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ORIGINAL INVESTIGATION

Systematic screening of lysyl oxidase-like (LOXL) family genes demonstrates that *LOXL2* is a susceptibility gene to intracranial aneurysms

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Abstract Four lysyl oxidase family genes (*LOXL1*, *LOXL2*, *LOXL3*, and *LOXL4*), which catalyze cross-linking of collagen and elastin, were considered to be functional candidates for intracranial aneurysms (IA) and were extensively screened for genetic susceptibility in Japanese IA patients. Total RNA was isolated from four paired ruptured IA and superficial temporal artery (STA) tissue and examined by real-time RT-PCR. The expression of *LOXL2* in the paired IA and STA tissues was elevated in the IA tissue. A total of 55 single nucleotide polymorphisms (SNPs) of *LOXL1-4* were genotyped for an allelic association study in 402 Japanese IA patients and 462 Japanese non-IA controls. Allelic associations were evaluated with the chi-square test and the permutation test especially designed for adjustment of multiple testing. SNPs of *LOXL1* and *LOXL4* were not significantly associated with IA, while several SNPs of *LOXL2* and *LOXL3* showed nominally significant associations in IA patients. We detected an empirically significant association with one SNP of *LOXL2* in familial IA patients after adjustment for multiple testing [$\chi^2 = 10.23$, empirical $P = 0.023$, OR (95% CI) = 1.49 (1.17, 1.90)]. Furthermore, multilocus interaction was evaluated by multifactor dimensionality reduction analysis. We found that the SNPs of *LOXL2* have an interactive effect with elastin (*ELN*) and LIM kinase 1 (*LIMK1*) that have been previously found to be associated with IA. In conclusion, one SNP of *LOXL2* showed a significant association with IA individually, and we also detected a gene–gene interaction of *LOXL2* with *ELN*/*LIMK1*, which may play an important role in susceptibility to IA.

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Introduction

The rupture of an intracranial aneurysm (IA) (MIM105800) results in a subarachnoid hemorrhage

with high morbidity and mortality, representing a major public health concern (Longstreth et al. 1993; Schievink et al. 1995; Inagawa et al. 1995). We have previously reported a genome-wide linkage study of IA in 104 affected Japanese sib pairs in which evidence of linkage at chromosome 5q22-31, 7q11, and 14q22 was detected (Onda et al. 2001). Lysyl oxidase, encoded by *LOX* located on a suggestive linkage region on chromosome 5q31, is an extracellular copper-containing enzyme that initiates cross-linking of collagen and elastin by oxidative deamination of lysine residues. It plays an essential role in the formation of extracellular matrix and connective tissue (Kagan and Li 2003). *LOX* is considered to enhance the strength of the blood vessel wall, and was therefore a plausible positional and functional candidate to IA formation. Previously, single nucleotide polymorphisms (SNPs) of *LOX* were analyzed for allelic and haplotype-based associations in Japanese and Central European IA patients, but no association was detected (Yoneyama et al. 2003; Hofer et al. 2004). Four novel genes encoding LOX-like proteins 1 through 4 (*LOXL1*, *LOXL2*, *LOXL3*, and *LOXL4*), assigned to chromosome 15q22, 8p21, 2p13, and 10q24, respectively, are categorized to be the "LOX family genes" (Kenyon et al. 1993; Jourdan-Le Saux et al. 1998; Asuncion et al. 2001; Jourdan-Le Saux et al. 2001; Molnar et al. 2003). Products of the LOX family genes have a highly conserved amino acid sequence at the C-terminus and have amine oxidase activity (Molnar et al. 2003). The LOX family genes could also be considered as plausible candidate genes for IA, thus, we evaluated allelic associations between IA patients and SNPs of *LOXL1-4*. Although we detected multiple SNPs of *LOXL2* and *LOXL3* that were significantly associated with IA, only one SNP of *LOXL2* was associated after adjustment of multiple testing with a permutation test. In addition, using the multifactor dimensionality reduction (MDR) method (Richie et al. 2001) to detect gene–gene interaction among multiple SNPs, we found that SNPs of *LOXL2* have an interactive effect with SNPs at an *ELN*/*LIMK1* locus that have been previously found to be, both statistically and functionally, associated with IA (Akagawa et al. 2006). This implicates that the SNPs of *LOXL2* could be a genetic risk factor for developing IA and account for a part of the pathogenesis of its formation.

Subjects and methods

Subjects

The Ethical Committees of Tokyo Women's Medical University, Chiba University, and Tokyo Metropolitan Fuchu Hospital, approved the study protocols, and all participants gave written informed consent. The clinical backgrounds of the Japanese subjects are summarized in Table 1. The DNA samples for the present study were 402 IA patients (mean age: 54.2 ± 10.0 years) and 462 controls (mean age: 63.5 ± 10.0 years), all of which are unrelated to each other. The IA patients included 185 familial IA patients (86 probands from nuclear families that had been used in our linkage study (Onda et al. 2001) and 99 patients with family history of IA) and 217 sporadic IA patients (onset of age < 60 years). The presence of IA was confirmed by conventional angiography, three-dimensional CT angiography, MR angiography, or surgical findings. The control subjects were outpatients with diseases other than IA, at the Department of Neurosurgery of the Tokyo Women's Medical University, Chiba University, and their nearby affiliated hospitals. The Japanese controls did not harbor an IA as verified

