Rare Coding Variants in *ANGPTL6* Are Associated with Familial Forms of Intracranial Aneurysm

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Intracranial aneurysms (IAs) are acquired cerebrovascular abnormalities characterized by localized dilation and wall thinning in intracranial arteries, possibly leading to subarachnoid hemorrhage and severe outcome in case of rupture. Here, we identified one rare nonsense variant (c.1378A>T) in the last exon of *ANGPTL6* (Angiopoietin-Like 6)—which encodes a circulating pro-angiogenic factor mainly secreted from the liver—shared by the four tested affected members of a large pedigree with multiple IA-affected case subjects. We showed a 50% reduction of ANGPTL6 serum concentration in individuals heterozygous for the c.1378A>T allele (p.Lys460Ter) compared to relatives homozygous for the normal allele, probably due to the non-secretion of the truncated protein produced by the c.1378A>T transcripts. Sequencing *ANGPTL6* in a series of 94 additional index case subjects with familial IA identified three other rare coding variants in five case subjects. Overall, we detected a significant enrichment ($p = 0.023$) in rare coding variants within this gene among the 95 index case subjects with familial IA, compared to a reference population of 404 individuals with French ancestry. Among the 6 recruited families, 12 out of 13 (92%) individuals carrying IA also carry such variants in *ANGPTL6*, versus 15 out of 41 (37%) unaffected ones. We observed a higher rate of individuals with a history of high blood pressure among affected versus healthy individuals carrying *ANGPTL6* variants, suggesting that ANGPTL6 could trigger cerebrovascular lesions when combined with other risk factors such as hypertension. Altogether, our results indicate that rare coding variants in *ANGPTL6* are causally related to familial forms of IA.

Introduction

Intracranial aneurysms (IAs) are acquired cerebrovascular abnormalities affecting 3% of the general population, at a mean age of 50 years.[1] They are characterized by a localized dilation and wall thinning in typical locations in intracranial arteries.[2] The most notorious and deleterious complication of an IA is the rupture, resulting in subarachnoid hemorrhage that can lead to severe disability and death.[3] Neither reliable biomarkers nor diagnostic tools are currently available to predict IA formation or evolution. Current treatments are mostly invasive and rely on microsurgical or endovascular treatment with a risk of procedural morbidity or mortality.[4]

Although the pathogenesis of IAs has been the subject of several studies for many years, the mechanisms underlying their formation, growth, and eventual rupture remain largely unknown.[5] IAs are mostly acquired lesions resulting from a defective vascular wall response to local hemodynamic stress.[6] The structural deterioration of the arterial wall involves inflammation and tissue degeneration with degradation of the extracellular matrix and smooth muscle cell apoptosis.[7] Risk factors such as hypertension, female sex, increasing age, smoking, excessive alcohol consumption, and familial history of aneurysm predispose to IA formation and rupture.[8] Furthermore, increasing evidence suggests a genetic component of IA formation.[9] Genome-wide association studies and subsequent case-control replication analyses have identified common risk alleles for IA formation, in particular on chromosome 9 within the cyclin-dependent kinase inhibitor 2B antisense inhibitor gene, on chromosome 8 near the transcription regulator gene *SOX17*, and on chromosome 4 near the endothelin receptor A gene.[10] However, these loci altogether may only explain around 5% of familial risk in Europe and Japan.[11]

Whole-exome sequencing approaches have recently been applied to families with multiple IA-affected case subjects, leading to the identification of genes associated with IA pathogenesis susceptibility, such as *RNF213* (MIM: 613768)[12] or *THSD1* (MIM: 616821).[13] While the Ring Finger Protein 213 had been previously involved in vascular-wall construction,[14,15] inactivation of the Thrombospondin Type 1 Domain Containing Protein 1 has been reported to impair the adhesion of endothelial cells to the extracellular matrix and to cause cerebral bleeding and increased mortality in zebrafish and mice.[13] These recent advances provide new insights into the pathophysiology of IA and demonstrate the usefulness of familial approaches based on whole-exome sequencing to improve

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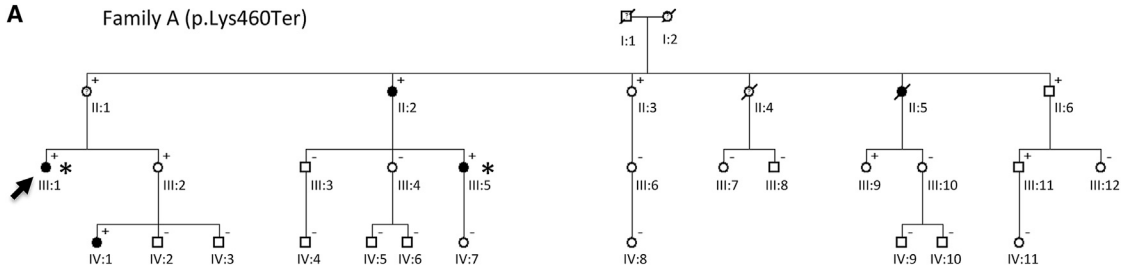

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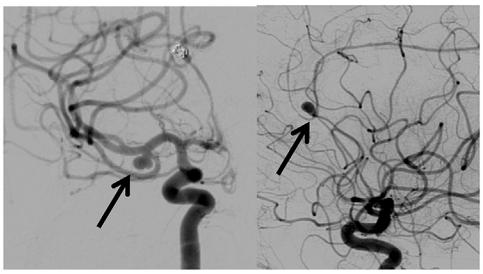

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Filtering steps	Remaining variants	
Individuals :	*III-1*	*III-5*
Functional variants	25,674	23,456
MAF < 0,1% in ExAC (NFE)	456	436
Shared by III.1 & III.5	29	
In IBD haplotypes	10	
Shared by all affected	8	
Predicted LOF	1 (*ANGPTL6*)	

Figure 1. Genetic Investigations in a Large Family with Multiple IA-Affected Case Subjects
(A) Pedigree of family A showing the segregation pattern of the variant *ANGPTL6* c.1378A>T. Filled boxes, empty boxes, and boxes with question marks indicate IA-affected case subjects, non-affected relatives, and individuals with unknown status; plus sign (+) indicates the presence of the *ANGPTL6* variant; minus sign (−) indicates its absence; arrow indicates the index case subject; and asterisks indicate the individuals included in WES analysis.
(B) Digital substracted angiographies showing two intracranial aneurysms carried by the index case III-1, one on the middle cerebral artery (left) and one on the anterior cerebral artery (right).
(C) Summary of the filtering steps applied to genetic variants detected by WES in individuals III-1 and III-5. Abbreviations: MAF, minor allele frequency; IBD, identity by descent; LOF, loss of function.

knowledge on the molecular mechanisms underlying IA formation and rupture.

