Original Article | Neurointervention

eISSN 2005-8330
https://doi.org/10.3348/kjr.2021.0467
Korean J Radiol 2022;23(1):101-111

Korean Journal of Radiology
KJR

Whole Exome Sequencing in Patients with Phenotypically Associated Familial Intracranial Aneurysm

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Objective: Familial intracranial aneurysms (FIAs) are found in approximately 6%–20% of patients with intracranial aneurysms (IAs), suggesting that genetic predisposition likely plays a role in its pathogenesis. The aim of this study was to identify possible IA-associated variants using whole exome sequencing (WES) in selected Korean families with FIA.

Materials and Methods: Among the 26 families in our institutional database with two or more IA-affected first-degree relatives, three families that were genetically enriched (multiple, early onset, or common site involvement within the families) for IA were selected for WES. Filtering strategies, including a family-based approach and knowledge-based prioritization, were applied to derive possible IA-associated variants from the families. A chromosomal microarray was performed to detect relatively large chromosomal abnormalities.

Results: Thirteen individuals from the three families were sequenced, of whom seven had IAs. We noted three rare, potentially deleterious variants (*PLOD3* c.1315G>A, *NTM* c.968C>T, and *CHST14* c.58C>T), which are the most promising candidates among the 11 potential IA-associated variants considering gene-phenotype relationships, gene function, co-segregation, and variant pathogenicity. Microarray analysis did not reveal any significant copy number variants in the families.

Conclusion: Using WES, we found that rare, potentially deleterious variants in *PLOD3*, *NTM*, and *CHST14* genes are likely responsible for the subsets of FIAs in a cohort of Korean families.

Keywords: *Whole exome sequencing; Genetics; Familial intracranial aneurysm*

INTRODUCTION

The global prevalence of intracranial aneurysms (IAs) is estimated to be 3.2% [1]. In 6%–20% of patients with IA, one or more of their family members also have an IA [2]. These cases are defined as familial intracranial aneurysms (FIAs) and are reported to have a more severe phenotype in terms of a higher number of aneurysms and a higher risk of rupture than those without a familial history [3-5].

Several linkage studies and genome-wide association studies (GWAS) have identified a large number of candidate loci associated with FIAs [6-8]. However, the potential genetic defects in these loci have a relatively small effect on the risk of developing IA and can only explain a small fraction of heritability [6,7]. Recently, several studies using next-generation sequencing (NGS) have suggested rare variants in 10 candidate genes (*ADAMTS15*, *THSD1*, *RNF213*, *ANGPTL6*, *LOXL2*, *ARHGEF17*, *C4orf6*, *SPDYE4*, *NFX1*, and *EDIL3*) with larger effects related to FIA; however, some of these variants require further validation. Although

Received: June 11, 2021 **Revised:** July 17, 2021 **Accepted:** August 3, 2021
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the variants of these genes may explain some aneurysms in certain ethnic groups, they are rarely replicated across different studies.

Previous studies have mostly focused on the presence of an aneurysm and not on its phenotypic presentation, such as its location, shape, and size. We hypothesized that if a specific gene was associated with IA, the characteristics of the aneurysm would be shared among members of the family. Therefore, to increase the possibility of gene identification, we reasoned that detailed information on the aneurysm should be obtained and considered when recruiting the family. The purpose of this study was to use NGS to identify potential IA-associated variants in families that share a specific phenotype.

MATERIALS AND METHODS

Study Population

The Institutional Review Board of Asan Medical Center approved this prospective study (IRB No. 2018-1106). Informed written consent for blood sampling and magnetic resonance angiography screening was obtained from all study participants.

IA was defined as a saccular dilatation of any size occurring in the intracranial arteries; FIA was defined as when at least two first-degree relatives in a family were diagnosed with IA. A family history of IA was identified in 28 (4.4%) patients among the 638 patients with IA in a tertiary hospital's prospectively collected database from between January 2011 and August 2018. We then selected families with FIA for further genetic testing according to the following inclusion criteria: 1) demonstration of the pedigree of the disease status in the family, 2) two or more affected members and one or more non-affected members are available for genetic testing, 3) available angiographic data for the participants (both affected and unaffected), 4) genetically enriched samples where the family has a severe phenotype of IA (multiple, early onset, ruptured) and common site involvement among families [3-5]; and 5) consent to participated provided by the patient and family members.

We excluded patients who had 1) fusiform, mycotic, or dissecting aneurysms in the intracranial artery, 2) aneurysms associated with an arteriovenous malformation, or 3) aneurysms associated with syndromic disorders (e.g., polycystic kidney disease, Ehlers-Danlos syndrome, Marfan syndrome, fibromuscular dysplasia, and moyamoya disease). Physical examination, ultrasonography, and/or computed tomography angiography were performed to rule out any known or unknown syndromes associated with IA. Other first-degree relatives who had not been screened for IA underwent magnetic resonance angiography.

