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Mutation Screen of *LOXL1* in Patients with Female Pelvic Organ Prolapse

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Abstract

Objectives—The *LOXL1* (lysyl oxidase-like 1) gene encodes a copper-dependent monoamine oxidase that catalyzes the deamination of a lysine residue in the crosslinking of tropoelastin monomers to form elastin. *LOXL1*-KO mice do not deposit normal elastic fibers in their genitourinary tract resulting in post-partum pelvic organ prolapse and lower urinary tract dysfunction with decreased bladder capacity and lower voiding pressure. We sought to identify which single nucleotide polymorphisms (SNPs) in the *LOXL1* coding sequence play a role in female pelvic organ prolapse (FPOP).

Methods—A total of 66 patients were screened, 48 in the case group and 18 in the control group. The 7 exons of *LOXL1* were evaluated for any polymorphisms.

Results—Three missense sequence changes (Arg141Leu, Gly153Asp, and Ser159Ala) and three silent mutations (Asp292Asp, Ala320Ala, and Ile521Ile) were identified. None of these polymorphisms were found to differ significantly in frequency in the case group compared to the control group.

Conclusion—Our findings do not support an association of any *LOXL1* exonal SNPs with the diagnosis of FPOP.

Keywords

LOXL1; Female pelvic organ prolapse; Urinary Incontinence

Introduction

Female pelvic floor dysfunction leading to urinary incontinence, anal incontinence, pelvic organ prolapse, and sexual and lower genitourinary dysfunction affects more than one-third of adult women in the United States.¹ Ultimately, the lifetime risk for women to undergo

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surgery for prolapse or incontinence is reported to be between 11-19%.^{2,3} Costs and quality of life concerns are significant within the context of an aging society, where surgical repair of prolapse is the most common inpatient procedure performed in women over 70 years of age.^{4,5,6}

Female pelvic organ prolapse (FPOP) is defined as abnormal descent or herniation of the pelvic organs protruding against the vaginal wall. The organs involved may include the uterus, vaginal apex, anterior vagina, or posterior vagina.⁷ Though the pathophysiology of FPOP is not yet well-defined, it is believed to be multifactorial, with genetically predisposed women developing prolapse after certain life events.⁸ The major risk factors for FPOP are increasing parity, aging, estrogen deficiency, obesity, smoking, and collagen and elastin deficiencies—particularly in patients with connective tissue disorders such as Marfan's or Ehlers-Danlos.^{1,9,10}

Anatomically, the pelvic floor holding the pelvic organs in place includes the endopelvic fascia, the levator muscles, and the perineal body in addition to the bony pelvis. The tensile strength provided by the connective tissues of these organs responds to abdominal pressures to prevent FPOP pathophysiology. These tissues are particularly rich in elastin in addition to collagen.¹¹ Elastin has an important role in providing resiliency and strength to the pelvic floor. A rat model has displayed elastin's high turnover and its important role in remodeling the pelvis during pregnancy and childbirth.¹²

Elastin attains its tensile strength and resiliency by forming a cross-linked polymer. The *LOXLI* (lysyl oxidase-like 1) gene, located at 15q24.1, encodes a copper-dependent monoamine oxidase that catalyzes the deamination of a lysine residue on tropoelastin monomers in the formation of crosslinks between monomers to generate elastin polymers.¹³ *LOXLI*-KO mice do not deposit normal elastic fibers in their genitourinary tract and show gross post-partum pelvic organ prolapse and lower urinary tract dysfunction with lower bladder capacity and voiding pressure.¹⁴ This finding of a mouse model for FPOP has sparked interest in the possible role of *LOXLI* in the pathophysiology of FPOP.

