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ORIGINAL ARTICLE

Differential gene expression of extracellular-matrix-related proteins in the vaginal apical compartment of women with pelvic organ prolapse

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Abstract

Introduction and hypothesis Pelvic organ prolapse (POP) is a multifactorial disorder that impairs the quality of life (QoL) of older women in particular. The purpose of this study was to elucidate the pathogenesis of POP by focusing on the extracellular matrix (ECM).

Methods Patients were classified into two groups—with or without cervical elongation—using the POP quantification system. Specimens were obtained from 29 women with POP during hysterectomy. The expression of fibulin-5, elastin, integrin β1 (ITGβ1), lysyl oxidase-like protein-1 (LOXL1) and collagen in the vagina, uterosacral ligament, and uterine cervix was investigated by quantitative real-time polymerase chain reaction (RT-PCR) and correlation between gene levels and severity of POP examined. The location of proteins was analyzed using immunohistochemical staining and expression of fibulin-5 protein analyzed by Western blotting.

Results Fibulin-5 and elastin were mainly expressed in lamina propria and fibromuscular layers of the vagina and uterosacral ligament. Gene levels of fibulin-5 and ITGβ1 in uterosacral ligaments increased with severity of POP in women with cervical elongation, while no correlation was observed in women with a normal cervix. In women with uterine cervical elongation, each ECM-related gene significantly increased with POP staging. Furthermore, fibulin-5 protein also increased in the uterosacral ligament and uterine cervix.

Conclusions The severity of POP and gene expression of ECM-related proteins were inversely correlated in vaginal tissue in a normal and elongated cervix. These results suggested that the differing progression of the two types of POP have a relationship with ECM-related protein.

Keywords Pelvic organ prolapse · Cervical elongation · Fibulin-5 · Integrin β1 · Lysyl oxidase-like protein-1 · Extracellular matrix

Introduction

Pelvic organ prolapse (POP) is a common, multifactorial disorder caused by many physical conditions, such as vaginal birth, mechanical pelvic trauma, aging, hormonal deficiency, heavy lifting, obesity, and a chronic cough [1]. The development of POP usually complicates lower urinary tract symptoms (LUTS) [incontinence, dysuria, overactive bladder OAB)], sexual dyspareunia, and/or fecal dysfunctions (constipation, fecal incontinence) and impairs the quality of life (QoL) and healthy aging of women. Pelvic floor organs, such as bladder, urethra, uterus, vagina, rectum, and anus, are connected by the endopelvic fascia pelvis, which is supported by the levator ani muscle. It is known that pregnancy and vaginal delivery cause laceration and nerve damage of the pelvic floor muscles and fascia, with descent of pelvic organs becoming clinically advanced [2]. However, analysis of the molecular biology during pathogenesis of POP has not been elucidated. Biochemical approaches toward understanding changes in pelvic floor support associated with parturition have not progressed, and there is still insufficient data to support the introduction of new treatments, such as those based on regenerative medicine.

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Physical damage to the pelvic floor during pregnancy and parturition as a cause of POP has been clarified by macroanatomical analysis, but the analysis of cellular and biochemical changes in the pelvic supportive tissue remains unclear. A secreted protein called fibulin-5 (alias DANCE) was identified in connective tissue, and fibulin-5 knockout mice showed abnormal formation of elastic fibers throughout the body [3]. Interestingly, fibulin-5 knockout mice exhibited pelvic organ descent after parturition. In this mouse, degradation of elastic fibers was not enhanced, and fibulin-5 was found to be essential for elastic fiber formation [4]. The extracellular matrix (ECM) responsible for tissue strength plays an important role in maintaining tissue morphology. Previous studies have reported that expression of fibulin-5 and ECM-related proteins decreased in POP patients. ECM-related proteins could be involved in POP, although the nature of this relationship is not clear.

Common POP is a multifactorial disorder. In addition to the predisposing factors, such as the strength of connective tissue and muscle, inducing factors such as vaginal childbirth and injury, promoting factors such as obesity, exercise, and hormone deficiency, and noncompensatory factors due to aging, are also involved. Cervical hypertrophy with elongation of the cervix was observed in relatively younger women, and POP with cervical elongation may be related and, in some cases, have a genetic predisposition. Clinically, POP patients can be categorized into two groups: women with a normal cervix and women with cervical elongation. However, differences in the expression of ECM-related protein in these two groups is not clear.

