LOXL3-sv2, a novel variant of human lysyl oxidase-like 3 (LOXL3), functions as an amine oxidase

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Abstract. Human lysyl oxidase-like 3 (*LOXL3*) functions as a copper-dependent amine oxidase toward collagen and elastin. The LOXL3 protein contains four scavenger receptor cysteine-rich (SRCR) domains in the N-terminus in addition to the C-terminal characteristic domains of the lysyl oxidase (LOX) family, such as a copper-binding domain, a cytokine receptor-like domain and residues for the lysyl-tyrosyl quinone cofactor. Using BLASTN searches, we identified a novel variant of *LOXL3* (termed *LOXL3-sv2*), which lacked the sequences corresponding to exons 4 and 5 of *LOXL3*. The *LOXL3-sv2* mRNA is at least 2,398 bp in length, encoding a 608 amino acid-long polypeptide with a calculated molecular mass of 67.4 kDa. The deletion of exons 4 and 5 do not change the open-reading frame of LOXL3 but results in deletion of the SRCR domain 2. The recombinant LOXL3-sv2 protein showed a β-aminopropionitrile-inhibitable amine oxidase activity toward collagen type I. In RT-PCR analysis, *LOXL3-sv2* was detected in all human tissues tested, along with *LOXL3* and *LOXL3-sv1*, a previously identified variant of *LOXL3*. These findings indicate that the human *LOXL3* gene encodes at least three variants, LOXL3, LOXL3-sv1 and LOXL3-sv2, all of which function as amine oxidases.

Introduction

The human lysyl oxidase-like 3 (*LOXL3*) gene was originally identified by EST database searches, encoding a 753 amino acid-long polypeptide with a deduced molecular mass of 83 kDa (1,2). LOXL3 belongs to an emerging family of lysyl oxidases (LOX), each of which functions as a copper-dependent amine oxidase for the formation of lysine-derived cross-links found in collagen and elastin fibrils (3). All LOX paralogues (LOX, LOXL, LOXL2, LOXL3 and LOXL4) contain a copper-binding domain, residues for lysyl-tyrosyl quinone (LTQ) and a cytokine receptor-like (CRL) domain in the C-terminus. In addition, four repeated copies of scavenger receptor cysteine-rich (SRCR) domains are found in the N-terminus of only LOXL2, LOXL3 and LOXL4, suggesting novel functional roles assigned to these three members (4-6).

LOXL3 has been reported to be associated with a diverse range of diseases in both humans and animals. LOXL3 was reported to interact with Snail to downregulate the expression of *E-cadherin* in carcinoma progression (7), to induce the autophagy of chondrocytes in osteoarthritis (8) and to play an essential role in the regulation of integrin signaling for correct positioning and anchoring of myofibers (9). Expression of LOXL3 was found to be significantly reduced at both mRNA and protein levels in the vaginal tissues of patients with pelvic organ prolapse (10). A recent genetic study reported a homozygous missense mutation (C676Y) in *LOXL3* as the cause of Stickler syndrome, a collagenopathy characterized by arthropathy and vitreoretinopathy with high myopia and cleft palate, in a consanguineous Saudi family (11). Several homozygous and compound heterozygous frame-shift mutations of *LOXL3* have been identified in patients with early-onset high myopia (12). In animal studies, *Loxl3*$^{-/-}$ mice showed perinatal lethality with craniofacial and spinal defects (13), and knockdown of *lox3b* in zebrafish resulted in craniofacial abnormalities (14).