Table 1 Clinical background of the subjects

	Familial IA	Sporadic IA	Total IA	P^*	Controls	$P^\dagger$
n	185	217	402		462	
Average of age (S.D.)	59.6 (10.2)	49.9 (7.3)	54.2 (10.0)	< 0.001	63.5 (10.0)	< 0.001
Female (%)	126 (68.1)	124 (57.1)	250 (62.2)	< 0.05	170 (36.8)	< 0.001
Ruptured IA patients (%)	121 (65.4)	178 (82.0)	299 (74.4)	< 0.001		
Multiple IA patients (%)	36 (19.5)	44 (20.3)	80 (19.9)	N.S.		
Site of IA						
ACoA (%)	45 (20.4)	65 (24.3)	110 (22.5)	N.S.		
MCA (%)	82 (37.1)	82 (30.6)	164 (33.5)	N.S.		
ICA (%)	41 (18.6)	74 (27.6)	115 (23.5)	< 0.05		
Other or unknown (%)	53 (24.0)	47 (17.5)	100 (20.4)	N.S.		
Total	221	268	489			

ACoA anterior communicating artery, *MCA* middle cerebral artery, *ICA* internal carotid artery

[a] Familial IA patients verses sporadic IA patients

[b] Total IA patients verses controls

by radiological examinations and had no familial history of SAH. All of the controls were over 50 years of age, in order to exclude most unmanifested IAs. Genomic DNA was extracted from peripheral blood or buccal swab according to a standard method.

Real-time RT-PCR analysis of the LOX family genes in paired IA and STA tissues

Four pairs of ruptured IA and superficial temporal artery (STA) specimens were excised from four female patients (aged from 34 to 80 years) who underwent surgical IA clipping at the Department of Neurosurgery of Tokyo Metropolitan Fuchu Hospital. Total RNAs from the arterial specimens were extracted using TRIzol reagent (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions, and used as a template in first-strand cDNA synthesis with SuperScript III First-Strand Synthesis System (Invitrogen). Real-time PCR was performed using SYBR *Premix Ex Taq* (Perfect Real Time) (TAKARA BIO, Otsu, Japan) on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. The PCR primers for LOX family genes and *GAPDH* were designed and synthesized by TAKARA BIO. The primer sequences can be obtained from the authors (see Supplementary Table 1). Relative expression levels for the respective LOX family genes were obtained by normalizing to *GAPDH* in all specimens. Statistical comparison in the quantitative analysis of the gene expression was performed by the paired *t*-test; $P < 0.05$ was considered significant.

SNP selection of LOXL1-4 and genotyping

The SNPs of *LOXL1-4* were obtained from the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP) or the IMS-JST JSNP DATABASE (http://snp.ims.u-tokyo.ac.jp/). SNPs with minor allele frequencies above 10% in an initial genotyping of 12 subjects were selected for further study. The SNPs were genotyped either by using TaqMan SNP Genotyping Assays on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) or by BigDye Terminator V1.1 Cycle Sequencing on ABI 3700 DNA Analyzer (Applied Biosystems) according to a standard protocol.

Statistical analysis

Differences in allelic frequencies were evaluated using a case–control design with the chi-square test, and the statistical power for association studies (1-β) was calculated using the chi-square test with arcsine approximation (Ambrosius et al. 2004). Haplotype frequencies for multiple loci and pairwise linkage disequilibrium (LD), using the standard definition of D' and r^2 (Lewontin 1964; Hill and Robertson 1968), were estimated in phase-unknown samples with SNPAlyze V3.2 software (DYNACOM, Mobara, Japan), in which the expectation-maximization (EM) algorithm (Excoffier and Slatkin 1995) is used. For an adjustment of multiple testing, Bonferroni's correction is known to be too conservative since all SNPs are assumed to be independent, and the false discovery rate (FDR) (Benjamini and Hochberg 1995), in which the most significant association is corrected in the same way as Bonferroni's correction, may also provide an unreasonably stringent threshold at least for the most significantly associated SNP. Thus, we applied a permutation test as proposed by Churchill and Doerge (1994) to determine empirical *P*-values. The individuals in an experimental dataset were labeled 1–*n* and each was scored at *m* SNPs. An affection status was also associated with each individual. In the permutation test, the affection statuses were shuffled *N* times among the *n* individuals to create permuted datasets that had only random genotype–phenotype associations and that were representative sample from an appropriate null distribution. A test statistic was computed at each of the SNPs and only its maximum value was recorded for each of the *N* permuted dataset. An empirical threshold value was obtained by computing the (1-α) percentile from the *N* maximum test statistics.

MDR for multi-locus interaction

In order to explore gene–gene interaction between the LOX family genes and *ELN*/*LIMK1* that had previously been identified as susceptibility genes (Akagawa et al. 2006), we performed MDR analysis (Richie et al. 2001). This method is based on cross-validation (CV) procedure, in which the dataset is divided into 10 equal parts, and the MDR model is developed using 9/10 of the data (training set). Each of the multilocus genotypes is categorized, depending on the case and control count, as either high- or low-risk groups and then the remaining 1/10 (testing set) is used to predict the affection status. The proportion of subjects whose affection status was correctly predicted is defined as testing balanced accuracy (TBA), and the number of times a particular model is identified as the best one is defined as CV consistency, and the two indexes are used to evaluate the goodness-of-fit of models. The procedure is repeated a total of 100 times, and TBA and CV consistencies were averaged for all combinations of SNPs.

