Supplemental Material

LOX Mutations Predispose to Thoracic Aortic Aneurysms and Dissections

Dong-chuan Guo, Ellen S. Regalado, Limin Gong, Xueyan Duan, Regie Lyn P. Santos-Cortez, Pauline Arnaud, Ren Zhao, Bo Cai, Ellen M. Hostetler, Rocio Moran, David Liang, Anthony Estrera, Hazim Safi, University of Washington Center for Mendelian Genomics, Suzanne M. Leal, Michael J. Bamshad, Jay Shendure, Deborah A. Nickerson, Guillaume Jondeau, Catherine Boileau, and Dianna M. Milewicz

Department of Internal Medicine (D.C.G., E.S.R., L.G., X.D., R.Z., B.C., E.M.H., D.M.M.) University of Texas Health Science Center, Houston, Texas; Center for Statistical Genetics, Department of Molecular and Human Genetics (R.L.P.S.C, S.M.L.) Baylor College of Medicine, Houston, Texas; Laboratory for Vascular Translational Science, INSERM U1148 (P.A., G.J., C.B.), Hôpital Bichat, Paris, France; Genomic Medicine Institute (R.M.), Cleveland Clinic, Cleveland, Ohio; Department of Medicine (D.L.), Stanford University Medical Center, Stanford, California; Department of Cardiothoracic and Vascular Surgery (A.E., H.S.), University of Texas Health Science Center, Houston, Texas; Department of Genome Sciences (M.J.B., J.S., D.A.N.), University of Washington, Seattle, Washington; Centre National de Référence pour le syndrome de Marfan et apparentés (G.J., C.B.), Hôpital Bichat, AP-HP, Paris, France.

Address reprint requests to: Dr. Milewicz at The University of Texas Medical School at Houston, 6431 Fannin, MSB 6.100, Houston, Texas 77030 (Dianna.M.Milewicz@uth.tmc.edu)

Materials and Methods

Exome and Sanger Sequencing

Genomic DNA was extracted from peripheral blood lymphocytes using standard protocols. Five micrograms of DNA from three affected individuals in family TAA602 were used for construction of the shotgun sequencing library as described previously using adaptors for paired-end sequencing. Exome sequences were captured by SeqCap EZ. Exome probes version 2.0 (Roche) and recovered according to manufacturer's directions. Enriched libraries were then sequenced on an Illumina GAIIx using manufacturer protocols. Reads were mapped to the reference human genome (UCSC hg19) with BWA (Burrows-Wheeler Aligner), and variant detection and genotyping are performed using the UnifiedGenotyper (UG) tool from GATK. Annotation of variants was performed using the SeattleSeq server (http://gvs.gs.washington.edu/SeattleSeqAnnotation/).[9] Sanger DNA sequencing assay was performed to validate exome sequencing results and test the segregation of variants with TAAD in families. *LOX* mutations were reported based on the RefSeq codes NM_002317.5.

Construction of LOX recombinant plasmids:

WT-LOX was amplified by using pcDNA-LOX (GenScript, Inc., Piscataway Township, NJ) with primers 5'-gcgaattcatgcgcttcgcctggaccgt-3' and 5'-acggtcgacatacggtgaaattgtgcagcct-3' (IDT, Inc., Coralville, Iowa). Three LOX mutants were generated by PCR-directed site mutagenesis with HiFi HotStart DNA polymerase (KAPA Biosystems, Wilmington, MA) and corresponding primers sets as follows: Ile248-Fw, 5'-ctgtctggccagtatagcatacagggcaga-3', Ile248-Rv, 5'-tctgccctgtatgctatactggccagacag-3', Arg280-Fw, 5'-cagatttcttacccatccgaccaagatattcc-3′ Arg280-Rv, 5'-ggaatatcttggtcggatgggtaagaaatctg-3', Arg348-Fw, 5'-cacagggattgagAcctggctgttatg-3', and Arg348-Rv, 5'-cataacagccaggtctcaatccctgtg-3'. The construct and mutations were verified by

Sanger sequencing. The cDNA of LOX-WT, -Ile248, -Arg280 and –Arg348 were inserted into pcDNA3 vector by digesting with EcoRI and SalI.

Lysyl Oxidase Assay and Western blot analysis

HeLa cells (10^5) were seeded into six-well plates. The recombinant plasmids were transfected into HeLa cells respectively using Lipofectamine 2000 reagent. After 72 hours, the dishes were scraped and the lysate homogenized (RIPA lysis buffer and protease inhibitor cocktail (Sigma-Aldrich) on ice). The lysates were spun and 50 ul of supernatant was assayed in triplicate. LOX activity was measured with a commercially available fluorimetric assay (AAT Bioquest, Sunnyvale,CA), which utilizes a proprietary LOX substrate that releases hydrogen in a horseradish peroxidase-coupled reactions. The assay was done following the manufacturer's recommendations. The reaction was incubated at 37°C for 30min, 60min, 90min, 120min, and 150min. Its signal was read by a fluorescence microplate reader at Ex/Em=540/590 nm.

Lysates (20 ug) were separated by 4-15% SDS polyacrylamide gel electrophoresis (TGX Mini Protean, Bio-Rad) with Tris-glycine running buffer. The proteins were transferred to a nitrocellulose membrane using the iBlot dry blotting system (Life Technologies). The membrane was blocked with 5% skim milk and incubated with rabbit polyclonal primary anti-LOX diluted 1:1,000 (abcam). The membrane was also incubated with rabbit monoclonal anti-GAPDH (1:5,000) to confirm equal loading of cellular proteins. The immune complexes were visualized using enhanced chemiluminescence reagent (Western Lightning Chemiluminescence ECL Pro, Perkin Elmer, Waltham, MA) and detected with ImageQuant LAS4000 (GE Healthcare).

Pathology Analysis

Aorta from patients and healthy control were sectioned and stained with Movat pentachrome and picro sirius red.

Supplementary Table I. Candidate genes identified by exome sequencing in the index family (TAA602).

Gene	Amino acid substitution	mRNA level[a]	Conservation[b]	Cscore	Predicted effect on function[c]	MAF in ExAC
LOX	S280R	3873.7	1	17.36	5/7	1.65E-05
ACER1	G41R	147.6	0.939	18.5	5/7	2.47E-05
ARHGAP33	P705L	144.5	0.002	2.809	1/7	0
HPGD	D80G	126.4	1	15.55	3/7	0
ISM1	D140N	126.3	1	22.9	3/7	1.69E-05
PCDHGA11	H650Q	159.5	0	14.61	3/6	0
PCSK1	S332N	176.3	1	29.5	4/7	0
RNF150	I395F	158.7	0.995	8.702	0/7	0
UBR5	R994C	341.6	1	14.42	5/6	0
ZNF609	R240H	211.1	1	19.75	5/7	2.56E-05

[a] The average level of mRNA expression in four smooth muscle cell lines explanted from control aorta. The levels of mRNA expression were quantified based on an arbitrary unit that was detected by the Illumina Sentrix Human Ref8 Beadchip Expression Array.
[b] PhastCons conservation score
[c] LRT, MutationTaster, Polyphen2 HDIV, Polyphen2 HVAR, SIFT, PROVEAN, and MutationAssessor.