The above studies emphasize the importance of further study of *LOXLI* as a potential candidate in a genetic or epigenetic etiology of FPOP. Most of the published studies investigating *LOXLI* involvement in FPOP have produced seemingly contradictory results. Three studies found reduced *LOXLI* expression in pre-menopausal, post-menopausal, or a mixed population of women with POP, suggesting a mechanism of sub-optimal elastin polymerization leading to FPOP.^{15,16,17} The group subsequently found increased CpG methylation in the *LOXLI* gene promoter in DNA from a mixed population of women with FPOP and lower *LOXLI* mRNA levels suggesting a possible epigenetic mechanism.¹⁸ Conversely, Jung et al found increased expression of *LOXLI* mRNA in post-menopausal women with FPOP, which they proposed was due to compensatory induction of *LOXLI* expression secondary to aberrant cross-linking of tropoelastin.¹⁹ Nevertheless, these studies support the idea that some forms of dysfunctional *LOXLI* expression or activity remain potential etiologies for FPOP.

A previous genetic study identified a single nucleotide polymorphism (SNP) in the promoter region of the *LOXLI* gene and showed that the minor allele yielded higher expression than the major allele in promoter-reporter assays in cultured cells.²⁰ However, they found no differences in frequencies of the promoter allelic pairs in FPOP patients compared to controls. To explore genetic factors that might impact LOXL1 activity or expression, we investigated the genotypes of the *LOXLI* coding sequence in women with Stage III/IV FPOP and non-asymptomatic women. Therefore goal of this study was to determine if women with and without FPOP demonstrate significant differences in *LOXLI* exon SNPs.

Materials and Methods

Study Population

This study conformed to the tenets of the Declaration of Helsinki and was approved by the Internal Review Boards (IRB 06-093) of the Cleveland Clinic Foundation. All blood samples were obtained after informed consent was secured.

The cases were comprised of women over the age of 18 with Stage III or IV FPOP and without any other type of pelvic floor dysfunction including stress urinary incontinence or urge urinary incontinence. The control group was comprised of women over 18 years of age without any pelvic floor dysfunction who were being seen for a benign vaginal surgical intervention.

Patients were excluded if they had a physical or mental disability that precluded them from informed consent. Patients were also excluded if they were diagnosed with one or more reproductive malignancies. An earlier study identified a sequence variant of the *LAMC1* gene, rs10911193, as associated with familial FPOP. LAMC1 is an extracellular matrix glycoprotein that is highly expressed in smooth muscle.^{21,22} To rule out the familial *LAMC1* genetic factor for FPOP, we screened our patients for this SNP and excluded them if they had the FPOP-associated variant. All patients were freely able to discontinue participation in the study at any time.

Mutation Screening

A 10 ml blood sample was collected from each patient. For mutation detection, PCR products corresponding to the *LOXLI* coding sequence (GenBank RefSeq NM_005576.2) were amplified from genomic DNA and analyzed by direct genomic sequencing. The 7 coding exons and immediately-flanking intron sequences of *LOXLI* were amplified utilizing the nine sense and anti-sense primer pairs shown in Table 1. Three primer pairs were designed for exon 1 to yield smaller overlapping amplicons. The buffer pH, Mg²⁺ concentration, annealing temperature, and presence or absence of 10% dimethyl sulfoxide were tailored to each primer pair to yield optimal amplification. ExoSAP reagent (Affymetrix; Santa Clara, CA) was utilized to remove unincorporated primers and dNTPs, and a Performa V3 Well Plate Kit (EdgeBio; Gaithersburg, MD) were utilized to remove gel loading dye. Direct sequencing was performed using a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems; Grand Island, NY) according to the manufacturer's protocol, and sequences were resolved on a CEQ-2000 automated sequencer (Beckman-

Coulter; Indianapolis, IN). Collected data were analyzed with SeqScape v2.5 software (Applied Biosystems; Grand Island, NY).