Results

Quantitative expression analyses of LOX family genes in paired IA and STA tissues

Real-time RT-PCR was performed to examine gene expression patterns in IA tissue compared with STA tissue (paired sample from the same individual) (Fig. 1). Consistently elevated expressions of *LOX* (3.9-fold, $P = 0.059$) and *LOXL2* (5.1-fold, $P = 0.011$) were observed in IA tissues in comparison with STA tissues. The expressions of *LOXL1*, *LOXL3*, and *LOXL4* were not significantly different. These results indicate a possible involvement of *LOX* and *LOXL2* in the pathophysiology of IA formation.

Allelic association study and linkage disequilibrium of LOXL1-4

Forty-nine SNPs, comprising 25, 11, 6, and 7 SNPs of *LOXL1*, *LOXL2*, *LOXL3*, and *LOXL4*, respectively, were first genotyped in 96 familial IA patients and 96 non-IA controls. The results of the screening were evaluated by the simple chi-square test as summarized in Table 2. Two SNPs of *LOXL2*, Exon5(34923)G/A (denoted as SNP7, see Table 2) and Intron6(41002)G/C (SNP8), and two SNPs of *LOXL3*, 3′FLR(20827)C/T (SNP5) and 3′FLR(21095)C/T (SNP6), both located next to each other, showed nominally significant association with IA ($P < 0.05$).

The physical and LD maps are shown in Fig. 2. LD patterns of the controls were depicted in the figure and essentially the same patterns were observed with the patients. All of the SNPs in either *LOXL1* or *LOXL3* were in near complete LD ($D' > 0.7$). In *LOXL2*, there was a structured LD block ($D' > 0.7$) from SNP1 to SNP6 and a scattered LD block from SNP7 to SNP16. In *LOXL4*, a LD block ($D' > 0.7$, $r^2 > 0.7$) from SNP3 to SNP7, which includes the middle to 3′-flanking region, was observed.

All of the subjects including the individuals in the first screening were then subjected to genotyping of the significant SNPs of *LOXL2* and *LOXL3* (Table 3). In *LOXL3*, significant association was replicated only in SNP5, which is located on the 3′ flanking region of the gene, with the familial patients ($\chi^2 = 5.33$, $df = 1$, and

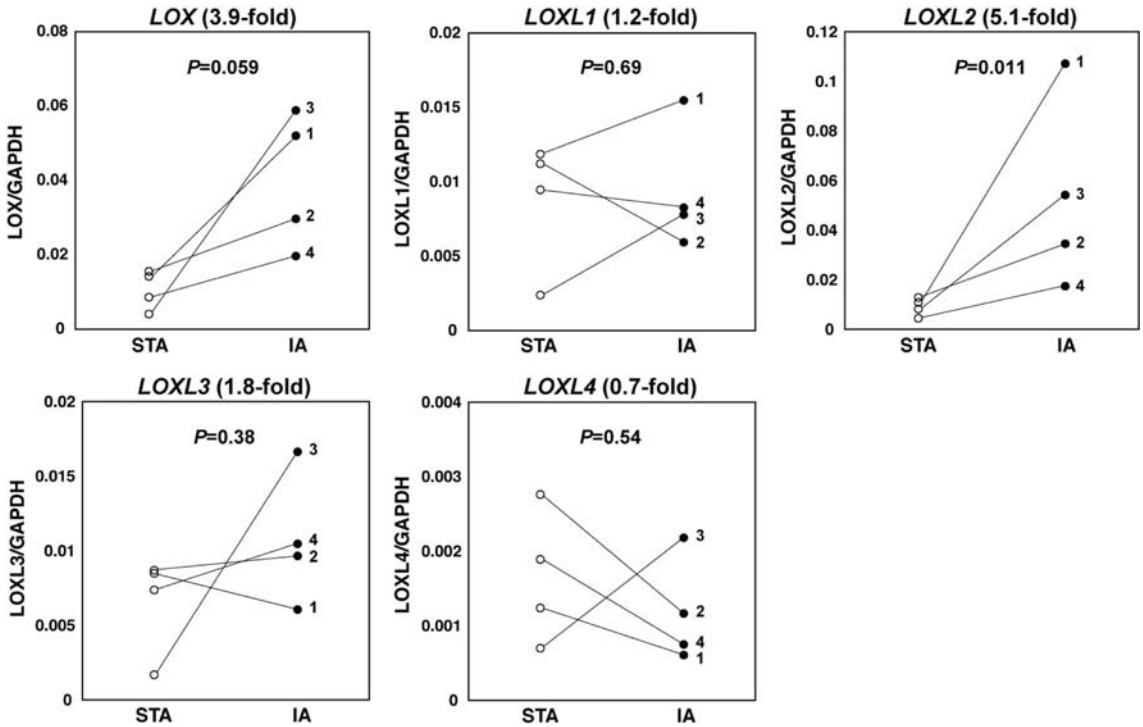

Fig. 1 Comparisons of expression levels of the LOX family genes in paired IA and STA tissues from the same IA patients. Expression levels of the LOX family genes relative to *GAPDH* (*Y*-axis) were evaluated by real-time RT-PCR analyses in four pairs of IA (*closed circle*) and STA (*open circle*) specimens that were surgically excised from four patients with IA(s). The differences in expression between IA tissues and the corresponding STA tissues were analyzed by paired *t*-test. The *P*-value obtained is shown in each panel. The numeral (1–4) in the panel represents patient's identification number. In each of paired IA and STA tissues, the increase in gene expression in IA tissue was obtained in comparison with the corresponding STA tissue, and the average fold-increases of the LOX family genes are shown in the upper part of the panels

