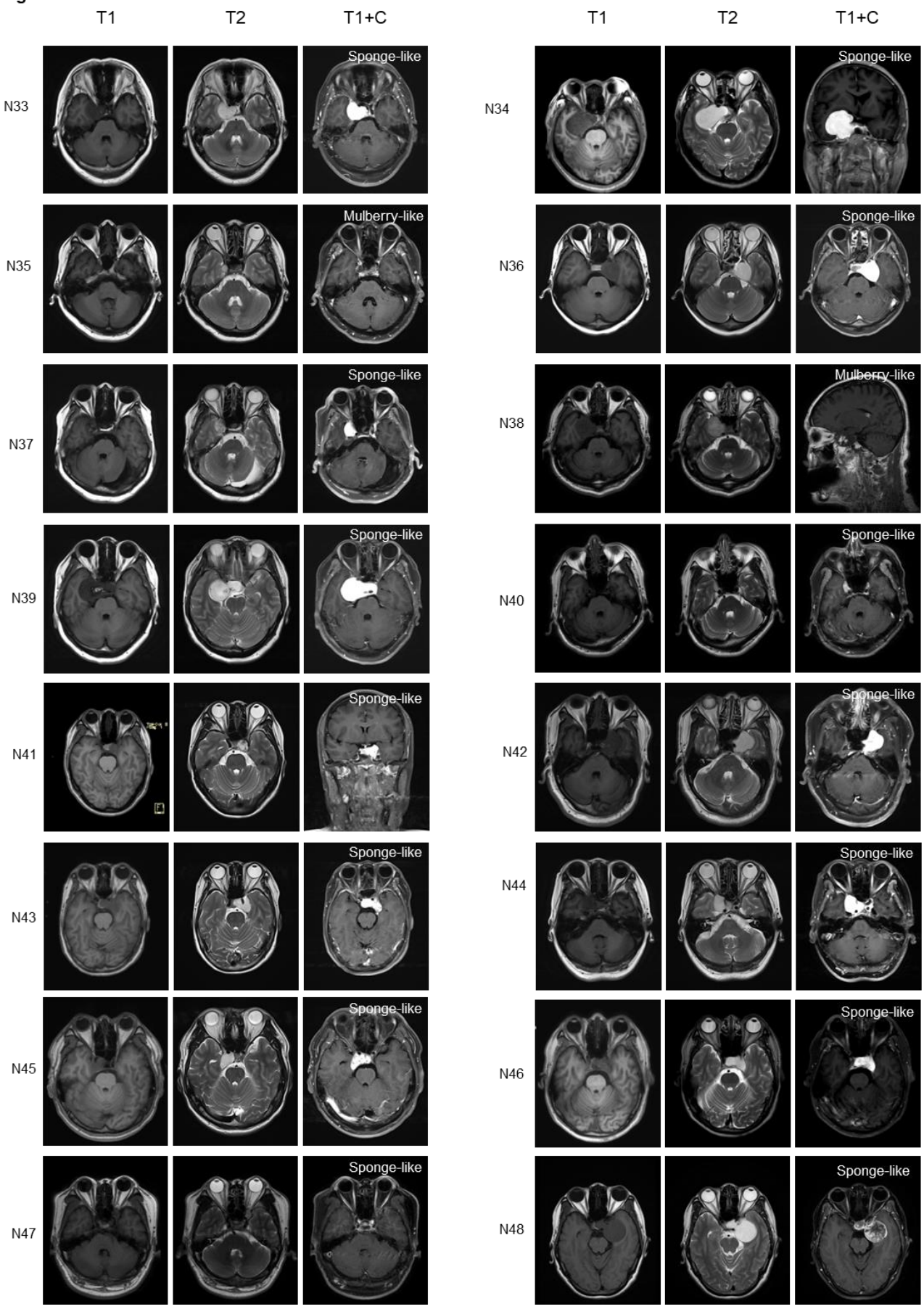

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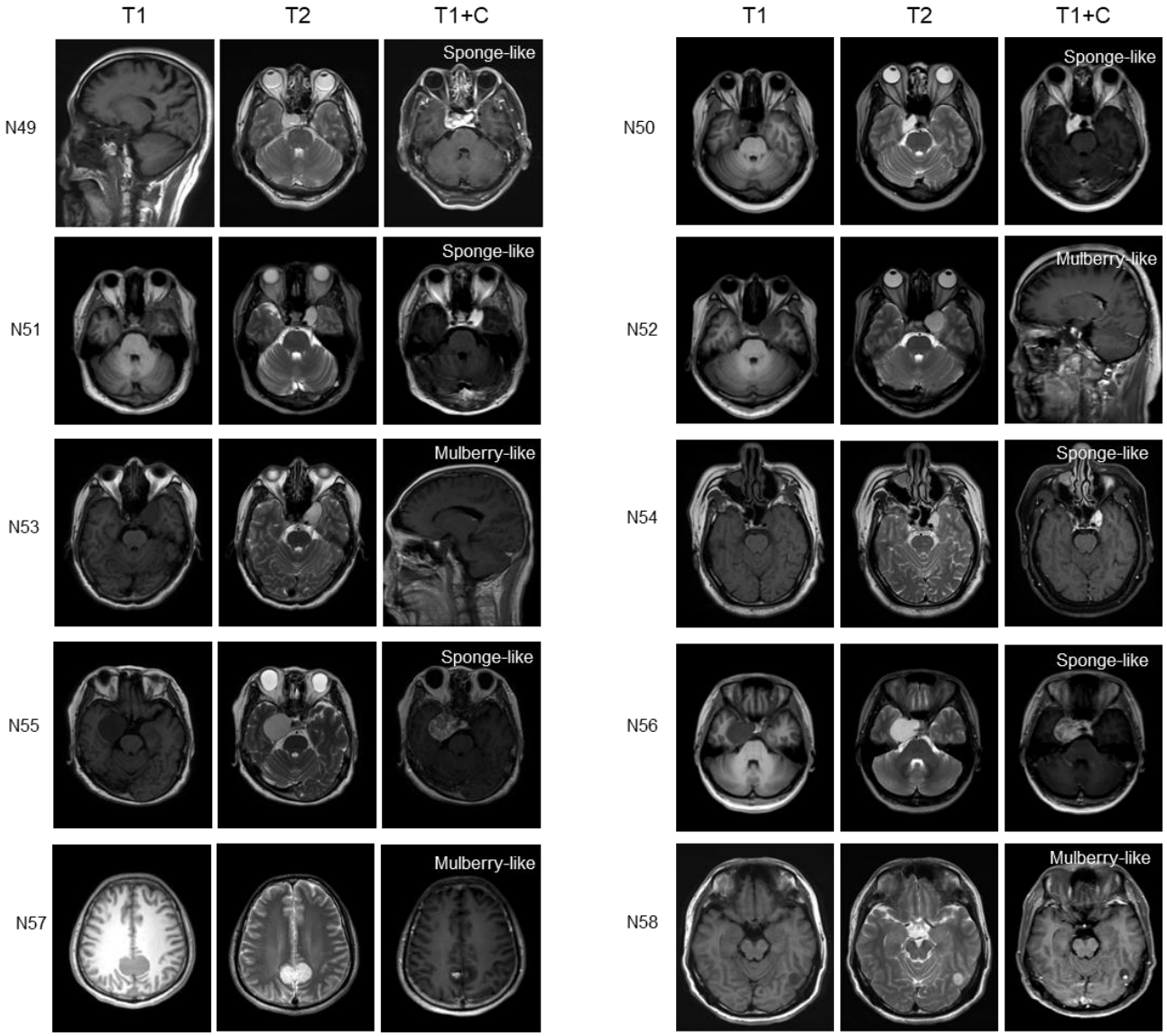

Figure S3. The MRI of 58 individuals in this study. 58 patients were categorized into sponge-like or mulberry-like according to the imaging appearance of contrast-enhanced T1-weighted (T1+C) imaging. Numbering of individuals was given at the left.

