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Loss of function mutations in *EPHB4* are responsible for vein of Galen aneurysmal malformation

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Vein of Galen aneurysmal malformation is a congenital anomaly of the cerebral vasculature representing 30% of all paediatric vascular malformations. We conducted whole exome sequencing in 19 unrelated patients presenting this malformation and subsequently screened candidate genes in a cohort of 32 additional patients using either targeted exome or Sanger sequencing. In a cohort of 51 patients, we found five affected individuals with heterozygous mutations in *EPHB4* including *de novo* frameshift (p.His191Alafs*32) or inherited deleterious splice or missense mutations predicted to be pathogenic by *in silico* tools. Knockdown of *ephb4* in zebrafish embryos leads to specific anomalies of dorsal cranial vessels including the dorsal longitudinal vein, which is the orthologue of the median prosencephalic vein and the embryonic precursor of the vein of Galen. This model allowed us to investigate *EPHB4* loss-of-function mutations in this disease by the ability to rescue the brain vascular defect in knockdown zebrafish co-injected with wild-type, but not truncated *EPHB4*, mimicking the p.His191Alafs mutation. Our data showed that in both species, loss of function mutations of *EPHB4* result in specific and similar brain vascular development anomalies. Recently, *EPHB4* germline mutations have been reported in non-immune hydrops fetalis and in cutaneous capillary malformation–arteriovenous malformation. Here, we show that *EPHB4* mutations are also responsible for vein of Galen aneurysmal malformation, indicating that heterozygous germline mutations of *EPHB4* result in a large clinical spectrum. The identification of *EPHB4* pathogenic mutations in patients presenting capillary malformation or vein of Galen aneurysmal malformation should lead to careful follow-up of pregnancy of carriers for early detection of anomaly of the cerebral vasculature in order to propose optimal neonatal care. Endovascular embolization indeed greatly improved the prognosis of patients.

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Introduction

Vein of Galen aneurysmal malformation is characterized by brain arteriovenous shunt, supplied by a variety of choroidal arterial feeding vessels that drain into the aberrantly persistent median prosencephalic vein, an embryonic precursor of the vein of Galen, and associated with abnormal deep venous drainage (Raybaud *et al.*, 1989; Alvarez *et al.*, 2007). The incidence of this condition is ~1 in 50 000 (Gabriel *et al.*, 2010) and it can be diagnosed during the prenatal stage with intrauterine ultrasound and MRI. Prenatal diagnosis is usually made during the third trimester, with colour Doppler ultrasonography. Foetal MRI is important to confirm vein of Galen aneurysmal malformation, to detect associated brain abnormalities and to rule out differential diagnoses including arachnoid, porencephalic or choroid plexus cysts, pineal tumours, choroid papilloma, intracerebral haematoma and dural sinus or pial arteriovenous malformations. The outcome is particularly poor when anomalies such as cerebral defects or signs of cardiac dysfunction are associated. This aneurysmal malformation can cause congestive heart failure and pulmonary hypertension in neonates, macrocrania, ventriculomegaly, seizures, mental retardation, haemorrhage or venous ischaemia. Endovascular embolization is the treatment of choice and greatly improved the prognosis. From a literature review (Khullar *et al.*, 2010), among 337 patients treated endovascularly between 2001 and 2010, 84.3% were found to have good or fair outcomes, and a 15.7% mortality rate was found, while 76.7% of untreated patients died.

Vein of Galen aneurysmal malformation is largely sporadic, affecting males and females. Several cases of cerebral arteriovenous shunts are known to be hereditary. Some are recognized as haemorrhagic hereditary telangiectasia or Osler–Weber–Rendu syndrome, a heterogeneous genetic autosomal dominant disorder associated with cerebral arteriovenous shunts and particularly with arteriovenous fistulae and caused by mutations in *ENG* (MIM: 187300), *GDF2* (MIM: 615506) or *ACVRL1* (MIM: 600376).

Affected individuals with neurofibromatosis type 1 caused by mutations in *NF1* (MIM: 162200) can also show arteriovenous fistulae after rupture of weak vessel walls. Among 140 affected individuals with the association capillary malformation-arteriovenous malformation (CM-AVM1, MIM: 608354), germline mutations in *RASA1* were identified in two individuals suffering from capillary malformation associated with vein of Galen aneurysmal malformation (Revenu *et al.*, 2008). The protein encoded by *RASA1*, p120RasGAP, is an inhibitor of RAS p21. Its critical role in vascular development has been established by the phenotype observed in *Rasa1* knockout mice (Henkemeyer *et al.*, 1995).

To gain further insight into the underlying cause of vein of Galen aneurysmal malformation, we took advantage of the added value of whole and targeted exome sequencing to study a cohort of 51 unrelated patients presenting this condition.

Materials and methods

Participants and study design

Fifty-one unrelated patients presenting vein of Galen aneurysmal malformation were included from 2015 through the National Reference Center for Paediatric Neurovascular Malformation of Bicêtre University Hospital. The diagnosis was established through MRI then digital subtracted angiography. Detailed neuroimaging features of the patients are presented in the ‘Results’ section.

The parents of all affected individuals provided written informed consent for genetic analysis of their children or fetuses and themselves in accordance with the ethical standards of our institutional review boards.