Table 2 Results of the allelic association study between IA patients and SNPs of *LOXL1-4* (First screening)

SNP No.	rs number	Location	Position	SNP type M/m	Amino acid Substitution	Minor allele frequency		χ^2	*P*
						Control (n)	Familial IA (n)		
LOXL1									
1	rs1550437	Intron 1	2174	C/T	–	0.371 (170)	0.343 (166)	0.27	0.603
2	rs6495085	Intron 1	2189	G/C	–	0.131 (168)	0.145 (166)	0.13	0.718
3	rs1550439	Intron 1	2515	T/A	–	0.262 (172)	0.193 (176)	2.32	0.128
4	–	Intron 1	4522	G/T	–	0.128 (164)	0.151 (166)	0.35	0.554
5	rs1992314	Intron 1	4643	G/C	–	0.482 (166)	0.435 (168)	0.76	0.385
6	rs1440101	Intron 1	5507	G/A	–	0.240 (150)	0.184 (174)	1.53	0.216
7	rs4886776	Intron 1	5872	G/A	–	0.463 (188)	0.425 (186)	0.55	0.459
8	rs8041642	Intron 1	5982	G/A	–	0.112 (188)	0.148 (182)	1.10	0.294
9	rs8041685	Intron 1	6069	G/A	–	0.071 (182)	0.102 (176)	1.08	0.300
10	rs4886778	Intron 1	6264	A/C	–	0.305 (128)	0.250 (172)	1.11	0.293
11	rs8042039	Intron 1	6394	C/T	–	0.148 (128)	0.151 (166)	0.00	0.959
12	rs8027022	Intron 1	7014	A/G	–	0.349 (172)	0.287 (178)	1.57	0.210
13	–	Intron 1	7021	A/G	–	0.466 (176)	0.420 (176)	0.74	0.391
14	rs28603291	Intron 1	7346	C/T	–	0.232 (138)	0.175 (166)	1.54	0.215
15	rs2028386	Intron 1	7584	G/C	–	0.357 (140)	0.299 (174)	1.20	0.273
16	rs4337252	Intron 1	7641	C/G	–	0.350 (140)	0.282 (174)	1.69	0.194
17	rs893820	Intron 1	10479	C/T	–	0.294 (170)	0.253 (174)	0.74	0.391
18	rs28594928	Intron 1	12324	C/T	–	0.419 (186)	0.450 (180)	0.35	0.554
19	–	Intron 1	12327	C/G	–	0.366 (186)	0.383 (180)	0.12	0.726
20	rs12441130	Intron 1	15778	T/C	–	0.343 (172)	0.280 (186)	1.68	0.195
21	rs1530169	Intron 2	17996	T/C	–	0.377 (162)	0.455 (154)	1.98	0.160
22	–	Intron 3	20183	G/A	–	0.440 (168)	0.494 (168)	0.97	0.325
23	–	Intron 4	20747	C/T	–	0.350 (140)	0.346 (162)	0.01	0.937
24	rs2304722	Intron 5	22625	T/C	–	0.369 (160)	0.361 (180)	0.02	0.884
25	–	Intron 6	22943	G/A	–	0.215 (130)	0.159 (164)	1.56	0.211
LOXL2									
1	rs11785442	Promoter	−40025	C/T	–	0.031 (192)	0.057 (192)	1.54	0.215
2	rs12544591	Intron 1	−1216	T/A	–	0.453 (192)	0.464 (192)	0.04	0.838
3	rs11992138	Intron 2	2952	G/A	–	0.302 (192)	0.318 (192)	0.11	0.741
4	rs954705	Intron 3	13640	T/G	–	0.484 (192)	0.453 (192)	0.38	0.540
5	rs11774789	Intron 3	23619	C/T	–	0.484 (192)	0.490 (192)	0.01	0.919
6	rs11781891	Intron 3	27132	G/T	–	0.031 (192)	0.052 (192)	1.04	0.307
7	rs1010156	Exon 5	34923	G/A	synonymous	0.432 (192)	0.563 (192)	6.51	0.011
8	rs9792301	Intron 6	41002	G/C	–	0.302 (192)	0.401 (192)	4.12	0.042
9	rs4291276	Intron 9	52273	C/T	–	0.031 (192)	0.047 (192)	0.62	0.429
10	rs1063582	Exon 10	58511	C/A	Met570Leu	0.318 (192)	0.333 (192)	0.11	0.744
11	rs2280941	Intron 12	66490	C/T	–	0.286 (192)	0.333 (192)	0.99	0.321
LOXL3									
1	–	Intron 2	1530	C/T	–	0.030 (164)	0.045 (156)	0.46	0.498
2	rs715407	Intron 4	14069	A/C	–	0.163 (190)	0.146 (192)	0.22	0.639
3	rs17010021	Exon 11	18223	T/A	Phe615Ile	0.435 (168)	0.472 (144)	0.45	0.505
4	rs6707302	Intron 11	18340	G/A	–	0.153 (176)	0.130 (162)	0.39	0.532
5	rs2241028	3' FLR	20828	C/T	–	0.297 (182)	0.176 (170)	6.99	0.008
6	rs2241027	3' FLR	21096	C/T	–	0.122 (180)	0.215 (158)	5.26	0.022
LOXL4									
1	rs2862296	Promoter	−9973	A/G	–	0.332 (190)	0.380 (192)	0.99	0.321
2	rs1983864	Exon 8	5323	G/T	synonymous	0.479 (192)	0.473 (188)	0.01	0.911
3	rs737657	Intron 10	9886	G/A	–	0.448 (192)	0.484 (184)	0.48	0.487
4	rs737656	Intron 10	10037	G/A	–	0.448 (192)	0.484 (182)	0.48	0.490
5	rs1048757	3′ FLR	18335	C/G	–	0.448 (192)	0.479 (192)	0.38	0.539
6	rs1359508	3′ FLR	18991	T/C	–	0.453 (190)	0.484 (190)	0.38	0.537
7	rs7899632	3′ FLR	22151	G/A	–	0.411 (192)	0.448 (192)	0.52	0.471