The purpose of this study was to identify the relationship between the two types of POP and changes in ECM-related proteins. Connective tissue involved in cervical elongation and responsible for the pathogenesis of uterine descent was also analyzed.

Materials and methods

Patient selection and tissue preparation

Tissue was obtained from Osaka City University Hospital from January 2012 to December 2015. All patients with POP were clinically evaluated and POP stage classified using the Pelvic Organ Prolapse Quantification (POP-Q) assessment [5]. Twenty-nine patients with stages III–IV POP underwent pelvic reconstructive surgeries. They were classified into two groups based on the presence or absence of uterine cervical elongation. Cervical length was defined as the distance between point C and D of the POP-Q assessment system [6]. Patients in whom point E was < 5 cm were allocated to the normal group ($n = 16$). and patients with cervical elongation group all had E measurements ≥5 cm ($n = 13$). Each tissue sample measuring 5 mm^3 was processed. Tissue was isolated from the upper vaginal area, bilateral uterosacral ligament, and uterine cervix. A uterine cervix sample was obtained from the anterior labium. Immediately after rinsing in saline, each specimen was fixed in formaldehyde solution for 24 h. Paraffin-embedded samples were sectioned for histological examination and separated tissues soaked in RNA stabilization reagent (RNAlater™ Thermo Fisher Scientific), fixed at 4 °C for 12 h, then stored at −30 °C in the soaking solution until use. This study was approved by the Osaka City University Ethics Committee (No.2199), and all patients provided informed consent.

Histology and immunohistochemistry

Tissue samples were fixed in 10% neutral formalin, embedded in paraffin using VIP5-Jr (Tissue-Tek, Japan), and sectioned at 4-μm thick. Morphological assessment was performed by hematoxylin and eosin stain (H&E). Antigen retrieval was carried out with 20 mM Tris-HCl buffer (pH 9.0) at 100 °C for 5 min. Immunohistochemistry for fibulin-5 (monoclonal, ab66339 Abcam, Cambridge, UK) and elastin (polyclonal, ab23747, Abcam) were undertaken for 1 h at room temperature. The sections were then incubated with the LSAB2 System-HRP (DaKo, Agilent, Glostrup, Denmark) for 1 h at room temperature and detected using the 3,3′-diaminobenzidine (DAB) system.

RNA extraction and quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (RT-PCR) using Taqman chemistry was performed. Primer and probes for ECM-related genes (fibulin-5: ABI Assay ID Hs00197064_m1, elastin: Hs00355783_m1, integrin β1 (ITGβ1): Hs00559595_m1, lysyl oxidase-like protein-1 (LOXL1): Hs00935937_m1, and collagen: Hs00899658_m1) were obtained from Thermo Fisher Scientific Inc. Total RNA was extracted from the vaginal wall, uterosacral ligament, and uterine cervix using the RNeasy Plus Universal Kit (QIAGEN GmbH. Hilden, Germany). Extracted RNA was reverse transcribed to complementary DNA (cDNA) using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The gene expression levels of fibulin-5, elastin, ITGβ1, LOXL1, and collagen were measured using the ABI7500 Fast System. The relative quantification was normalized to glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH, Hs99999905_m1) expression.

Western blotting analysis

Crude protein was extracted from the vaginal wall, uterosacral ligament, and uterine cervix. Samples from the normal cervix group (B point 2 or 3) and the cervical elongation group (C point 5 or 6) were matched according to donor age. Extracted protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophores (SDS-PAGE) and transferred to the polyvinylidene difluoride (PVDF) membrane. After blocking, the membrane was incubated with anti-fibulin-5 antibody (1:1000 dilution) for 1 h at room temperature. The bound antibodies were detected after incubation with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin G (IgG) using ImmunoStar LD (Wako Pure Chemical Industries, Ltd.). Adjustment of each band for concentration was performed with anti β-actin antibody (monoclonal A5441, SIGMA-ALDRICH, St. Louis, MO, USA).