Previously, we reported a variant of *LOXL3*, termed *LOXL3-sv1* (15), which contained the characteristic C-terminal domains of the LOX family but lacked the characteristic N-terminal region including SRCR domains 1-3. The LOXL3-sv1 showed amine oxidase activity toward different types of collagens with distinct substrate specificity from LOXL3 (15). In search of more human *LOXL3* variants, we identified a novel variant, termed *LOXL3-sv2*, which lacked the sequences corresponding to exons 4 and 5 of *LOXL3*. The deduced amino acid sequence of LOXL3-sv2 does not have the SRCR domain 2 but contains all the other functional domains of LOXL3. The recombinant LOXL3-sv2 protein showed β-aminopropionitrile (BAPN)-inhibitable amine oxidase activity toward collagen type I. These findings indicate that *LOXL3* encodes at least three variants, LOXL3, LOXL3-sv1 and LOXL3-sv2, each functioning as an amine oxidase toward collagen.

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Materials and methods

EST database search. The human *LOXL3* (GenBank accession no. NM_032603.3) cDNA sequence was used to search the human EST database through the BLASTN program (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST). EST clones showing significant sequence homology to human *LOXL3* were purchased from GE Dharmacon (Lafayette, CO, USA) and were completely sequenced using an ABI 310 automated DNA sequencer.

RT-PCR analysis in human tissues. Human MTC (Multiple Tissue cDNA) Panels (Clontech Laboratories, Inc., Mountain View, CA, USA) were PCR-amplified with primers specific to *LOXL3-sv1* (GenBank accession no. DQ378059) and *LOXL3-sv2* (GenBank accession no. NM_001289164) using *Ex-Taq* polymerase (Takara Bio, Inc., Otsu, Japan). The *LOXL3-sv1*-specific primers were: forward, 5'-TGGAAG TCAGGCTTCCTGAC-3' and reverse, 5'-CTCAGAGCTTCT CGAGCACT-3'. The *LOXL3-sv2*-specific primers were: 5'-GTGTGACTGAATGTGCCTCC-3' and reverse, 5'-CAG TCATCCCCACAGATGAG-3'. Both the *LOXL3-sv1*-specific and *LOXL3-sv2*-specific primer sets were also designed to detect the *LOXL3* mRNA. The PCR conditions consisted of 30 cycles at 94˚C for 45 sec, 56-58˚C for 45 sec, and 72˚C for 45 sec with a predenaturation at 94˚C for 5 min and a final extension at 72˚C for 7 min. The amplified PCR products were analyzed by electrophoresis on a 2% agarose gel. *GAPDH* was used as an internal control. All RT-PCR analyses were performed in the linear range of amplification, and quantification was performed using Kodak Gel Logic Imaging System (Eastman Kodak, Rochester, NY, USA).

Expression and purification of the LOXL3-sv1 and LOXL3-sv2 proteins. For expression of the LOXL3-sv1 protein, the pET21-LOXL3-sv1 construct that was previously reported by us (15) was used. For expression of LOXL3-sv2, an EST clone (clone ID no. 6650423; GE Dharmacon) was used as a PCR template, and the entire coding region of *LOXL3-sv2* was PCR-amplified by PfuTurbo™ DNA polymerase (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. The sequences of the oligonucleotide primers used for the construction of the expression plasmids were 5'-GCGGCTAGCATGCGACCT GTCAGTGTCTG-3' and 5'-GCGAAGCTTGATAATCTGGT TGCTGGTCTG-3'. A unique restriction site, either *Nhe*I or *Hin*dIII, was introduced in each primer for convenient subcloning into the pET21 vector. The PCR conditions consisted of 30 cycles at 94˚C for 60 sec, 58˚C for 60 sec and 72˚C for 60 sec with a predenaturation at 94˚C for 5 min and a final extension at 72˚C for 7 min. The sequence of the resulting expression constructs was confirmed by DNA-sequencing analysis using a Cycle Sequencing Ready Reaction kit (Life Technologies, Paisley, UK) according to the manufacturer's instructions. The *Escherichia coli* (*E. coli)* strain BL21 (DE3) (Novagen, Inc., Madison, WI, USA) was used for transformation of the LOXL3-sv1 or LOXL3-sv2 expression construct. The recombinant proteins were expressed, purified and refolded into an enzymatically active form as previously reported (15).