In the present study, by combining whole-exome sequencing, identity-by-descent (IBD) analysis, gene burden testing, and functional investigations, we identified rare coding variants in the angiopoietin-like 6 gene (*ANGPTL6* [MIM: 609336]) as causally related to familial forms of IA.

Material and Methods

Clinical Recruitment

Familial cases of IA are defined as at least two first-degree relatives both diagnosed with typical IA (defined as a saccular arterial dilatation of any size occurring at a bifurcation of the intracranial vasculature), without any age limitation. Index case subjects and their relatives were recruited following the French ethical guidelines for genetic research and under approval from the French Ministry of Research (no. DC-2011-1399) and the local ethical committee. Informed written consent was obtained from each individual agreeing to participate in the genetic study, to whom MRI screening and blood sampling were proposed.

The full recruiting process has been described previously.[16] In brief, neuroradiological phenotyping was performed in each recruiting center by interventional neuroradiologists, neurologists, and neurosurgeons in order to recruit only case subjects with typical saccular bifurcation IA. Mycotic, fusiform-shaped, or dissecting IAs were systematically excluded, as well as IA in relation with an arteriovenous malformation and IA resulting from syndromic disorders such as Marfan disease or vascular forms of Elhers Danlos. Eye fundus, transthoracic echocardiography, non-invasive analysis of endothelial dysfunction, and Doppler echography analysis of peripheral arteries (sub clavians, radials, femorals, renals, and digestives) were carried out to check for any other vascular malformation or variation potentially linked to the presence of IA, thus constituting a syndrome yet unknown.

Whole-Exome Sequencing (WES)

Genomic DNA was extracted from peripheral blood lymphocytes using the NucleoSpin Blood kit XL (Macherey Nagel). In brief, coding exons from 3 μg of genomic DNA were captured using the SureSelect Human All Exon V4 Kit (Agilent Technologies), following the manufacturer's protocol. DNA was sheared by acoustic fragmentation (Bioruptor Diagenode) and purified with the magnetic beads Agencourt AMPure XP (Beckmann Coulter genomics), and fragment quality was assessed (TapeStation 2200 Agilent). Exome-enriched genomes were paired-end sequenced (100-bp reads) on Illumina HiSeq 1500 (Illumina) to a mean depth above 30×. Sequence reads were mapped to the human reference genome (Broad Institute human_g1k_v37) using the Burrows-Wheeler Aligner.[17] Duplicates were flagged using Picard software. Reads were realigned and recalibrated using the Genome Analysis Toolkit (GATK).[18] Variant detection was performed with GATK HaplotypeCaller. Functional annotation of high-quality variants was performed using Ensembl VEPv7.4. The sequencing quality was determined with the Depth Of Coverage Walker provided in GATK. Knime4Bio[19] was used for all merging and filtering steps. Variants with a sequencing depth of less than 10 or a genotype quality below 90 were excluded, as well as synonymous variants with no predicted effect on splicing sites. At last, from the resulting set of "functional" variants (as reported in Figure 1), we filtered

out any variant with a minor allele frequency (MAF) higher than 0.1% in the non-Finnish European (NFE) population from the ExAC database, as well as few remaining variants reported with a minor allele frequency (MAF) higher than 10% in our in-house database of 260 whole-exome sequences from individuals with various cardiac phenotypes.

Identity-by-Descent Analysis

SNP genotyping was performed on population-optimized Affymetrix Axiom Genome-Wide CEU 1 array plates following the standard manufacturer's protocol. Fluorescence intensities were quantified using the Affymetrix GeneTitan Multi-Channel Instrument, and primary analysis was conducted with Affymetrix Power Tools following the manufacturer's recommendations. After genotype calling, all individuals had a genotype call rate above 97%. SNPs with an MAF $< 10\%$, a call rate $< 95\%$, or $p < 1 \times 10^{-5}$ when testing for Hardy-Weinberg equilibrium were excluded. IBD estimation was performed with IBDLD v.3.34, NoLD method.[20] Shared regions were obtained by analyzing a set of independent SNPs ($R^2 < 0.2$) using genotypes from French individuals[21] as a reference panel. The IBD status at every SNP locus was obtained for each pair of individuals, based on a hidden Markov model implemented in the IBDLD program. Still using IBDLD, we estimated the kinship coefficients between pairs of IA-affected case subjects from distinct pedigrees. We invariably found values around 0.025, thus excluding non-documented close relatedness between individuals from distinct pedigrees carrying the same rare variant in *ANGPTL6*.

Capillary Sequencing and Burden Testing

Validation experiments for each selected variant, familial segregation analyses, and further screening for *ANGPTL6* coding mutations were performed by capillary sequencing on an Applied Biosystems 3730 DNA Analyzer, using standard procedures. Sequence analyses were performed with SeqScape v.2.5. *ANGPTL6* variants were numbered according to the canonical transcript (GenBank: NM_031917.2). Burden test was performed using SKAT[22] and CAST,[23] by comparing the proportion of individuals carrying at least one rare coding variant within *ANGPTL6* among the 95 index case subjects with IA versus 404 healthy individuals with French ancestry. *ANGPTL6* status in control individuals was determined by whole-genome sequencing with a mean depth of coverage above 30×. Rare variants were defined as variants with an MAF below 1% among the 7,509 whole-genome-sequenced individuals with NFE ancestry from the gnomAD database. The count of alleles with rare coding variants in *ANGPTL6* among case subjects was also compared with the same allele count among the 7,509 whole-genome sequenced individuals with NFE ancestry from gnomAD,[24] through the use of Fisher's exact test.

