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Exome Sequencing Reveals a Novel Variant in *NFX1* Causing Intracranial Aneurysm in a Chinese Family

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Abstract

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The authors declare that they have no competing interests.

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All exome data in this study are available upon request to the corresponding author.

Background: Genetic risk factors play an important role in the pathogenesis of familial intracranial aneurysm (FIA), however, the molecular mechanisms remain largely unknown. We investigate potential FIA-causing genetic variants by rare variant interrogation and a family-based genomics approach in a large family with an extensive multigenerational pedigree with FIA.

Method: Exome sequencing (ES) was performed in a likely dominant family with IA disease. Variants were analyzed by an in-house developed pipeline and prioritized using various filtering strategies, including population frequency, variant type, and predicted variant pathogenicity. Sanger sequencing was also performed to evaluate the segregation of the variants with the phenotype.

Results: Based on the ES data obtained from five individuals from a family with 7/21 living members affected with IA, a total of 14 variants were prioritized as candidate variants. Familial segregation analysis revealed that *NFX1* c.2519T>C (p.Leu840Pro) segregated in accordance with Mendelian expectations with the phenotype within the family; i.e. all IA-affected cases and absent from all unaffected members of the second-generation. This missense variant is absent from public databases (1000genome, ExAC, gnomAD, ESP5400), and has damaging predictions by bioinformatics tools (Gerp++ score =5.88, CADD score =16.43, MutationTaster score =1, LRT score =0). In addition, 840Leu in *NFX1* is robustly conserved in mammals and maps in a region before the RING-type zinc finger domain.

Conclusion: *NFX1* c.2519T>C (p.Leu840Pro) likely contributes to the pathogenesis of FIA.

Keywords

familial intracranial aneurysm; exome sequencing; genetics; *NFX1*

Introduction

Intracranial aneurysm (IA [MIM: 105800]) is a complex disorder characterized by dilation or ballooning of the cerebral artery. IA affects 3.2% of the general population, with a mean age-of-onset of 50 years [1]. IAs are generally asymptomatic, but the rupture of an IA may result in life-threatening subarachnoid hemorrhage (SAH, incidence rate in IA cases=0.7% [2]), which could lead to death in half of the patients within 1 month [3]. The pathogenesis of IA remains enigmatic. The wall of IAs is often characterized by lack of elastic laminas, leading to the collagen fibers being exposed to more mechanical load [4,5]. Besides the known risk factors (hypertension, cigarette smoking, and alcohol consumption [6,7]), mounting evidence suggests that genetic risk factors play an important role in the pathogenesis of IAs; IA may be considered as a complex trait and understanding potential gene X environmental (G X E) interactions might possibly elucidate modifiable risk. It is known that first degree relatives of patients with the disorder have up to seven times greater risk than the general population, and about 10% of patients with aneurysmal SAH have first or second-degree relatives with unruptured intracranial aneurysms [8]. Korja et al. have reported that the estimated heritability for aneurysmal SAH is 41% in the Nordic Twin Cohort, suggesting that there is a moderate role for genetic factors in the etiology of SAH [9].

Of note, IAs are observed in a subset of families with dominant polycystic kidney disease (ADPKD) due to pathogenic variants in *PKD1/2*, Ehlers-Danlos syndrome IV caused by

mutations in collagen type 3, Loeys–Dietz syndrome associated with variants in the transforming growth factor beta (TGFβ) signaling pathway genes, most frequently *TGFBR1* and *TGFBR2*, and Marfan syndrome caused by pathogenic variants in *FBN1* [10]. These latter Mendelizing disease traits are all syndromic examples with IA as an associated endophenotype, but clearly support an underlying genetic etiology for the pathobiology of IAs.

Both strategies of common and rare variant identification have been used in detecting disease-associated or disease-causing genetic factors potentially contributing to IAs. Genome-wide association studies (GWAS) have been applied extensively in sporadic IAs which have focused on the role of common variants that may have a minor effect on disease risk [11–16]. In addition, replicated associations have identified some susceptibility loci for IAs including 4q31.23 (*EDNRA*), 8q12.1 (*SOX17*), 9p21.3 (*CDKN2A/CDKN2B/CDKN2BAS*), 10q24.32 (*CNNM2*), 12q22, 13q13.1(*KL/STARD13*), 18q11.2 (*RBBP8*), and 20p12.1 [17–19]. But although the locus association is robust to replicate, the actual potential gene involved at distinct locus is less clear. Moreover, these loci can only explain a small fraction (~5%) of the population-attributable risk for IAs [17,20]. Thus, the genes contributing to the genetic predisposition of IAs is largely unknown. The detection of rare variants which are expected to have a larger effect size on disease risk is potentially one approach to unravel disease biology and the pathogenesis of the disorder.