Amine oxidase assays. The amine oxidase activity of the LOXL3-sv1 and LOXL3-sv2 recombinant proteins were assessed using a peroxidase-coupled fluorometric assay with the Amplex® Red hydrogen peroxide assay kit (Molecular Probes, Eugene, OR, USA) as previously described (16). Each reaction contained 10 μg of the purified LOXL3-sv1 or LOXL3-sv2 recombinant protein and 20 pmol of calfskin type I collagen (Sigma-Aldrich, St. Louis, MO, USA) as a substrate in a reaction volume of 200 μl. Parallel assays were performed in the absence or presence of 1 mM BAPN for 30 min at 37˚C. Fluorescence was measured using a fluorescence spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) with excitation and emission wavelengths of 500 nm and 650 nm, respectively. Total amine oxidase activity was expressed as nM of H_2O_2 produced per μg of the LOXL3-sv1 or LOXL3-sv2 recombinant protein, calculated by interpolation with fluorescence values from an H_2O_2 calibration curve.

Statistical analysis. Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by a multiple-comparison Tukey's test. All statistical analyses were performed using SPSS software version 12 (SPSS, Inc., Chicago, IL, USA). Statistical significance was determined at P-values <0.05.

Results

Distinct exon-intron structure of LOXL3-sv2 from LOXL3 and LOXL3-sv1. Searches for novel variants of the human *LOXL3* gene resulted in the identification of ESTs (accession nos. EL736465 and CN422297) in the GenBank database, which showed a different exon-intron structure from *LOXL3*. Furthermore, this novel variant, termed *LOXL3-sv2*, showed an exon-intron structure distinct from the *LOXL3-sv1* that we previously reported (15), lacking exons 4 and 5 of *LOXL3* (Fig. 1A). The novel variant corresponded to the LOXL3 transcript variant 2 (*LOXL3-v2*) that was recently reported in the GenBank (accession no. NM_001289164). However, there was a difference in the length of the 3'-untranslated region (3'-UTR); our *LOXL3-sv2* contains 476 bp 3'-UTR, whereas the previously reported *LOXL3-v2* contains 1,348 bp 3'-UTR. LOXL3-sv2 also corresponded to an unverified cDNA sequence of LOXL3 (accession no. BC071865), but BC071865 contained an additional 30 bp sequence at the 5'-end, originating from an unknown source.

The *LOXL3-sv2* mRNA is at least 2,398 bp in length, encoding a 608 amino acid-long polypeptide with a calculated molecular mass of 67.4 kDa. The deletion of exons 4 and 5 do not change the open-reading frame of LOXL3 but results in deletion of the SRCR domain 2 (Fig. 1B). The deduced amino acid sequence of LOXL3-sv2 contains the characteristic C-terminal domains of the LOX family, including the copper-binding domain, the residues for the LTQ cofactor, and the CRL domain (Fig. 2).

Expression analysis of LOXL3, LOXL3-sv1 and LOXL3-sv2 in human tissues. To compare the expression of *LOXL3*, *LOXL3-sv1* and *LOXL3-sv2* in human tissues, RT-PCR analyses were performed with poly(A)$^+$ RNA isolated from 16 different human tissues. For specific detection of *LOXL3-sv1*