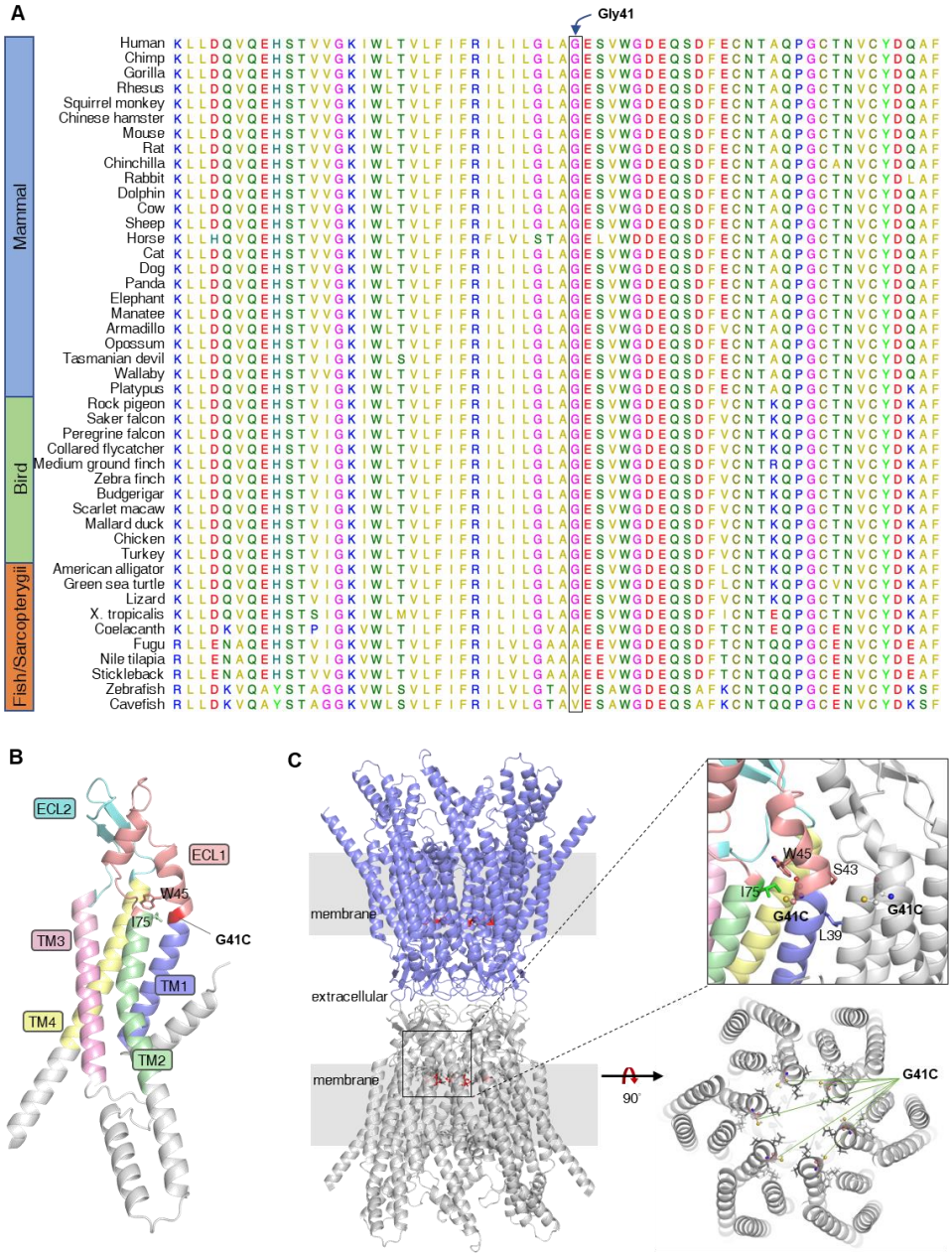

Figure S4. P. Gly41Cys variant occurred on a conserved site of the cx37 protein. A, Multiple sequence alignment of Cx37 protein showed that the observed variant position Gly41 is 100% conserved among Mammals and birds. **B,** Alphafold2 predicted structure of Cx37WT monomer. The 3D structural model showed that the variant site Gly41 was located on margin of transmembrane helical domain (TM1) and extracellular loop domain (ECL1). **C,** Alphafold2 predicted 3D structure of *GJA4* G41C hexamers and docking results of 2 hexamers from 2 different cells form a gap junction channel. The structure of two Cx37 hexamers formed gap junction channel showed the mutant residue is located on the interface of any two monomers and close to inner side of the channel, which might influence its normal function.