Exome sequencing (ES) has emerged as a robust technology for identifying coding variation at the genome-wide level and enabling researchers to identify causative rare variants predisposing to disease. Broad application of ES has led to a better understanding of the genetic architecture of predisposition to some Mendelian diseases such as intracranial vertebral-basilar artery dissecting aneurysm (IVAD) [21], neurogenetic disorders [22,23], and brain arteriovenous malformations (BAVM) [24,25], as well as familial intracranial aneurysms (FIAs), in recent years. With the approach of ES, variants in *ADAMTS15* [26], *TMEM132B* [27], *THSD1* [28], *RNF213* [29], *ANGPTL6* [30] and *LOXL2* [31] were associated with FIAs. These recent advances provide new insights into the genetics of IA and demonstrate the usefulness of pedigree analysis and family-based ES to explore pathogenesis underlying IA formation.

In the present study, a large four-generation family with multiple cases of IA presenting an autosomal dominant (AD) likely inheritance pattern was ascertained and studied. By ES of selected family member samples and further segregation analysis using Sanger methodology, we identified a novel missense variant in Nuclear Transcription Factor X-box Binding 1 (*NFX1* [MIM: 603255]) as the causative mutation in the family.

Materials and methods

Family Recruitment

We enrolled a family of Chinese Han ethnicity (Figure 1A) with 7/21 living members affected by IA. Participants were reviewed by two experienced neuroradiologists independently to validate the diagnosis of IAs by radiology imaging of the cerebrovascular system (MRA/DSA) and to rule out IAs resulting from syndromic disorders such as autosomal dominant polycystic kidney disease (ADPKD, MIM: 173900), Ehlers-Danlos

syndrome type IV (MIM: 130050), Loeys–Dietz syndrome (MIM: 610192), Marfan syndrome (MIM: 154700), or brain arteriovenous malformations (BAVM, [MIM: 108010]) by physical examination and medical records review (past medical history, ultrasound, biochemical examination, X-ray). Peripheral blood samples were obtained from available family members.

This research was approved by the ethics committee of Beijing Tiantan Hospital under 2016YFC1300800. Informed written consent was obtained from all of the participants.

Genomic DNA Preparation and ES

Genomic DNA for each individual was extracted from peripheral blood lymphocytes using a standard phenol-chloroform method. Exome sequencing (ES) was performed on three IA cases and two phenotypically normal members from the family (Figure 1A, marked with "S"). DNA samples were prepared in Illumina libraries and then underwent whole-exome capture with the SureSelect Human All Exon V6+UTR r2 core design (91 Mb, Agilent), followed by sequencing on the Illumina HiSeq 4000 platform with 150-bp paired-end reads mode.

Variant-calling and Annotation

All reads were mapped to the human reference sequence (hg 19) using BWA-MEM (version 0.7.12). Picard (version 2.5.0, http://picard.sourceforge.net) and SAMtools (version 0.1.19) were used to mark duplicate reads and process the alignment file. Genome Analysis Toolkit (GATK version 3.4.0) was then used to refine the alignments by performing local indel realignment and subsequent base quality recalibration. Single-nucleotide variants (SNVs) and insertions/deletions (indels) were called with the Haplotype Caller of the GATK. Filtering of variant quality was performed by variant quality score recalibration (VQSR) measurement using GATK's recommended parameters incorporating 892 in-house exome data (available upon request). Retained variants were annotated by the in-house 'PUMCH' annotation pipeline [32] which applied ANNOVAR (Annotation of Genetic Variants), VEP (Variant Effect Predictor) and additional annotation tools and clinical databases. Computational prediction tools (SIFT [33], Polyphen-2 [34], MutationTaster [35], LRT [36], Gerp++ [37] and CADD [38]) were used to predict the conservation and pathogenicity of candidate variants. All variants were compared against publicly available databases such as the 1000 Genomes Project (http://internationalgenome.org/), the Exome variant server, NHLBI GO Exome Sequencing Project (ESP, http://evs.gs.washington.edu/EVS/), the Exome Aggregation Consortium database (ExAC, http://exac.broadinstitute.org/), and Genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org/).