Expression Analyses of ANGPTL6

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Stable HEK293 cell lines were obtained by transfection of pcDNA3.1 vector encoding WT ANGPTL6 and p.Lys460Ter ANGPTL6 (G418 selection). Recombinant proteins are expressed as Nter-FLAG fusion proteins. In HEK293, recombinant human proteins were detected by both anti-FLAG (Sigma Aldrich, F-3165), anti-ANGPTL6 antibodies raised against AA 114-130 of ANGPTL6 (Adipogen, AG-25A-0030), and ELISA (kit supplied by Adipogen). In human subjects, serum ANGPTL6 levels were measured by ELISA. For transcript analysis, total RNA from stably transfected HEK293 cells was purified using Trizol (Life technology) according to the manufacturer's instructions and then reverse-transcribed. Real-time quantitative PCR was performed using the TaqMan 7900 Sequence Detection System (Applied Biosystems). Primers used to assess *ANGPTL6* mRNA expression were designed using the Primer Express 3.1 software (sequences available on request).

Results

A Nonsense Variant in ANGPTL6 Shared by Family Members with IA

The index case subject of family A (individual III-1; Figure 1A) was diagnosed after a subarachnoid hemorrhage (SAH) at the age of 51. This event revealed a ruptured anterior cerebral artery aneurysm and a second middle cerebral artery aneurysm (Figure 1B). She completely recovered from the subarachnoid hemorrhage and because of known familial history of ruptured IAs (II-2, II-5), a systematic screening was performed among relatives. Her cousin (III-5) and her niece (IV-1) were both diagnosed with two and one IAs, respectively. Her uncle (II-4) had an episode suggestive of aneurysmal SAH at the age of 36 and died before a CT scan or angiography could be performed. Her mother (II-1), who carries an ectasia measuring less than 2 mm and diagnosed as uncertain,[16] was classified as phenotype unknown.

Clinical information was collected for 28 individuals from family A (Table S1). IA was diagnosed on CT angiography or conventional angiography. DNA was available for 27 of them (DNA was unavailable for II-5 who died in 1974 after a rupture of IA). Individuals with IAs (II-2, II-5, III-1, III-5, and IV-1) were all female. Noteworthy, all IA-affected case subjects except IV-1 suffered from high blood pressure.

We combined WES and IBD analysis to identify any rare genetic variant likely explaining this familial form of IA. Whole-exome sequencing applied to the first cousins III-1 and III-5 led to the detection of 25,674 and 23,456 functional sequence variants, respectively, in comparison to the human reference genome assembly (Figure 1C). After filtering out genetic variants reported with an MAF above 0.1% in the non-Finnish European (NFE) population from the ExAC database,[24] we ended up with 29 rare variants shared between the first cousins, which were all manually reviewed by visual inspection of sequence reads using the Integrative Genomics Viewer.[25]

In parallel, IBD analysis of the complete pedigree identified 12 haplotypes shared by the 4 affected relatives. Within these chromosomal intervals, individuals III.1 and III.5 shared 10 rare, non-synonymous variants (Figure 1C). By capillary sequencing, we determined that the 4 affected relatives share 8 of these variants (Table 1), among which one nonsense variant, c.1378A>T (p.Lys460Ter), was in *ANGPTL6*.

Table 1. Rare Non-synonymous Variants Shared by All Affected Members in Family A, and Rare Non-synonymous Variants in *ANGPTL6* Detected in Other Families

Family	Genomic Position (GRCh37/hg19)	Gene	Nucleotide Change[a]	Protein Consequence[a]	MAF in ExAC (NFE)	GERP Score	Predicted Functional Impact		Carriers / Total	
							SIFT	PolyPhen-2	Affected	Unaffected
A	chr6:52701135C>A	*GSTA5*	c.171G>T	p.Met57Ile	0.00002	2.63	deleterious	benign	4/4	5/22
A	chr10:73046467G>A	*UNC5B*	c.574G>A	p.Asp192Asn	0.00001	5.43	deleterious	possibly_damaging	4/4	10/22
A	chr11:64756981G>A	*BATF2*	c.373C>T	p.Leu125Phe	0.00002	3.54	tolerated	possibly_damaging	4/4	6/22
A	chr11:65113459G>T	*DPF2*	c.122G>T	p.Gly41Val	0.00013	−7.09	tolerated	unknown	4/4	6/22
A	chr11:65746304C>T	*SART1*	c.2303C>T	p.Thr768Ile	0.00000	4.76	deleterious	probably_damaging	4/4	6/22
A	chr19:10610439C>T	*KEAP1*	c.271G>A	p.Ala91Thr	0.00013	4.68	tolerated	benign	4/4	5/22
A	chr19:14001265G>A	*C19orf57*	c.404C>T	p.Ala135Val	0.00025	−3.41	deleterious	benign	4/4	5/22
A	chr19:10203300T>A	*ANGPTL6*	c.1378A>T	p.Lys460Ter	0.00001	4.39	LOF		4/4	5/22
B + C	chr19:10206848T>A	*ANGPTL6*	c.392A>T	p.Glu131Val	not found	4.03	deleterious	benign	4/4	4/10
D	chr19:10204205G>A	*ANGPTL6*	c.1042C>T	p.Leu348Phe	not found	3.67	tolerated	probably_damaging	1/1	0/1
E + F	chr19:10206782G>GC+	*ANGPTL6*	c.439_457dup	p.Ala153ValfsTer66	0.00060	4.36	LOF		3/4	6/8

Abbreviations are as follows: GERP, Genomic Evolutionary Rate Profiling; MAF, minor allele frequency; LOF, loss of function; ExAC (NFE), Exome Aggregation Consortium (Non-Finnish Europeans); SIFT, Sorting Intolerant From Tolerant.