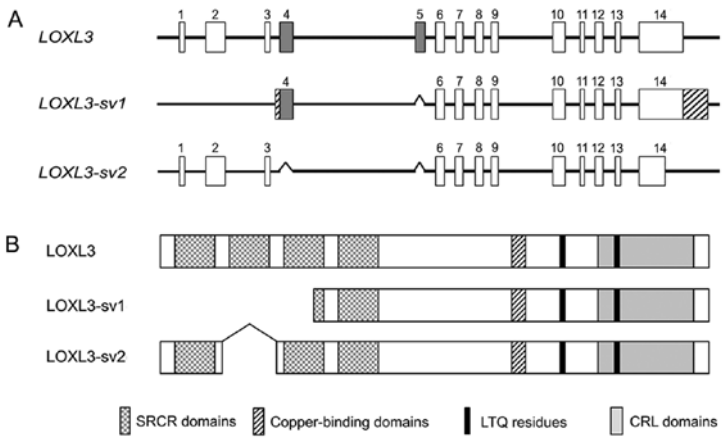

Figure 1. Structures of exon-intron and functional domains. (A) Schematic diagrams of the exon-intron structure of human lysyl oxidase-like 3 (*LOXL3*), *LOXL3-sv1* and *LOXL3-sv2*. Exons are shown as boxes, and introns as bold lines. In *LOXL3-sv1*, the hatched boxes indicate the regions found only in the *LOXL3-sv1* cDNA but not in the *LOXL3* and *LOXL3-sv2* cDNAs. (B) Schematic diagrams of the functional domains of LOXL3, LOXL3-sv1 and LOXL3-sv2. The scavenger receptor cysteine-rich (SRCR) domains, the copper-binding domains, the lysyl-tyrosyl quinone (LTQ) residues and the cytokine receptor-like (CRL) domains are indicated. The slanted lines in LOXL3-sv1 indicate the SRCR domain 2 deleted in LOXL3-sv2.