Statistics

Genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium by Pearson's χ^2 test. Allele frequencies were compared in case vs. control groups by Fisher's exact test, along with calculations of odds ratios and 95% confidence intervals (CI), using Graphpad Prism 6. Post hoc power analyses of the missense polymorphisms were conducted using Graphpad Statmate 2 and Prism 6 to determine: a) the power to detect the observed differences in allele frequencies as significant ($p < 0.05$) with the group sizes and observed allele frequencies in the study, b) the differences that could be detected as significant in the study groups with 80% power, and c) the increases in sample sizes necessary for the observed difference to be considered significant. Additionally, for nonsynonymous SNPs, we utilized two amino acid substitution prediction methods to assess their potential as candidate mutations responsible for Mendelian and complex diseases; the "Sorting Tolerant from Intolerant" (SIFT) algorithm and Polyphen-2.^{23,24}

Results

Sequence changes in LOXL1

A total of 66 patients were screened for *LOXL1* SNPs, 48 in the case group and 18 in the control group. The case group's mean age was 65.4 with a standard deviation (SD) of 9.1 years. The control group's mean age was 56.9 with a SD of 9.0 years. Both case and control groups had similar BMI, means of 26.8 and 26.6 respectively. The majority of patients were white, 45 out of 48 in the case group and 14 out of 18 in the control group. The mean parity of cases was 1.4 with a SD of 1.5 while the mean parity of the controls was 1.0 with a SD of 1.4. See Table 2 for further details.

Three missense sequence changes (Arg141Leu-rs1048661, Gly153Asp-rs3825942, and Ser159Ala-rs78803776) and two silent SNPs (Asp292Asp-rs41429348 and Ala320Ala-rs41435250) were identified in *LOXL1* exon 1, and one silent SNP (Ile521Ile-rs369758147) was found in exon 5. None of those polymorphisms were found to differ significantly in frequency in the case group compared to the control group in comparisons of overall allele frequencies or of the diploid genotypes. All of the SNP frequencies in both the patient and control groups were in Hardy-Weinberg equilibrium. Post hoc power analyses of the allele frequencies of the three missense polymorphisms revealed that the study had low power to detect the minor differences observed between FPOP patients and controls as significant, but that it had about 80% power to detect differences of about 2-fold in the more common SNPs (Arg141Leu and Gly153Asp) ("Minimal detectable difference" in Table 3).

The Gly153Asp and Ser159Ala sequence changes were predicted to be "tolerated changes based on homologues in protein alignment" by SIFT algorithm and "benign changes with sensitivities greater than 0.9 by the PolyPhen-2 algorithm. The Arg141Leu sequence change resulted in the SIFT algorithm predicting a "damaging" change based on homologues in protein alignment and a "possibly damaging" change by the Polyphen-2 algorithm.

The Arg141Leu and Gly153Asp polymorphisms were by far the most common of the six SNPs found in our study population; the CTG (Leu141) allele was detected in 30.2% and 30.6% of the patients and controls, respectively, and the GAC (Asp153) allele was detected in 20.8% and 16.7% of the patients and controls, respectively (Table 3). Those allelic frequencies are similar to what others have found in control groups of other *LOXLI* SNP studies.^{25,26} None of the other SNPs were found homozygously in any of the FPOP patients or control individuals. The missense SNP Ser159Ala was found heterozygously in one patient in the case group and one patient in the control group. The silent polymorphisms Asp292Asp, Ala320Ala, and Ile521Ile were each found heterozygously in one control individual; Asp292Asp was found in two FPOP patients, while none of the FPOP patients harbored either of the other silent SNPs.

Discussion

FPOP is a common and costly condition with severe reductions in the quality of women's lives. The exact pathophysiology of FPOP remains to be determined. Herein, we sought evidence for a genetic predisposition to developing FPOP. Though fairly stable in other tissues, elastin has a high turnover rate in the pelvis as a result of its significant role in remodeling during pregnancy and childbirth.¹² There is evidence that *LOXLI* localizes specifically to the sites of elastogenesis.¹³ The potential that *LOXLI* mutations may play a role in human FPOP has been displayed by *LOXL1*-KO mice, which model the many symptoms of FPOP in humans.¹⁴