Statistical analysis

Statistical analysis was carried out using SAS9.2 software (SAS Institute, Cary, NC, USA). The Wilcoxon rank-sum test was used to compare clinical data and was the nonparametric version of the two-sample t test, similar to the Mann—Whitney U test. $P < 0.05$ was considered statistically significant. A correlation analysis test was used to determine relationships between quantitative and categorical variables. When the correlation coefficient r value indicates plus, it can be judged that there was a positive correlation and indicates that with minus, there was a negative correlation. Generally, when the correlation coefficient is >2, it can be judged that the correlation between groups is significant. We used a correlation analysis to judge between the relationship of gene expression and degree of POP progression.

Results

Localization of fibulin-5 and elastin in the vagina and uterosacral ligament

The vaginal wall consisted of squamous epithelium, lamina propria, muscularis, and adventitia (Fig. 1a), and the uterosacral ligament was made up of smooth muscle bundles, abundant medium and small blood vessels, and small nerve bundles (Fig. 1d). Immunohistochemical analysis showed fibulin-5 (Fig. 1b, e) and elastin (Fig. 1c, f) predominately expressed in the lamina propria and fibromuscular layer of the vaginal wall. Fibulin-5 and elastin had a similar localization pattern in the vaginal wall and uterosacral ligament.

Correlation analysis between mRNA of ECM-related proteins and progression of POP

Basic clinical information of the two groups is shown in Table 1. Patients in the elongated cervix group (57.54 ± 11.7 years) were younger than those in the normal cervix group (66.69 ± 4.83 years). Cervix was longer in the cervical elongation group (5.62 ± 2.2), in the normal-cervix group (2.75 ± 1.09). However, POP stage score of those in the normal group (3.25 ± 0.43) was greater than for those in the cervical elongation group (2.85 ± 0.36). There was no significant difference with BMI, gravidity, and parity.

Correlation between gene expression of ECM-related proteins (fibulin-5 and ITGβ1) and the degree of progression of POP (most distal point on POP-Q assessment) was examined. Generally, the most distal point of POP in patients without cervical elongation was point B, while point C was most distal in POP patients with cervical elongation. The correlation value (r value) of fibulin-5 and ITGβ1 expression in vaginal wall and uterosacral ligament tissue was −0.274, −0.304, and − 0.206, −0.297, respectively. In the normal cervix group, expression of fibulin-5 and ITGβ1 and degree of POP progression showed a weak negative correlation (Fig. 2). On the other hand, expression of both genes in patients in the cervical elongation group was significantly different from the normal cervix group (Fig. 3). The expression of both genes was similar in vaginal tissue from the normal cervix group (fibulin-5: −0.274, ITGβ1: −0.206) (Fig. 2a, b) and elongation group (fibulin-5: −0.081, ITGβ1: −0.265) (Fig. 3a, b). However in ligament tissue, expression patterns in the elongation group (fibulin-5: 0.302, ITGβ1: 0.152) (Fig. 3b, d) differed to expression in the normal cervix group (fibulin-5: −0.304, ITGβ1: −0.297) (Fig. 2b, d). There was a statistically significant positive correlation between gene expression in tissue at the most distal point.

Differential ECM gene expression in the uterine cervix

To further understand the mechanism of uterine cervix elongation, we investigated uterine cervical tissue obtained from POP patients with or without cervical elongation. There was no correlation between fibulin-5 expression and the progression of POP in the normal cervical group (−0.015). However, fibulin-5 increased with POP severity in the elongation group (0.302) (Fig. 4A a, b). There was a relationship between ITGβ1, LOXL1 elastin gene expression, and progression of POP. ITGβ1and LOLX1 expression in both the normal and elongation groups were 0.210, 0.504 and 0.216, 0.830 respectively (Fig. 4A c–f), showing marked increased expression in cervical elongation patients. The major components that constitute cervical connective tissue are elastin fibers and collagen. The correlation value of elastin and collagen expression in normal and elongation groups were 0.099, 0.915 (Fig. 4B a, b) and 0.319, 0.938 (Fig. 4B c, d), respectively. Expression of