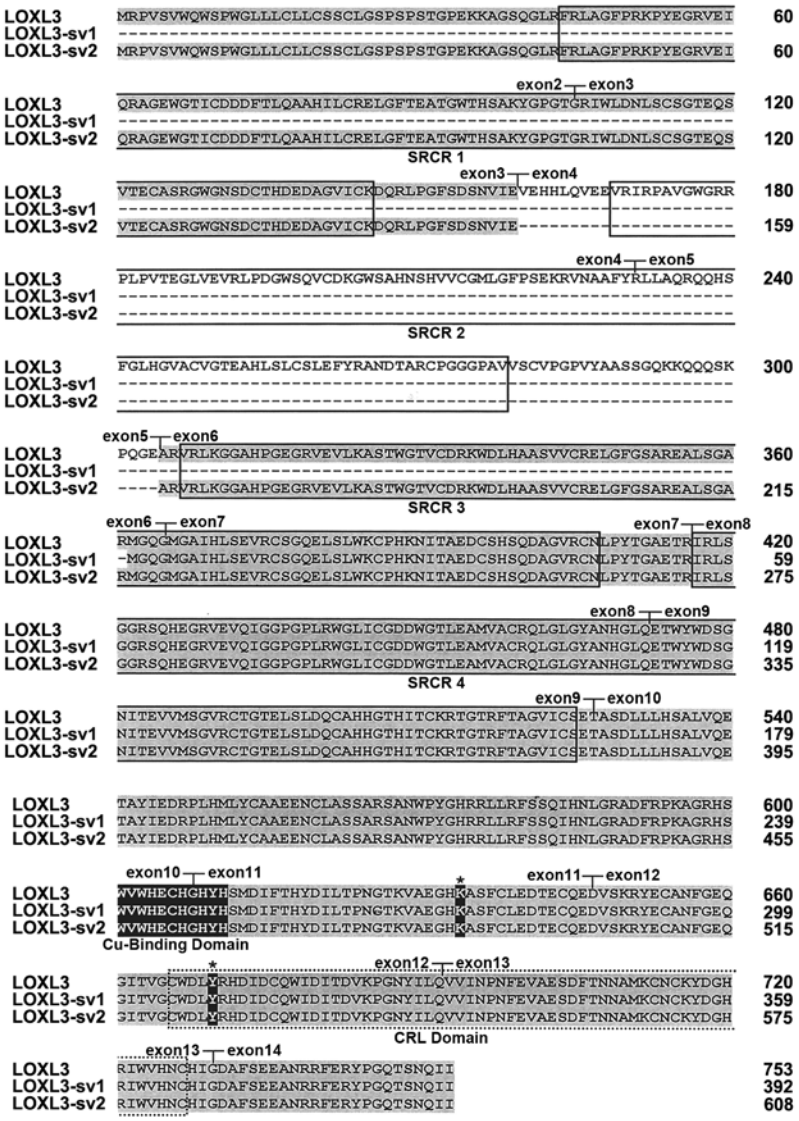

Figure 2. Deduced amino acid sequences of human lysyl oxidase-like 3 (LOXL3), LOXL3-sv1 and LOXL3-sv2. Amino acids are indicated as a single letter code and numbered from the N-terminus. The conserved amino acid residues are indicated by shading. The copper-binding motif is indicated in reverse-font letters in a box of darker shading, the lysyl-tyrosyl quinone (LTQ) residues are in reverse-font letters with an asterisk mark, and the cytokine receptor-like (CRL) domain is in a box with dashed lines. The exon junctions are indicated by T-bars. Scavenger receptor cysteine-rich (SRCR) domains 1-3 are deleted in LOXL3-sv1, whereas only SRCR domain 2 is deleted in LOXL3-sv1.