Whole Exome Sequencing (WES) Analysis

Genomic DNA was extracted from peripheral blood cells using the Chemagic Magnetic Separation Module I (Chemagic MSM I) extraction robot with a DNA Blood 200 μL Kit. SureSelect Human All Exon V5 (Agilent Technologies) was used for library preparation, and sequencing was performed on the Illumina NextSeq500 platform (Illumina Inc.), which generated 2 x 150 bp paired-end reads. The averages of the 30 x and 20 x coverage for the target regions were 89.18% and 94.34%, respectively. Trimmomatic v.0.36 was used to trim sequences of sequencing adapters and suffixes of low quality (i.e., Phred quality score < 10).

Variant Calling and Filtering Strategy

All reads were aligned to the human reference genome (GRCh37/hg19) using the Burrow-Wheeler Aligner (BWA version 0.7.12). The Picard tool (version 1.96, http://picard.sourceforge.net) was used to remove duplicate reads, and the Genome Analysis ToolKit (GATK version 3.30) was used for variant calling. The Annotation of Genetic Variants program (ANNOVAR, http://annovar.openbioinformatics.org) was used to annotate alterations using information from the following public databases: the Single Nucleotide Polymorphism database (dbSNP 147, https://www.ncbi.nlm.nih.gov/snp/), 1000 Genomes Project (https://www.internationalgenome.org/), Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org/), and Genome Aggregation Database (gnomAD, https://gnomad.broadinstitute.org/).

Variants with less than 10 x coverage and an allele frequency of more than 0.01 in public databases (1000 Genomes Project, Exome Aggregation Consortium, and gnomAD) were removed. Additionally, variants affecting protein-altering and splicing (e.g., non-synonymous amino acid changes, start codon alterations, stop loss changes, in-frame insertions/deletions, frameshifts, nonsense variants, and changes affecting consensus splice site sequences) were included.

In the family-based approach, we selected variants segregating as occurring only in affected members, and variants shared with unaffected members. For knowledge-

https://doi.org/10.3348/kjr.2021.0467

Korean Journal of Radiology
KJR

based prioritization, the variants were screened among the 450 genes associated with aneurysm or vascular/connective tissue disorders in the Online Mendelian Inheritance in Man (OMIM) database (https://www.omim.org/), 16 IA candidate genes reported in PubMed-indexed studies (https://pubmed.ncbi.nlm.nih.gov/), and 77 genes from the GWAS catalog (https://www.ebi.ac.uk/gwas/) for brain aneurysms.

Variant Interpretation

The pathogenicity of the variants was predicted using Sorting Intolerant From Tolerant (SIFT) [9], Polyphen2 [10], Genomic Evolutionary Rate Profiling (GERP) [11], Combined Annotation Dependent Depletion (CADD) [12], rare exome variant ensemble learner (REVEL) [13], Mendelian Clinically Applicable Pathogenicity (M-CAP) [14], pLI (the probability of being loss-of-function intolerant) [15], and % haploinsufficient (HI) [16]. Pathogenicity thresholds were chosen according to the respective authors' recommendations (SIFT, ≤ 0.05; Polyphen2, ≥ 0.9; GERP, ≥ 2; CADD, ≥ 20; REVEL, ≥ 0.5; M-CAP, ≥ 0.025; pLI, ≥ 0.9; and %HI, ≤ 10%).

Based on the standards and guidelines for the interpretation of sequence variants from the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) [17], the candidate variants were classified into five types: pathogenic variant (PV), likely pathogenic variant (LPV), variant of uncertain significance (VUS), likely benign variant (LBV), and benign variant (BV). We assigned PP3 (pathogenic supporting) as a variant if at least two out of three meta-predictors (CADD, REVEL, M-CAP) and SIFT or Polyphen2 calculated a pathogenicity score above their respective thresholds. All clinically significant and novel variants were confirmed using independent Sanger sequencing [18].

Chromosomal Microarray

To identify submicroscopic deletions or duplications that are difficult to assess using whole exome sequencing (WES), copy number analysis was performed using CytoScan HD (Affymetrix) according to the manufacturer's protocol. Regions of homozygosity and copy number variants (CNVs) shared between affected and unaffected siblings were eliminated as potential candidate regions. Thresholds for the detection of candidate pathogenic CNVs in affected subjects were set to 25 CNV markers for deletions and 50 CNV markers for duplications. CNVs were interpreted based on the technical standards of a joint consensus recommendation of the ACMG and the Clinical Genome Resource (ClinGen) [19].

