A haplotype spanning two genes, ELN and LIMK1, decreases their transcripts and confers susceptibility to intracranial aneurysms

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The rupture of an intracranial aneurysm (IA) results in subarachnoid hemorrhage, a catastrophic neurological condition with high morbidity and mortality. Following-up on our previous genome-wide linkage study in Japanese population, we extensively analyzed a 4.6 Mb linkage region around D7S2472 on 7q11 by genotyping 168 single nucleotide polymorphisms (SNPs). SNP association and window scan haplotype-based association studies revealed a susceptibility locus for IA on a single LD block covering the 3'-untranslated region (3'-UTR) of ELN and the entire region of LIMK1. An association study with 404 IA patients and 458 non-IA controls revealed that the ELN 3'-UTR G(1659)C SNP has the strongest association to IA (P = 0.000002) and constitutes a tag-SNP for an at-risk haplotype, which contains two functional SNPs, the ELN 3'-UTR (1502) A insertion and the LIMK1 promoter C(-187)T SNP. These allelic and haplotype-based associations were confirmed in a Korean population. Ex vivo and in vitro analyses demonstrate that the functional impact of both SNPs is the decrease of transcript levels, either through accelerated ELN mRNA degradation or through decreased LIMK1 promoter activity. Elastin and LIMK1 protein are involved in the same actin depolymerization signaling pathway; therefore, these lines of evidence suggest a combined effect of the SNPs in the at-risk haplotype possibly by weakening the vascular wall and promoting the development of IA.

INTRODUCTION

Subarachnoid hemorrhage (SAH) is most often the result of a ruptured intracranial aneurysm (IA). Patients who survive the acute phase are often left with a substantial disability and impaired quality of life as a result of major neurological deficits. The annual incidence of SAH due to ruptured IA is 18 to 23 per 100,000 (1-3), whereas the prevalence of unruptured IA detected by MR angiography, CT angiography or digital subtraction angiography in the Japanese population has been reported to be much higher, around 6-7% (4,5). The prevalence of unruptured IAs detected at autopsy ranges from

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0.2–8.9\% (6,7). Despite continuing improvement of diagnostic methods, treatment techniques and medical care, the 30-day mortality of aneurysmal SAH is unchanged and remains a major public health concern (8).

Several factors such as smoking, ethnicity, female gender (especially in menopause) and hypertension are involved in the pathogenesis of IA. Epidemiological studies demonstrate a strong genetic influence: the prevalence of unruptured IA is significantly higher (10.5–13.5\%) in a Japanese subgroup with a family history of IA (4,5). The risk of SAH is four times higher in first degree relatives and six times higher in siblings of patients with SAH than in the general population (9,10). However, the etiology and pathogenesis of IA formation remain largely unknown. Several studies have attempted to identify genes contributing to the susceptibility to IA, focussing on candidates for allelic association in genes that encode mostly matrix proteins such as endoglin, lysyl oxidase and matrix metalloproteases. But they did not yield consistent results (11–17). Genetic linkage study of IA families represents a more systematic approach. In the first genome-wide linkage study of IA with 104 Japanese affected sib pairs, we identified a significant linkage to chromosome 7q11 (MLS = 3.22 near D7S2472) (18). Although this result was confirmed in Utah families of European origin (19), another Japanese study failed to find this linkage (20). The elastin gene (ELN), a positional and functional candidate gene located within the linkage region, has been investigated for allelic association with IA, but the results were divergent (18,21,22). Another candidate gene, collagen α2(I) (COL1A2), has been identified as a genetic risk factor in IA patients with a family history (14), but there has been no subsequent replication study.