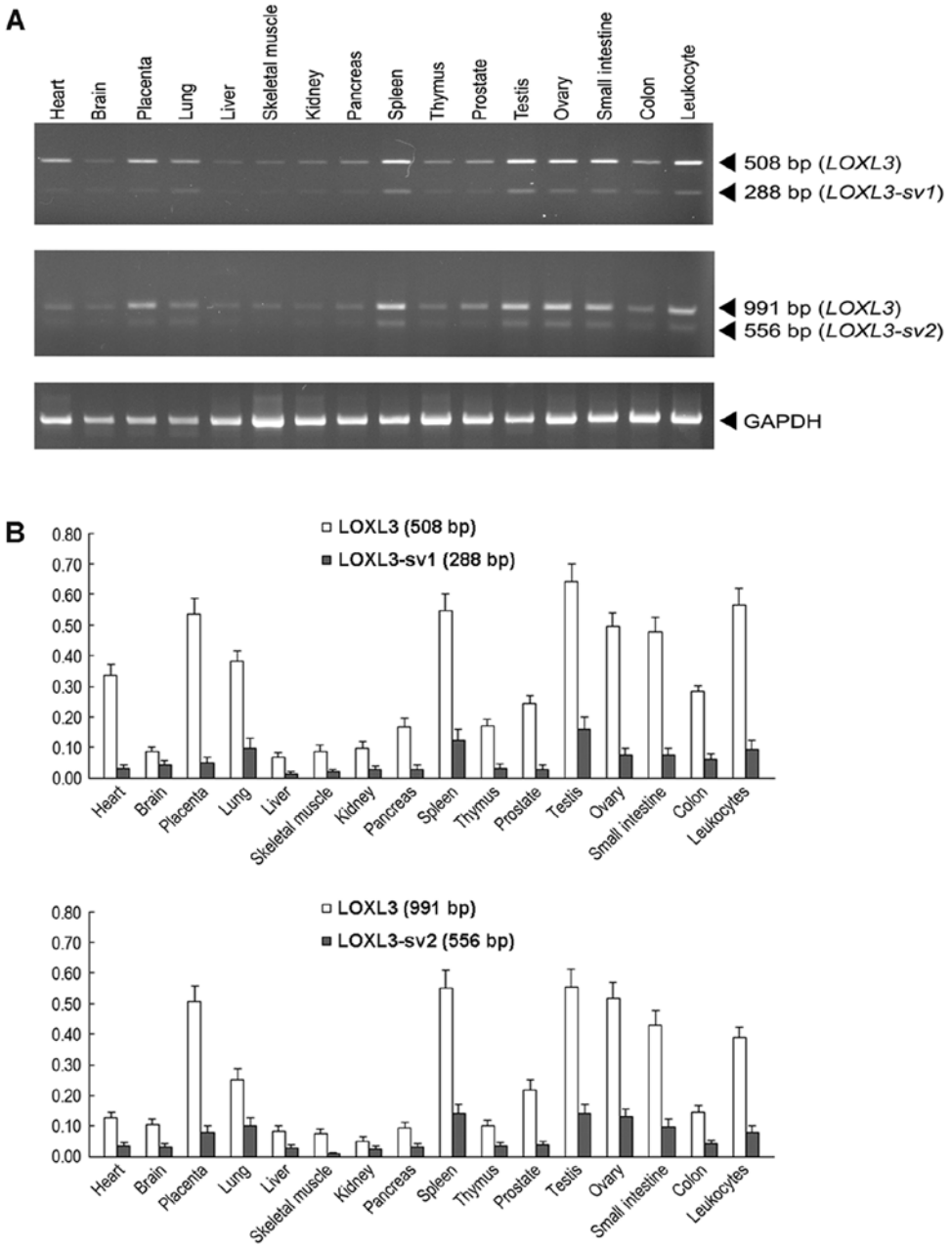

Figure 3. RT-PCR analysis of human lysyl oxidase-like 3 (*LOXL3*), *LOXL3-sv1* and *LOXL3-sv2* in human tissues. (A) A typical example of RT-PCR analysis of *LOXL3*, *LOXL3-sv1* and *LOXL3-v2* using poly(A)$^+$ RNA from 16 different human tissues. The analysis was repeated in triplicate, and all analyses showed similar expression patterns. *GAPDH* was used as an internal control. (B) The intensities of the PCR amplicons were expressed as a densitometric ratio to the intensity of the *GAPDH* amplicon. The standard deviations are indicated as small T-bars (error bars) on the graph.

and *LOXL3-sv2* amplicons, the different exonic structures of the *LOXL3-sv1* and *LOXL3-sv2* mRNAs were used to design the primers. Both the *LOXL3-sv1*- and *LOXL3-sv2*-specific primer sets were also expected to detect the *LOXL3* mRNA that contains all the exonic sequences. For the *LOXL3-sv1*-specific amplicon, the forward primer was derived from exon 4, which was deleted in the *LOXL3-sv2* mRNA, and the reverse primer was derived from exon 6. This primer set was expected to amplify a 288 bp band from nucleotide sequences 169-456 of *LOXL3-sv1* and a 508 bp band from nucleotide sequences 638-1,145 from *LOXL3*. For the *LOXL3-sv2*-specific amplicon, the forward primer was derived from exon 3, which was deleted in the *LOXL3-sv1* mRNA, and the reverse primers were derived from exon 8. This primer set was expected to amplify a 556 bp band from nucleotide sequences 460-1,015 of *LOXL3-sv2* and a 991 bp band from nucleotide sequences 431-1,421 of *LOXL3*. In all tissues tested, amplicons of expected sizes were detected for both *LOXL3-sv1* and *LOXL3-sv2*, but the expression levels were significantly lower than that of *LOXL3* (Fig. 3). The expression patterns of *LOXL3* and the two variants were similar in all tissues tested (Fig. 3).

Amine oxidase activity of LOXL3-sv2. We previously reported that LOXL3-sv1 functions as an amine oxidase (15). To determine if LOXL3-sv2 functions as an amine oxidase, we expressed and purified the LOXL3-sv2 protein in a hexa-histidine recombinant form as previously described for LOXL3 and LOXL3-sv1 (15). The LOXL3-sv1 protein was also