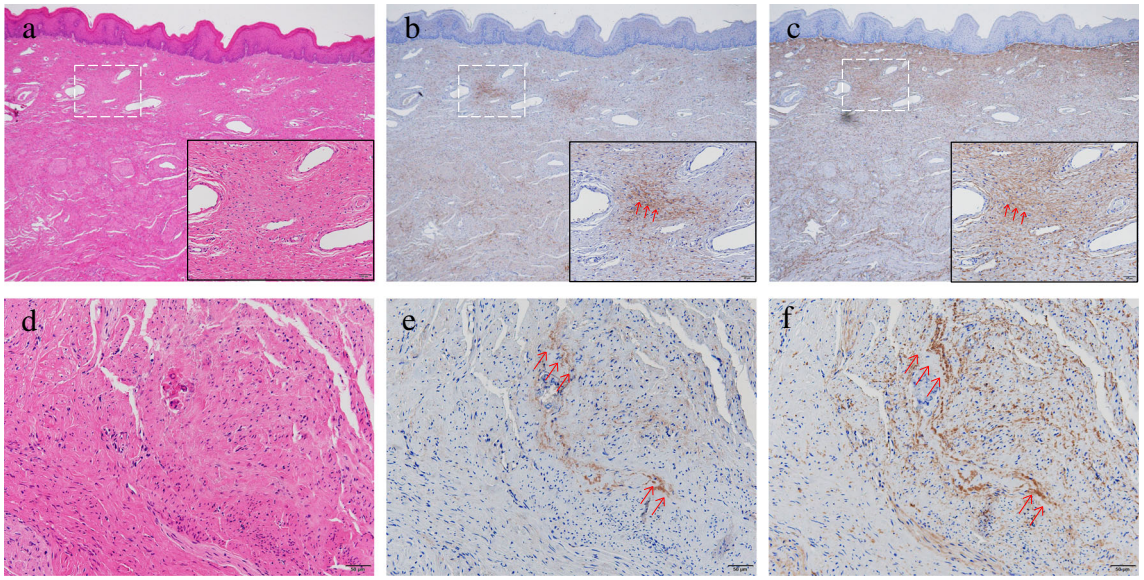

Fig. 1 Histology of the vaginal wall (**a, b, c**) and uterosacral ligament (**d, e, f**) tissues. Hematoxylin-eosin staining (**a** and **d**) and immunohistochemical stain of fibulin-5 (**b** and **e**) and elastin (**c** and **f**). Magnification, a, b, c = ×4; inner frame ×20; d, e, f, = ×20

elastin and collagen also significantly increased in patients with cervical elongation.

Expression of fibulin-5 protein

We compared fibulin-5 protein expression levels between the vaginal wall, uterosacral ligament, and uterine cervix in women with or without cervical elongation. Western blot analysis showed that fibulin-5 protein levels in the vaginal wall did not change significantly (Fig. 5a). This was in contrast to the significant increase of fibulin-5 protein levels in the uterine cervix of patients with cervical elongation (Fig. 5b, c).

Discussion

POP in women has a number of causes, including weak pelvic floor support function, organizational structural

Table 1 Clinical characteristics of pelvic organ prolapse (POP)

Characteristics	Normal cervix group $n = 16$	Cervical elongation group $n = 13$	*P* value
Age (years) (mean ± SD)	66.69 ± 4.83	57.54 ± 11.76	0.0229
Body mass index (kg/m^2) (mean ± SD)	25.30 ± 3.83	23.67 ± 4.28	0.3080
Gravidity (median, SD)	2.75 ± 0.97	2.69 ± 0.82	0.8704
Parity (median, SD)	2.25 ± 0.00	2.15 ± 0.77	0.7458
POP-Q assessment			
Aa (median, SD)	2.13 ± 0.70	0.69 ± 2.16	0.0446
Ba (median, SD)	4.31 ± 1.45	2.38 ± 1.86	0.0054
Point C (median, SD)	2.94 ± 2.61	3.38 ± 1.55	0.6033
Point D (median, SD)	0.19 ± 2.16	−2.54 ± 1.15	0.0003
Ap (median, SD)	0.00 ± 1.00	−1.00 ± 1.00	0.0003
Bp (median, SD)	0.81 ± 1.67	−1.00 ± 0.68	0.0010
Tvl (median, SD)	8.00 ± 0.70	8.25 ± 0.72	0.7524
Cervical length/cervical elongation (cm) (C-D)	2.75 ± 1.09	5.62 ± 2.20	0.0007
POP stage (median, SD)	3.25 ± 0.43	2.85 ± 0.36	0.0151