In this study, we performed systematic single nucleotide polymorphism (SNP) fine-mapping of the 4.6 Mb linkage region surrounding the peak linkage marker D7S2472. One hundred and sixty-six SNPs of 26 positional genes were initially detected and genotyped. Disease-related linkage disequilibrium (LD) was assessed using the sliding window method. Positive association with IA was found in Utah families of European origin (19), but these results were not harbor an IA as verified by radiological examination. One hundred and sixty-six out of the genotyped 188 SNPs were polymorphic in our Japanese population (allelic frequency > 0.01) and within expectations of the Hardy–Weinberg equilibrium. In the pairwise LD analysis, mainly gene-specific LD blocks were observed (Fig. 1A). The 166 SNPs were tested for allelic association evaluated by a permutation test with 10 000 iterations. Fifteen SNPs showed significant association (permutation P < 0.05), and nine were clustered within a region of ~400 kb centromeric to D7S2472 (Fig. 1B). The locus contains ELN, LIMK1 and the cytoplasmic linker 2 gene (CYLN2). To increase the statistical power to detect genetic association, systematic haplotype analyses in the 4.6 Mb region were performed using the sliding window method with two to four adjacent SNPs as shown in Figure 1C and D. Haplotypes comprising two to four adjacent SNPs with significant association (permutation P < 0.05) were also clustered within the locus containing ELN, LIMK1 and CYLN2, thus confirming the single-SNP associations. After setting the threshold level for significance of the permutation P-value to below 0.01, associations were observed only in one distinct LD block, which included the 3’ part of ELN and LIMK1 in both single and global haplotype-based comparisons (Fig. 1C and D).

**Search for susceptibility SNPs**

In the initial screening, the boundaries of the ELN–LIMK1 LD block were observed between intron 21 T(+17)C and intron 31 A(+242)G SNPs of ELN (|D′| = 0.16), and between intron 2 G(–1626)C and intron 12 T(–156)C SNPs of LIMK1 (|D′| = 0.57). Within the LD block, five SNPs were over-represented in IA patients with statistical significance, but all of them were intronic or intergenic. To identify the causal SNPs in the LD block, the genomic DNA of the 96 familial IA patients initially screened were re-sequenced at exons 22–33 and 3’-UTR of ELN. We also re-sequenced a 1.5 kb promoter region and exons 1–12 of LIMK1 to identify novel SNPs. Several intronic or intergenic SNPs available from the public database were also genotyped. Within the region, we confirmed a total of 16 SNPs including the six SNPs used for the initial screening. They formed highly structured LD blocks spanning from at least intron 29 of ELN to intron 10 of LIMK1 (|D′| > 0.85). Although this region did not contain SNPs in the coding regions, we identified two 3’-UTR SNPs in ELN and three promoter SNPs in LIMK1. We then genotyped these five SNPs in 404 cases and 458 non-IA control individuals including the initially screened subjects. Allelic association was tested by chi-square and permutation tests with 1 000 000 iterations. All five SNPs showed significant association with IA (permutation P < 0.05) as summarized in Table 1. The most significant association was observed with the ELN 3’-UTR G(+659)C SNP (χ² = 22.5, df = 1, P = 0.000002, permutation P = 0.000001, odds ratio = 3.11). In chicken specimen, the 3’-UTR of ELN is known to play a key role in post-transcriptional regulation by binding cytosolic protein to the specific sequences (23,24). Thus, the 3’-UTR SNPs might directly affect the stability of the transcripts, despite the fact that ELN 3’-UTR sequence involving the ELN 3’-UTR G(+659)C SNP and...