Manual Review and Prioritization

From the set of variants passed from quality control (QC) (Step 1) performed by VQSR measurement using GATK's recommended parameters, we retained novel variants using Exome Aggregation Consortium_East Asian (ExAC_EAS) population (Step 2). Then all protein-altering or splice region variants were retained (Step 3). Variants segregated with disease, which present in the three affected individuals and absent in the phenotypically normal participants sent for ES were retained for further analysis (Step 4). Then, predicted

deleterious (truncating or CADD (phred-like) score≥10) variants were list as the candidate variants (Step 5). These variants were manually reviewed by visual inspection of sequence reads using the Integrative Genomics Viewer (IGV) [39].

Variant Confirmation and Familial Segregation Analysis

To validate the variants identified by ES with an orthogonal sequencing technology, and evaluate the segregation of the variants with the phenotype, we sequenced all the available family members using direct Sanger sequencing. Variants segregated fully with definite IA phenotype in the family (Step 6), and absent from the second-generation unaffected members (Step 7) were considered as the final etiologic variant. Polymerase chain reaction (PCR) was performed with the primers summarized in Table S1, and products were purified with Axygen-AP-GX-50 Toolkit and sequenced on an ABI Prism 3730 Avant DNA sequencer (Applied Biosystems).

Results

Clinical Information

The proband (individual III-1; Figure 1A) was diagnosed after a subarachnoid hemorrhage (SAH) at age 31y, with a ruptured anterior communicating artery aneurysm (Figure 1B). In the following years, his aunt (Ⅱ−4) and his mother (Ⅱ−2) were sequentially stricken by SAH at age 52y and 63y, notably older than the proband's age at diagnosis. Because of the significant recurrence of ruptured IAs in this family (III-1, II-2, II-4), a systematic cerebral artery screening was performed among all available relatives by digital subtraction angiography (DSA)/ magnetic resonance angiography (MRA). The proband's another aunt (II-10) and two uncles (II-8, II-11) were observed to carry IAs (Figure 1B) without any clinical symptoms. His grandfather (I-1) died in his 60s after an episode suggestive of aneurysmal SAH. In combination with his unaffected sister (III-4), all findings consist with Mendelian expectation for AD likely IA disease trait in this family. Interestingly, the proband's father (Ⅱ−1), without a familial history of IA, was also found to be carrying an IA in the right middle cerebral artery (Figure 1B). Characteristics and aneurysm images of IA cases are shown in Table 1 and Figure 1B independently. Demographic information of the other members is shown in Table S2. Three affected individuals (II-2, II-4, III-1) and two unaffected relatives (II-5, III-4) were selected for ES.

Exome Sequencing Identified 14 Candidate Variants

ES on DNA isolated from blood of the selected five individuals generated approximately 10 billion bases for each individual with an average depth-of-coverage of 99.73×. And 96.35% (95.85 % to 96.65 %) of target exon regions were covered by at least 20× (Table S3). After alignment and a series of quality control procedures, 384102 single nucleotide variants (SNVs) and 45898 insertions/deletions (indels) were identified. We primarily focused on the novel, heterozygous variants in the coding region predicted by conceptual translation to affect protein-coding sequences. After filtering against reference from public databases (ExAC_EAS), 1589 non-synonymous SNVs and 478 indels were retained (Table 2, Step 3). Among them, 14 SNVs and 3 indels co-segregated with the IA phenotype among the 5 family members sent for ES (Table 2, Step 4). Taking into consideration of the

pathogenicity, 14 variants were prioritized as candidate variants (Table 3). The rare variant filtering steps and results are illustrated in Table 2.

Missense Variant in *NFX1* Co-segregating with the Disease

The 14 candidate variants were validated using Sanger sequencing in all fifteen individuals (7 cases, 8 controls) in the family. Co-segregation analysis identified a missense variant in *NFX1* shared by all the affected IA cases and absent from the second-generation unaffected members. In the third-generation, *NFX1* c.2519T>C was detected in only one 29 year old member (Ⅲ−7) who may not manifest IA at the time probably due to the late age of onset for IA (Figure 1C); these data suggested the causal role of *NFX1* c.2519T>C in this family.