Position the number of nucleotides from the first nucleotide (A) of the start codon, *M/m* major and minor alleles, *n* the number of alleles genotyped, *3′-FLR* 3′ flanking region

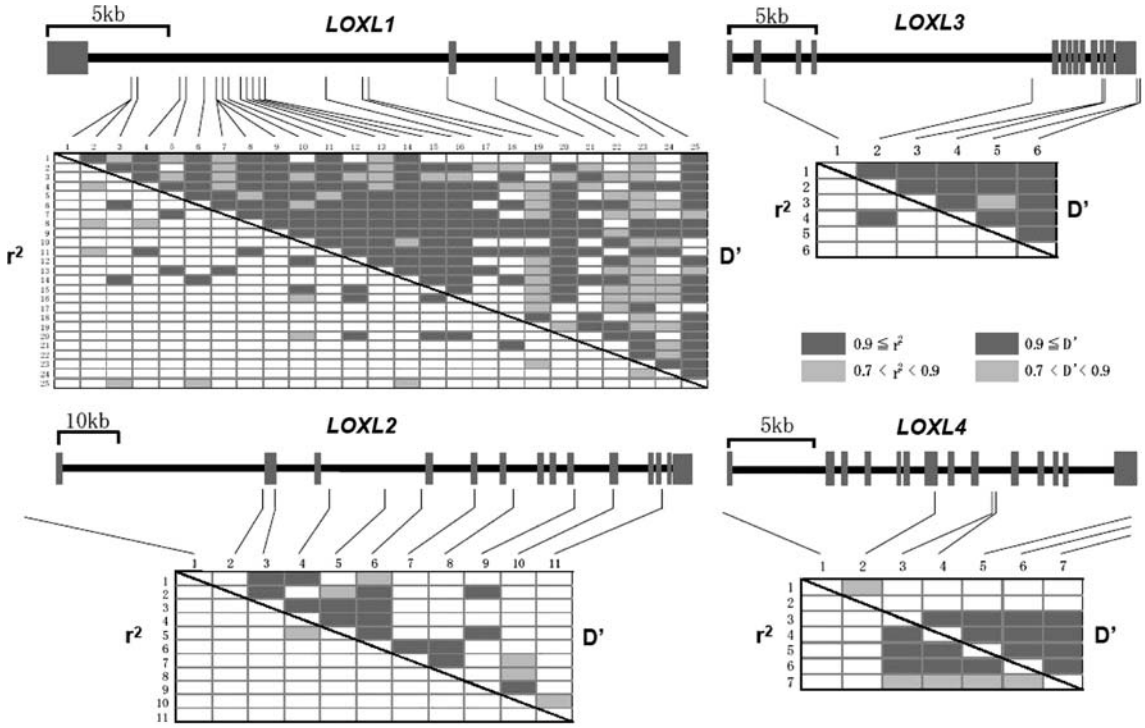

Fig. 2 Linkage disequilibrium pattern of *LOXL1-4*. The gene structure together with the position of SNPs is shown for each gene. Pairwise LD coefficient, D' and r^2, were determined and expressed as a block structure. In the schematic block, *dark shaded boxes* indicate pairwise LD of D' or $r^2 > 0.9$ and *light gray* D' or $r^2 = 0.7$–0.9. *Blank boxes* represent D' or $r^2 < 0.7$

$P = 0.021$). As the functional impact of SNPs outside of *LOXL3* is not easily evaluated and the evidence of association had disappeared after adjustment for multiple testing, subsequent studies focused on SNPs of *LOXL2*. We resequenced *LOXL2* and identified six additional known SNPs. Among a total of 17 SNPs of *LOXL2* we detected a nominally significant allelic association with SNP7 in all IA patients [$\chi^2 = 7.00$, $df = 1$, $P = 0.008$, and OR (95% CI) = 1.29 (1.07, 1.56)]. It is synonymous and located on exon 5. Exon 5(34869)A/G (SNP6b), Exon5(34938)G/A (SNP7a), and Intron9(52273)C/T (SNP9) also showed weak but nominally significant associations (Table 3). To avoid false positive results, we applied the permutation test for an adjustment of multiple testing (Churchill and Doerge 1994). However, except for the SNP7 with the most significant association in the familial IA patients [$\chi^2 = 10.23$, empirical $P = 0.023$, and OR (95% CI) = 1.49 (1.17, 1.90)], none of the associations met the empirical significance level according to the permutation test (Table 3). SNP7 also showed a significant association with IA under the genotypic models, especially under the recessive model for the major allele in the familial patients ($\chi^2 = 13.74$, $df = 1$, $P = 2.1 \times 10^{-4}$) (see Supplementary Tables 2, 3). None of the SNPs in the control samples showed deviation from Hardy–Weinberg's equilibrium (data not shown). The statistical power for the association study with this dataset was >0.98, with difference in allele frequencies between the cases and controls of 0.1.