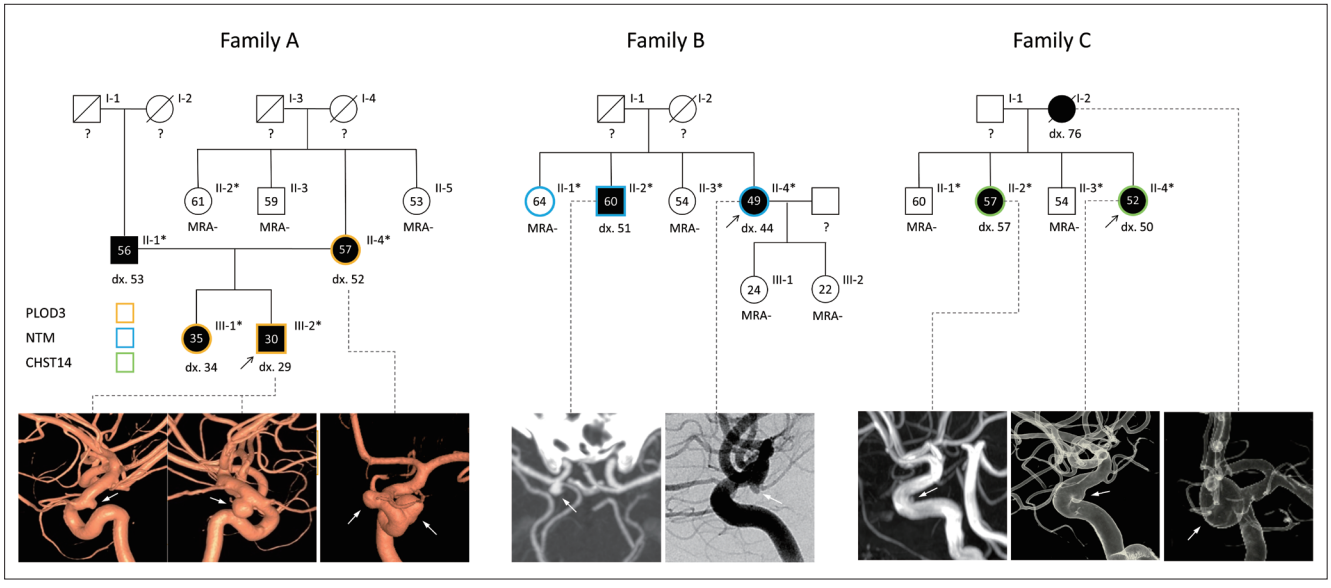

Fig. 1. Pedigree of the families with genetic and phenotypical findings. Asterisks indicate individuals that were subjected to whole-exome sequencing. black arrows = probands, black symbols = IA-affected cases, circles = females, diagonal line through a symbol = deceased, dx. number = age at IA diagnosis, IA = intracranial aneurysm, MRA- = negative result on magnetic resonance angiography, number in symbols = current age, Squares = males, white arrows = aneurysms on angiography, ? = unknown aneurysm status

https://doi.org/10.3348/kjr.2021.0467

RESULTS

Clinical Phenotypes of Three Families with FIA Used in This Study

Thirteen individuals from three families were selected for WES. In all three families, two or more members had IAs at a common location (Fig. 1). In family A, the proband and his mother had paraclinoid aneurysms. The characteristics of this family included early onset (III-1, 2), presence of multiple aneurysms (average number of aneurysms ≥ 2) in a common location (II-4, III-1), and relatively few risk factors (Table 1). The father of the siblings also had an IA in the middle cerebral artery. Family B had two affected siblings and two unaffected siblings in the second generation. The proband (II-4) had two small unruptured aneurysms in the right internal carotid artery at the origin of the posterior communicating artery (P-COM) and at the top of the basilar artery, and her older brother (II-2) also had a small internal carotid artery aneurysm at the origin of the P-COM artery. Family C had three affected females (I-1, II-2, and II-4) whose IAs were commonly located in the paraclinoid region of the internal carotid artery.

WES Analysis and Variant Filtering

WES was performed in all living affected individuals and at least one unaffected first-degree relatives of the probands. Among the > 90000 variants initially discovered in the WES, an average of approximately 500 variants for each individual were selected after excluding those with insufficient coverage, a frequency of 0.01 or more in the population, and variants that did not affect the protein (Fig. 2). Through a family-based approach according to Mendelian inheritance patterns, 40, 38, and 27 variants (autosomal dominant) and 222, 76, 162 variants (autosomal