SD standard deviation, *POP-Q* Pelvic Organ Prolapse Quantification,*Aa* anterior vaginal wall 3 cm proximal to the hymen, *Ba* most distal position of the remaining upper anterior vaginal wall, *C* most distal edge of cervix or vaginal cuff scar, *D* posterior fornix, *Ap* posterior vaginal wall 3 cm proximal to the hymen, *Bp* most distal position of the remaining upper posterior vaginal wall, *Tvl* total vaginal length

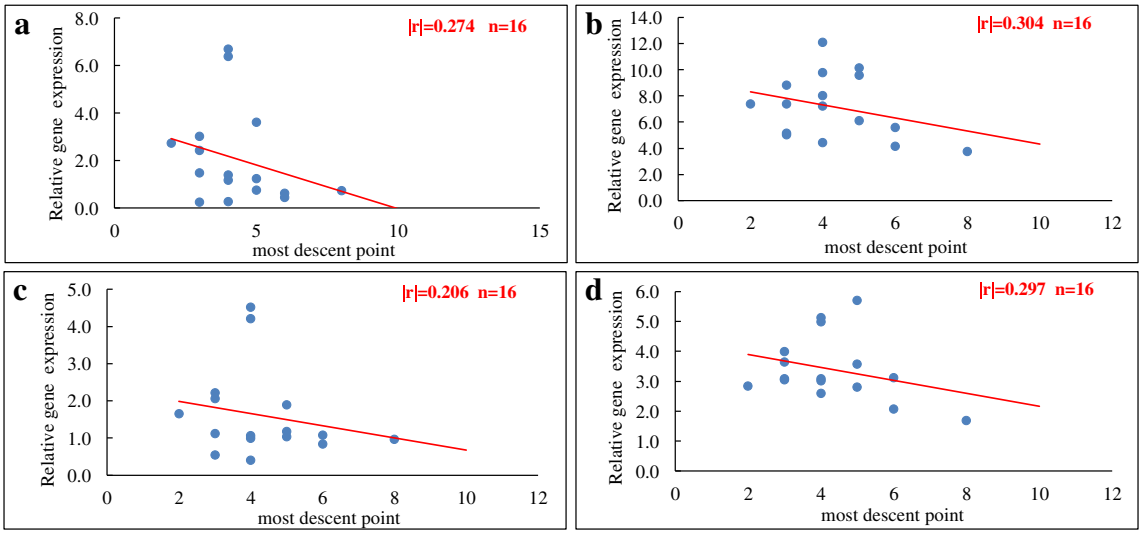

Fig. 2 Correlation analysis of Pelvic Organ Prolapse Quantification (POP-Q) staging and fibulin-5 (**a** and **b**) and integrin β1 (ITGβ1) (**c** and **d**) gene expression in vaginal wall (**a** and **c**) and uterosacral ligament (**b** and **d**) tissue in the normal cervix group

defects resulting in the pelvic floor moving downward, and urinary, fecal, and sexual dysfunction. POP seriously affects patient QoL and its prevalence has gradually being seen in younger women [7]. The etiology of POP has been researched for a half century, with many studies examining changes in connective tissue and an association with POP development [8–10]. The hammock theory, introduced in the early 1990s, accurately explained the support mechanism for pelvic floor organs [2] as being on three levels, with apical (level I), middle (level II), and distal (level III) levels. The vaginal apical suspension (level I), near uterosacral and cardinal ligament complex, provides most support for the pelvic floor [11]. In this study, samples from the upper vaginal wall and uterosacral ligament were analyzed to clarify the supporting role of uterovaginal suspension. POP severity and gene expression of ECM-related proteins were inversely correlated. Elastin was the main component of the complex elastic fibers composed of elastin microfibrils in connective tissue [12]. Fibulin-5 promotes original elastase through formation of ITGβ1 and plays a vital role in elastin formation by guiding the assembly of elastic fibers [13, 14]. Integrin adhesion specifies the direction of arrangements on the surface of ECM through catalysis and deamination by LOXL1 [15, 16]. In this reaction, lysine in elastin is oxidized to aldehyde lysine, thereby synthesizing highly stable, covalently cross-linked