Procedures

Whole exome sequencing

Genomic DNA for each individual was extracted from blood or frozen tissue with the use of a QiAamp® DNA midi or mini kit, respectively (Qiagen). Whole exome sequencing was performed in 19 index cases using the Exome Capture Agilent

SureSelect XT V5 kit for library preparation and exome enrichment as previously described (Zhou *et al.*, 2012). Sequencing was performed on a Genome Analyzer Iix Illumina instrument in paired-end mode with a read length of 2×100 bp. The median coverage of the whole exome sequencing was 67. Reads were aligned to the human reference genome sequence (UCSC hg19, NCBI build 37.3) using BWA software (Li *et al.*, 2009a). Variants were selected using the SAMtools (Li *et al.*, 2009b) then annotated using Annovar software (Wang *et al.*, 2010). Variants in coding regions (including non-synonymous and nonsense mutations), intron-exon junctions or short coding insertions or deletions were selected when the minor allele frequency (MAF) was <0.003 . Non-synonymous mutations were selected when the prediction of pathogenicity gave a score ≥ 0.5 using PolyPhen-2 (Adzhubei *et al.*, 2010) or ≤ 0.05 with SIFT (Kumar *et al.*, 2009).

Predicted deleterious variants in genes shared by two or more affected individuals were selected as candidates. After variant selection of the best candidate, the coding regions of *EPHB4* were sequenced in a cohort of 32 additional unrelated affected individuals.

Targeted exome sequencing

Targeted exome sequencing of *EPHB4* was performed using the Agilent SureSelectXT Custom kit for library preparation and exome enrichment. Sequencing was performed on an Illumina MiSeq System using paired-end 150 bp reads and following Illumina's protocol using the MiSeq Reagent Micro Kit, v2. The average of the median coverage was 490. Variants were selected using the same filters as those used for whole exome sequencing data.

Sanger sequencing

All variants identified through whole or targeted exome sequencing were further validated by Sanger sequencing. PCR primer pairs were designed from genomic DNA to amplify and sequence each *EPHB4* exon (Supplementary Table 1). PCR amplification was carried out as previously described (Zhou *et al.*, 2012). PCR products were purified then sequenced using the forward or reverse primers (Eurofins Genomics). The obtained DNA sequences were compared with published sequences (BLAST, NCBI). Sanger sequencing was also performed to establish the genotype of each family member and to analyse the segregation of the variant within each family.

Real time PCR amplification

Real time PCR amplification was conducted using genomic DNA on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the SsoAdvancedTM Universal SYBR[®] Green Supermix (Bio-Rad). Genomic deletion was defined when the ratio of tested DNA to control DNA was ≤ 0.5 . Real time PCR amplification of each sample was performed in duplicate using primers within *EPHB4* exons 2, 5, 9, 10, 11, 12, 13, 16 and *ALB* (albumin) as internal control (Supplementary Table 1).

Reverse transcription-PCR amplification

Total RNAs were extracted from lymphoblastoid cell lines by using TRI Reagent LS method (Sigma). Four micrograms of

RNA was used to synthesize cDNA by using random primers following the manufacturer's manual (SuperScript[®] III reverse transcriptase, Invitrogen) in a final volume of 20 μ l. PCR amplification was carried out as previously described (Zhou *et al.*, 2012). RT-PCR products were separated by agarose gel electrophoresis and labelled with ethidium bromide. To determine the effect of mutations closed to intron-exon junctions, PCR amplification analysis from single strand cDNA was performed using primers flanking exons of *EPHB4* (Supplementary Table 1). As internal control for PCR amplification, *ACTB* (β -actin) cDNA was co-amplified (Supplementary Table 1). Sanger sequencing was performed from the RT-PCR products.

Zebrafish studies

Embryo care

Embryos were cared for according to standard protocols (Westerfield, 1995). All animal experiments were conducted with approved protocols at Inserm.

The transgenic line (*Tg[fli1:EGFP]y1:Tg[gata:dsRed]*) allowed *in vivo* simultaneous imaging of blood vessel architecture by visualizing endothelial cells (*fli1:EGFP*) and erythrocytes (*gata1:dsRed*) in the same animal during embryonic vascular development and was used for the knockdown experiments (Lawson *et al.*, 2002; Traver *et al.*, 2003). Embryos were collected by natural spawning, and raised in fish water with methylene blue at 28.5°C.

Knockdown of *ephb4* in transgenic zebrafish using antisense morpholino oligonucleotide

Antisense morpholino oligonucleotides (MO) *ephb4a* (splice, *ephb4a*-MO) and 5mis-*ephb4a* (splice, *ephb4a*-MisMO) were purchased from Gene Tools LLC as previously described (Kawasaki *et al.*, 2014 and Supplementary Table 1). MO (0.4 pmoles) were injected into 1–4 cell stage embryos using picospritzer injector (Nasevicius and Ekker, 2000). Knockdown efficiency for *ephb4a*-MO was previously reported by Kawasaki *et al.* (2014).

Ephb4 mRNA synthesis and microinjection

Human *EPHB4* cDNA (GenBank: BC052804.1), corresponding to full-length *EPHB4* mRNA (NM_004444.4) was purchased from Source BioScience, linearized and capped RNAs were *in vitro* transcribed using the mMESSAGE mMACHINE Kit (Ambion Inc.) following the manufacturer's instructions. A truncated form of cDNA was generated from BC052804.1 and cloned to express a truncated protein retaining the first 269 amino acids mimicking the frameshift deletion observed in affected individuals AA5614. *In vitro* transcription was performed as described above. Sixty picograms of each mRNA were co-injected with *ephb4a*-MO.