Haplotype-based association study of LOXL2

The three SNPs, SNP6b, SNP7, and SNP7a which were located on exon 5 of *LOXL2*, showed significant association ($P < 0.05$) in all cases of IA. Because these three SNPs are in tight LD with each other ($D' > 0.9$), the SNPs were selected to construct haplotypes for a haplotype-based association study. Three major haplotypes and their estimated frequencies are shown in Table 4 for IA patients and their controls. Bonferroni's correction was used with the number of the haplotypes, since they were independent from one another. The most common haplotype, Hap2, was over-represented with statistical significance both in total ($\chi^2 = 7.48$, $df = 1$, nominal $P = 0.006$, and corrected $P = 0.018$) and familial IA patients ($\chi^2 = 10.69$, $df = 1$, nominal $P = 0.001$, and corrected $P = 0.003$). In contrast, Hap1 was more frequent in the controls than in the familial IA patients ($\chi^2 = 6.46$, $df = 1$, nominal $P = 0.011$, and corrected $P = 0.033$), which indicates that Hap1 is a protective haplotype. The haplotype-based association showed that Hap2 could be regarded as an at-risk haplotype and that the SNP7 constitutes a tag-SNP for Hap2.

Table 3 Results of the allelic association study between IA patients and SNPs in *LOXL2* and *LOXL3* (Second screening)

SNP No.	rs number	Location	Position	SNP type M/m	Amino acid substitution	Minor allele frequency		Versus total IA			Minor allele frequency	Versus familial IA		
						Control (n)	IA (n)	χ^2	*P*	Empirical *P**	Familial IA (n)	χ^2	*P*	Empirical *P**
LOXL2														
1	rs11785442	Promoter	−40025	C/T	–	0.048 (398)	0.065 (542)	1.20	0.273	0.985	0.077 (310)	2.69	0.101	0.754
2	rs12544591	Intron 1	−1216	T/A	–	0.480 (400)	0.443 (542)	1.28	0.257	0.980	0.439 (310)	1.20	0.274	0.984
3	rs11992138	Intron 2	2952	G/A	–	0.295 (400)	0.275 (542)	0.46	0.499	1.000	0.268 (310)	0.64	0.424	0.999
4	rs954705	Intron 3	13640	T/G	–	0.470 (400)	0.456 (542)	0.19	0.664	1.000	0.429 (310)	1.18	0.277	0.985
5	rs11774789	Intron 3	23619	C/T	–	0.477 (392)	0.470 (540)	0.04	0.840	1.000	0.490 (310)	0.12	0.727	1.000
6	rs11781891	Intron 3	27132	G/T	–	0.050 (400)	0.065 (540)	0.92	0.339	0.996	0.074 (310)	1.80	0.180	0.925
6a	rs7819977	Intron 4	34676	C/T	–	0.182 (888)	0.187 (680)	0.05	0.826	1.000	0.182 (324)	0.00	0.989	1.000
6b	rs2294127	Exon 5	34869	A/G	synonymous	0.262 (906)	0.218 (740)	4.31	0.038	0.426	0.226 (354)	1.71	0.191	0.934
7	rs1010156	Exon 5	34923	G/A	synonymous	0.477 (924)	0.541 (804)	7.00	0.008	0.122	0.576 (370)	10.23	0.001	0.023
7a	rs2294128	Exon 5	34938	G/A	synonymous	0.264 (906)	0.218 (740)	4.73	0.030	0.353	0.226 (354)	1.92	0.165	0.904
7b	rs1051146	Exon 6	39857	A/G	synonymous	0.365 (356)	0.389 (568)	0.53	0.466	1.000	0.328 (256)	0.90	0.343	0.996
8	rs9792301	Intron 6	41002	G/C	–	0.310 (400)	0.345 (678)	1.40	0.237	0.972	0.394 (330)	5.62	0.018	0.229
8a	rs3736016	Exon 9	51365	C/T	synonymous	0.273 (862)	0.262 (686)	0.20	0.652	1.000	0.276 (304)	0.02	0.901	1.000
8b	rs7357444	Exon 9	51383	C/T	synonymous	0.079 (870)	0.106 (708)	3.34	0.068	0.620	0.114 (316)	3.44	0.064	0.585
9	rs4291276	Intron 9	52273	C/T	–	0.033 (400)	0.063 (678)	4.88	0.027	0.330	0.073 (330)	6.08	0.014	0.182
10	rs1063582	Exon 10	58511	C/A	Met570Leu	0.309 (904)	0.316 (776)	0.10	0.754	1.000	0.333 (354)	0.72	0.397	0.999
11	rs2280941	Intron 12	66490	C/T	–	0.353 (400)	0.338 (542)	0.23	0.635	1.000	0.365 (310)	0.11	0.740	1.000
LOXL3														
5	rs2241028	3′ FLR	20828	C/T	–	0.245 (878)	0.215 (794)	2.04	0.153	0.888	0.185 (368)	5.33	0.021	0.262
6	rs2241027	3′ FLR	21096	C/T	–	0.196 (874)	0.189 (790)	0.13	0.716	1.000	0.223 (364)	1.14	0.286	0.987
Korean group														
LOXL2														
6b		Exon 5	5982	A/G	synonymous	0.246 (496)	0.193 (378)	3.46	0.063	0.329	0.333 (6)	–	–	–
7		Exon 5	34923	G/A	synonymous	0.444 (514)	0.525 (398)	5.98	***0.015***	0.087	0.500 (6)	–	–	–
7a		Exon 5	6264	G/A	synonymous	0.249 (490)	0.198 (374)	3.16	0.076	0.381	0.333 (6)	–	–	–
8		Intron 6	41002	C/G	–	0.301 (372)	0.370 (392)	4.05	***0.044***	0.244	0.167 (6)	–	–	–
9		Intron 9	52273	T/C	–	0.091 (374)	0.070 (394)	1.23	0.267	0.854	0.000 (6)	–	–	–
LOXL3												–	–	–
5		3′ FLR	20827	C/T	–	0.196 (484)	0.235 (388)	1.88	0.171	0.688	0.167 (6)	–	–	–
6		3’ FLR	21095	C/T	–	0.262 (488)	0.269 (390)	0.05	0.817	1.000	0.333 (6)	–	–	–