Table 1. Baseline Characteristics of the Participants

Family (Member)	Age (Year)	Sex	Risk Factors	Diagnosis (Age; Year)	Aneurysm Location (Size; mm)	Treatment (Age; Year)	WES
A (II-1)*	56	M	DM, HL, Smoking 90PY	DSA (53)	Rt. MCA (3.8)	Clipping (53)	Yes
A (II-2)	61	F	None	MRA	Negative	N/A	Yes
A (II-3)	59	M	Smoking 30PY	MRA	Negative	N/A	No
A (II-4)*	57	F	HL	DSA (52)	Lt. paraclinoid (8.5, 5.1)	Coiling (52)	Yes
A (II-5)	53	F	None	MRA	Negative	N/A	No
A (III-1)*	35	F	None	MRA (34)	Rt. P-COM (1.7), Lt. P-COM (1.8)	No	Yes
A (III-2)*	30	M	Smoking 5PY	DSA (29)	Lt. paraclinoid (6.0), Rt. paraclinoid (3.2) Lt. AchA (2.0)	Coiling (29)	Yes
B (II-1)	64	F	None	MRA	Negative	N/A	Yes
B (II-2)*	60	M	HTN, DM, HL, smoking 25PY	CTA (51)	P-COM (7.7)	Coiling (51)	Yes
B (II-3)	54	F	DM, HL	MRA	Negative	N/A	Yes
B (II-4)*	49	F	HTN, DM	DSA (44)	Basilar top (3.1), P-COM (2.1)	Coiling (44) (basilar top)	Yes
C (I-2)*	82	F	Cardiac (angina), HTN, HL	DSA (76)	Paraclinoid (14.3)	Coiling (76)	No
C (II-1)	60	M	Smoking 15PY, cardiac (MI)	MRA	Negative	N/A	Yes
C (II-2)*	57	F	Cardiac (arrhythmia)	MRA (57)	Paraclinoid (1.0)	No	Yes
C (II-3)	54	M	None	MRA	Negative	N/A	Yes
C (II-4)*	52	F	None	DSA (50)	Paraclinoid (4.5)	No	Yes

*IA-affected subjects. AchA = anterior choroidal artery, An = aneurysm, CTA = computed tomography angiography, DM = diabetes mellitus, DSA = digital subtraction angiography, HL = hyperlipidemia, HTN = hypertension, Lt = left, MCA = middle cerebral artery, MI = myocardial infarction, MRA = magnetic resonance angiography, N/A = not applicable, P-COM = posterior communicating artery, PY = pack-year, Rt = right, WES = whole exome sequencing