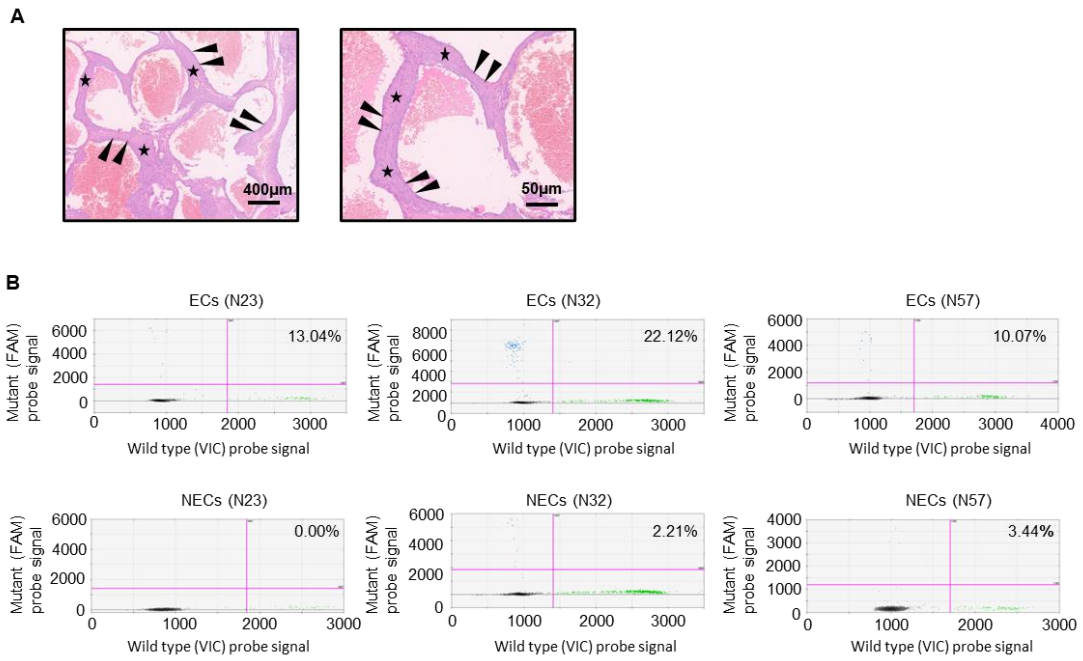

Figure S5. Detection of *GJA4* c.121G>T mutation in extra-axial cavernous hemangiomas endothelium. A, HE staining of the extra-axial cavernous hemangioma tissue. The arrows indicate vascular endothelial cells, and the asterisks indicate mesenchymal cells. **B,** Amplitude scatter plots of *GJA4* c.121G>T, p.G41C ddPCRs of mutant samples. Dots represent single well data. Blue: the droplet encloses at least one copy of mutant template. Green: the droplet encloses at least one copy of wild-type template. Orange: the droplet encloses at least one copy of wild-type and one copy of mutant template. Black: the droplet encloses no target molecular; x axis: the signal intensities of HEX probe for the reference allele; y axis: the signal intensities of FAM probe for the mutant allele. ECs: endothelial cells; NECs: non endothelial cells.