* Empirical *P* values generated by 100,000 iterations

Table 4 Haplotype-based association study of *LOXL2*

Haplotype	*LOXL2*			Allele frequency			Versus total IA			Versus familial IA		
	SNP6b	SNP7	SNP7a	Control	Total IA	Familial IA	χ^2	*P*-value	Corrected *P* *-value	χ^2	*P*-value	Corrected *P**-value
				(n = 924)	(n = 804)	(n = 370)						
Hap1	A	G	G	0.259	0.231	0.192	1.73	0.189	0.467	6.46	0.011	0.033
Hap2	A	A	G	0.475	0.541	0.576	7.48	0.006	0.018	10.69	0.001	0.003
Hap3	G	G	A	0.262	0.228	0.233	2.73	0.099	0.269	1.21	0.271	0.613

* Corrected *P*-values obtained using Bonferroni's correction

MDR analysis

Subsequently, we performed MDR analysis to explore gene–gene interaction between *LOXL2* and *ELN*/*LIMK1* that had previously been identified as susceptibility genes (Table 5). The 3′-UTR (+502) and 3′-UTR (+659) SNPs of *ELN*, the Pro.(−961), Pro.(−428) and Pro.(−187) SNPs of *LIMK1* that showed significant associations with IA in the previous study (Akagawa et al. 2006) were tested with SNP6b, SNP7, SNP7a, SNP8, and SNP9 of *LOXL2*. In the MDR analysis, SNPs in strong LD evaluated by r^2 statistics with each other tend to result in unreasonably low-CV consistency because the SNPs have an equal chance to be selected and one SNP may falsely be selected instead of the highly associated SNP (Heidema et al. 2006). Thus, it would be appropriate to select only the most significantly associated SNP among SNPs in strong LD. Also, we removed SNPs with low minor allele frequency (<0.05). Eventually, we selected six SNPs, i.e., 3′-UTR (+502) and 3′-UTR (+659) SNPs of *ELN*, Pro.(−428) and Pro. (−187) SNPs of *LIMK1*, and SNP6b and SNP7 of *LOXL2*, and one more *LOXL2* SNP, Exon10(58511)A/C (SNP10), was also added because it was non-synonymous. Table 5 shows that the 5-SNP (3′-UTR (+502) SNP of *ELN*, Pro. (−428) SNP of *LIMK1*, SNP6b, SNP7, and SNP10 of *LOXL2*) model was supported (TBR = 0.5988 and $P < 0.001$), which indicates that although *LOXL2* itself does not have such a strong effect on susceptibility to IA, it has an interactive and significant effect with *ELN*/*LIMK1*.

Discussion

Since the first genome-wide linkage study of IA with affected siblings (Onda et al. 2001), several genome-wide and candidate locus linkage studies have been reported (Olson et al. 2002; Yamada et al. 2003; Farnham et al. 2004; Roos et al. 2004; van der Voet et al. 2004; Yamada et al. 2004; Nahed et al. 2005), further reports have been summarized by Krischek and Inoue (2006). The linkage results of these studies, however, have not been consistent presumably reflecting the heterogeneous nature of the disease (Yamada et al. 2003, Krischek et al. 2006). As widely accepted, association study with candidate genes is an alternative approach to identify genetic susceptibility of complex diseases. Thus far, candidate genes encoding components of extracellular matrix and connective tissue or involved in vascular remodeling have been studied for IA in distinct populations. Significant associations were reported in genes such as *ELN*/*LIMK1*, collagen 1A2, endoglin, and angiotensin converting enzyme

Table 5 The best model for each number of SNPs estimated by MDR analysis with the IA dataset