This is the first study to analyze the human *LOXLI* coding sequence for SNPs in FPOP patients in comparison with asymptomatic women. Of the six sequence variations identified in *LOXLI*, none were found to differ significantly in frequency between the case and control groups. Our findings, therefore, suggest that the basis of FPOP in our patients is not associated with polymorphisms in the *LOXLI* coding sequence. The authors acknowledge the possibility that the size of our study population was not large enough to detect a significant difference between FPOP patients and controls if *LOXLI* SNPs contribute to a small proportion of FPOP cases. However, if any of the SNPs played a major role in the majority of FPOP cases, it is likely that the difference between groups would have been large enough to be detected as significant in this study (Table 3). Another potential limitation of the study is that the mean age of the controls was 8.5 years less than the cases and therefore as control patients may develop prolapse at a later age. Additionally, we do not report our patients' menopausal status, though the majority of our patient's ages would suggest they are post-menopausal. While menopause does not affect distribution and frequencies of SNPs, it is possible that menopause and estrogen status could impact prolapse.

The mutation Ser159Ala, found heterozygously in one case patient and in one control patient, is currently listed as a single nucleotide variation of unknown clinical significance and merits further study. The silent mutations Asp292Asp, Ala320Ala, and Ile521Ile would also benefit from a larger study to note if their associations would have any clinical implications.

LOXL1's roles in several pathologies have been investigated by SNP analyses. Of particular interest to the current study, a possible association of the homozygous CTG (Leu141) polymorphism with stress urinary incontinence (SUI) was reported in a study of Turkish women.²⁷ SUI and FPOP often present concurrently due to reasons such as urethral hypermobility and similar risk factors.^{28,29} We believe it to be likely that SUI, FPOP and LOXL1 are interconnected and that LOXL1 may be one of the links and that it warrants further study in the future. Missense and silent SNPs have also been found in *LOXL1* exons in the study of glaucoma. Two missense SNPs in exon 1 have been associated with pseudoexfoliation (PEX) syndrome. Specifically, the strongly linked CGG (Arg141) and GGC (Gly153) alleles, which predominate over the respective CTG (Leu141) and GAC (Asp153) alleles in most populations, have been associated with an increased risk of PEX syndrome.³⁰

The different associations of rs1048661 (Arg141Leu) alleles with pseudoexfoliation syndrome/glaucoma and SUI suggest a pleiotropic and complex function of LOXL1 in cross-linking elastin, and emphasize the importance of investigating its role in FPOP.^{27,30} These associations also provide justification for the SIFT and PolyPhen-2 results predicting disease-causing amino acid substitutions based on sequence and structural changes. Similar to our results, other studies of *LOXL1* SNPs found no significant association with Alzheimer's disease³¹ or adolescent idiopathic scoliosis,³² despite suggestive evidence from studies of animal models.^{33,34} LOXL1's cross-linking elastin has been suggested to be a mechanism for vessel stability and therefore protective during development of vascular disease such as abdominal aortic aneurysm.³⁵ Other pathologies implicated in human studies include deregulation of LOXL1 in endometriosis and spontaneous cervical artery dissection.^{36,37} These various pathologies are based on the evidence that LOXL1 has a role in breakdown and re-synthesis of elastic fibers and connective tissue. If LOXL1 is unable to rebuild properly after damage by factors such as pregnancy, parturition and aging, this may lead to prolapse and hypermobility of pelvic organs, as well as other pathologies from damage to respective organ systems.¹¹ Though our study did not demonstrate SNPs of LOXL1 to be associated with FPOP, we do believe there is ample evidence for a role of LOXL1 in the pathophysiology of FPOP. Further research of different patient cohorts with larger sample numbers is warranted.

Conclusion

In this study, we did not find the frequency of *LOXL1* polymorphisms to be significantly different in the FPOP patients compared to the control subjects. However, much evidence points to development of FPOP from elastin and the expressed protein LOXL1 dysfunction, and our results do not exclude this etiology. Extracellular matrix and connective tissue protein expression can be modulated by forces such as body mass index, bony pelvic anatomy, pelvic trauma, pelvic injury, and pelvic stressors such as cough, exercise, and pregnancy. Therefore, these factors remain important to study. Future studies should investigate these mechanisms influencing LOXL1 expression with particular regard to epigenetic control, as well as post-transcriptional and post-translational processes. Those processes may determine elastin content through an effect on LOXL1, and therefore may influence predisposition for FPOP.