Zebrafish phenotype scoring

Two types of assays to score distinct vascular phenotypes of the dorsal cranial vessels were used at 3 days post-fertilization (dpf). To evaluate dorsal longitudinal vein anatomy, the phenotype was categorized into either normal (a single vein indistinguishable from wild-type controls) or abnormal (duplicated or triplicated vein). We also evaluated the mesencephalic vein anatomy: in wild-type controls, the two mesencephalic veins can be seen projecting rostrally from the dorsal

longitudinal vein and then diving ventrally in two branches. The morphological aspect of paired mesencephalic veins was evaluated as either normal or abnormal (absent or interrupted in one or both mesencephalic veins). Scoring for normal or abnormal embryos for either dorsal longitudinal veins or mesencephalic veins in wild-type, *ephb4a*-MO, *ephb4a*-misMO, embryos co-injected with *ephb4a*-MO with full-length or truncated *ephb4* mRNA was undertaken. This quantification was carried out blindly by two examiners. All assays were performed using transgenic embryos with endothelial cells labelled in green. A two-tailed Student's *t*-test was used for all statistical analyses. In each case, the *P*-value is provided.

Confocal image analysis

Image acquisition was performed using a Leica SP8 confocal microscope. Image analysis was performed offline using ImageJ.

Results

Neuroimaging features of patients

Digital subtracted angiography established vein of Galen aneurysmal malformation diagnosis based on the following criteria: arterial choroidal feeders of the arteriovenous shunt, aneurysmal dilatation of the so-called vein of Galen (actually its precursor, the median vein of the prosencephalon) and deep venous drainage (such as internal cerebral veins) not connected to the vein of Galen but to venous alternative pathways such as lateral mesencephalic, sub temporal veins or lateral sinus (Fig. 1; Raybaud *et al.*, 1989; Lasjaunias *et al.*, 2006). Other disease entities such as vein of Galen dilatations characterized by arteriovenous malformations draining into a dilated mature vein of Galen, which is connected to the internal cerebral veins, dural arteriovenous shunts draining into a dilated mature vein of Galen, or vein of Galen varix characterized by a dilated vein of Galen without arteriovenous shunt were not included.

Identification of *EPHB4* heterozygous mutations in patients

Whole exome sequencing was performed in 19 unrelated patients with vein of Galen aneurysmal malformation. Predicted deleterious variants in genes shared by two or more affected individuals were selected as candidates. Under the hypothesis of autosomal recessive inheritance, no candidate variants were identified. Under the hypothesis of autosomal dominant inheritance, three affected individuals were carrying deleterious mutations in *EPHB4*. In affected individual AA5614, a heterozygous 1 bp duplication in *EPHB4* (Genbank: NM_004444.4, c.570dupG) leading to frameshift and premature stop codon (p.His191Alafs*32, Fig. 2) was identified. Sanger sequencing of PCR products using primers flanking the mutation

(Supplementary Table 1) confirmed the mutation in the affected individual (Fig. 2). Analysis of both parents showed that the mutation had occurred *de novo* (data not shown, available on request). This variant was not annotated in the 1000 Genomes or ExAC Browser databases. In the affected individual, vein of Galen aneurysmal malformation was associated with a large cutaneous capillary malformation on the face (Supplementary Fig. 1).

In two unrelated affected individuals, heterozygous *EPHB4* mutations were located in intron 14 close to the junction with exon 14. In the affected individual AA5615, the mutation was located at the first nucleotide of intron 14 (c.2484+1G>T, Fig. 2), suggesting a potential impact on splicing. This variant was not annotated in the 1000 Genomes or ExAC Browser databases. Analysis of both parents showed that the mutation was inherited from the mother (data not shown). In the affected individual, vein of Galen aneurysmal malformation was associated with two cutaneous capillary malformations on the body (Supplementary Fig. 1). Clinical examination of the healthy mother revealed similar cutaneous malformation of the skin (data not shown) without any clinical history of neurological defect. RT-PCR products of *EPHB4* cDNA from lymphoblastoid cell RNA of the affected individual and his mother compared to control revealed, in addition to the wild-type, an abnormal RT-PCR product (Fig. 2). Sequencing of the RT-PCR products revealed that the smaller PCR product corresponded to truncated transcript leading to the lack of amino acids 814 to 829 (p.Met814_Val829del) establishing the deleterious effect of the c.2484+1G>T mutation (Fig. 2). In the affected individual Patient AA5616, a splice mutation similar to the previous one was identified (c.2484+2insT, Fig. 2). This mutation was inherited from the healthy father (data not shown). Clinical examination of the father was not available as well as cell lines for RNA analysis. This variant was not annotated in the 1000 Genomes or ExAC Browser databases. No germline mutation was identified in *RASA1*, *ENG*, *ACVRL1*, *GDF2* or *NF1* in our cohort of 19 affected individuals.

EPHB4 was therefore selected as high candidate for the disease-causing gene. Targeted exome sequencing or Sanger sequencing of the coding regions of *EPHB4* were performed on the DNA sample of a cohort of 32 additional unrelated affected individuals with vein of Galen aneurysmal malformation. This approach further revealed two unrelated affected individuals (Patients AA5717 and AA5718) carrying distinct heterozygous missense mutation in *EPHB4* (Fig. 2). These mutations (c.2609T>A: p.Val870Glu and c.319T>C: p.Cys107Arg) were not annotated in the 1000 Genomes or ExAC Browser databases and are predicted to be pathogenic with a high score (PolyPhen-2 score of 1 for both mutations). These heterozygous mutations were inherited from healthy mothers (data not shown). No neurological defect was reported in the mothers.