[a]The following reference sequences were used in naming mutations. They correspond to RefSeq transcripts, unless otherwise specified: GSTA5, NM_153699.1; UNC5B, NM_170744.4; BATF2, NM_001300807.1; DPF2, ENST00000531989 (from Ensembl; the variant is synonymous based on the RefSeq transcripts); SART1, NM_005146.4; KEAP1, NM_012289.3; C19orf57, NM_024323.3; ANGPTL6, NM_031917.2.

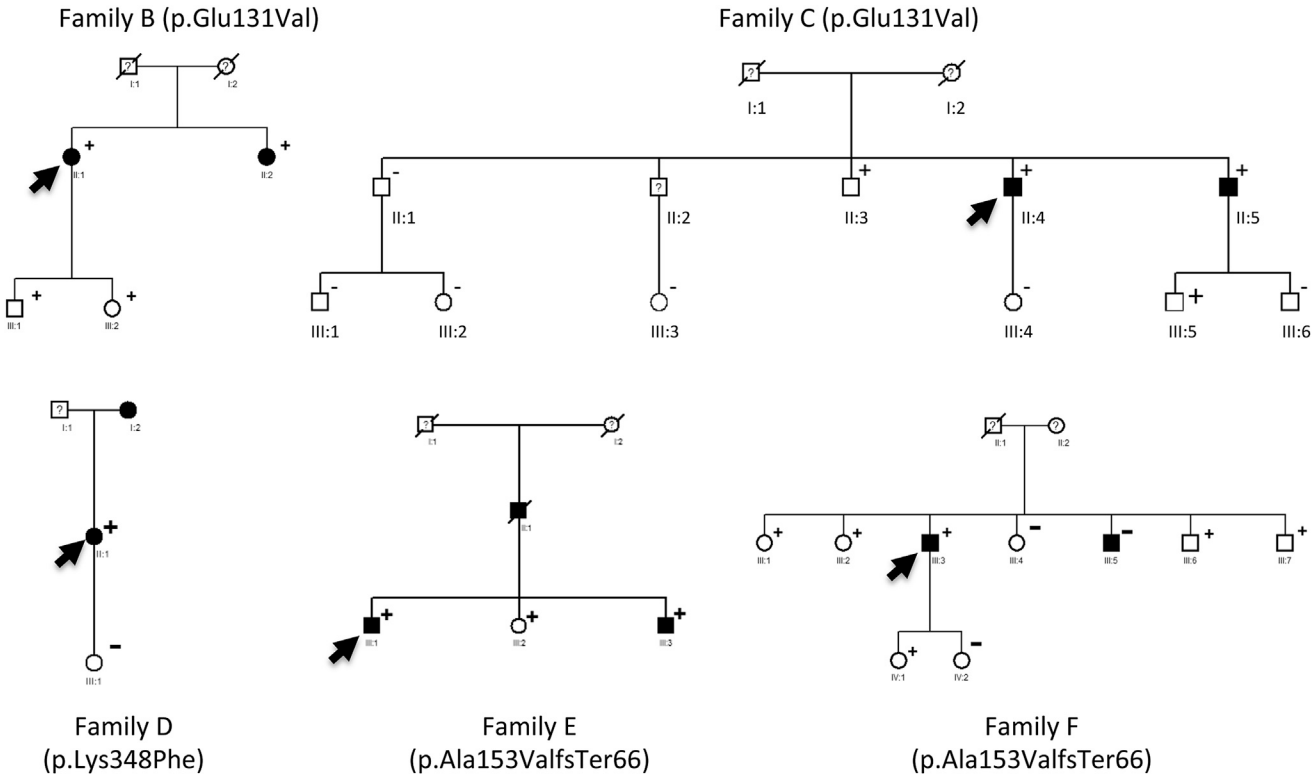

Figure 2. Familial Case Subjects of IA in the Presence of Rare Coding Variants in *ANGPTL6*
Filled boxes, empty boxes, and boxes with question marks indicate IA-affected case subjects, non-affected relatives, and individuals with unknown status; plus sign (+) indicates the presence of the *ANGPTL6* variant; minus sign (−) indicates its absence; and black arrows indicate the index case subjects.

Enrichment in Rare Coding Variants within ANGPTL6 Among IA-Affected Case Subjects

We then extended genetic screening on the coding portion of *ANGPTL6* to 94 additional index case subjects with familial IA. We identified 5 additional individuals carrying rare, non-synonymous variants in *ANGPTL6* predicted as damaging *in silico* by PolyPhen-2 and/or SIFT (Table 1): two case subjects with the same missense mutation in exon 1 leading to the c.392A>T substitution (p.Glu131Val) in *ANGPTL6*, one case subject with a missense mutation in exon 4 leading to the c.1042C>T substitution (p.Leu348Phe), and two case subjects with the same CGCGCTGAGCCTCGGCGGA-bp insertion leading to one premature stop codon in exon 2 (c.439_457dup [p.Ala153ValfsTer66]).

Overall, from the 6 index case subjects, family screening led to the identification of 16 relatives with diagnosed IAs (Tables 1 and S1; Figures 1A and 2). Out of the 13 family members carrying IAs and agreeing to participate in genetic research, 12 (92%) carry rare non-synonymous variants in *ANGPTL6* versus 15 out of 41 (34%) unaffected ones. The only affected individual who does not carry any rare coding variant in *ANGPTL6* is a 54-year-old male (III-5, family F, Figure 2) presenting with an aneurysm on the anterior communicant artery, with no reported history of smoking, high blood pressure, or any relevant associated disease.