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LOXL1, Lysyl Oxidase-like 1

Figure 1. LOXL1 Sequence changes

Table 1
LOXL1 PCR-details of sequence primers, annealing temperature and amplicon size for LOXL1

Exon (LOXL1)	Sense Primer	Antisense Primer	Annealing Temp (C)	MgCl ₂	Buffer pH	DMSO
1-1	5'-CCGTGGGAAGAGACACGCCAG-3'	5'-AAGGCCGAGCCGAGACCCAGGA-3'	62	1.5mM	8.6	Y
1-2	5'-TCGGCTTTGGCCAGGTGCCCGACA-3'	5'-TAGAAGCCCTGAGGGGGTACTCG-3'	64	1.5mM	8.6	Y
1-3	5'-TGGCCTCGGCGGGGTCACTACC-3'	5'-CGCTGCTCCGTGCTTAGCAGCTCC-3'	64	1.5mM	8.6	Y
2	5'-TAGCAGTGGCCAACTGATGCTCT-3'	5'-AGTAGGCTGGGTCTTGGGTGACT-3'	60	1.5mM	8.4	N
3	5'-TGGGGCTGCAGAACAGTGGCCAA-3'	5'-TCTCCCAGAGTGTCTCATGGTGGGA-3'	58	1.0mM	8.6	N
4	5'-TCACCTGAGCCCATCTCAGGATGG-3'	5'-GAGTGGCCAGAAAGCCTTGGCAGCTT-3'	60	1.5mM	8.6	N
5	5'-AGGTGGGCCAGAACTCTGAAAGG-3'	5'-AGATGCTGCCCTCTGCTCGGCAGCT-3'	60	1.5mM	8.6	N
6	5'-CACCTCTCTGTCTGTCTGTGCC-3'	5'-AGGTATCTCAGGTGGCCTTGCCTG-3'	60	1.5mM	8.6	N
7	5'-CTGCCACGAGGATCTTGGGCTGTG-3'	5'-TTTGGAAAGGGGAGAGATTGGCCAT-3'	60	1.5mM	8.6	N

Condition Mix:

MgCl – based on conc in 1.2mL
 Amp Buffer – 200ul in 1.2mL
 dNTPs (0.2mM) – 200ul in 1.2mL
 DMSO – 200ul in 1.2mL
 Water – up to 1.2mL

Amp Buffer:

2.5mL of 2M KCl
 2.0mL of 1M Tris pH
 8.4/8.6
 1.0mL of 100x BSA
 4.5mL of dH₂O

PCR Cycles:

1. 94C for 5min
2. Annealing Temp for 30sec
3. 72C for 30 sec

4. 94C for 30 sec
5. Repeat 2-4 for 35 cycles
6. Annealing Temp for 1min30sec
7. 72C for 5min
8. 4C Storage

Standard Reaction Mix:

- 12ul of Condition Mix (MgCl, Buffer, DMSO, water, dNTPs (0.2mM))
- 6ul of DNA (20ng/ul)
- 1ul of Sense Primer
- 1ul of Anti-sense Primer
- 1ul of Taq polymerase

Table 2**Demographics**

	Age	Height (m)	Weight (kg)	BMI (kg/m ²)	Parity	Race (%)
FPOP Cases						45 white (94%) 3 non-white (6%)
<i>Mean</i>	65.4	1.57	69	26.8	1.4	
<i>Standard deviation</i>	9.1	0.07	12	4.5	1.5	
<i>Range</i>	41-78	1.42-1.73	42-98	15.4-35.1	0-5	
Control						14 white (78%) 4 non-white (22%)
<i>Mean</i>	56.9	1.64	72	26.6	1.0	
<i>Standard deviation</i>	9.0	0.06	15	5.1	1.4	
<i>Range</i>	41-73	1.57-1.73	56-116	21.3-41.3	0-4	