From either whole, targeted exome or Sanger sequencing data, when homozygosity was found in three successive intragenic *EPHB4* single nucleotide polymorphisms

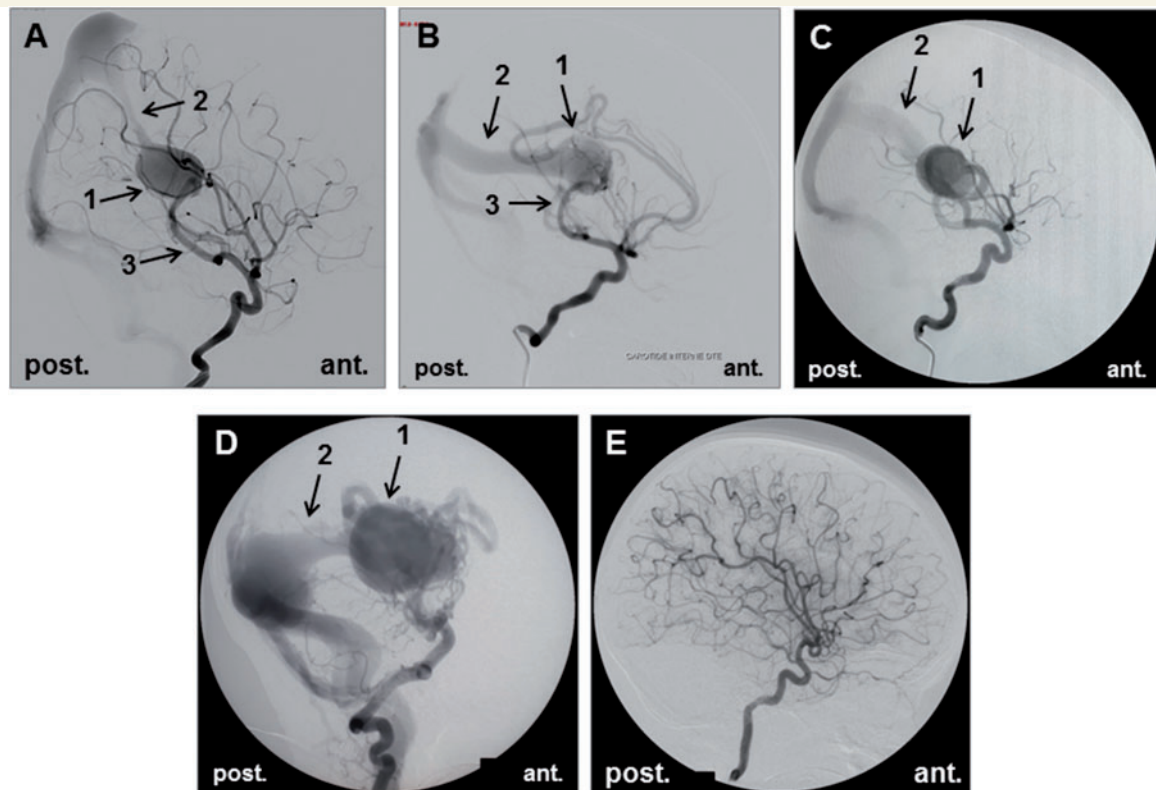


Figure 1 Digital subtracted angiogram of the affected individuals (A–D) and control (E). On the arterial phase, vein of Galen aneurysmal malformation diagnosis was established based on the observation of marked dilatation of vein of Galen (1) draining into a falx sinus (2), and arterial choroidal feeders of the arteriovenous shunt (3) in Patients AA5718 (A), AA5614 (B), AA5615 (C), and AA5616 (D) when compared to control (E). The angiogram was not performed in Foetus AA5717 as the pregnancy was terminated at the request of the parents.

having a minor allele frequency in the 1000 Genomes database between 0.2 to 0.6, a heterozygous deletion was suspected. Homozygous regions were found in 14 patients. Real time PCR amplification was performed on DNA from these patients using primers located in exons 2, 5, 9, 10, 11, 12, 13 and 16 of the *EPHB4* gene. No heterozygous deletion was observed. This approach also enabled one to exclude duplication of these exons.

The main clinical and genetic features of the five patients carrying *EPHB4* heterozygous mutations are summarized in Table 1.

Knockdown of *ephb4* in transgenic zebrafish embryos leads to marked anomalies of dorsal cranial vessels

Antisense morpholino (*ephb4a*-MO) oligonucleotides and their corresponding ‘mismatch’ controls (*ephb4a*-MisMO) were designed to specifically knockdown the *EPHB4* orthologue (*ephb4a*) in transgenic zebrafish (Tg[*fli1*:EGFP]y1; Tg[*gata1*:dsRed]). The embryos were analysed at 3 dpf when vascular morphogenesis is well established. Transgenic embryos injected with *ephb4a*-MisMO were indistinguishable from non-injected control embryos