Number of SNPs	SNPs included in the model[a]	Testing balanced accuracy	Cross-validation consistency
1	Pro.(−428)	0.5719	10.0
2	Pro.(−428), SNP7	0.5812	8.8
3	Pro.(−428), SNP7, SNP10	0.5570	5.1
4	3′-UTR (+502), Pro.(−428), SNP7, SNP10	0.5752	9.5
5	3′-UTR (+502), Pro.(−428), SNP6b, SNP7, SNP10	0.5988*	10.0
6	3′-UTR (+502), Pro.(−428), Pro.(−187), SNP6b, SNP7, SNP10	0.5869	6.9
7	3′-UTR (+502), 3′-UTR (+659), Pro.(−428), Pro.(−187), SNP6b, SNP7, SNP10	0.5888	–

[a] 3′-UTR (+502) and 3′-UTR (+659) belong to *ELN*, Pro.(−428) and Pro.(−187) to *LIMK1*, and SNP6b, SNP7, and SNP10 to *LOXL2*

* $P < 0.001$

(Takenaka et al. 1999; Onda et al. 2001; Ruigrok et al. 2004; Slowik et al. 2004; Yoneyama et al. 2004; Akagawa et al. 2006). In the present study, the LOX family genes, *LOXL1-4*, were extensively and systematically investigated. They are plausible functional candidates since lysyl oxidase activities that cross-link collagen and elastin are essential for mechanical stability and maintenance of vascular structure. The LOX family gene-products appear to have not only common lysyl oxidase activities but also diverse biological functions including tumor suppression, cell adhesion, and senescence (Saito et al. 1997; Csiszar 2001). However, functional assignments of the family members remain largely undetermined. Mice lacking *Lox* showed perinatal lethality despite the redundancy of lysyl oxidase activity suggesting a critical role of the gene. In addition, saccular aneurysms in the thoracic aorta were observed in the *Lox* deficient mice suggesting an important role of the gene in aneurysm formation, possibly by reducing the strength of the vascular structure (Maki et al. 2002). Distinct functional significance was shown in mice lacking *Loxl1* that were viable and showed a specific role of *LOXL1* in elastogenesis (Liu et al. 2004). *LOXL2* is highly expressed in metastatic breast cancer-derived cell lines (Akiri et al. 2003) and it was shown that *LOXL2* interacts and cooperates with snail, a transcription factor, to down-regulate E-cadherin expression (Peinado et al. 2005) that might play a role in tumor progression.

In the present study, 55 SNPs in the LOX family genes were examined for allelic association with Japanese IA patients. Significant association was identified with SNP7 of *LOXL2* in the familial IA cases (nominal $P = 0.001$ and empirical $P = 0.023$) (Table 3). Because SNP7, located in the center of exon 5 of *LOXL2*, is synonymous, we first examined the possibility whether SNP7 functions as a splicing enhancer but obtained negative results with human umbilical artery smooth muscle cells (data not shown). A possibility remains that an unknown SNP in tight LD with SNP7 is a *bona-fide* causality to IA formation.

As we previously reported that SNPs in the *ELN*/*LIMK1* locus were associated with IA (Akagawa et al. 2006), we tested possible gene–gene interaction between *LOXL2* and *ELN*/*LIMK1*. Multilocus analysis was performed by using MDR and we found that SNP6b, SNP7, and SNP10 of *LOXL2* have a statistically interactive effect with SNPs of *ELN*/*LIMK1*. In addition to the evidence of genetic association, *LOXL2* showed a significantly elevated expression in IA tissue (5.1-fold increase) (Fig. 1), whereas *LOXL1*, *LOXL3*, and *LOXL4* did not. These results reinforce the possibility that *LOXL2* plays an important role in IA formation. Elevated levels of *LOXL2* could be explained by a compensation mechanism to strengthen the vascular wall of aneurysm and also indicate an important role of *LOXL2* in aneurysmal formation. It was reported that *LOX* and *LOXL2* target collagen molecules to cross-link while *LOXL1* preferentially targets elastin as a substrate (Liu et al. 2004; Vadas et al. 2005). Thereby, a physiological interaction between *LOXL2* and *ELN*/*LIMK1* is still unclear and further investigation is evidently needed.

In general, inconsistent results are often yielded for linkage and association studies, presumably because of genetic heterogeneity, sample size, and the different ways of determining a significance level, etc. Thus, it is necessary to perform genetic analyses more carefully, e.g., by setting an integrated and appropriate significance level or by confirming an association in several populations, and to improve the availability of statistical methods. Also the MDR method is not the ultimate solution for identifying gene–gene interactions, it still has some issues that need to be solved; e.g., how to overcome the fact that SNPs in extremely strong LD can lead to inadequate results, and how to find out in which way each of SNPs included in the model contributes to susceptibility. Thus, more sophisticated statistical methods to detect gene–gene interaction will also become increasingly important for understanding the complicated mechanisms of human common complex diseases.

In conclusion, SNP7 (rs1010156) of *LOXL2* showed an empirically significant association with IA, and this SNP together with SNP6b (rs2294127) and SNP10 (rs1063582), of *LOXL2* showed a significantly interactive effect with *ELN* and *LIMK1*. Currently, the functional impact of SNP7 of *LOXL2* is uncertain. However, elevated expression of *LOXL2* in IA tissues suggests that the protein plays an important role in IA formation.

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