**RESULTS**

**Initial screening of the 4.6 Mb linkage region on 7q11**

We initially screened 188 SNPs in the 4.6 Mb linkage region surrounding the peak linkage marker, D7S2472, in 96 familial IA patients and 160 non-IA controls of Japanese ethnicity. The SNPs were selected according to the criteria (allelic frequency > 0.01, gene-based) of the database of Assays-on-Demand™ SNP Genotyping Products (Applied Biosystems, Tokyo, Japan). The 96 IA patients consisted of 78 probands from nuclear families that had participated in our genome-wide linkage study (18) and 18 patients with a family history of IA. The 160 selected controls, all over 50 years of age, did...
Figure 1. Results for the pairwise LD analysis and the sliding window scan of the 4.6 Mb linkage region around D7S2472 on 7q11. (A) Genes, in which at least three SNPs were genotyped, are depicted in the upper panel. The pairwise LD block of the tested region evaluated by the $D'$ or $r^2$ statistics is shown. (B) Single SNP association analyses using 96 familial cases and 160 non-IA controls. SNPs are shown in single columns along a horizontal line without taking the intervals into account. The statistical value is represented by the logarithm of $P$-value ($-\log P$) in the vertical line. (C) and (D) Haplotypes were constructed using the sliding window method with two to four adjacent SNPs. Frequencies of all estimated haplotypes were compared between 96 familial cases and 160 non-IA controls and haplotype-based association was tested using a single haplotype comparison (C) or global test (D). All $P$-values were generated after 10 000 iterated permutations.
To examine the functional impacts of the ELN promoter activity of one or more of the SNPs on insertion SNP is responsible for the reduction in the amount of the stability of the mRNA were investigated (Fig. 3A, upper panel). The constructs corresponded to three common ELN 3’-UTR sequences that covered 99% of the tested population (M/M, m/M and m/m—representing minor (m) and major (M) allele at the +502 and +659 sites) (Fig. 3A). They were transfected in parallel into HEK293 cells. All inserted sequences resulted in reduced luciferase activity compared with the control vector without insertion, indicating that the inserted ELN 3’-UTR sequences influenced mRNA stability. The constructs containing the ELN 3’-UTR (+502) A insertion SNP (m at +502), regardless of the +659 genotype, showed significantly reduced luciferase activity: the M/m haplotype had 48.0% and the m/m haplotype 53.5% activity compared with the M/M haplotype. There was no significant difference between the m/M and the m/m haplotypes, suggesting that the ELN 3’-UTR G(+659)C SNP does not affect the stability of the mRNA (Fig. 3B, left). A stretch of AU-rich sequence between the first and second poly(A) signals of ELN 3’-UTR was found, which might accelerate the degradation of mRNA (25). To eliminate the effect of the AU-rich sequence, we generated two additional reporter constructs (Fig. 3A, the two poly(A) signals of ELN were removed and a poly(A) signal derived from a pGL3 vector was used). These constructs were transfected into both HEK293 and HCT116 cells. When compared with the previous constructs, elevated luciferase activity was observed in the HEK293 cells (61.3% for the M/M haplotype). The construct containing the ELN 3’-UTR (+502) A insertion SNP showed that the ELN 3’-UTR (+502) A insertion SNP have a reduced level of ELN transcription (Fig. 2A and B). The cells heterozygous for the ELN 3’-UTR G(+659)C SNP did not exhibit any additional impact (Fig. 2A). Although there was only one carrier of the ELN 3’-UTR G(+659)C SNP, these results suggest that the ELN 3’-UTR (+502) A insertion SNP is responsible for the reduction in the amount of ELN transcripts. Analysis of the LIMK1 transcripts showed that cells heterozygous for all three promoter SNPs of LIMK1 also have significantly reduced LIMK1 transcription, suggesting the impact of one or more of the SNPs on the promoter activity of LIMK1 (Fig. 2C).