(wild-type) ($n = 29$; Fig. 3). In contrast, by looking at different brain layers, transgenic zebrafish embryos injected with *ephb4a*-MO exhibited marked vascular anomalies of the dorsal cranial vessels including both dorsal longitudinal and mesencephalic veins by 3 dpf (Figs 3 and 4). Abnormal number of dorsal longitudinal vein (duplicated or triplicated vein) was observed in 82% of *ephb4a*-MO embryos (14 of 17 embryos) compared to 0% in either wild-type ($n = 21$) or *ephb4a*-MisMO ($n = 8$, $P < 0.0001$; Figs 3 and 4). Similarly, abnormal morphology of the mesencephalic vein (absent or interrupted in both or one mesencephalic vein) was observed in 76% of *ephb4a*-MO (13 of 17 embryos) when compared to 0% in either wild-type ($n = 21$) or *ephb4a*-MisMO ($n = 8$, $P < 0.0001$; Figs 3 and 4). Interestingly, the upper and lower middle layers of cranial vessels did not exhibit an abnormal vascular pattern (Supplementary Fig. 2) indicating that the anomalies were restricted to specific vessels of the brain. To determine whether the *ephb4a*-MO abnormal phenotype of dorsal cranial vessels was specific to *ephb4a* knockdown, we co-injected *ephb4a*-MO with full-length human *EPHB4* mRNA (wild-type mRNA). The percentage of vascular anomalies was significantly reduced for both mesencephalic vein (1 of 13 embryos showed an anomaly, 8%, $P < 0.0001$) and dorsal longitudinal vein (3 of 13 embryos

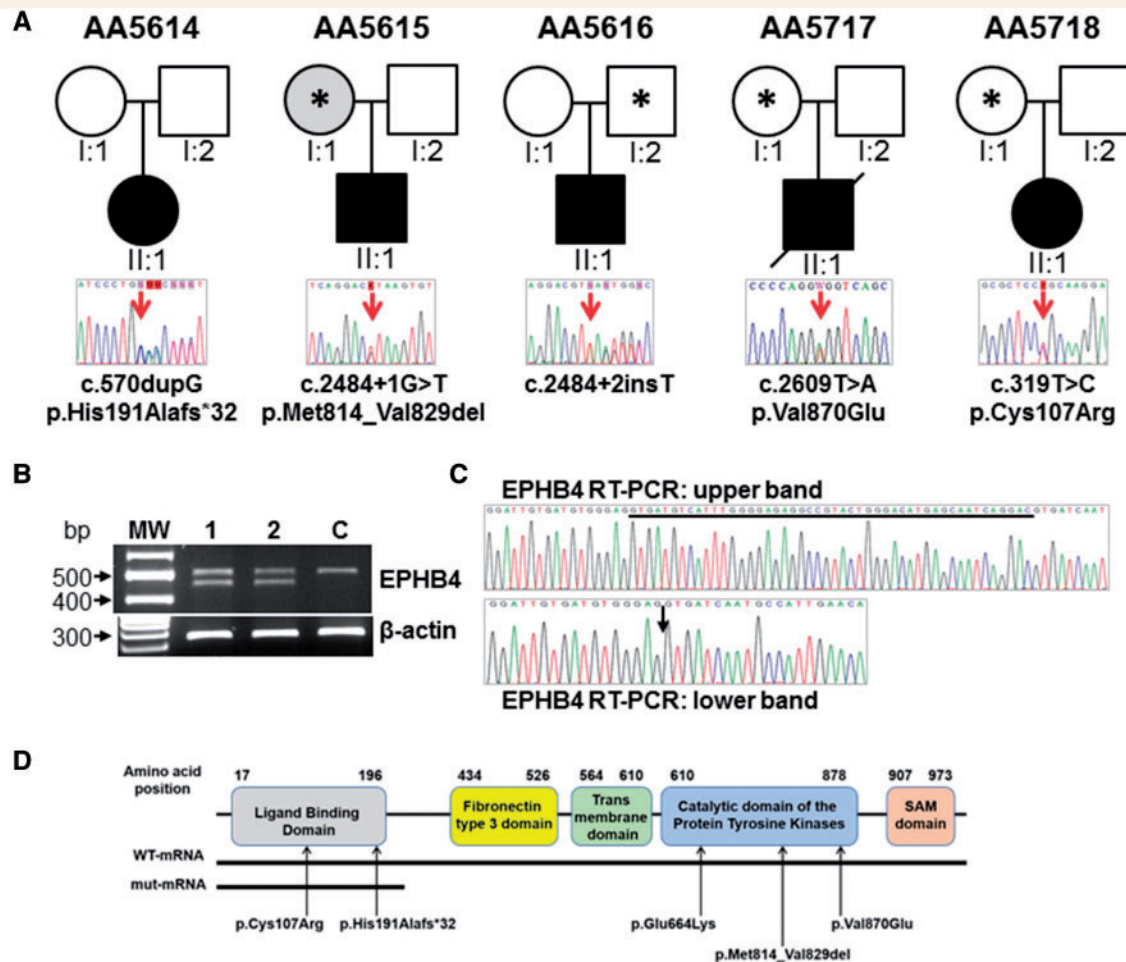


Figure 2 *EPHB4* mutations identified in patients presenting vein of Galen aneurysmal malformation. (A) Pedigrees for Families AA5614, AA5615, AA5616, AA5717 and AA5718 are shown. Arrows indicate mutant nucleotide positions. The affected individuals carry heterozygous mutation. The nucleotide and amino acid changes based on NM_004444.4 and NP_004435.3 reference sequences respectively are indicated. Open symbols: unaffected; filled symbols: affected. In Family AA5615, the carrier mother (in grey) displays cutaneous capillary malformation. Asterisk indicates that the parent is carrying the mutation. (B) RT-PCR analysis was performed from RNA after reverse transcription in affected individual Patient AA5615 (lane 1), his mother (lane 2) and a control (lane C) and revealed two bands on gel electrophoresis in lane 1 and 2 when compared to (C) and internal control (β -actin). MW = molecular weight marker. (C) Sanger sequencing was performed from the RT-PCR products using primers EPHB-Ex12-15-F and EPHB-Ex12-15-R (Supplementary Table 1). Sequence analysis revealed, in addition to the wild-type sequence (upper band), a truncated transcript (lower band, arrow) leading to the lack of amino acids 814 to 829 in both affected individual and his mother (underlined, p.Met814_Val829del). (D) Location of mutations found in the affected individuals from our cohort and in the family reported by Amyere et al. (2017; p.Glu664Lys). The amino acid sequences encoded by wild-type-mRNA or mut-mRNA used in zebrafish experiments are shown. SAM domain = sterile alpha motif domain.