The clinical characteristics of the remaining 12 case subjects are reported in Tables 2 and S1. Seven of them (58%) carry multiple IAs (with a maximum of three). IA is located on the middle cerebral artery bifurcation in 7 case subjects (58%), on the anterior communicant artery, the anterior cerebral artery, and the internal carotid artery in 3 case subjects (25%), and on the posterior communicant artery in 2 case subjects (17%).

To further test the association of *ANGPTL6* rare variants with susceptibility to familial IA, we also compared the proportions of individuals carrying at least one rare, non-synonymous variant across this gene among the 95 index case subjects enrolled in the present study (6/95; 6.32%) versus 404 healthy individuals with French ancestry (8/404; 1.98%). We found a significant enrichment in individuals carrying non-synonymous variants with an MAF below 1% in the NFE reference population, among IA-affected case subjects (SKAT, $p = 0.023$; see Tables S2 and S3). Similar results were found when comparing allele counts among the 95 index case subjects versus the 7,509 non-Finnish European individuals with whole-genome sequences available in the gnomAD database (Tables S2 and S3).

Reduced ANGPTL6 Secretion in the Presence of the c.1378A>T Variant

ANGPTL6 is one of the eight members of the secreted glycoprotein ANGPTL family, which share a common

Table 2. Clinical Characteristics and Exposition to Risk Factors for IA-Affected Case Subjects and Unaffected Relatives, According to *ANGPTL6* Status

	Healthy Individuals; No ANGPTL6 Variant (n = 16[a])	Healthy Individuals; ANGPTL6 Variant (n = 15)	IA Carriers; ANGPTL6 Variant (n = 12)
Median age	57	56	59
Female sex	56%	53%	58%
History of smoking	56% (8 p.y.[b])	73% (10 p.y.[b])	67% (31 p.y.[b])
High blood pressure	7%	9%[c]	50%[c]
Alcohol intake > 150 g/w	19%	13%	33%
Median BMI	22	24	24
Diabetes - dyslipidemia	19%	18%	33%

[a]Healthy individuals aged over 35 years, without ANGPTL6 variant
[b]p.y., median consumption in pack/years among smokers
[c]Significant difference between affected versus healthy individuals carrying ANGPTL6 variants; p = 0.013 (chi-square test)

structure consisting of an amino-terminal coiled-coil domain, a linker region, and a carboxy-terminal fibrinogen-like domain. The c.1378A>T *ANGPTL6* variant leads to the occurrence of a premature stop codon in the last exon. The corresponding transcript may thus escape the nonsense-mediated mRNA decay and is predicted to result in a protein truncated by the last 11 amino acids (p.Lys460Ter ANGPTL6). To analyze the functional properties of p.Lys460Ter ANGPTL6, we established stable cell lines expressing the wild-type (WT ANGPTL6) and mutant ANGPTL6 (p.Lys460Ter ANGPTL6). The two types of cell lines expressed similar levels of transcripts suggesting that c.1378A>T *ANGPTL6* variant mRNA was not degraded (Figure 3A). Western blot using anti-FLAG antibody showed that WT ANGPTL6 was secreted in the culture medium while p.Lys460Ter ANGPTL6 was nearly undetectable in the supernatant of cells transfected with the variant (Figure 3B). Quantification of ANGPTL6 concentration by ELISA confirmed the significant reduction of the secretion of p.Lys460Ter ANGPTL6 compared to WT ANGPTL6 (Figure 3B). In agreement with this defective secretion, immunofluorescence labeling and quantification in permeabilized cells clearly showed the retention of p.Lys460Ter ANGPTL6 in the cytoplasm (Figure 3C). Although we did not directly demonstrate that c.1378A>T *ANGPTL6* variant mRNA escaped nonsense-mediated mRNA decay, our data strongly suggest that the c.1378A>T *ANGPTL6* variant leads to effective expression of the truncated p.Lys460Ter ANGPTL6 protein, which is not secreted. Accordingly, heterozygous subjects for the c.1378A>T *ANGPTL6* variant are expected to present with decreased levels of circulating ANGPTL6. To assess this hypothesis, we performed ELISA to compare the serum concentration of ANGPTL6 in subjects from family A reported as homozygous for the WT *ANGPTL6* (n = 5) versus heterozygous for the c.1378A>T *ANGPTL6* (n = 7) and found a 50% reduction in the serum level of ANGPTL6 in heterozygous subjects (Figure 3D).

A similar analysis of the serum concentration of ANGPTL6 has been performed in subjects from family C. We found no reduction in the serum concentration of ANGPTL6 between heterozygous subjects for p.Glu131Val ANGPTL6 versus homozygous for the WT ANGPTL6 (Figure S1).

Discussion

In the present study, we identified one rare nonsense variant, c.1378A>T, in the last exon of angiopoietin-like 6 (*ANGPTL6*), which was carried by the four tested affected members of a large pedigree with multiple IA-affected case subjects. Because (1) the c.1378A>T nonsense variant is the only loss-of-function variant shared by all affected members in the family, (2) it is carried by only 5 out of the 22 unaffected relatives, and (3) ANGPTL6 is a functionally relevant candidate for playing a role in IA pathophysiology, we thereafter focused our investigations on this particular gene.