In vitro functional analyses of ELN 3’-UTR SNPs

To confirm the ex vivo findings on the effect on ELN transcripts, we first cloned three types of ELN 3’-UTR sequences into the downstream region of an SV40 promoter-luciferase gene transcriptional unit, and the effect of the insertions on the mRNA stability were investigated (Fig. 3A, upper panel). The constructs corresponded to three common ELN 3’-UTR haplotypes that covered 99% of the tested population (M/M, m/M and m/m—representing minor (m) and major (M) allele at the +502 and +659 sites) (Fig. 3A). They were transfected in parallel into HEK293 cells. All inserted sequences resulted in reduced luciferase activity compared with the control vector without insertion, indicating that the inserted ELN 3’-UTR sequences influenced mRNA stability. The constructs containing the ELN 3’-UTR (+502) A insertion SNP (m at +502), regardless of the +659 genotype, showed significantly reduced luciferase activity: the M/m haplotype had 48.0% and the m/m haplotype 53.5% activity compared with the M/M haplotype. There was no significant difference between the m/M and the m/m haplotypes, suggesting that the ELN 3’-UTR G(+659)C SNP does not affect the stability of the mRNA (Fig. 3B, left). A stretch of AU-rich sequence between the first and second poly(A) signals of ELN 3’-UTR was found, which might accelerate the degradation of mRNA (25). To eliminate the effect of the AU-rich sequence, we generated two additional reporter constructs (Fig. 3A, the two poly(A) signals of ELN were removed and a poly(A) signal derived from a pGL3 vector was used). These constructs were transfected into both HEK293 and HCT116 cells. When compared with the previous constructs, elevated luciferase activity was observed in the HEK293 cells (61.3% for the M/M haplotype). The construct containing the ELN 3’-UTR (+502) A insertion SNP showed that the ELN 3’-UTR (+502) A insertion SNP have a reduced level of ELN transcription (Fig. 2A and B). The cells heterozygous for the ELN 3’-UTR G(+659)C SNP did not exhibit any additional impact (Fig. 2A). Although there was only one carrier of the ELN 3’-UTR G(+659)C SNP, these results suggest that the ELN 3’-UTR (+502) A insertion SNP is responsible for the reduction in the amount of ELN transcripts. Analysis of the LIMK1 transcripts showed that cells heterozygous for all three promoter SNPs of LIMK1 also have significantly reduced LIMK1 transcription, suggesting the impact of one or more of the SNPs on the promoter activity of LIMK1 (Fig. 2C).
protein was fractionated with anion exchange chromatography in which the protein was eluted at about 0.4 M NaCl (data not shown). Thus, the reduction in ELN transcripts associated with the ELN 3'UTR (+502) A insertion SNP may well be due to the binding of an unidentified cytoplasmic factor that accelerates degradation of the transcript.

**In vitro functional analyses of the LIMK1 promoter SNPs**

In the promoter region of LIMK1, two common haplotypes were observed, the triple minor −961A/−428A/−187T (A/A/T) haplotype and the triple major −961G/−428G/−187C (G/G/C) haplotype, both of which covered >95% of the tested population. To confirm the ex vivo findings derived from the RT–PCR study of the UASMC, we cloned these two promoter haplotypes into the upstream region of the luciferase gene (Fig. 4A, top). The effects of the promoter sequences on transcriptional activity were examined in HEK293 cells and HCT116 cells. Both inserted sequences showed strong promoter activity (Fig. 4B). The construct containing the A/A/T haplotype showed reduced transcriptional activity compared with the G/G/C haplotype (31.9% reduction, P = 0.00003, Figure 2. Genotype-specific differences in ex vivo expression of ELN and LIMK1. RNA derived from UASMCs, with the genotypes shown in the figure, was subjected to RT–PCR or real-time PCR using SYBR Green to estimate the amount of genotype-specific transcripts of ELN and LIMK1. (A and B) The amount of ELN transcripts due to genotype was evaluated either by RT–PCR or by real-time RT–PCR. The ELN 3'UTR (+502) A insertion allele is shown as A-IN, whereas the major allele at the site is shown as ‘−’. (C) The amount of LIMK1 transcripts according to the genotype was evaluated with real-time RT–PCR. Genotypes at the three sites of LIMK1 as seen in the tested cells were all heterozygous. The Welch’s t-test was applied for statistical evaluation.
Figure 3. *ELN* 3'-UTR allele-dependent transcriptional regulation in HEK293 and HCT116 cells and EMSA with riboprobes containing the *ELN* 3'-UTR (+502) A insertion SNP. (A) *ELN* 3'-UTR with or without the poly(A) signal and AU-rich sequence was subcloned into the downstream region of the reporter gene pGL3-promoter vector, as described in ‘Materials and Methods’. M/M, m/M and m/m denote the minor (m) and major (M) allele at the +502 and +659 sites. +502 Major and +502 A-IN denote the major and minor allele at the +502 site. (B) Each reporter vector was transfected into HEK293 or HCT116 cells, and the firefly luciferase activity was normalized with the Renilla luciferase activity of co-transfected phRL-TK. The data are expressed as relative activity, mean ± SE of six dishes. (C) DIG-labeled riboprobes containing the (+502) A insertion or the major type (A-IN and Major, respectively) were incubated with HEK293 cytoplasmic extract. The arrows point to specific binding of the A-IN riboprobe.
Welch’s t-test). To identify the critical SNP responsible for the difference in transcriptional activity, we generated deletion constructs removing the −961 site (Δ-A/T and Δ-G/C) and subsequently the −428 site (ΔΔ-T and ΔΔ-C) (Fig. 4A).