showed an anomaly, 23%, $P = 0.0005$) in embryos co-injected with both *ephb4a*-MO and wild-type mRNA compared with those injected with *ephb4a*-MO only (Figs 3 and 4). In contrast, co-injection of *ephb4a*-MO with mut-mRNA, mimicking the mutation of the affected individual AA5614, did not result in statistically significant difference with *ephb4a*-MO injected embryos for both mesencephalic vein (15 of 20 showed anomalies, 75%, $P = 0.66$) and dorsal longitudinal vein (16 of 20 showed anomalies, 80%, $P = 0.86$; Figs 3 and 4). These data indicate that knockdown of *ephb4a* was specific. In addition, they strongly suggest that the *EPHB4* mutation found in affected individual AA5614 (c.570dupG) leads to a loss

of function since the *EPHB4* wild-type mRNA but not mut-mRNA was able to markedly improve the vascular phenotype of *ephb4a*-MO zebrafish. These data demonstrate that *EPHB4* plays an essential role in the development of specific brain vessels.

Discussion

We report herein the identification of germline loss-of-function *EPHB4* mutations in vein of Galen aneurysmal malformation. In a cohort of 51 unrelated affected individuals, we found five affected individuals with heterozygous

Table 1 Main clinical features and characteristics of *EPHB4* mutations found in patients with VGAM

Patient	AA5614	AA5615	AA5616	AA5717	AA5718
Clinical characteristics					
Age at diagnosis	Prenatal: 3rd trimester ultrasound	Prenatal: 3rd trimester ultrasound	Postnatal: 4 days of life (cardiac failure)	Prenatal: 3rd trimester ultrasound	Prenatal: 3rd trimester ultrasound
Prenatal MRI additional findings	None	Unknown	N/A	Hydrocephaly and leukomalacia ^a	Unknown
Head circumference at delivery (cm, percentile)	35, 90	38, 97	37, 90	37.5, 97	32, 20
Neonatal cardiac failure	No	No	Yes: stable with medical treatment	N/A	No
Age at first embolization (months)	2	6	4	N/A	3.5
Associated cutaneous capillary malformations	Yes	Yes	No	Unknown	No
Psychomotor development (age of evaluation)	Normal (3 years)	Normal (8 years)	Normal (10 years)	N/A	Normal (2 years)
<i>EPHB4</i> mutations					
cDNA change	c.570dupG	c.2484+1G>T	c.2484+2insT	c.2609T>A	c.319T>C
Inheritance	<i>De novo</i>	Mother	Father	Mother	Mother
Predicted protein change	p.His191Alafs*32	p.Met814_Val829del	Unknown	p.Val870Glu	p.Cys107Arg
Protein domain	Truncated protein	Catalytic domain of PTK	Catalytic domain of PTK	Catalytic domain of PTK	Ligand binding domain
PolyPhen-2 (score)	N/A	N/A	N/A	Probably damaging (1)	Probably damaging (1)
SIFT (score)	N/A	N/A	N/A	Deleterious (0.0)	Deleterious (0.0)
1000 Genomes MAF	Absence	Absence	Absence	Absence	Absence
ExAc Browser MAF	Absence	Absence	Absence	Absence	Absence

aa = amino acid; MAF = minor allele frequency; N/A = not applicable; PTK = protein tyrosine kinases.

^aTermination of pregnancy.

mutations in *EPHB4* including *de novo* frameshift or inherited deleterious splice or predicted pathogenic missense mutations, strongly suggesting that *EPHB4* mutations are responsible for this disease. Mutations occurred in various domains of *EPHB4* (Fig. 2). No germline mutation of *RASA1* was found in our cohort.

In recent years, the zebrafish has emerged as an important vertebrate model system for studying vascular development. The molecular mechanisms underlying vessel formation in zebrafish and its anatomical vascular architecture are highly similar to those in higher vertebrates (Isogai *et al.*, 2001). As a model system, the zebrafish offers unique advantages for studying vascular development *in vivo* (McKinney *et al.*, 2008; Isogai *et al.*, 2009). Although knockdown of *ephb4a*, the *EPHB4* orthologue, was already performed in zebrafish (Kawasaki *et al.*, 2014), previous study has only analysed spinal cord vascular development but not vascular development in the brain. Here, we show a critical role of *ephb4a* in the development of specific veins in the brain by the observation of marked venous anomalies of the dorsal cranial vessels including both dorsal longitudinal and mesencephalic veins in *ephb4a*-MO zebrafish larvae. Our data showed that *EPHB4* has a critical role in brain vascular development in both zebrafish and humans. This model allowed us to demonstrate that *EPHB4* loss-of-function is responsible for vein of Galen aneurysmal malformation since we were able to rescue the vascular brain defect of *ephb4a*-MO zebrafish

by overexpressing a human wild-type form of *EPHB4* but not truncated *EPHB4* mimicking the frameshift mutation found in Patient AA5614. In addition, our data strongly suggest that loss-of-function mutations in *EPHB4* result in specific brain vascular development anomalies in both species. Interestingly, comparative anatomy in vertebrates suggested that the dorsal longitudinal vein is the orthologue of the median prosencephalic vein of Markowski (Aurboonyawat *et al.*, 2007), which is aberrantly persistent in vein of Galen aneurysmal malformation and shows abnormal morphology in *ephb4a*-MO zebrafish.

