Activated leukocyte cell adhesion molecule/cluster of differentiation 166 rs10933819 (G>A) variant is associated with familial intracranial aneurysms

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Abstract. Rupture of intracranial aneurysms (IAs) is the most common cause of subarachnoid hemorrhage (SAH). Currently, there is sufficient evidence to indicate that inflammatory responses contribute to aneurysm rupture. Moreover, the familial occurrence of SAH suggests that genetic factors may be involved in disease susceptibility. In the present study, a clinically proven case of IA in a patient who is a heterozygous mutation carrier of the activated leukocyte cell adhesion molecule (ALCAM)/cluster of differentiation 166 (CD166) gene, is reported. Genomic DNA was extracted from two siblings diagnosed with SAH and other available family members. A variant prioritization strategy that focused on functional prediction, frequency, predicted pathogenicity, and segregation within the family was employed. Sanger sequencing was also performed on the unaffected relatives to assess the segregation of variants within the phenotype. The verified mutations were sequenced in 145 ethnicity-matched healthy individuals. Based on whole exome sequencing data obtained from three individuals, two of whom were diagnosed with IAs, the single-nucleotide variant rs10933819 was prioritized in the family. Only one variant, rs10933819 (G>A), in ALCAM co-segregated with the phenotype, and this mutation was absent in ethnicity-matched healthy individuals. Collectively, ALCAM c1382 G>A p.Gly229Val was identified, for the first time, as a pathogenic mutation in this IA pedigree.

Introduction

Subarachnoid hemorrhage (SAH) due to rupture of intracranial aneurysms (IAs) is a devastating type of stroke (1). Despite progress in diagnosis and treatment, the incidence and 30-day mortality rates of SAH have remained stable for more than three decades (2).

However, the primary cause of IA rupture is not clearly understood. Previous studies have shown that chronic vascular inflammation, hemodynamic stress, and other factors lead to arterial wall remodeling, smooth muscle cell proliferation, and aneurysm rupture (3). In addition to known risk factors, genetic predisposition and family history play a significant role in aneurysm formation and progression (4). Population-based studies have reported that first-degree relatives of patients with IAs have up to eight times greater risk of SAH than the general population (5). Van Hoe et al. reported that individuals with ≥2 affected first-degree relatives had a greater prevalence of IA (average 13.1% vs. 3% in the general population) (6). Whole-exome sequencing (WES) is the most effective and rational approach for determining rare genetic variants and identifying the genetic basis of diseases through the investigation of family forms. Rare variants with relatively large individual effects need to be detected, and recent studies have identified associations in family cases with only a small number of genes (7-14).

The objective of the present study was to identify the genetic risk factor of IAs/SAH in an affected Kazakh family using WES while comparing them to 145 ethnicity-matched healthy individuals.

Materials and methods

Clinical phenotyping. The present study was approved (Approval no. 1/16.02.2015) by the Human Research Ethics Committee of the National Center for Neurosurgery (Nur Sultan, Kazakhstan).

In accordance with the Familial Intracranial Aneurysm (FIA) study protocol (15), the following inclusion and exclusion criteria were applied: Eligible families: i) Families with...
at least two living affected siblings; ii) families with at least two affected siblings, one of whom is living and the other whose genotype can be reconstructed through the collection of closely-related living family members (i.e., spouses and children); iii) Families with ≥3 affected family members (such as cousin, uncle, aunt), two of whom are alive and have living connecting relatives; and iv) families with ≥3 affected family members, with one living affected and at least one other affected relative whose genotype can be reconstructed through the collection of closely related living family members.

The exclusion criteria included a family history of polycystic kidney disease, Ehlers-Danlos syndrome, Marfan syndrome, fibromuscular dysplasia, Moya-Moya syndrome, or failure to obtain informed consent from the patient or family members. Patients with a history of neurological or connective tissue diseases were excluded.

Eventually, a large family of Kazakh ethnicity (Fig. 1A) with two IA- and SAH-affected individuals among the 20 living members were recruited. IA cases and SAH-affected individuals were examined independently by three experienced neurologists from the Department of Vascular and Functional Neurosurgery of the National Center for Neurosurgery (Nur Sultan, Kazakhstan) using computed tomography or magnetic resonance angiography (Fig. 1B). Peripheral blood samples were obtained from two IA-affected individuals (II:4 and II:6) and two available family members. The clinical characteristics of these four participants are shown in Table I. Written informed consent was obtained from all participants.