The effects on transcriptional activity were examined in the HEK293 cells. Each deletion construct showed elevated luciferase activity when compared with both the three-SNP (A/A/T and G/G/C) constructs. Furthermore, the differences
in transcriptional activity between alleles still remained (24.6% reduction, \( P = 0.0006 \), Welch’s \( t \)-test) even after the -961 and -428 sites were deleted. Similar results were also observed in the HCT116 cells (\( P = 0.004 \), Welch’s \( t \)-test) (Fig. 4B, right). These results indicate that the \( LIMK1 \) promoter C(-187)T SNP is responsible for the difference in transcriptional activity.

To examine nuclear factor binding, two DIG-labeled double-stranded oligonucleotides containing the -187C and -187T alleles (Fig. 5A) were designed for EMSA using HEK293 nuclear extract. -187C allele-specific DNA-protein binding was observed after incubation with the HEK293 nuclear extract, and there was no specific competition with the -187T allele (Fig. 5B and C). EMSA was also performed for two other SNP sites, the -961G or -961A and the -428G or -428A SNPs, but there was no allelic difference in DNA–protein binding (data not shown). The TFSEARCH program showed an 8 bp sequence containing -187C appears to be the AP2-alpha-binding motif. (B) HEK293 cell nuclear extract was incubated with -187C and -187T probes. The arrow points to specific binding. (C) The specific interaction with -187C probe was competed with various amounts of non-labeled -187C or -187T competitors (5-, 10- and 20-fold from left to right). (D) The -187C probe was reacted with AP2-enriched extract (AP2 ext., Promega) and the specific binding supershifted with the anti-AP2 antibody was observed. The specific binding with nuclear extract from HEK293 cells was not supershifted with the antibody.

![Figure 5. EMSA with oligonucleotide containing the \( LIMK1 \) promoter SNPs. (A) The double-stranded oligonucleotide probe containing either the -187C or the -187T allele is shown. The computer program TFSEARCH based on TRANSFAC databases, available at website (http://www.cbrc.jp/research/db/TFSEARCH.html), was used to predict potential binding sites of transcription factors in the regulatory region. The 8 bp sequence containing -187C appears to be the AP2-alpha-binding motif. (B) HEK293 cell nuclear extract was incubated with -187C and -187T probes. The arrow points to specific binding. (C) The specific interaction with -187C probe was competed with various amounts of non-labeled -187C or -187T competitors (5-, 10- and 20-fold from left to right). (D) The -187C probe was reacted with AP2-enriched extract (AP2 ext., Promega) and the specific binding supershifted with the anti-AP2 antibody was observed. The specific binding with nuclear extract from HEK293 cells was not supershifted with the antibody.](https://academic.oup.com/hmg/article/15/10/1722/2355911)

Analyses of the combinational effect of the \( ELN \) and \( LIMK1 \) SNPs

We identified two functional SNPs from the \( ELN-LIMK1 \) LD block, the \( ELN \) 3'-UTR (+502) A insertion and the \( LIMK1 \) promoter C(-187)T SNPs, both significantly associated with IA. However, the strongest association was observed with the \( ELN \) 3'-UTR G (+659) C SNP having the highest odds ratio (Table 1), which had no detectable functional impact on the \( ELN \) transcript. To examine the relationships among these three SNPs, they were combined to construct haplotypes. The haplotype-based associations were tested with a 1 000 000 iterated permutation test. Four major haplotypes (each frequency >5%) were observed in 404 IA patients (Table 2). One haplotype, the triple minor (A/C/T) haplotype, was over-represented in IA patients, showing a highly significant difference in frequency between the IA and non-IA control group (\( \chi^2 = 19.2, \ df = 1, \ P = 0.000012 \), permutation
A global haplotype comparison also showed significant differences between the IA and non-IA control group (permutation $P = 0.00033$), further indicating the involvement of the $ELN$–$LIMK1$ haplotypes in susceptibility to IA. Thus, the A/C/T haplotype both represents individuals statistically at-risk with susceptibility to IA and implicates two SNPs that reduce $ELN$ and $LIMK1$ transcripts in the pathology of the disease. The most significantly associated SNP, the $ELN$ 3′-UTR G(þ659)C SNP, can be regarded as a tag-SNP for the at-risk haplotype.