Indeed, members of the ANGPTL family have been reported as regulators of angiogenesis through their carboxyl terminus.[26] In human, ANGPTL6 has been identified as a circulating pro-angiogenic factor mainly secreted from the liver, which increases endothelial permeability and stimulates endothelial cell migration.[27,28] Although members of the ANGPTL family have also been described as regulators of glucose and lipoprotein metabolisms through their amino terminus,[13] a large-scale population study has shown that ANGPTL6 does not affect lipoprotein profile.[29]

Interestingly, it is known that genes associated with vascular malformations of the brain have demonstrated or plausible roles in angiogenesis and vascular remodelling.[30] The development of the vasculature involves both vasculogenesis during embryogenesis and angiogenesis. Vasculogenesis of the cerebral vasculature occurs outside the brain, followed by capillary sprouting from the perineural plexus to penetrate the neural tube. Growth of the cerebral vasculature then entirely results from angiogenesis that requires vascular endothelial cell proliferation and migration, then vascular stabilization, corresponding to the formation of capillary tubes by endothelial cells and the recruitment of pericytes, the smooth muscle cell precursors, to their walls. All these timely organized events are tightly controlled by angiogenic factors, and aberrant expression or function of these mediators, including ANGPTL6, may lead to abnormal cerebral artery wall formation.[31–33] For example, defective recruitment of pericytes leads to vessel dilation and microaneurysm.[31]

We then extended our genetic screen on *ANGPTL6* to 94 additional index case subjects with familial IA and identified additional rare missense or frameshift variants in this

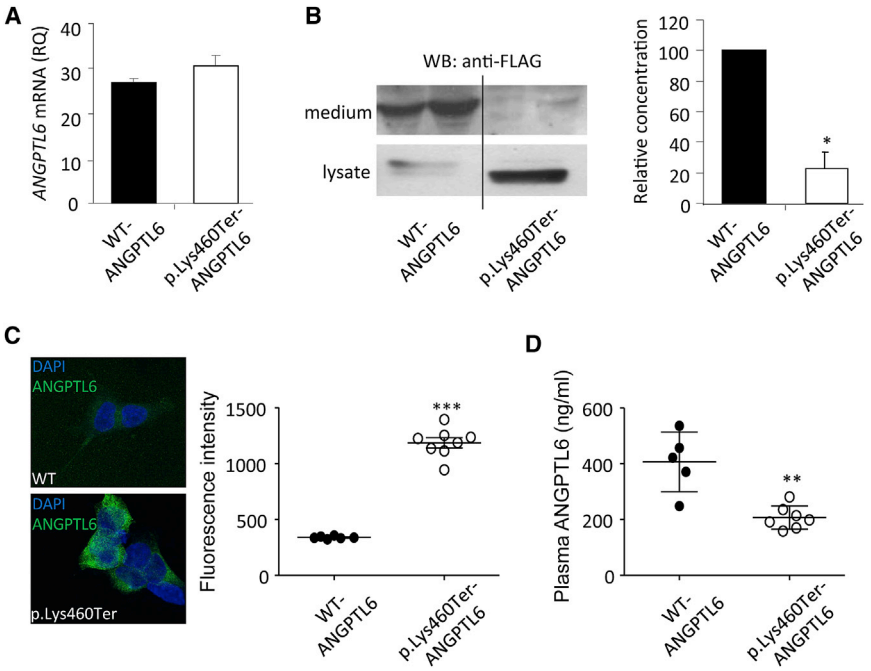

Figure 3. Analysis of WT and p.Lys460Ter ANGPTL6 in Cultured Cells and Individual Sera

(A) Analysis by qPCR of *ANGPTL6* transcripts in HEK293 cells encoding WT and p.Lys460Ter ANGPTL6.

(B) Analysis by western blot (anti-FLAG Ab) and ELISA of the levels of WT and p.Lys460Ter ANGPTL6 in culture media and lysates from stably transfected HEK293.

(C) Immunofluorescence labeling with anti-ANGPTL6 Ab and corresponding quantification in permeabilized HEK293 cells expressing WT and p.Lys460Ter ANGPTL6.

(D) Analysis of serum levels of ANGPTL6 in control subjects (WT ANGPTL6) and individuals harboring the p.Lys460Ter ANGPTL6 variant (heterozygous)

All results are expressed as means and error bars indicate standard deviation. Significant differences between independent groups were assessed by the Mann-Whitney test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

gene carried by index case subjects from 5 additional families (Figure 2 and Table 1). In total, among the 6 families, 12 out of 13 (92%) family members carrying IA also carry such variants in *ANGPTL6*, versus 15 out of 41 (37%) unaffected ones. The only affected individual who does not carry any rare coding variant in *ANGPTL6* (III-5, family F; Figure 2) presents with an IA in the anterior communicant artery, a location where IAs have been reported with probably weaker hereditability.[34,35]

By burden test, we showed a significant enrichment in rare non-synonymous variants within this gene in our population of 95 index case subjects with familial IA, compared to a reference population of 404 individuals with French ancestry. This result confirms that rare coding variants in *ANGPTL6* are associated with familial forms of IA.

In human, ANGPTL6 is described as a circulating protein mainly secreted from the liver and acting as an endocrine factor in the peripheral tissues.[36] Here we showed a 50% reduction of ANGPTL6 serum concentration in heterozygous c.1378A>T subjects compared to their relatives who do not carry the variant. Our results in stably transfected cell lines suggest that this reduction may be caused by the inability of the truncated protein lacking the last 11 amino acids (p.Lys460Ter ANGPTL6) to be secreted. In contrast, we showed that heterozygous subjects for the c.392A>T (p.Glu131Val) variant present with similar levels of circulating ANGPTL6 as subjects who do not carry the variant. This result does not exclude that ANGPTL6 may be associated with IA through dysfunction of the secreted form of the protein. We could not assess the levels of circulating ANGPTL6 in the remaining IA-affected case subjects and relatives, due to sample unavailability (Table S1).

In this study, we have observed some individuals (n = 15) carrying possibly deleterious variations in ANGPTL6 but without IA at the moment of the study. Since all affected individuals in our study have been subjected to at least one environmental risk factor, it is possible that familial rare variants in *ANGPTL6* require additional inauspicious factors to trigger IA development. A prominent and well-established environmental factor associated with the IA formation is the history of high blood pressure.[8] Indeed, we found in our study a significant difference in the rate of case subjects with a history of high blood pressure in affected versus healthy individuals heterozygous for *ANGPTL6* variants (Table 2). We hypothesize that *ANGPTL6* variants render cerebral arterial wall vulnerable to deformation, thus promoting lesion formation and/or progression, when challenged with other deleterious genetic or environmental factors such as high blood pressure, either through direct mechanical effects on vessel walls or by triggering inflammation. In the present study, we have identified rare coding variants in *ANGPTL6* causally related to IA in 6 families with multiple affected relatives. Our results point to a novel pathogenic pathway for a disease whose etiology is still poorly understood. Currently no molecular test has been established to help the diagnosis of IA. Developing biomarkers could undoubtedly facilitate early detection and risk assessment of IA. In this context, correlating the activity level of ANGPTL6 with IA risk could be of great interest. A knock-in mouse model is currently under development in order to address these prominent questions about *ANGPTL6* and IA susceptibility. Discovering genetic risk factors and better understanding the molecular mechanisms responsible for IA formation will be prerequisites for the identification of new therapeutic targets.