A total of 145 healthy individuals were selected from the control group in our previous study of sporadic IA cases (16). The control group comprised healthy individuals aged 18-80 years old with no personal or family history of IA, SAH, or other neurological disorders (such as arteriovenous malformations of the brain, cavernous angiomas, brain tumors, craniocerebral trauma, and connective tissue diseases including Marfan syndrome and Ehlers-Danlos syndrome). All the individuals included in the present study were unrelated. In all cases, IA was diagnosed using computed tomography angiography (CTA) or selective cerebral angiography (SCA). The participants were only of Kazakh nationality.

WES analysis. Initially, only three family members [the proband (II:4) and two siblings (II:2, II:6)] were available for WES (Fig. 1A). From the collected pedigree with IAs, genomic DNA was isolated from two affected siblings (II:4, II:6) and one unaffected individual (II:2) using the Promega Wizard Genomic DNA Purification Kit (cat. no. A1125; Promega Corporation) according to the manufacturer’s protocol. DNA quantification was performed using PicoGreen method (cat. no. P7589; Invitrogen; Thermo Fisher Scientific, Inc.) and sequenced on a Novaseq 6000 platform (Illumina, Inc.) with 150 bp paired-end reads. Single-nucleotide variants (SNVs) and insertion/deletions (indels) were detected using the GATK best practice guidelines (17). ANNOVAR (annotation of genetic variants) tools were used to annotate the variants for location and the corresponding gene and transcript length (18).

A flowchart detailing variant filtering is illustrated in Fig. 2.

SNVs located in the intron region and synonymous variants that did not affect the splicing site were excluded. Variants with a minor allele frequency (MAF) >0.01 in the 1000 Genomes database (http://www.1000genomes.org) and exome aggregation consortium (ExAC; http://exac.broadinstitute.org) were discarded. Programs were used to evaluate the potential pathogenicity of the SNVs, including SIFT (https://sift.jcvi.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2), LRT (http://www.genetics.wustl.edu/jlab/lrt_query.html) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar). 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/), ExAC (http://exac.broadinstitute.org/), and the genome aggregation database (gnomAD; http://gnomad.broadinstitute.org/).

Variant confirmation. To confirm the variants identified by WES, Sanger sequencing was performed on the DNA obtained from peripheral blood samples of four members of the participating family. Additionally, the DNA from the son of the proband (the only one available in the next generation) was sequenced by the same method. Moreover, the prevalence of the variant in 145 ethnicity-matched healthy individuals from our previous study on sporadic cases of IA, was analyzed (16). Specifically, polymerase chain reaction (PCR) primers (forward and reverse) were designed, and their sequences are listed in Table I. Amplified PCR was performed with a final volume of 20 µl, containing 50 ng/µl of the genomic DNA, 10 pmol of forward and reverse primers (ALCAM_F and ALCAM_R), 0.4 µl of Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Inc.), 2.5 mM of dNTPs (Fermentas; Thermo Fisher Scientific, Inc.), and 4 µl of 5X PCR buffer. The thermal cycling conditions included initial denaturation for 30 sec at 98°C, followed by 25 cycles of denaturation at 98°C for 10 sec, annealing at 58°C for 10 sec, elongation at 72°C for 20 sec, and a final elongation for 10 min at 72°C. PCR products confirmed by gel electrophoresis were purified using Exo-Sap enzymes (cat. nos. EN0581 and EF0651; Thermo Fisher Scientific, Inc.). DNA sequencing was performed using the BigDye Terminator Cycle Sequencing v3.1 kit (cat. no. 4337455; Applied Biosystems; Thermo Fisher Scientific, Inc.). DNA sequencing analysis was performed using an ABI 3730XL Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Using co-segregation analysis, SNVs with phenotypes confirmed in the affected family members and absent in the non-family member controls were identified as susceptibility genes for IAs.

The Conserved Domains Database of NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and UniProt (https://www.uniprot.org/uniprot/Q13740#family_and_domains) were searched to analyze conservation of the protein in evolution.

Results

Clinical data. The proband (II:4, Fig. 1A) was a 59-year-old Kazakh man diagnosed with SAH. CTA revealed multiple
aneurysms, with one ruptured aneurysm in the anterior communicating artery and two unruptured IAs in the middle cerebral artery (II:4; Fig. 1B). No other cerebrovascular disease was found in the proband, except for aneurysms. Previously, his brother (II:6) was affected by SAH at 50 years of age. His brother was 52 years old at the time of the study. The patient had a giant aneurysm in the middle cerebral artery (II:6; Fig. 1B). The diagnosis was independently confirmed by two neurologists.

Furthermore, one of the older sisters (II:1) of the proband died at the age of 48 years after an episode suggestive of a hemorrhagic stroke. The other sister (II:2) had hypertension.