Among the Ephrin family, the *EPHB4* receptor plays a unique role in vascular development, where it regulates venous differentiation (Adams *et al.*, 1999; Gerety *et al.*, 1999). EphB4 is highly enriched in veins, and is the only EphB receptor that specifically binds to EphrinB2 (Herbert *et al.*, 2009). During early stages of vertebrate embryogenesis, coordinated sorting and segregation of arterial/venous-fated angioblasts into distinct networks of arteries and veins is essential to establish a functional vasculature. The ligand EphrinB2 and its receptor EphB4, which are expressed in arterial and venous progenitors, respectively, interact to establish a directional progenitor migration. Here, we provide evidence for a critical role of *EPHB4* in embryo/foetal development of specific brain vessels in humans.

Recently, a functional *EPHB4*/ *RASA1*/mTORC1 signaling axis in endothelial cells has been proposed based on

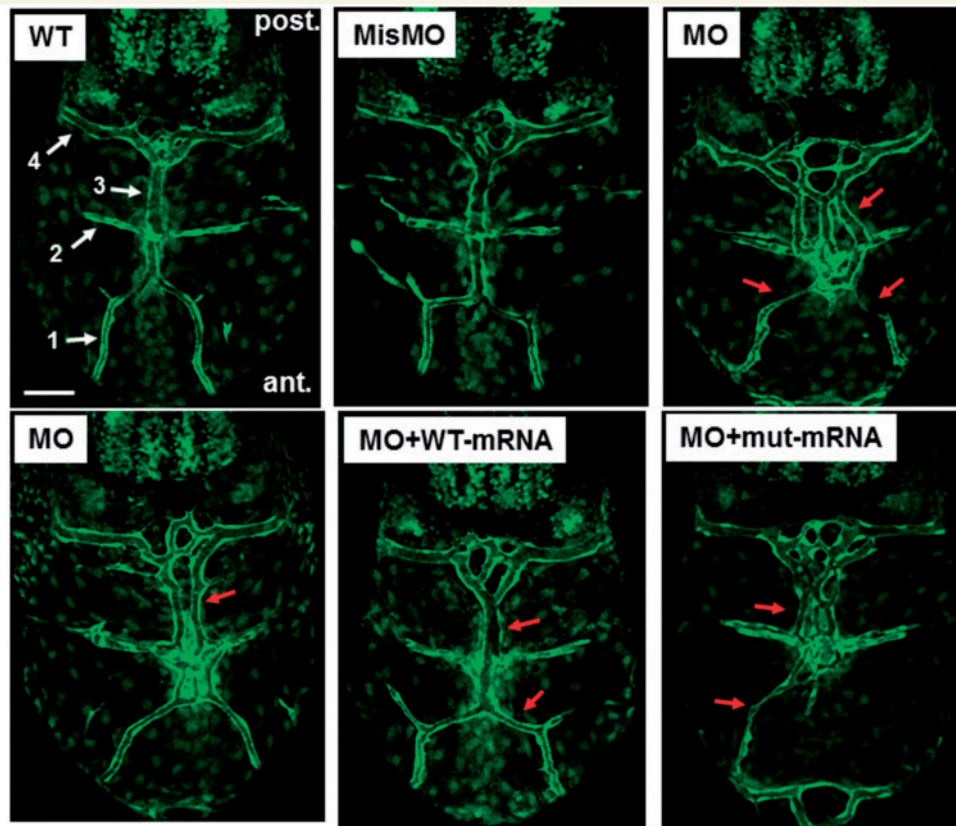


Figure 3 *Ephb4a* knockdown in transgenic zebrafish embryos leads to brain vascular defects. Morphological views of the most dorsal cranial vessels using the transgenic line *fli1:EGFP* in wild-type (WT) or embryos injected with either *ephb4a*-MO (MO) or 5-base mismatch control *ephb4a*-MO (MisMO) at 3 dpf. Anterior is to the bottom and posterior is to the top in all images. Note that *ephb4a*-MisMO phenotype was indistinguishable from wild-type. In contrast, transgenic zebrafish embryos injected with *ephb4a*-MO exhibited marked vascular anomalies of the dorsal longitudinal vein (DLV) and mesencephalic vein (MsV) veins at 3 dpf (red arrows). Abnormal number of dorsal longitudinal vein (duplicated or triplicated veins) was observed. Similarly, abnormal morphology of mesencephalic vein (absent or interrupted in one or both mesencephalic vein) was observed in *ephb4a*-MO. Co-injection of *ephb4a*-MO with full-length human *EPHB4* mRNA (WT-mRNA) can rescue the mesencephalic and dorsal longitudinal vein anomalies in most embryos. In contrast, when *ephb4a*-MO is co-injected with truncated *EPHB4* transcript (mut-mRNA), mesencephalic and dorsal longitudinal vein anomalies remain similar to those observed in *ephb4a*-MO. Scale bar = 60 μ m. 1 = mesencephalic vein; 2 = middle cerebral vein; 3 = dorsal longitudinal vein; 4 = posterior (caudal) cerebral vein (PCeV).