https://doi.org/10.3348/kjr.2021.0467

Korean Journal of Radiology
KJR

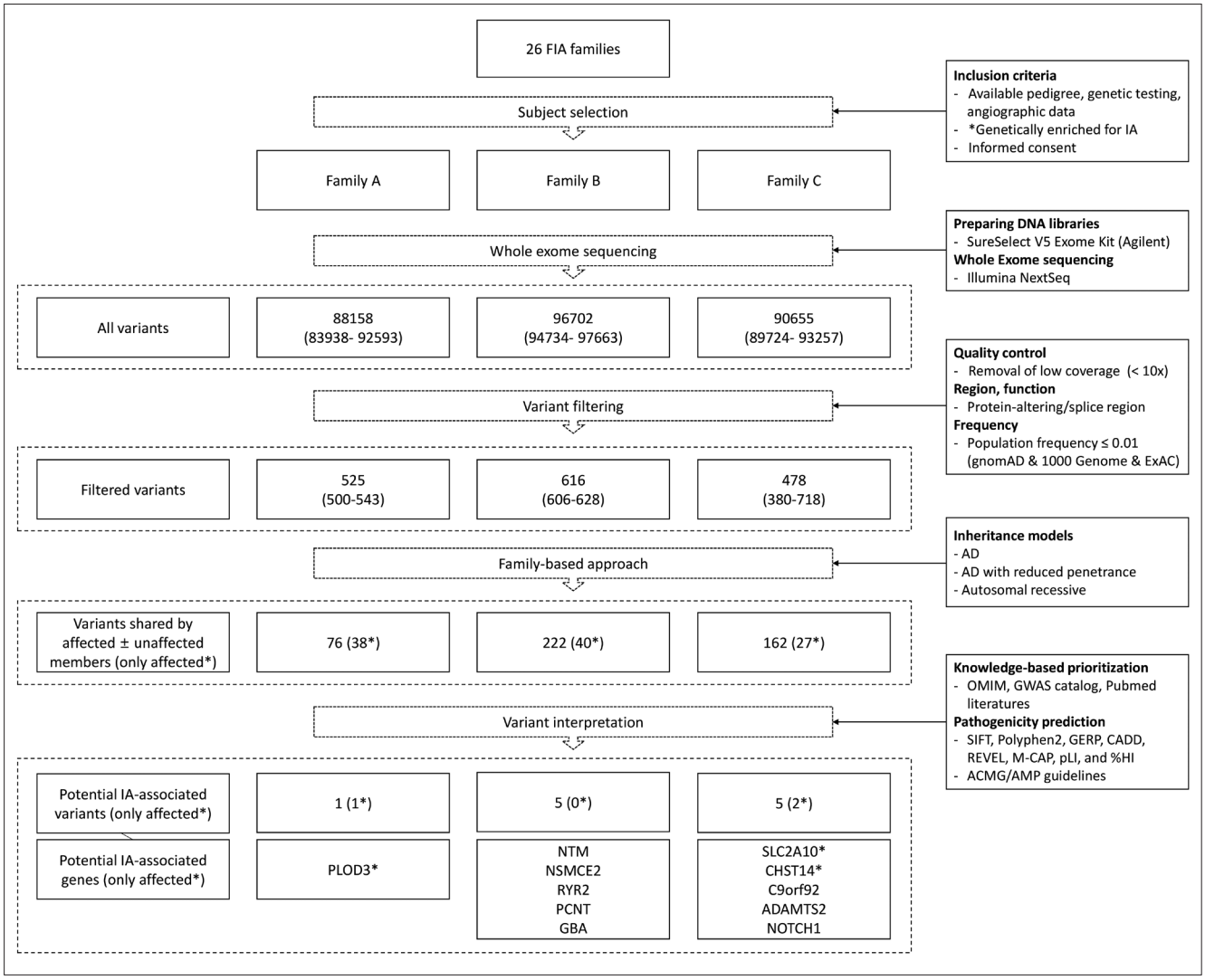

Fig. 2. Flowchart of variant filtering steps and results by family. *Genetically enriched for IA: severe IA phenotype (multiple, early onset, or ruptured) and common site involvement within families. ACMG = American College of Medical Genetics and Genomics, AD = autosomal dominant, AMP = Association for Molecular Pathology, CADD = Combined Annotation Dependent Depletion, ExAC = exome aggregation consortium, FIA = familial intracranial aneurysm, GERP = Genomic Evolutionary Rate Profiling, GWAS = genome-wide association studies, HI = haploinsufficient, IA = intracranial aneurysm, M-CAP = Mendelian Clinically Applicable Pathogenicity, OMIM = Online Mendelian Inheritance in Man, pLI = the probability of being loss-of-function intolerant, REVEL = rare exome variant ensemble learner, SIFT = Sorting Intolerant From Tolerant

dominant reduced penetrance) were selected in each family, respectively. There were no variants that showed segregation of autosomal recessive patterns among the three families. Finally, 11 pathogenic or damaging variants potentially associated with IA were derived through pathogenicity prediction algorithms and knowledge-based prioritization from previous genetic studies.

Potential IA-Associated Genes

All variants were heterozygous and missense variants, except for the nonsense mutation in the *C9orf92* gene (Table 2). *GBA* and *C9orf92* genes have been reported as susceptible genes associated with brain aneurysm in the GWAS catalog, and the remaining genes were reported to be associated with aneurysm or vascular/connective tissue disorders in the OMIM. The genes found in recent NGS studies were not identified in this study [8,20-27].

Of the 11 genes, *PLOD3*, *NTM*, *GBA*, *CHST14*, *SLC2A10*, and *C9orf92* genes have been reported to be related to IA or intracranial hemorrhage [28-31]. When assuming complete penetrance of the autosomal dominant variants, one variant of the *PLOD3* gene in family A, no variants in family B, and two variants of the *SLC2A10* and *CHST14* genes in family C remained. Table 3 summarizes the function of all candidate

 https://doi.org/10.3348/kjr.2021.0467

Table 2. Variants Identified in Patients with Familial Intracranial Aneurysms according to the Application of Filtering Strategy and Segregation

Gene	Transcript ID	c. notation p. notation	rs ID	MAF	Pathogenicity Predictions†								ACMG Interpretation‡	Subjects	
				gnomAD/ KRGDB	SIFT	Polyphen2	GERP	CADD	REVEL	M-CAP	pLI	%HI	Classification	Fm	Member*
PLOD3	NM_001084.4	c.1315G>A p.Ala439Thr	rs138610113	0.000403/ 0.002105	0.011	0.646	5.13	21.9	0.087	0.06	0.06	0.65	VUS PM1 + PP3	A	II-2*, III-1*, III-2*
NTM	NM_001048209.1	c.968C>T p.Thr323Met	rs755518659	0.000018/ 0.000000	0.223	0.958	5.21	23.6	0.247	0.138	1	14.03	VUS PM2 +PP3	B	II-1, II-2*, II-4*
RYR2	NM_001035.2	c.3595G>A p.Asp1199Asn	rs752376759	0.000065/ 0.000000	0.153	0.733	0.235	8.116	0.028	0.003	0	80.67	LPV PM1 + PM2 + PP2 + PP3	B	II-2*, II-3, II-4*
PCNT	NM_006031.5	c.545G>A p.Arg182His	rs193268784	0.000113/ 0.003143	0.001	1	5.62	32	0.22	0.037	0.04	23.92	LBV PM2 + BP1 + BP4	B	II-2*, II-3, II-4*
NSMCE2	NM_173685.2	c.217C>T p.Arg73Trp	rs201903722	0.000191/ 0.007079	0.241	0.001	1.25	13.01	0.493	0.241	0	52.28	VUS PM2 + PP3 + BP1	B	II-1, II-2*, II-3, II-4*
GBA	NM_000157.3	c.680A>G p.Asn227Ser	rs364897	0.000074/ 0.000262	0.001	0.979	4.3	23.6	0.244	N/A	0	51.13	LPV PS3 + PM1 + PM2 + PP5	B	II-1, II-2*, II-3, II-4*
CHST14	NM_130468.3	c.58C>T p.Arg20Trp	rs577809616	0.000216/ 0.003584	0	0.998	3.8	27.9	0.37	0.771	0.45	35.81	VUS PM2 + PP2 + PP3	C	II-2*, II-4*
SLC2A10	NM_030777.3	c.931G>A p.Val311Ile	rs139932041	0.000287/ 0.000786	0.283	0.084	3.88	14.58	0.14	0.018	0	71.81	VUS PM1 + PM2	C	II-2*, II-4*
ADAMTS2	NM_014244.4	c.268G>A p.Ala90Thr	rs776393146	0.000095/ 0.005500	0.595	0.212	4.18	19.61	0.044	0.008	0.97	25.76	VUS PM1 + PM2 + BP1	C	II-2*, II-3, II-4*
NOTCH1	NM_017617.4	c.5422G>A p.Asp1808Asn	rs571739078	0.000043/ 0.000786	0.091	0.676	4.55	23.7	0.381	0.512	1	0.15	VUS PM2 + PP2 + PP3	C	II-2*, II-3, II-4*
C9orf92	NM_001271829.1	c.106C>T p.Gln36Ter	N/A	0.000000/ 0.000000	N/A	N/A	1.98	N/A	N/A	N/A	0	0.52	VUS PM2	C	II-2*, II-3, II-4*