The association study with Korean samples

Korean samples comprising 195 IA patients and 250 controls were used to confirm the associations observed in the Japanese population. Although the two functional $ELN$ 3′-UTR (+502) A insertion and $LIMK1$ promoter C(−187)T SNPs were not significantly associated with IA, the association with the $ELN$ 3′-UTR G(þ659)C SNP reached a statistical significance ($\chi^2 = 4.89$, df = 1, $P = 0.027$, permutation $P = 0.018$, odds ratio = 1.81) (Table 3). The haplotype-based association study also showed that the A/C/T haplotype was significantly associated with IA ($\chi^2 = 5.56$, df = 1, $P = 0.018$, permutation $P = 0.019$) (Table 4).

### DISCUSSION

Previous linkage and association studies of IA from two different ethnic groups, Japanese and Utah white populations, showed evidence of linkage in a region on chromosome 7q11, although inconsistent results were also reported (18–20). In the current study, the previously found 4.6 Mb linkage region with an LOD score >1 was extensively examined using systematic haplotype-based sliding window scan analyses and LD mapping (18). We were able to pinpoint a susceptibility locus to IA on a single LD block spanning the 3′-UTR of $ELN$ and the entire region of $LIMK1$ in the Japanese population.
samples. Further examination of the SNPs in the LD block using all of the available subjects with IA showed that two functional SNPs were significantly associated with IA. One of them, the ELN 3′-UTR (+502) A insertion SNP (χ² = 4.75, df = 1, P = 0.029, permutation P = 0.029, odds ratio = 1.38), decreased the amount of ELN transcripts by altering the mRNA–protein interaction (Fig. 3). The other, the LIMK1 promoter C(−187)T SNP (χ² = 17.2, df = 1, P = 0.000033, permutation P = 0.000019, odds ratio = 1.97), reduced the promoter activity of LIMK1 by altering AP2 or an unidentified nuclear factor binding (Figs 4 and 5). These functional impacts on the transcripts of the two SNPs were similar in both ex vivo and in vitro findings. In addition, we identified the ELN 3′-UTR G(+659)C SNP, which had the strongest association and highest odds ratio (χ² = 22.5, df = 1, P = 0.000002, permutation P = 0.000001, odds ratio = 3.11), as a tag-SNP for the three-SNP haplotype harboring the ELN 3′-UTR (+502) A insertion and LIMK1 promoter C(−187)T SNPs (Table 2). Similar associations were observed in Korean IA samples (Tables 3 and 4). Thus, the polymorphisms in the haplotype contribute to reduction of both the ELN and LIMK1 transcripts. Although each SNP itself represents an increased risk of development of IA, the two SNPs together may exert a synergistic effect on susceptibility in considering that the risk of development of IA may only play a partial role in genetic risk for IA. In conclusion, we have identified the ELN 3′-UTR G(+659)C SNP as a genetic susceptibility allele for IA in Japanese and Korean populations. It occurs in an at-risk haplotype containing two functional SNPs that reduce the transcript levels of ELN and LIMK1, respectively. Biofunctional analyses show that elastin and LIMK1 protein affect the stability and synthesis of vascular walls by sharing the same elastin signaling pathway. Although the effects observed throughout these experiments were significant and consistent, further replication in independent family-based and case–control samples in different populations is required to confirm the role of the haplotype in genetic risk for IA. In addition, as the carrier frequency of the ELN/LIMK1 haplotype is relatively low, it may only play a partial role in genetic susceptibility to IA.