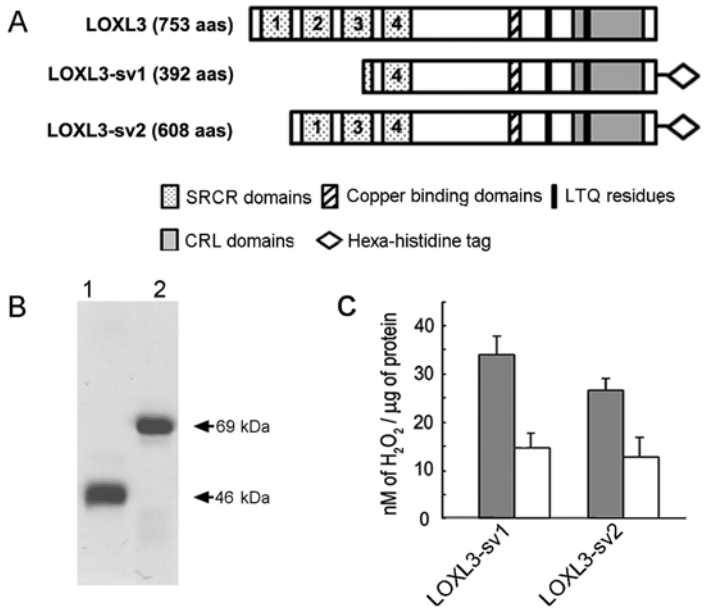

Figure 4. Amine oxidase activity of human lysyl oxidase-like 3 variants LOXL3-sv1 and LOXL3-sv2. (A) Schematic diagrams of the recombinant LOXL3-sv1 and LOXL3-sv2 proteins. Both proteins are tagged with a hexa-histidine at the C-terminus. (B) The recombinant LOXL3-sv1 and LOXL3-sv2 proteins were expressed and purified as recombinant proteins of 46 and 69 kDa, respectively. (C) The amine oxidase assays were repeated in quadruplicate. The amine oxidase activity was expressed as nM of H_2O_2 produced per μg of the LOXL3-sv1 or LOXL3-sv2 recombinant protein. The gray and white bars indicate the reactions without and with β-aminopropionitrile (BAPN), respectively. Standard deviations are indicated as small T-bars on the graph.

expressed and purified in a hexa-histidine recombinant form for comparison of the amine oxidase activity with LOXL3-sv2 (Fig. 4A). However, the full-length LOXL3 protein was not expressed in the *E. coli* overexpression system, as we previously reported (15). It was probably due to the presence of rare codons clustered in the N-terminus of LOXL3, such as CCC (Pro^{11}, Pro^{50}, Pro^{53}, Pro^{173}, Pro^{181}, Pro^{183} and Pro^{220}) and AGG (Arg^{51}, Arg^{192} and Arg^{224}) that are infrequently used in *E. coli*. Clusters of rare codons were reported to reduce the expression level in *E. coli* (17). The apparent sizes of the purified recombinant proteins were in agreement with the deduced molecular mass; 46 and 69 kDa for the recombinant LOXL3-sv1 and LOXL3-sv2 proteins, respectively (Fig. 4B).

The recombinant LOXL3-sv1 and LOXL3-sv2 proteins were assessed for amine oxidase activity toward collagen type I using a peroxidase-coupled fluorometric assay. Both the recombinant LOXL3-sv1 and LOXL3-sv2 proteins showed significant levels of amine oxidase activity toward collagen type I in a manner sensitive to β-aminopropionitrile (BAPN), an irreversible inhibitor of the LOX-derived amine oxidase activity (Fig. 4C). The LOXL3-sv1 protein showed slightly higher amine oxidase activity than the LOXL3-sv2 protein; however, the difference was not statistically significant (Fig. 4C). These results indicate that LOXL3-sv2, as do LOXL3 and LOXL3-sv1, also functions as an amine oxidase toward collagen type I, a physiological substrate of the LOX family proteins.