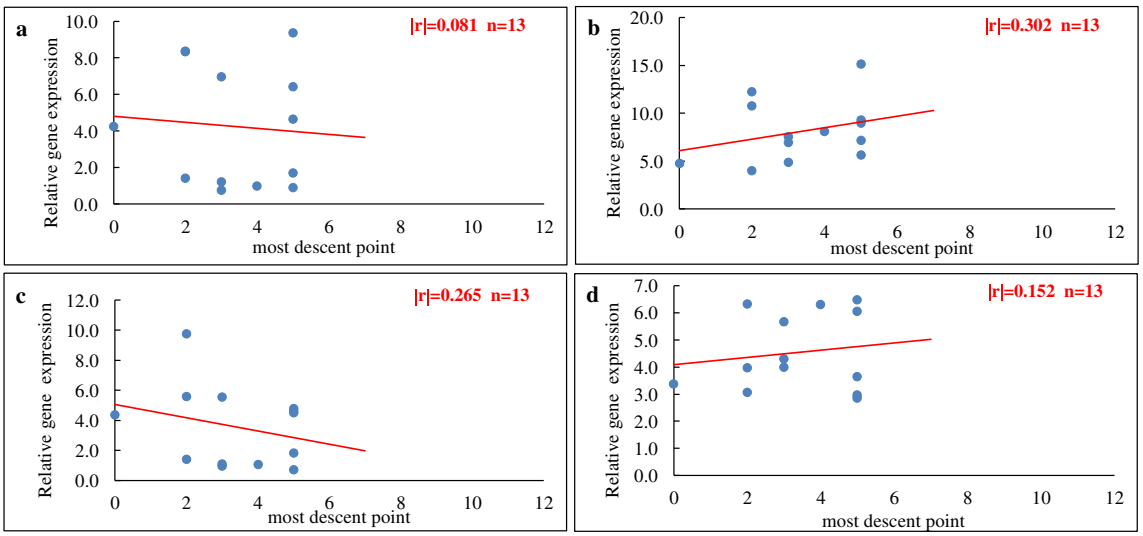

Fig. 3 Correlation analysis of Pelvic Organ Prolapse Quantification (POP-Q) staging and fibulin-5 (**a** and **b**) and integrin β1 (ITGβ1) (**c** and **d**) gene expression in vaginal wall (**a** and **c**) and uterosacral ligament (**b** and **d**) tissues in the cervical elongation group