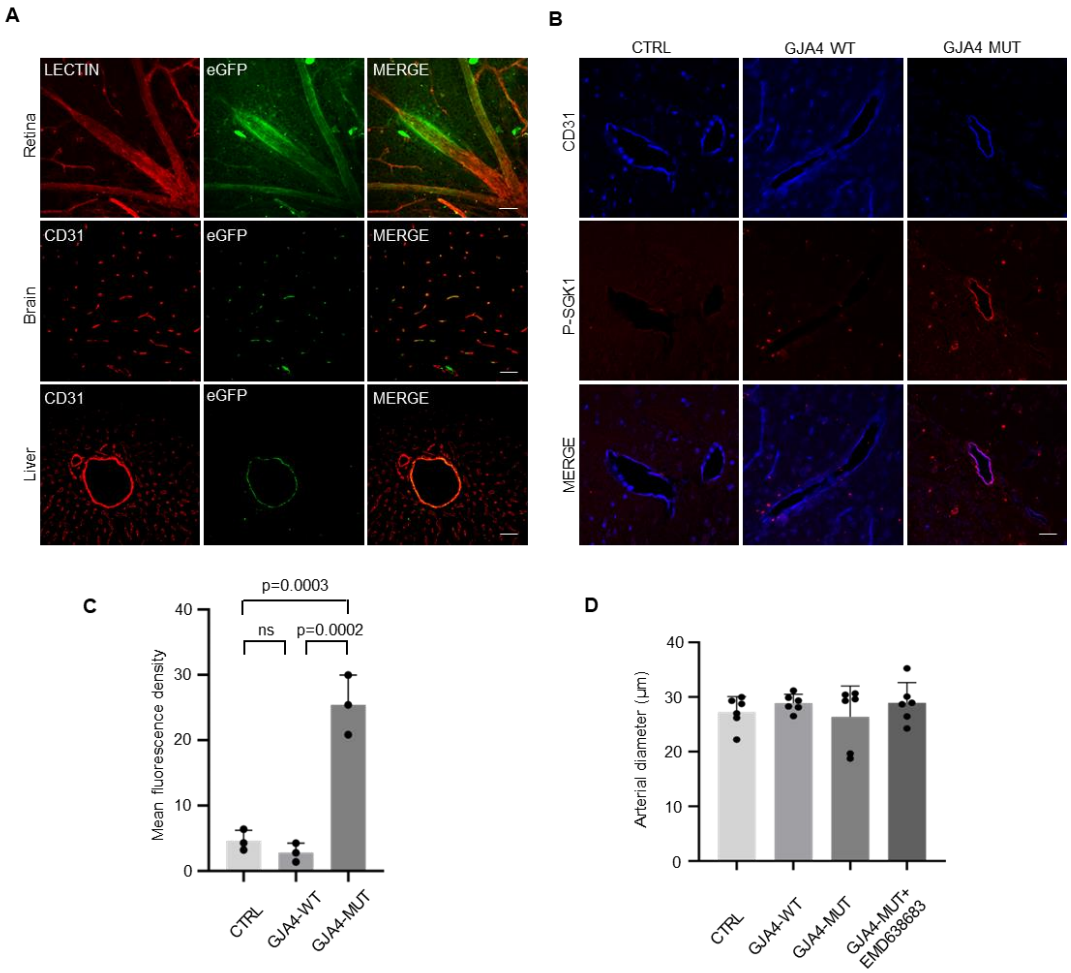

Figure S6. *GJA4* mutation regulates retinal angiogenesis in mice through SGK-1 signaling. A, Expression of eGFP (green) within endothelial cells (red) in the retina, brain, and liver of control mice at 3rd week post injection. Scale bar: retina 100μm; brain and liver 50μm. **B** and **C,** Immunostaining showed an increased mean fluorescence density of P-SGK1 in the brain endothelial of *GJA4*-Mutant mice compared to that observed in *GJA4*-WT and CTRL mice. n=3. Scale bar: 50μm. **D,** Quantitative assessment of venous diameter of vessels in the superficial plexuses in the retina of *GJA4*-mutant, *GJA4*-WT and CTRL mice. MUT+EMD638683 represents *GJA4*-mutant mice treated with EMD638683. n=6.

Supplemental Tables

Table S1: Clinical information of the 58 ECH patients

[Included as a separate Excel file]

Table S2: All somatic mutations identified in N55-N58

[Included as a separate Excel file]

Table S3: ddPCR detection results for *GJA4* c.121G>T

[Included as a separate Excel file]

References

1. Li H, Nam Y, Huo R, Fu W, Biaobin J, Zhou Q, Song D, Yang Y, Jiao Y, Weng J, Yan Z, Di L, Li J, Wang J, Xu H, Wang S, Zhao J, Wen Z, Jiguang W, Cao Y (2021) De Novo Germline and Somatic Variants Convergently Promote Endothelial-to-Mesenchymal Transition in Simplex Brain Arteriovenous Malformation. Circulation research. doi:10.1161/circresaha.121.319004

2. Li H, Durbin R (2010) Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics (Oxford, England) 26 (5):589-595. doi:10.1093/bioinformatics/btp698

3. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R (2009) The Sequence Alignment/Map format and SAMtools. Bioinformatics (Oxford, England) 25 (16):2078-2079. doi:10.1093/bioinformatics/btp352

4. Trifonov V, Pasqualucci L, Tiacci E, Falini B, Rabadan R (2013) SAVI: a statistical algorithm for variant frequency identification. BMC systems biology:S2. doi:10.1186/1752-0509-7-s2-s2

5. Weng J, Yang Y, Song D, Huo R, Li H, Chen Y, Nam Y, Zhou Q, Jiao Y, Fu W, Yan Z, Wang J, Xu H, Di L, Li J, Wang S, Zhao J, Wang J, Cao Y (2021) Somatic MAP3K3 mutation defines a subclass of cerebral cavernous malformation. American journal of human genetics 108 (5):942-950. doi:10.1016/j.ajhg.2021.04.005

6. Varadi K, Michelfelder S, Korff T, Hecker M, Trepel M, Katus HA, Kleinschmidt JA, Muller OJ (2012) Novel random peptide libraries displayed on AAV serotype 9 for selection of endothelial cell-directed gene transfer vectors. Gene Ther 19 (8):800-809. doi:10.1038/gt.2011.143

7. Sun S, Qin W, Tang X, Meng Y, Hu W, Zhang S, Qian M, Liu Z, Cao X, Pang Q, Zhao B, Wang Z, Zhou Z, Liu B (2020) Sirt7Vascular endothelium-targeted gene therapy rejuvenates blood vessels

and extends life span in a Hutchinson-Gilford progeria model. Science advances 6 (8):eaay5556. doi:10.1126/sciadv.aay5556

8. Yardeni T, Eckhaus M, Morris H, Huizing M, Hoogstraten-Miller S (2011) Retro-orbital injections in mice. Lab animal 40 (5):155-160. doi:10.1038/laban0511-155