NFX1 encodes a nucleic acid-binding protein that interacts with the conserved X1 box *cis*-element, and is conserved through yeast, *Drosophila*, *Caenorhabditis elegans*, *Arabidopsis*, mice, and humans [40]. Studies of *NFX1* homologues demonstrate its importance in normal cell growth, function, and homeostasis across species[40]. In addition, 840Leu in *NFX1* is robustly conserved in mammals (Figure 1D) indicating its evolution may have preserved function. *NFX1* c.2519T>C (p.Leu840Pro) maps where no domain structure has been delineated in a region before the RING-type zinc finger domain (Figure 1E); variation to Pro may produce a kink in the protein secondary structure. This missense variant is absent from public databases (1000genome, ExAC, gnomAD, ESP5400), and has substantial damaging predictions by bioinformatics tools (Gerp++ score =5.88, CADD score =16.43, MutationTaster score =1, LRT score =0).

Identification of a Paternally Inherited Missense Variant in *NOTCH3*

Since the father of the proband also developed an IA (individual Ⅱ−1; Figure 1B), we also analyzed paternally inherited rare variants in the proband. After filtering with the conditions of low minor allele frequency and inheritance modal, we found a deleterious missense variant (c.1760G>A) in *NOTCH3* which was confirmed in his affected father while absent in his unaffected sister using Sanger method (Figure 2A). The *NOTCH3* c.1760G>A (p.Arg587His) variant is located in the EGF-like 15 domain (Figure 2B). With the predictions by bioinformatics tools (Gerp++ score =3.22, CADD score =11.78, MutationTaster score =0.996), this missense variant is present in only one individual in ExAC_EAS. Meanwhile, 587Arg in *NOTCH3* is also strongly conserved in mammals, including human, rhesus, mouse, dog and elephant (Figure 2C). However, we are unable to testify the participation of this variant in the pathogenesis of IA in the proband or his affected father due to the lack of genetic evidence.

Discussion

In the present study, we identified one novel missense variant, c.2519T>C (p.Leu840Pro), in the 16th exon of Nuclear Transcription Factor X-box Binding 1 (*NFX1*), which was heterozygous in all six IA-affected members and only one out of the eight unaffected relatives in the pedigree. This variant is absent from public databases, and predicted to be deleterious by bioinformatics tools.

In addition, we found a deleterious missense variant c.1760G>A (p.Arg587His) in *NOTCH3* which was detected in the proband (Ⅲ−1) and his affected father but not in his mother and sister. This variant leads to the same residue change c.1759C>T (p.Arg587Cys) enrolled in HGMD as causative for CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) [41], a vascular degenerative disease that is the most common form of hereditary stroke disorder, leading to dementia due to systemic vascular degeneration. While intracranial aneurysms are present in both the proband and father's cerebral vascular imaging, there are no clinical manifestations of migraine, recurrent cerebral ischemia, emotional disturbances, and dementia, or any evidence of leukoencephalopathy to suggest a CADASIL diagnosis.

Concurrent variants in the two genes suggest the possibility of epistasis or mutational burden effects. The proband (Ⅲ−1) who is heterozygous for both of the deleterious variants had more severe IA characterized by an early age of onset of IA rupture. For example, the age of the proband on his IA rupture (31 years old) is much younger than that of the other two affected members on his maternal side of the family (52 and 63 years old). The impact of mutational burden on phenotypic expression and severity of disease has been described in families with peripheral neuropathy demonstrating intrafamilial phenotypic variability [42]. Such second hit mutations in another gene that trigger the disease process in the region of the lesion also exist in several vascular diseases [28,43–45]. Genetic heterogeneity, phenocopy, age dependent penetrance, and gene-environment interactions are all factors that make it complicated to identify the pathogenesis of IA. *NFX1* has not been previously implicated in cerebrovascular disease. Therefore, this finding may represent a novel disease association for *NFX1.*

The major limitation of our study is the lack of investigation of the functional significance of the identified *NFX1* and *NOTCH3* variants. Our study design focused on rare, deleterious variants in potential novel IA-related genes. Intronic and regulatory region were not covered by ES, thus were unable to be studied.