*IA-affected subject, †Pathogenicity thresholds: SIFT (≤ 0.05), Polyphen2 (≥ 0.9), GERP (≥ 2), CADD (≥ 20), REVEL (≥ 0.5), M-CAP (≥ 0.025), pLI (≥ 0.9), and %HI (≤ 10%), ‡ACMG standards and guidelines: VUS, LBV, LPV, each pathogenic criterion is weighted as very strong (PVS1), strong (PS1–4); moderate (PM1–6), or supporting (PP1–5), and each benign criterion is weighted as stand-alone (BA1), strong (BS1– 4), or supporting (BP1–6). ACMG = American College of Medical Genetics and Genomics, CADD = Combined Annotation Dependent Depletion, c.notation = nucleotide change, Fm = family, GERP = Genomic Evolutionary Rate Profiling, HI = haploinsufficient, KRGDB = Korean reference genome database, LBV = likely benign variant, LPV = likely pathogenic variant, MAF = minor allele frequency, M-CAP = Mendelian Clinically Applicable Pathogenicity, N/A = not available, pLI = the probability of being loss-of-function intolerant, p.notation = amino acid change, REVEL = rare exome variant ensemble learner, rs = reference, SIFT = Sorting Intolerant From Tolerant, SNP = single nucleotide polymorphism, VUS = variant of uncertain significance