MATERIALS AND METHODS

Subjects

The Ethics Committees of Tokyo Women’s Medical University, Chiba University, Chonbuk National University and University of Tokyo approved the study protocols, and all participants gave written informed consent. The Japanese DNA samples for the present study were from 404 IA patients (age: 53.9 ± 10.2 years; 254 females/150 males) and 458 controls (age: 61.4 ± 13.0 years; 283 females/495 males). The IA patients included both familial IA patients [78 probands from nuclear families that had been used in our linkage study (18) and 107 patients who had a family history of IA] as well as 219 sporadic IA patients under the age of 60 at diagnosis. The presence of IA was confirmed by digital subtraction...
angiotherapy, three-dimensional CT angiography, MR angiography or surgical findings. The 458 unrelated controls were outpatients of the Department of Neurosurgery of Tokyo Women’s Medical University, Department of Neurosurgery of Chiba University and their nearby affiliated hospitals with diseases other than IA. The controls did not harbor an IA as verified by radiological examination and none had a family history of SAH. All were over 50 years of age to exclude most early-onset diseases.

To confirm allelic and haplotype-based associations observed in Japanese population, we performed a Korean case–control study with DNA samples of patients recruited at Chonbuk National University. The DNA samples were from 195 Korean IA patients (age: 55.2 ± 11.2 years; 141 females/54 males) and 250 Korean controls (age: 65.7 ± 6.4 years; 117 females/133 males). The IA patients were mainly sporadic patients except three familial cases, confirmed by surgical findings. The controls were mainly outpatients without a medical history of SAH, including a few trauma cases. All were over 55 years of age.

Selection and genotyping of SNPs
For initial screening, 188 SNPs located around the marker D7S2472 were chosen from a series of available Assays-on-Demand SNP Genotyping Products (Applied Biosystems). Genomic DNA was examined by TaqMan assay according to the supplier’s manual using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems).

For the second screening, additional SNPs were picked out from the NCBI dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) and IMS-JST JSNP (http://snp.ims.u-tokyo.ac.jp/) databases or identified by direct sequencing. Genotyping was then performed by direct sequencing using BigDye terminator cycle sequencing on an ABI PRISM 3700 DNA analyzer (Applied Biosystems).

Primary culture of UASMCs
We obtained 44 umbilical cords during delivery at the Department of Obstetrics of Tokyo Women’s Medical University and Kosei General Hospital. All of the participants gave written informed consent, and the study was performed under the approval of the Ethics Committee of Tokyo Women’s Medical University and Kosei General Hospital. Umbilical arteries were excised from the cords and cut into small pieces. UASMCs were separated using Hanks buffer containing 2 mg/ml collagenase and cultured in HuMedia-SG medium (Kurabo, Osaka, Japan) supplemented with epithelial growth factor (0.5 ng/ml), basic fibroblast growth factor (2 ng/ml), insulin (5 μg/ml), antibiotics and 5% fetal bovine serum (FBS).

RT–PCR and quantitative real-time PCR analysis
We extracted total RNA from UASMCs using TRIzol reagent according to manufacturer’s instruction (Invitrogen, Tokyo, Japan). RT–PCR was performed with the SuperScript one-step RT–PCR system (Invitrogen). Real-time PCR was carried out on an ABI PRISM 7700 sequence detection system using SYBR Green PCR Master Mix (Applied Biosystems) according to supplier’s manual. Copy numbers of ELN and LIMK1 transcripts were calculated by referring to standard curves and normalized by the total RNA using GAPDH as an internal control.

Transfection and reporter assays
The entire 3′-UTR of ELN containing each observed haplotype was produced by PCR synthesis. Genomic DNA of corresponding genotype was used as a template. These ELN 3′-UTR inserts were subcloned into the XbaI/BamHI-digested pGL3-promoter vector (Promega). Deletion constructs lacking the poly(A) signal sequences were also produced by PCR synthesis using the former constructs as templates. The deletion constructs were subcloned into the XbaI-digested pGL3-promoter vector and verified by direct sequencing.