Discussion

LOXL3-sv2, the newly identified variant of *LOXL3*, encodes a 608 amino acid-long polypeptide with a calculated molecular mass of 67.4 kDa. Previously, we reported an approximately 67 kDa protein in several human tissues using a polyclonal antibody against LOXL3 and proposed that the 67 kDa protein could be derived from post-translational modification of the full-length LOXL3 (15). However, judging from the deduced molecular mass of LOXL3-sv2, the previously reported 67 kDa band may correspond to a novel LOXL3-sv2 protein, although further detailed characterization is required. By promoter analysis, we previously demonstrated that an alternative promoter element present in the 5'-flanking intronic region of exon 4 of the *LOXL3* gene regulates the transcriptional activation of *LOXL3-sv1*. The promoter region responsible for expression of the *LOXL3* mRNA contained no TATA and CAAT boxes but abundant CpG-rich sequences, as shown in a number of eukaryotic housekeeping genes (18,19). In contrast, the *LOXL3-sv1*-specific promoter contained a TATA box with a number of potential transcription factor binding sites (15). The presence of exon 1 in the newly identified *LOXL3-sv2* mRNA indicates that the promoter element located in the upstream region of exon 1 regulates the transcriptional activation of *LOXL3-sv2*. Identification of these LOXL3 variants suggest that at least three different regulatory mechanisms exist for the transcription of the *LOXL3* gene, being responsible for the differential expression of *LOXL3*, *LOXL3-sv1* and *LOXL3-sv2*.

The LOX family proteins have been proposed to exert different functional roles through distinct substrate specificity in the formation and maintenance of extracellular matrix. LOX and LOXL1 are more likely involved in the formation of fibrillar collagen fibers, such as types I-III, whereas LOXL2 is responsible for cross-linking of collagen type IV associated with the basement membranes (20). In peroxidase-coupled fluorometric assays with different types of collagens, LOXL3 showed higher amine oxidase activity toward collagen type I, II and VIII, whereas LOXL3-sv1 showed higher amine oxidase activity toward collagen types III and IV (15), further suggesting that the LOX family proteins may play different

functional roles through distinct substrate specificity. In our amine oxidase assays, the recombinant LOXL3-sv2 protein showed a significant level of amine oxidase activity toward collagen type I in a BAPN-sensitive manner, indicating that LOXL3-sv2 functions as an active amine oxidase, although substrate specificity of LOXL3-sv2 has yet to be determined. Considering the diverse structural divergence and tissue distribution of different collagen types, detailed amine oxidase assays and colocalization studies with different types of collagens are required for further understanding of the functional significance of this novel LOXL3 variant.

LOXL3 contains four repeated copies of SRCR domains in the N-terminus, which are characterized by 6-8 cysteine residues in 90-110 amino acid long regions. SRCR domains are found either on the cell surface proteins or on secreted proteins, mediating protein-protein interactions for cell adhesion and cell signaling (21,22). The deduced amino acid sequence of LOXL3-sv2 shows deletion of the SRCR domain 2 but contains all the other SRCR domains as well as the characteristic C-terminal domains of the full-length LOXL3. In contrast, LOXL3-sv1 does not have the N-terminal SRCR domains 1-3 of LOXL3. Previously, using a series of deletion constructs of the N-terminal SRCR domains, we showed that the SRCR domains did not affect the amine oxidase activity of LOXL4, a close paralogue of LOXL3 (23). In this study, the purified recombinant proteins of both LOXL3-sv1 and LOXL3-sv2 showed BAPN-inhibitable amine oxidase activities toward collagen type I, further indicating that deletion of SRCR domains do not influence the amine oxidase activity at least in *in vitro* assays. Recently, LOXL2, another SRCR domain-containing LOX paralogue, was shown to affect keratinocyte differentiation through the SRCR domains but not by amine oxidase activity (24), suggesting unique functional roles of the SRCR domains present in the LOXL proteins. The partial absence of N-terminal SRCR domains in LOXL3-sv1 and LOXL3-sv2 suggests that these two variants may possess distinct interactive profiles from LOXL3 in protein-protein interactions. Further studies on the interactive properties of LOXL3, LOXL3-sv1 and LOXL3-sv2 will be important for understanding the functional differences of these three variants generated from the single gene in the biogenesis of extracellular matrix proteins.

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