Supplemental Data

Supplemental Data include one figure and three tables and can be found with this article online at https://doi.org/10.1016/j.ajhg.2017.12.006.

Consortia

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Web Resources

Ensembl Genome Browser, human genome, http://useast.ensembl.org/Homo_sapiens/Info/Index
ExAC Browser, http://exac.broadinstitute.org/
GenBank, https://www.ncbi.nlm.nih.gov/genbank/
gnomAD Browser, http://gnomad.broadinstitute.org/
OMIM, http://www.omim.org/
Picard, http://broadinstitute.github.io/picard/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq
SIFT, http://sift.bii.a-star.edu.sg/
Variant Effect Predictor, http://useast.ensembl.org/Homo_sapiens/Tools/VEP

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Supplemental Data

Rare Coding Variants in *ANGPTL6* Are Associated with Familial Forms of Intracranial Aneurysm

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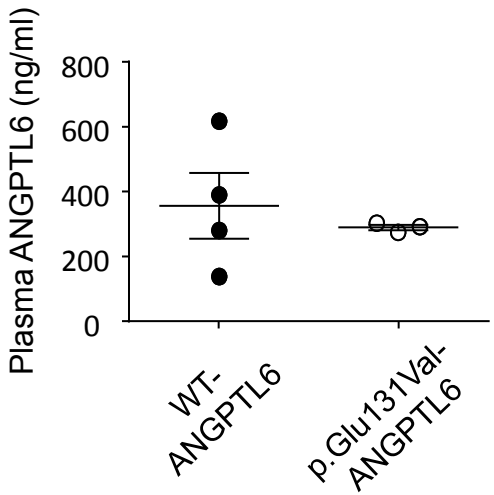
Supplemental Figure 1

Analysis of serum levels of ANGPTL6 in controls (WT-ANGPTL6) and individuals expressing the p.Glu131Val-ANGPTL6 (heterozygous).



Supplemental Table 2: Association tests on rare coding variants in *ANGPTL6*

Statistical test	Number of carriers		Allele count		p-value	OR	CI 95%
	IA cases	Internal controls	IA cases	gnomAD NFE individuals			
SKAT	6/95	8/404	-	-	0.0233	-	-
CAST	6/95	8/404	-	-	0.0331	3.326	[0.927-11.242]
FISHER	-	-	6/190	169/15018	0.0227	2.865	[1.023-6.485]

Supplemental Table 3: Full list of rare coding variants* detected in *ANGPTL6* among IA cases and reference individuals