LIMK1 promoter constructs containing three observed SNPs and 5′-UTR were produced by PCR synthesis using genomic DNA as templates, and ligated into the KpnI/BglIII-digested pGL3-basic vector (Promega). Deletion constructs were also produced by PCR synthesis using the former constructs as templates and ligated into the KpnI/BglII-digested pGL3-basic vector.

HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and antibiotics and HCT116 cells in McCoy’s 5A medium supplemented with 10% FBS and antibiotics. The cells (10⁵ cells/well) were transfected with 0.2 μg of each expression vector and 6 ng of pRL-TK vector (Promega) as an internal control for transfection efficiency, using Fugene-6 (Roche Diagnostics, Tokyo, Japan). At 48 h post-transfection, the cells were solubilized and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Statistical analyses
Differences in allelic frequencies were examined using a case–control design and the chi-square test. Haplotype frequencies for multiple loci were estimated using the expectation–maximization method (SNPAlvyz v3.2 software, DYNACOM, Mobar, Japan). Additionally, the permutation test was performed to test deviation of allelic frequencies of SNPs and haplotypes (35). Distribution of the test statistic was estimated by evaluating the statistics for a random sampling of 10 000 to 1 000 000 iterated permutations by fixing the total numbers of both cases and controls to avoid false-positive results of multiple testing, which is incorporated in SNPAlvyz v3.2 software. We calculated LD between pairs of SNPs using the standard definition of D’ (36) and r² (37).

For all ex vivo and in vitro experiments, mean and standard errors were calculated and statistical analyses were carried out with Welch’s t-test or analysis of variance (ANOVA). For the luciferase assay that compared the three ELN 3′-UTR constructs (M/M, m/M and m/m, as shown in the legend of Fig. 4), post hoc comparisons following ANOVA were carried out by Scheffe’s test.
Electrophoretic mobility shift assay

EMSA was performed with a DIG Gel Shift Kit (Roche Diagnostics). The nuclear and cytoplasmic extracts were prepared from HEK293 cells using a NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce Biotechnology Inc., Rockford, IL, USA). HEK293 cytoplasmic extract was fractionated by Q-Sepharose ion exchange chromatography using HiTrap column (GE, Tokyo, Japan) in 5 mM HEPES buffer, pH 7.9, containing 0.1 mM EDTA with 0–0.6 M NaCl gradient on an ÄKTA explorer (GE). An AP2-enriched nuclear extract was purchased from Promega.

The DIG-labeled 53 nt RNA transcript containing the ELN 3'-UTR (+502) A insertion or the major type alleles were synthesized using a DIG RNA Labeling Kit (T7/SP6) (Roche Diagnostics). In vitro transcription was performed using T7 promoter sequence-attached PCR products synthesized from the former ELN promoter sequence-attached PCR products synthesized from the former ELN reporter constructs. The RNA transcript was heated for 5 min at 85°C and then cooled down to room temperature for 15 min before the following reaction. The HEK 293 cytoplasmic extracts and the fractionated proteins were incubated at 20°C for 30 min with the RNA transcripts in 5 mM HEPES buffer, pH 7.9, containing 0.5 mM MgCl₂, 7.5 mM KCl, 0.5 mM DTT, 0.12 mM EDTA, in a total volume of 15 µl. Protein–RNA complexes were separated by electrophoresis on a 6% polyacrylamide gel in 0.5x TBE running buffer. The DNA–protein complexes were electroblotted onto nylon membrane and the band shift was visualized according to user’s manual for DIG Gel Shift Kit.

For the analyses of the LIMK1 promoter SNPs, we generated six DIG-labeled double-stranded oligonucleotides for either −961G or −961A (21 nt), −428G or −428A (21 nt), and −187C or −187T (26 nt). DIG-labeled probes were incubated with HEK293 nuclear extracts for 20 min at 25°C and separated by electrophoresis on a 6% non-denaturing polyacrylamide gel with 0.5 x TBE running buffer. The DNA–protein complexes were electroblotted onto nylon membrane and the band shift was visualized according to user’s manual for DIG Gel Shift Kit. For the competition assay, we pre-incubated the HEK293 extracts with unlabeled oligonucleotide probes before adding labeled oligonucleotide probes.

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Conflict of Interest statement. None declared.

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