Conclusions

In conclusion, *NFX1* c.2519T>C (p.Leu840Pro) is likely contributing to the pathogenesis of FIA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations:

SAH	subarachnoid hemorrhage
ES	exome sequencing
IVAD	intracranial vertebral-basilar artery dissecting aneurysm
BAVM	brain arteriovenous malformations
AD	autosomal dominant
NFX1	Nuclear Transcription Factor X-box Binding 1
DSA	digital subtraction angiography
MRA	magnetic resonance angiography
SNV	single nucleotide variant
GWAS	genome-wide association study
indels	insertions/deletions
ExAC_ EAS	Exome Aggregation Consortium_East Asian
MAC	Minor allele count
pLI	probability of loss of function (LoF) intolerance
PCR	Polymerase chain reaction
VSMCs	vascular smooth muscle cells
DTAAD	descending thoracic aortic aneurysm and dissection
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CCM	cerebral cavernous malformation

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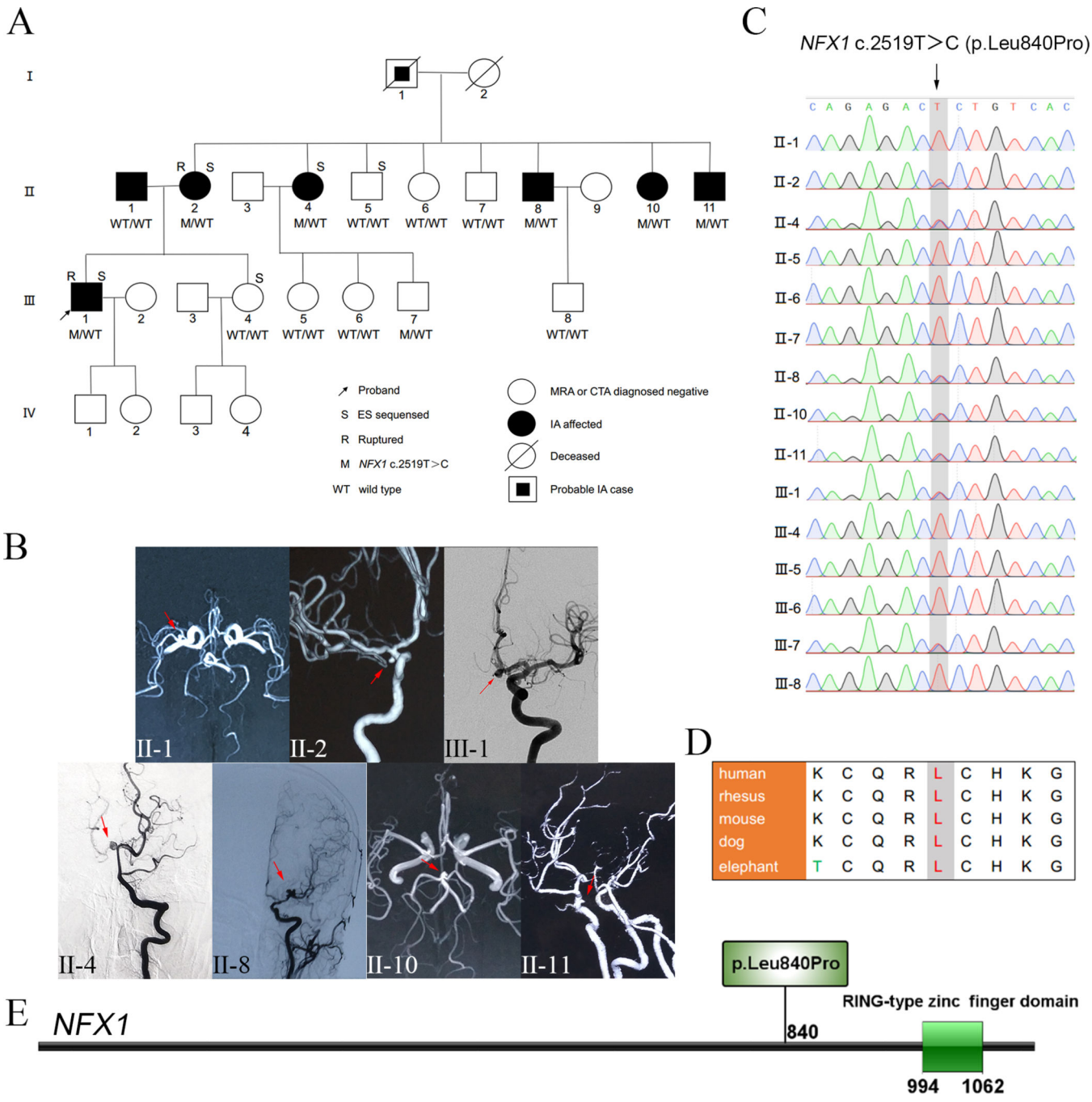