Table 3. Summary of the Potential IA-associated Genes

Gene	Gene Full Name	Gene Function	OMIM Disease	OMIM Inheritance	Phenotype
PLOD3	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	Forms hydroxylysine residues in -Xaa-Lys-Gly- sequences in collagens. These hydroxylysines serve as sites of attachment for carbohydrate units and are essential for the stability of the intermolecular collagen cross-links	Lysyl hydroxylase 3 deficiency, 612394	AR	Stickler syndrome, variable features of Ehlers-Danlos syndrome, epidermolysis bullosa with vascular complications, intracranial arterial dilatation [34,35]
NTM	Neurotrimin	Neural cell adhesion molecule	Aneurysm, intracranial berry, 7, 612161	AD	IA, cerebral hemorrhage, thoracic aortic aneurysm, aortic rupture [29]
RYR2	Ryanodine receptor 2 (cardiac)	Calcium channel that mediates the release of Ca (2+) from the sarcoplasmic reticulum into the cytoplasm and thereby plays a key role in triggering cardiac muscle contraction. Aberrant channel activation can lead to cardiac arrhythmia. Required for embryonic heart development	Arrhythmogenic right ventricular dysplasia 2, 600996; Ventricular tachycardia, catecholaminergic polymorphic, 1, 604772	AD	Arrhythmogenic right ventricular dysplasia, ventricular tachycardia [47]
PCNT	Pericentrin	Integral component of the filamentous matrix of the centrosome involved in the initial establishment of organized microtubule arrays in both mitosis and meiosis. Plays a role, together with *DISC1*, in the microtubule network formation. Is an integral component of the PCM	Microcephalic osteodysplastic primordial dwarfism, type II, 210720	AR	Extreme SS, dysmorphism
NSMCE2	Non-SMC element 2, MMS21 homolog (S. cerevisiae)	E3 SUMO-protein ligase component of the SMC5-SMC6 complex, a complex involved in DNA double-strand break repair by homologous recombination. The complex is required for telomere maintenance via recombination in ALT (alternative lengthening of telomeres) cell lines	Seckel syndrome 10, 617253	AR	Extreme SS, dysmorphism
GBA	Glucosidase, beta, acid	Lysosomal enzyme that catalyzes the breakdown of the glycolipid glucosylceramide to ceramide and glucose	Susceptibility to Parkinson disease, late-onset, 168600; Gaucher disease, 230800	AR, AD, Multifactorial	Susceptible to IA [28] and Parkinson's disease [48], Gaucher disease
CHST14	Carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 14	Catalyzes the transfer of sulfate to position 4 of the N-acetylgalactosamine (GalNAc) residue of dermatan sulfate. Plays a pivotal role in the formation of 4-0-sulfated IdoA blocks in dermatan sulfate. Appears to have an important role in the formation of the cerebellar neural network during postnatal brain development	Ehlers-Danlos syndrome, musculocontractural type 1, 601776	AR	Intracranial hemorrhage [31], Craniofacial dysmorphism, musculoskeletal abnormality
SLC2A10	Solute carrier family 2 (facilitated glucose transporter), member 10	Facilitative glucose transporter glucose transporter 10 (GLUT10)	Arterial tortuosity syndrome, 208050	AR	IA [30], arterial tortuosity, CNS stroke, dysmorphism, skin and joint abnormality
ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	Cleaves the propeptides of type I and II collagen prior to fibril assembly. Does not act on type III collagen. May also play a role in development that is independent of its role in collagen biosynthesis	Ehlers-Danlos syndrome, type VIIC, 225410	AR	Dermatosparaxis (tearing of skin), SS, dysmorphism
NOTCH1	Notch 1	Functions as a receptor for membrane-bound ligands Jagged1, Jagged2, and Delta1 to regulate cell-fate determination. May play an essential role in post-implantation development, probably in some aspect of cell specification and/or differentiation. May be involved in mesoderm development, somite formation, and neurogenesis	Adams-Oliver syndrome 5, 616028; Aortic valve disease 1, 109730	AD	Developmental disorder
C9orf92	Chromosome 9 open reading frame 92	No function information available	None	Unknown	Susceptible to IA in a Korean IA GWAS study [28]

AR = autosomal recessive, AD = autosomal dominant, CNS = central nervous system, GWAS = genome-wide association study, IA = intracranial aneurysm, OMIM = Online Mendelian Inheritance in Man, PCM = pericentriolar material, SS = short stature, SMC = structural maintenance of chromosomes

https://doi.org/10.3348/kjr.2021.0467

Korean Journal of Radiology KJR

genes and their related diseases.

Chromosomal Microarray

Several chromosomal losses or gains were found in each family, but most of the CNVs were benign or likely benign. One copy number gain of unknown significance was detected in family A, which did not segregate with the phenotype. In addition, no genes were potentially related to IA in the corresponding regions.

DISCUSSION

In this study, WES was performed in three selected FIA families to identify genetic variants associated with IAs. A total of 13 participants were sequenced, of whom 7 had IAs. Among the 11 potential IA-associated variants, we noted three rare, potentially deleterious variants (*PLOD3* c.1315G>A, *NTM* c.968C>T, and *CHST14* c.58C>T) after considering gene-phenotype relationships, gene function, co-segregation, and variant pathogenicity.

The *PLOD3* gene encodes lysyl hydroxylase 3 (LH3), which is involved in post-translational modification of collagens, including type IV collagen [32,33]. As such, pathogenic variation of this gene can lead to complex connective tissue disorders resembling Stickler syndrome, Ehlers-Danlos syndrome, and epidermolysis bullosa [34-36]. Although vascular complications are rare manifestations of these syndromes, some cases of aneurysms or arterial dissection have been reported [34,35]. In addition, embryonic lethality with intracranial hemorrhage has been reported in LH3-knockout mice [33]. Although the *PLOD3* mutation found in family A was a heterozygous variant, it could be a potential IA-associated variant considering the severe variability of the phenotype of PLOD3-related diseases [34].

Neurotrimin (NTM) belongs to the IgLON family of glycosylphosphatidylinisotol (GPI)-anchored cell adhesion molecules and has been implicated in the promotion of neurite outgrowth and adhesion [37]. Luukkonen et al. [29] reported that the *NTM* gene is associated with IA and thoracic aortic aneurysm and suggested that truncations in the *NTM* gene caused IA and thoracic aortic aneurysm in a family. The 11q25 chromosomal region has been suggested as a susceptibility locus for both IA and aortic aneurysms in several independent linkage studies [38,39]. Although the individual (II-1 in family B) unaffected by IA had a rare PV of the *NTM* gene, it is still considered a potential IA-associated variant when considering the reduced penetrance or late onset of the aneurysm phenotype.