GRCh37/ hg19 position	rsID	REF allele	ALT allele	Protein Consequence (ENSP00000253109)	Transcript Consequence (ENST00000253109)	Annotation	Allele Count in cases (n=95)	Allele Count in internal controls (n=404)	Allele Count (gnomAD NFE)	MAF (gnomAD NFE)
19:10203293	.	G	C	p.Ala462Gly	c.1385C>G	missense	0	0	1	6.68E-05
19:10203300	rs769022609	T	A	p.Lys460Ter	c.1378A>T	stop gained	1	0	0	-
19:10203324	rs200825170	G	A	p.Arg452Cys	c.1354C>T	missense	0	0	1	6.68E-05
19:10203363	rs201518909	G	A	p.Arg439Ter	c.1315C>T	stop gained	0	0	2	1.33E-04
19:10203375	rs150235799	C	T	p.Gly435Ser	c.1303G>A	missense	0	0	1	6.67E-05
19:10203390	rs769478685	C	T	p.Gly430Ser	c.1288G>A	missense	0	0	1	6.67E-05
19:10203416	rs548381762	T	C	p.His421Arg	c.1262A>G	missense	0	0	1	6.69E-05
19:10203432	rs146282548	C	G	p.Gly416Arg	c.1246G>C	missense	0	1	9	6.02E-04
19:10203434	rs372960335	C	T	p.Arg415Gln	c.1244G>A	missense	0	1	1	6.69E-05
19:10203435	rs377498859	G	A	p.Arg415Trp	c.1243C>T	missense	0	0	1	6.69E-05
19:10203444	.	G	C	p.Leu412Val	c.1234C>G	missense	0	0	2	1.34E-04
19:10204027	rs776568403	G	T	p.Ser407Tyr	c.1220C>A	missense	0	0	1	6.67E-05
19:10204052	rs568396237	C	T	p.Val399Met	c.1195G>A	missense	0	0	1	6.67E-05
19:10204083	.	AAGAG	A	p.Ser387PhefsTer81	c.1160_1163delCTCT	frameshift	0	0	1	6.67E-05
19:10204132	.	T	C	p.His372Arg	c.1115A>G	missense	0	0	1	6.67E-05
19:10204137	rs761235613	G	T	p.Ser370Arg	c.1110C>A	missense	0	0	1	6.67E-05
19:10204142	rs754265148	C	T	p.Glu369Lys	c.1105G>A	missense	0	0	1	6.67E-05
19:10204187	rs779222736	C	G	p.Gly354Arg	c.1060G>C	missense	0	0	3	2.00E-04
19:10204205		G	A	p.Leu348Phe	c.1042C>T	missense	1	0	0	-
19:10204215	rs769287477	C	G	p.Glu344Asp	c.1032G>C	missense	0	1	0	-
19:10204229	rs138652863	G	A	p.Arg340Cys	c.1018C>T	missense	0	0	3	2.00E-04
19:10204239	.	C	G	p.Gln336His	c.1008G>C	missense	0	0	1	6.67E-05
19:10204377	.	G	C	p.His315Asp	c.943C>G	missense	0	0	1	6.66E-05
19:10204419	rs748744561	G	A	p.Arg301Trp	c.901C>T	missense	0	0	2	1.33E-04
19:10204440	rs201494217	C	T	p.Gly294Arg	c.880G>A	missense	0	0	1	6.66E-05
19:10204464	rs774043154	A	G	p.Trp286Arg	c.856T>C	missense	0	0	2	1.33E-04
19:10204469	rs201622589	G	C	p.Ser284Ter	c.851C>G	stop gained	0	0	24	1.60E-03
19:10204476	rs760163416	C	T	p.Val282Ile	c.844G>A	missense	0	0	1	6.66E-05
19:10204510	rs778164883	C	A	p.Gln270His	c.810G>T	missense	0	0	1	6.67E-05
19:10204512	rs372998779	G	C	p.Gln270Glu	c.808C>G	missense	0	0	3	2.00E-04
19:10204529	rs150117768	C	A	p.Arg264Leu	c.791G>T	missense	0	0	1	6.67E-05
19:10204530	rs182345321	G	A	p.Arg264Cys	c.790C>T	missense	0	0	5	3.33E-04
19:10205491	.	C	T	p.Glu236Lys	c.706G>A	missense	0	0	2	1.33E-04
19:10205553	rs138519545	T	C	p.Asp215Gly	c.644A>G	missense	0	0	1	6.66E-05
19:10205607	rs145558307	G	A	p.Pro197Leu	c.590C>T	missense	0	0	1	6.67E-05
19:10206662	rs200836122	T	C	p.Gln193Arg	c.578A>G	missense	0	0	13	8.68E-04
19:10206680	rs771739756	C	G	p.Gly187Ala	c.560G>C	missense	0	0	1	6.72E-05
19:10206686	.	C	A	p.Cys185Phe	c.554G>T	missense	0	0	1	6.68E-05
19:10206729		C	G	p.Val171Leu	c.511G>C	missense	0	1	0	-
19:10206761	rs771917209	A	C	p.Leu160Arg	c.479T>G	missense	0	0	1	6.70E-05
19:10206763	rs369847598	C	A	p.Gln159His	c.477G>T	missense	0	0	5	3.35E-04
19:10206774	rs770895499	G	A	p.Arg156Trp	c.466C>T	missense	0	0	1	6.72E-05
19:10206780	rs199549770	C	T	p.Ala154Thr	c.460G>A	missense	0	0	1	6.72E-05
19:10206782	rs770263825	G	GCGCGCTGAG CCTCGGCGGA	p.Ala153ValfsTer66	c.439_457dupTCCGCC GAGGCTCAGCGCG	frameshift	2	0	1	6.72E-05
19:10206787	rs776060868	C	CTGAGCCTCGG CGGACGCGT	p.Ala153ValfsTer66	c.434_452dupACGCGT CCGCCGAGGCTCA	frameshift	0	0	2	1.34E-04
19:10206801	rs779899913	A	G	p.Ser147Pro	c.439T>C	missense	0	0	1	6.76E-05
19:10206824	rs554909694	A	G	p.Leu139Pro	c.416T>C	missense	0	0	1	6.82E-05
19:10206824	rs554909694	A	T	p.Leu139His	c.416T>A	missense	0	0	1	6.82E-05

19:10206848	rs576667683	T	A	p.Glu131Val	c.392A>T	missense	2	2	39	2.67E-03
19:10206854	.	C	G	p.Gly129Ala	c.386G>C	missense	0	1	0	-
19:10206875	.	G	A	p.Ala122Val	c.365C>T	missense	0	0	1	6.92E-05
19:10206896	.	T	C	p.Gln115Arg	c.344A>G	missense	0	0	1	6.94E-05
19:10206915	.	G	A	p.Gln109Ter	c.325C>T	stop gained	0	0	1	7.00E-05
19:10206993	.	C	A	p.Ala83Ser	c.247G>T	missense	0	0	1	6.82E-05
19:10206995	.	A	T	p.Leu82Gln	c.245T>A	missense	0	0	1	6.81E-05
19:10206997	.	C	G	p.Arg81Ser	c.243G>C	missense	0	1	3	2.03E-04
19:10207001	.	T	C	p.Gln80Arg	c.239A>G	missense	0	0	1	6.77E-05
19:10207020	.	C	G	p.Glu74Gln	c.220G>C	missense	0	0	1	6.73E-05
19:10207035	.	C	T	p.Val69Ile	c.205G>A	missense	0	0	3	2.05E-04
19:10207035	.	C	A	p.Val69Phe	c.205G>T	missense	0	0	1	6.82E-05
19:10207053	.	C	G	p.Ala63Pro	c.187G>C	missense	0	0	2	1.35E-04
19:10207059	.	C	T	p.Glu61Lys	c.181G>A	missense	0	0	1	6.76E-05
19:10207076	rs560311003	T	G	p.Glu55Ala	c.164A>C	missense	0	0	1	6.77E-05
19:10207083	rs746538090	T	A	p.Thr53Ser	c.157A>T	missense	0	0	1	6.76E-05
19:10207103	rs560892001	C	T	p.Gly46Asp	c.137G>A	missense	0	0	2	1.34E-04
19:10207104	.	C	T	p.Gly46Ser	c.136G>A	missense	0	0	1	6.73E-05
19:10207225	rs751565560	C	T	p.Trp5Ter	c.15G>A	stop gained	0	0	1	6.69E-05

[*] *Rare variants were defined as having an MAF below 1% among the whole-genome sequenced individuals with European Non-Finnish ancestry available in gnomAD*