Figure 1.

Family pedigree, clinical images, and segregation results (A-C). Protein location and evolutionary conservation of the NFX1 c.2519T>C variant (D-E). (A) Pedigree and NFX1 genotype segregation in the family. WT/WT represents both wild type alleles, i.e. bi-allelic, while M/WT designates NFX1 c.2519T>C pathogenic variant as heterozygous allele. (B) MRA/DSA images of the presenting family members IA; i.e. clinically affected cases. Red arrows indicate the site of IAs. (C) Validation of the NFX1 c.2519T>C variant via Sanger sequencing. (D) Protein sequence alignments indicate that 840Leu in NFX1 is robustly

conserved in mammals. (E) NFX1 p.Leu840Pro mapping, located in a region where no domain structure has been delineated before the RING-type zinc finger domain of NFX1 (http://ibs.biocuckoo.org/).

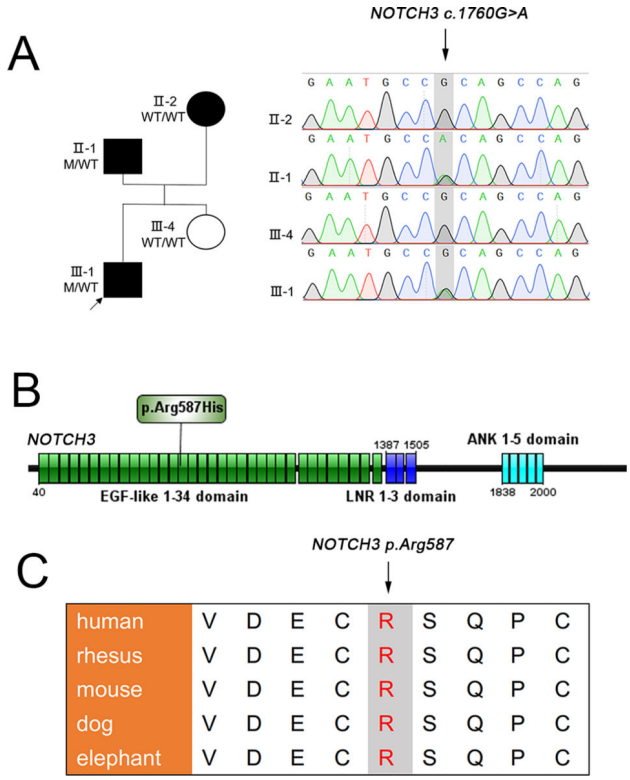

Figure 2.

Pedigree of the proband's family, and Sanger sequencing, protein location and evolutionary conservation of the NOTCH3 c.1760G>A variant (A-C). (A) Validation of NOTCH3 c.1760G>A variant via Sanger sequencing in the family. WT/WT represents both two wild type alleles, i.e. bi-allelic, while M/WT designates NOTCH3 c.1760G>A pathogenic variant as heterozygous allele. (B) NOTCH3 p.Arg587His is located in the EGF-like 15 domain (http://ibs.biocuckoo.org/). (C) Protein sequence alignments indicate that 587Arg in NOTCH3 is robustly conserved in mammals.

Table 1.

Clinical Characteristics of patients with intracranial aneurysm.