The niece (IV:1, 22 years old) of the proband was diagnosed with early-onset hypertension. The father of the proband had no familial history of IA and died at 70 years of age, whereas the mother of the proband was diagnosed with ischemic heart disease and hypertension and died at 57 years of age.

Figure 1. Family pedigree and clinical images. (A) Pedigree of the family of the proband used for whole-exome and Sanger sequencing. (B) Magnetic resonance angiography images of the family members with an intracranial aneurysm (identified by the arrow) [clinically affected cases: II:4 (left) and II:6 (right)].
Therefore, blood samples from the parents of the proband were unavailable for mutation analysis. Two IA-affected (II:4 and II:6) and one unaffected (II:2) individual, from whom sufficient DNA samples were available, were selected for WES analysis (Table II).

WES analysis. WES analysis was conducted using DNA samples isolated from the blood of three selected individuals. A total of 13.6-17.5 billion bases were generated for each individual, with an average coverage depth of 97.9X. In addition, 96.35% (95.85-96.65%) of the target exon regions were covered by at least 200X (Table III). A flowchart detailing variant filtering is illustrated in Fig. 2. After alignment and a series of quality control procedures, a total of 2,426,633 SNVs were identified (from three individuals). Novel heterozygous variants in the coding region, as predicted by conceptual translation, which affect protein-coding sequences, were investigated. After filtering against references from the public ExAC database <0.01, 1,330 SNVs were retained. Furthermore, benign or tolerated variants, as predicted by PolyPhen-2, were removed. This resulted in 713 variants (Table SI). A dominant mode of inheritance was then assumed. This inheritance mode assumes that one risk allele is sufficient to be affected by the disease. In our case, the criteria were to have a wild-type genotype in the unaffected individual and a heterozygous or homozygous mutant genotype in individuals with IA.

Finally, one candidate genetic variant, activated leukocyte cell adhesion molecule (ALCAM) c1382 G>A (p.Gly229Val) was selected, by considering known disease genes and ontology associations with SAH morphogenesis or other known diseases. Considering its pathogenicity, this variant was prioritized as a putative candidate, although it was not found in the ClinVar database. The score predictor of c1382 G>A substitution pathogenicity was 0.994 for the PolyPhen2 algorithm, and this mutation was predicted as 'probably damaging'. The allele A MAF of rs10933819 polymorphism was 0.0010 in the ExAC database.

Validation and co-segregation analysis of candidate variants. Candidate genetic variants in the four family members were directly sequenced using Sanger sequencing. Moreover, the variant was validated in 145 healthy individuals to eliminate any false-positive findings.

The missense variant c1382 G>A (p.Gly229Val) in ALCAM was confirmed to be heterozygous in the proband (pedigree II:4; Fig. 3). A heterozygous mutation in his brother with IA (pedigree II:6; Fig. 3A) was also identified, whereas his elder sister and son had the wild-type gene and a normal phenotype (pedigrees II:2 and III:7, respectively; Fig. 3A). These results indicated that the c1382 G>A (p.Gly229Val) gene co-segregated with the disease phenotype in the family members that were tested and were absent from the unaffected second-generation members. Furthermore, this mutant was absent in 145 normal ethnically-matched individuals. Collectively, these findings showed complete co-segregation.

Table I. Primer sequences and characteristics.

| Primer name | Sequence (5’–3’) | T<sub>a</sub> (˚C) | Amplicon length (bp) |
|-------------|------------------|----------------|---------------------|
| ALCAM_F     | tctctctgtgatattcgt | 58  | 560                 |
| ALCAM_R     | attcagaagagcaagt | 58 |                     |

ALCAM RefSeqGene on chromosome 3: NCBI Reference Sequence NG_029729.1, (172829…..173370). ALCAM, activated leukocyte cell adhesion molecule; Ta, annealing temperature; F, forward; R, reverse.
of the mutation in the pedigree of the IA/SAH-affected family, indicating its role in the pathogenesis of this disease.

Conservation of the protein in evolution. The cluster of differentiation 166 (CD166) antigen (ALCAM) has five conserved Ig-like domains (Fig. 3B): V-type 1, V-type 2, C2-type 1, C2-type 2, and C2-type 3. The missense mutation, p.Gly229Val, is located in the Ig-like V-type 2 domain of the CD166 antigen. The CD166 antigen protein sequences from several species, including humans, chimps, mice, rats, rabbits, bovines, and pigs, were compared. Multiple sequence alignment analysis revealed that the Gly229 residue was strongly conserved (Fig. 3B).