The *CHST14* gene encodes carbohydrate sulfotransferase 14/dermatan 4-O-sulfotransferase 1 (CHST14/D4ST1), which is required for the maturation of dermatan sulfate that is involved in collagen formation [40]. Along with variants in *DSE*, biallelic PVs in *CHST14* are one of the causes of musculoskeletal Ehlers-Danlos syndrome [41]. Moreover, intracranial hemorrhage was reported in 9% of patients with mcEDS-*CHST14* [31].

Among the other candidates, *GBA* and *C9orf92* genes were suggested to be IA-susceptible genes in a recent GWAS study of the Korean population [28]. Biallelic PVs of the *GBA* gene cause Gaucher disease, and a heterozygous variant is a well-known risk factor for PD [42,43]. In a previous study, the rs75822236 in *GBA* gene showed the strongest association with the risk of IA formation (odds ratio = 161.46) with sufficient statistical power (1.1×10^{-19}), whereas the SNP in the *C9orf92* gene was underpowered because of the small sample size [28].

Another candidate gene in family C, *SLC2A10*, encodes the facilitative glucose transporter glucose transporter 10 (GLUT10). Homozygous or compound heterozygous PVs of this gene cause arterial tortuosity syndrome, which is characterized by tortuosity, elongation, stenosis, and aneurysm formation in major arteries [44]. In contrast, heterozygous carriers of this gene variant are asymptomatic and do not show any notable vascular anomalies [45]. The heterozygous carriers (II-2 and II-4 in family C) in our study also did not show any arterial abnormalities that indicated arterial tortuosity syndrome.

In our study, we selected families that would be most genetically enriched for IAs considering the phenotypes, which include common locations of the IA among family members, multiple IAs, early onset, and fewer risk factors. In particular, our study is distinct from other studies in terms of the selection criteria that the affected members in each family should share the same aneurysm location. We assume that the intuition of the physicians who diagnosed and treated the patients played an important role in identifying their genetic predisposition.

Many genetic studies have been performed on FIA, and several genetic variations have been identified through linkage studies, GWAS, and NGS; however, these can explain only a small proportion of the total IAs in certain ethnic groups [7]. The current literature suggests that marked genetic heterogeneity may exist in distinct populations, and

https://doi.org/10.3348/kjr.2021.0467

Korean Journal of Radiology
KJR

only two genetic studies, a linkage study and a GWAS, have been performed on the Korean population to date [28,46]. Our study is the first FIA study using NGS in Korea and may serve as a basis for establishing a genetic database for Korean patients with aneurysms. If sufficient data on FIA is accumulated through genetic studies, simple genetic testing using an NGS panel could offer great clinical benefits in terms of risk stratification, treatment decisions, and the screening of unaffected family members.

Multiple factors are intricately involved in aneurysm development [3-5]. Gene-environment interaction and phenocopy hinder genetic studies on this matter, especially in patients with multiple risk factors such as hypertension, smoking, old age, and female sex. Therefore, it is difficult to determine whether the genetic variations in our study were entirely responsible for FIAs. Further validation using replication studies and expression or functional analyses are required to support our results.

The limitations of this study are as follows. First, this study suggested several candidate genes, but these have not been fully validated. Further validation studies, such as replication studies for sporadic IA groups or functional analysis of the corresponding genes are needed. Second, the basic assumption of this study was that there would be some rare variants with strong effects that could explain the IAs of each family. However, the IAs in the families may be caused by environmental factors or common genetic variants, rather than rare variants, even though we have selected the most genetically enriched families with FIA in our database. Third, there were no candidate variants that were only found in the affected members of family B, and we thus had to find the most probable candidate (*NTM*) by assuming reduced penetrance. In addition, although the variants in *PLOD3* and *CHST14* genes were segregated in families A and C, the number of affected members may not be sufficient to exclude the possibility of false-positive results. Lastly, some participants only underwent magnetic resonance angiography, which may have produced false-negative or false-positive findings, especially for tiny aneurysms. Despite these limitations, our study presented the use of a methodology for finding rare PVs using WES for IAs, a relatively common multifactorial disease. Further familial studies with more severe phenotypes and more affected members would be able to identify additional candidate genes with higher confidence.

In conclusion, we studied three families that were genetically enriched for IA and performed WES to identify possible IA-associated variants. We found that the rare, potentially deleterious variants in *PLOD3*, *NTM*, and *CHST14* are likely responsible for a subset of FIAs. Our findings may contribute to the understanding of IA pathogenesis, the establishment of an FIA genetic database in Korea, and further validation of IA candidate genes.

Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

Acknowledgments

We would like to thank Romain Bourcier, Gervaise Loirand, and Hubert Desal from INSERM, CNRS, UNIV Nantes, l'institut du thorax for sharing valuable information, meetings, and discussions on the topic of familial aneurysm.

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Funding Statement

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government

(MSIT) (No. 2018R1A2B6003143).

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