	Ⅱ−1	Ⅱ−2	Ⅱ−4	Ⅱ−8	Ⅱ−10	Ⅱ−11	Ⅲ−1
Age at diagnosis or last evaluation /sex	68/M	63/F	52/F	49/M	46/F	41/M	31/M
Presentation	Asymptomatic	SAH	SAH	Asymptomatic	Asymptomatic	Asymptomatic	SAH
IA Location	MCA M4	ICA C7	BA terminus	MCA M4	BA terminus	ICA C4	ACoA
Size (mm)	3×3	4×4	5×5	5×5	3×3	3×3	4×4
Smoking	N	N	N	Y	N	Y	Y
Alcohol drinking	Y	N	N	Y	N	Y	Y
Hypertension	N	Y	Y	N	N	N	N
Diabetes	N	N	N	N	N	N	N
Hyperlipidemia	N	N	N	N	N	N	N

M, man; F, female; MCA, middle cerebral artery; ICA, internal carotid artery; BA, basilar artery; ACoA, anterior communicating artery; Y, yes; N, no

Table 2.

Exome-Variant Filtration Steps and Results of the Pedigree

Filtration Steps	SNVs	indels
Step 1): QC passed variants [a]	**384102**	**45898**
Step 2): novel variants [b]	204610	33009
Step 3): Protein-altering [c] or splice region variants	1589	478
Step 4): Variants segregated with disease [d]	14	3
Step 5): Variants predicted to be deleterious [e]	11	3
Candidate variants list	**11**	**3**
Step 6): familial segregation analysis [f]	4	2
Step 7): absent from the second-generation unaffected members	1	0
The final etiologic variant	***NFX1* c.2519T>C**	

[a] SNVs and indel variants filtered by variant quality score recalibration (VQSR) measurement using GATK's recommended parameters;

[b] Novel variants, absent from public databases (ExAC_EAS);

[c] Nonsynonymous, stop-gain, frameshift, start-lost, stop-lost;

[d] Variants present in the three affected individuals (II-2, II-4, III-1) and absent in the phenotypically normal participants (II-5, III-4) sent for ES;

[e] Variants predicted to be deleterious with CADD≥10 or truncating variants;

[f] Variants segregated fully with definite IA phenotype in the family.

Table 3.

Information of Candidate Variants

Gene Symbol	Variant Type	Zygosity	Chr_Position	Ref mRNA	Amino Acid Change	Gerp++ Score	CADD Score	pLI Score	ExAC_EAS Count
EFHD1	Missense	Het	2_233498511	NM_025202.3	c.97G>A(p.Ala33Thr)	−1.08	11.56	–	0
FNDC1	Missense	Het	6_159654771	NM_032532.2	c.3227A>G(p.Gln1076Arg)	−2.84	13.63	–	0
IFNA2	Missense	Het	9_21385094	NM_000605.3	c.235C>T(p.Leu79Phe)	−0.19	14.89	–	0
NFX1	Missense	Het	9_33351652	NM_002504.4	c.2519T>C(p.Leu840Pro)	5.88	16.43	–	0
MYOCD	Missense	Het	17_12666834	NM_153604.2	c.2690C>T(p.Pro897Leu)	6.08	18.70	–	0
RHO	Missense	Het	3_129247637	NM_000539.3	c.61C>T(p.Arg21Cys)	4.59	26.2	–	0
PLK3	Missense	Het	1_45271250	NM_004073.2	c.1841C>T(p.Thr614Ile)	5.70	29.9	–	0
LENG8	Missense	Het	19_54967909	NM_052925.2	c.1540G>T(p.Ala514Ser)	3.07	21.5	–	0
SERAC1	Missense	Het	6_158535967	NM_032861.3	c.1538C>T(p.Thr513Met)	2.39	35	–	0
HYDIN	Missense	Het	16_71127814	NM_001198542	c.1433G>C(p.Arg478Pro)	4.95	17.69	–	0
KIAA1524	Missense	Het	3_108279578	NM_020890.2	c.1745A>T(p.Lys582Met)	5.68	12.66	–	0
ESYT3	Frameshift	Het	3_138153945	NM_031913.3	c.308delG(p.Gly103AlafsTer30)	2.19	13.28	0	0
TIGD7	Frameshift	Het	16_3350476	NM_033208.3	c.135_139delTAAAA(p.Lys47Ter)	4.29	36	0	0
HEATR4	Deletion	Het	14_73989704	NM_203309.2	c.151_153delTTC(p.Phe51del)	0.45	12.04	0	0
NOTCH3	Missense	Het	19_15297996	NM_000435.2	c.1760G>A(p.Arg587His)	3.22	11.87	–	1

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