Discussion

Previous research has shown that the sex and hormonal background of a patient, alcohol abuse, and the presence of arterial hypertension (AH) are general risk factors for the development of aneurysmal SAH (19). Environmental and genetic factors play important roles in the pathogenesis and rupture of IAs (20).

A practical WES method was used to systematically explore rare coding variants and identify potentially causative variants in a family case of IA. After sequencing two affected individuals and one unaffected member, bioinformatic filtering revealed that only ALCAM c1382 G>A was a candidate variant. Sanger sequencing showed that this variant was fully co-segregated with definite IA phenotypes in the family.

Very little information is available on the rs10933819 polymorphism of ALCAM. ALCAM (or CD166) is an immunoglobulin-like protein belonging to the cell adhesion molecule family with three C2 and two V extracellular domains. It functions by mediating homotypic and heterotypic cell-to-cell contacts via the CD166-CD6 interaction (21). Notably, the heterotopically interaction affinity with CD6 is much higher than the homotypic interaction with itself. Hassan et al (21) reported that the interaction with CD166 was 100-fold lower than that with CD6. Together, the heterotypically interacting CD6 and CD166 play a role in T cell activation. CD6 is a lymphocyte membrane protein that contains an scavenger receptor cysteine-rich (SRCR) domain that interacts with CD166. Interestingly, soluble monomeric forms inhibit T cell activation.

Cell adhesion molecules (CAMs) are essential for inflammatory processes. They are highly expressed in vascular endothelial tissues (22). CD166-CD6 interactions form immunological synapses at T cells and CD166-presenting cells. ALCAM contributes to the movement of T cells and monocytes through the endothelium and blood-brain barrier (18). ALCAM and vascular cell adhesion molecule (VCAM) levels were high in patients with systemic lupus erythematosus (SLE) vs. healthy individuals, and there is a theory that elevated expression of ALCAM may breach T cell tolerance (23). Willrodt et al (24) investigated the role of ALCAM in corneal allograft rejection. The cornea is usually avascular, but inflammation-induced neovascularization in the cornea of the receptor increases the risk of allograft rejection. ALCAM blockade reduced the angiogenic process and T cell activation, which adds to the evidence regarding the dual role of ALCAM in vasculogenesis and mediation of dendritic cells.

In 2001, Ohneda et al (25) described the role of ALCAM in hematopoietic and endothelial cell development. However, they reported that ALCAM is highly expressed in the YSCL-72 endothelial cell line (derived from the yolk sac) and not in EOMA (derived from the adult aorta). Moreover, they experimentally examined the role of ALCAM in vascular tube formation. They found that ALCAM facilitates cord-like endothelial cluster formation but has no effect on sheet-like clusters.
Thus, ALCAM physically interacts with the T cell-expressed scavenger receptor CD6, endothelial L1CAM, and galectin-8 (24). Notably, the other lectins, namely galectin-1 and galectin-3, have been reported to be associated with abdominal aortic aneurysms (26,27).

However, secreted ALCAM (sALCAM) performs differently. Galectin-8 has been reported to play a role in blood and lymph vessel angiogenesis (28). Noticeably, galectin-8 interacts with podoplanin in lymphatic angiogenesis and with ALCAM in vascular endothelial cells. Galectin-3 inhibits galectin-8 (29). Cheng et al reported higher galectin-3 expression and lower galectin-8 expression in endothelial cells than in the epithelial cells (29).

In summary, a rare variant, c1382 G>A (p.Gly229Val), in ALCAM, which has not been previously reported in the Kazakh population was identified. The present study has the limitation that ALCAM c1382 G>A was fully co-segregated with definite IA phenotypes in only one family. Therefore, further studies are required to evaluate the functional impact of ALCAM c1382 G>A, which may warrant further replication and biological investigations related to IA.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the SRA database (https://www.ncbi.nlm.nih.gov/sra) repository under accession no. PRJNA819841 (BioProject) (SRR18475941, SRR18475942 and SRR18475943).
Authors' contributions
AA, KM, YM and EZ conceived the study. GK, AA, EZ, KM and YR wrote the first draft of the manuscript. YM, BD and SA performed the sample collection, processed the clinical data and test results. IA conducted the design and synthesis of primers. KK performed the laboratory experiments. TU, KK and AA validated the data. GK, SA and YR analyzed data. TU and KM prepared the figures. EZ and KM confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate
The present study was approved (approval no. 1/16.02.2015) by the Human Research Ethics Committee of the National Center for Neurosurgery (Nur Sultan, Kazakhstan). Written informed consent was obtained from all participants.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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