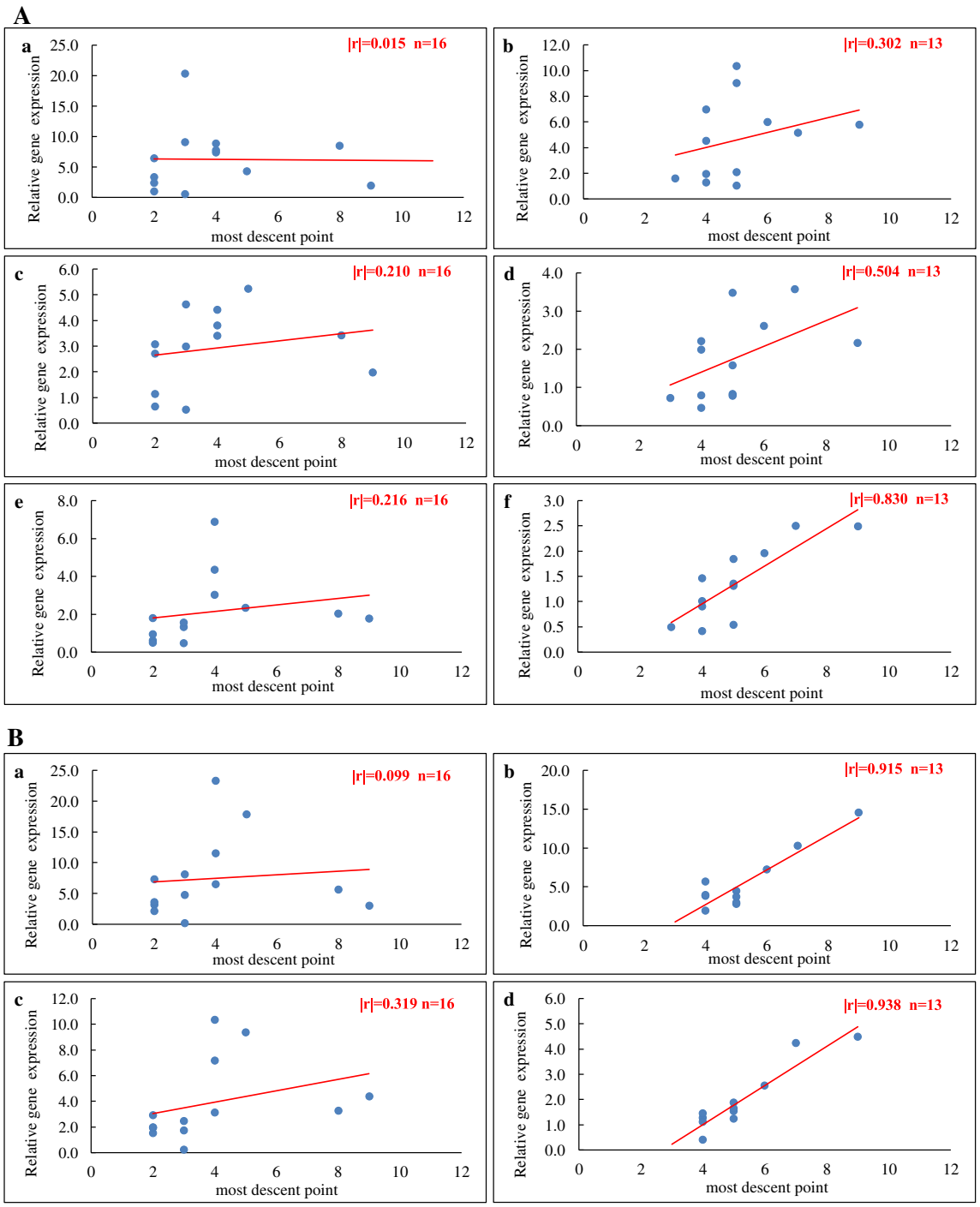

Fig. 4 Correlation analysis of Pelvic Organ Prolapse Quantification (POP-Q) staging and fibulin-5 (**A**, *a* and *b*), integrin β1 (ITGβ1) (**A**, *c* and *d*), lysyl oxidase-like protein-1 (LOXL1) (**A**, *e* and *f*), elastin (**B**, *a* and *b*), and collagen (**B**, *c* and *d*) expression in cervix tissues; *a*, *c*, and *e* are normal cervix group; *b*, *d*, and *f* are cervical elongation group

elastomeric fibers, resulting in a firm, mesh structure. Usually, integrin combines with LOXL1 and fibulin-5 to stabilize elastic fibers. The ECM-related proteins promote the composition of fibrils and maturation and development of elastic fibers. The differential gene expression of ECM-related proteins in the vaginal and uterosacral ligament in POP patients suggests there were other inducing factors affecting integrin adhesion and fibulin-5 stabilization.

Multiple etiological factors are involved in the development of POP. In addition to mechanical and hormonal