the observation that dysregulation of this pathway in endothelial cells promotes caudal vasculature malformation (Kawasaki *et al.*, 2014). mTORC1 inhibitors such as rapamycin could rescue or prevent the caudal vascular defects (Kawasaki *et al.*, 2014). This approach should be further explored as a new therapeutic research strategy in brain vascular malformations. Mutations of *EPHB4* have been reported in two distinct clinical disease entities. In two unrelated families with autosomal dominant lymphatic-related hydrops fetalis with atrial septal defect (MIM: 617300), Martin-Almedina *et al.* (2016) reported heterozygous missense variants in *EPHB4*. Among capillary malformation-arteriovenous malformation (CM-AVM2), inherited heterozygous mutations in *EPHB4* have been reported in 54 families with cutaneous capillary malformation either isolated (63%) or associated with arteriovenous malformation (35%, Amyere *et al.*, 2017). Among them, capillary malformation was associated with vein of Galen aneurysmal malformation in a single family. These results revealed

that heterozygous germline mutations of *EPHB4* result in a large clinical spectrum ranging from non-immune hydrops fetalis, capillary malformation without neurological expression to vein of Galen aneurysmal malformation. Interestingly, in two of five patients presenting vein of Galen aneurysmal malformation and carrying heterozygous *EPHB4* mutations, no cutaneous capillary malformation was observed. In addition, the clinical penetrance of the disease mutation is incomplete within the same family as shown in Family AA5615 where the affected child suffers from vein of Galen aneurysmal malformation with cutaneous capillary malformation while the carrier mother has cutaneous capillary malformation without neurological defect. These data strongly suggest a role of other genes linked or not to the *EPHB4* locus in the clinical expression of disease phenotype. This situation has been reported in other autosomal dominant diseases with incomplete penetrance including capillary malformation-arteriovenous malformation. Recently, a somatic inactivating *RASA1*

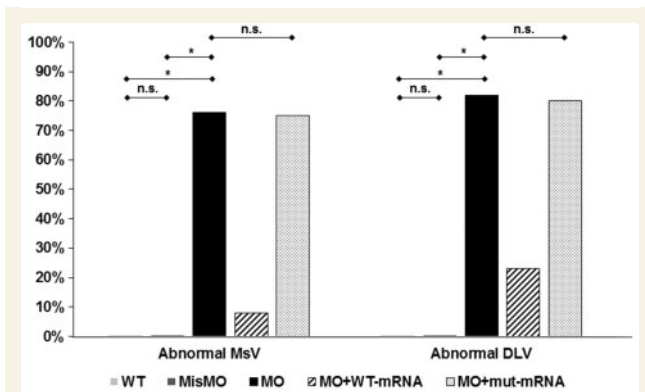


Figure 4 Graph indicating the percentage of brain venous anomalies in transgenic zebrafish in each condition.

Transgenic zebrafish embryos were non-injected (wild-type, WT, $n = 21$) or injected with *ephb4a*-MO (MO, $n = 17$), *ephb4a*-MisMO (MO, $n = 8$), or co-injected with *ephb4a*-MO and either full-length *EPHB4* mRNA (MO + WT-mRNA, $n = 13$) or truncated *ephb4* (MO + mut-mRNA, $n = 20$). Zebrafish embryos were mounted for microscopy and then scored for the number of abnormal dorsal longitudinal vein (abnormal DLV) or abnormal mesencephalic vein (abnormal MsV). * $P \leq 0.0005$; n.s. = not significant.

mutation was additionally identified in capillary malformation lesion tissue in a patient with germline *RASA1* mutation, providing the first evidence for a second hit model to explain disease development (Lapinski *et al.*, 2018). This genetic model might account for the incomplete penetrance of *EPHB4* mutation. Under this hypothesis, Sanger sequencing of *EPHB4* was performed in brain vascular malformation of the AA5717 affected foetus and did not reveal somatic mutation of *EPHB4* in addition to the germline identified mutation (c.2609T>A, data not shown, available on request) suggesting a role of another gene in the expression of the disease phenotype.

In summary, we show herein that 10% of vein of Galen aneurysmal malformations are associated with deleterious heterozygous mutations of *EPHB4*. A search for intragenic mutations of *EPHB4* should be proposed in patients presenting with this condition. It should be also proposed in affected individuals with cutaneous capillary malformation only. The identification of *EPHB4* pathogenic mutation should provide better information of families on the autosomal dominant mode of inheritance of the trait, the large clinical spectrum, and the need for careful follow-up of pregnancy of *EPHB4* mutation carriers for early detection of vein of Galen aneurysmal malformation in order to propose optimal care through endovascular embolization, which greatly improves the prognosis of patients.

Web resources

The URLs for data presented herein are as follows: BLAST, <http://blast.ncbi.nlm.nih.gov/Blast.cgi> dbSNP150, <http://www.ncbi.nlm.nih.gov/snp> 1000 Genomes, <http://www.1000genomes.org/>

Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org/>
 ExAC, <http://exac.broadinstitute.org/>
 ANNOVAR, <http://www.openbioinformatics.org/annovar/>
 PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>
 Sift, <http://sift.jcvi.org/>

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Supplementary material

Supplementary material is available at *Brain* online.

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