Table 3
SNP Distribution and frequencies between FPOP case and control

SNP			Case	Control
Position 141; CGG/CTG; Exon 1	Distribution of genotype	(CGG/CGG)	23/48 (47.9%)	10/18 (55.6%)
		(CGG/CTG)	21/48 (43.8%)	5/18 (27.7%)
		(CTG/CTG)	4/48 (8.3%)	3/18 (16.7%)
		HWE Pearson- χ^2	0.068	2.15
		P-value	0.8	0.14
	Analysis of allele frequency	Allele Frequency Arg141Leu (CGG → CTG) (rs1048661)	29/96 (30.2%)	11/36 (30.6%)
		χ^2	0.001	
		P-value	0.969	
		Odds Ratio	0.984	
		95% CI of Odds Ratio	0.40-2.46	
	Power analysis	Power to detect observed difference ^a	<3%	
		Minimal detectable difference ^b	24.9%	
		Sample size increase for significance ^c	2500-fold	
	Structural Analysis	SIFT: Damaging change based on homologues in protein alignment		
		Poly-Phen-2: possibly damaging change with specificity > 0.9		
Position 153; GGC/GAC; Exon 1	Distribution of genotype	153 (GGC/GGC)	30/48 (62.5%)	13/18 (72.2%)
		153 (GGC/GAC)	16/48 (33.3%)	4/18 (22.2%)
		153 (GAC/GAC)	2/48 (4.2%)	1/18 (5.6%)
		HWE Pearson- χ^2	0.0053	0.7199
		P-value	0.9419	0.396
	Analysis of allele frequency	Allele Frequency Gly153Asp (GGC → GAC) (rs3825942)	20/96 (20.8%)	6/36 (16.7%)
		χ^2	0.29	
		P-value	0.59	
		Odds Ratio	1.32	
		95% CI of Odds Ratio	0.44-4.08	
	Power analysis	Power to detect observed difference ^a	8%	
		Minimal detectable difference ^b	21.1%	
		Sample size increase for significance ^c	14-fold	
	Structural Analysis	SIFT: Damaging change based on homologues in protein alignment		
		Poly-Phen-2: possibly damaging change with specificity > 0.9		
Position 159; TCG/GCG; Exon 1	Distribution of genotype	159 (TCG/TCG)	47/48 (97.9%)	17/18 (94.4%)
		159 (TCG/GCG)	1/48 (2.1%)	1/18 (5.6%)
		HWE Pearson- χ^2	0.0053	0.0146
		P-value	0.9419	0.90383

SNP			Case	Control
	Analysis of allele frequency	Allele Frequency Ser159Ala (TCG → GCG) (rs78803776)	1/96 (1.0%)	1/36 (2.8%)
		χ^2	0.53	
		P-value	0.47	
		Odds Ratio	0.37	
		95% CI of Odds Ratio	0.01 - 13.9	
	Power analysis	Power to detect observed difference ^a	10%	
		Minimal detectable difference ^b	7.4%	
		Sample size increase for significance ^c	8-fold	
	Structural Analysis	SIFT: Tolerated change based on homologues in protein alignment		
		Poly-Phen-2: benign change with sensitivity > 0.9		

^aPower to detect the observed difference as significant (p<0.05 by two-tailed Fisher's exact test) with the study group sizes and observed allele frequency.

^bDifference between the percentages of cases and controls that could be detected as significant (p<0.05 by two-tailed Fisher's exact test) with 80% power and the given group sizes.

^cFold increase in sample size (maintaining the same ratio of cases to controls) necessary for the observed difference to be considered significant (p<0.05 by two-tailed Fisher's exact test).

Abbreviations: FPOP, Female Pelvic Organ Prolapse; SNP, single nucleotide polymorphism; HWE, Hardy-Weinberg Equilibrium; CI, Confidence Interval; SIFT, "Sorting Tolerant from Intolerant" algorithm