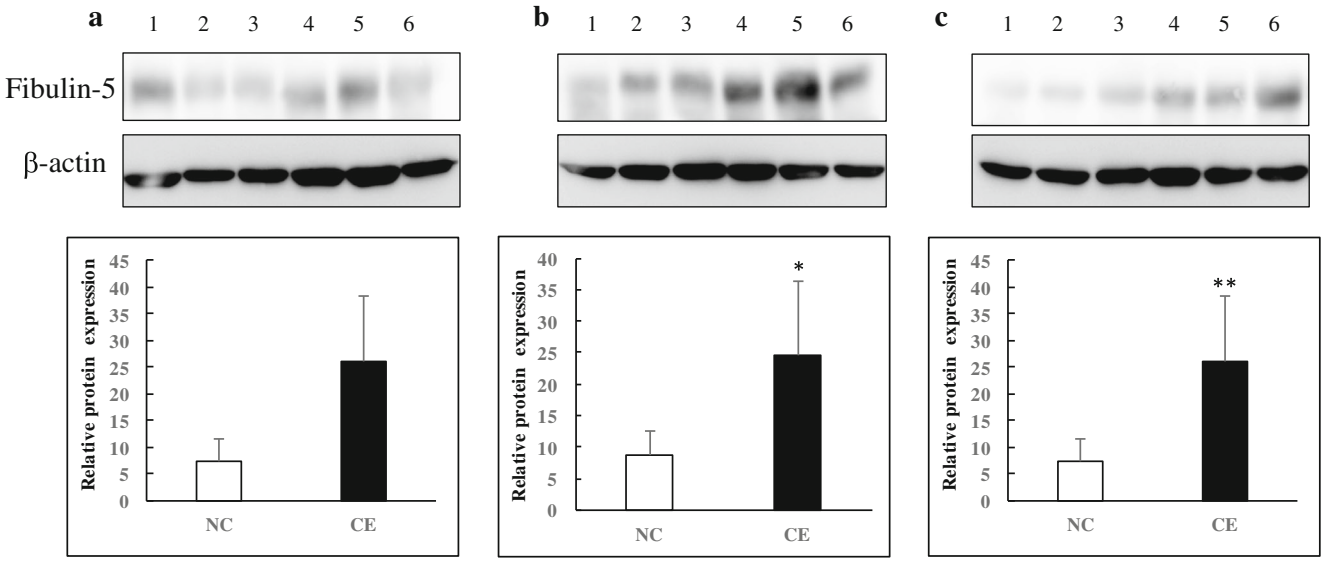

Fig. 5 Western blot analysis of fibulin-5 in vaginal wall (**a**), uterosacral ligament (**b**), and uterine cervix (**c**). 1–3: normal cervix group (NC), 4–6: cervical elongation group (CE). * $P = 0.04$, ** $P = 0.03$

inducers, such as pelvic floor injury, menopause, and aging, risk factors that predispose to POP include race, sex, and genetics. A strong family history of POP is also a risk factor and is probably related to differences in connective tissue structure in different subpopulations [17]. In the elderly, POP predominates mainly with anterior and apical defects of the vagina. Whereas relatively younger women with less parity have uterine prolapse characterized by elongation of the uterine cervix [18]. The pathophysiologic mechanisms underlying the development of cervical elongation are currently not well understood. Our study also could not find differences, such as BMI, between women with and without cervical elongation (see Table 1). Expression of connective tissue proteins was compared between groups according to the presence or absence of cervical elongation. Similar to previous reports, mean age was younger and parity numbers lower in women with cervical elongation. In this study, the focus was on POP progression and uterine cervical elongation. Uterine cervical tissue was composed of connective tissue (85–90%) and smooth muscle (10–15%), with connective tissue having the main role in uterine cervical function [6, 19]. However, few studies looked at biological analysis of uterine cervical elongation and gene expression. This is the first study of POP in terms of progression and uterine cervical elongation.

In the cervical elongation group, there was a positive correlation between POP progression and ECM-related protein expression, whereas no correlation was observed in women without cervical elongation. In the cervical elongation group, fibulin-5 protein expression in the uterosacral ligament and uterine cervix was significantly increased than in the normal cervical group. This suggests that the pathogenesis of cervical extension differed from common POP progression. Although POP stages were II–III in this study, point D of the cervical elongation group was smaller than that of the normal cervical group (see Table 1). Because point D indicates support at the apical part of the vagina, this point will be considered the greatest difference between groups. As POP progressed, reactive ECM proteins were compensated for by the reactive ECM protein in the cervix tissue. Uterine cervical connective tissue was made up of collagen, elastic, and reticular fibers. Collagen fibers are tough enough to maintain the shape and function of the uterine cervix. Elastic fibers can be extended and restored, following external tension. Elastin expanded the uterine cervix through expansion of elastic fibers [15, 20]. Fibulin-5 in reticular fibers affected the toughness of the uterine cervix. Fibulin-5 was not only a key component in formation of elastic fibers but also a critical component of elastic fiber assembly [21].

With the increase in our aging population, greater numbers of older women are susceptible to POP, which could seriously impact their QoL. This study differs from previous investigations in which data from POP patients was compared with those of anatomically normal controls. It is very difficult to establish control tissues to study the biochemical pathogenesis of POP because of differing ages of women with POP as well as contamination of normal tissue with uterine myoma and uterine cancer, as tissue was obtained during gynecological surgery. Therefore, we classified POP patients into groups: with or without cervical elongation. The pathogenesis of POP in women with cervical elongation clearly differed from POP in women with a normal cervix [22]. This study specifically analyzed the role of ECM-related proteins between the two groups and the correlation of their expression patterns with POP progression. Damage and repair of each gene may be associated with POP pathogenesis. We strongly suggest that POP is preventable and patients can be managed through use of molecular biology.

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Compliance with ethical standards

Conflicts of interest None.

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