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Original Research

Expression of pelvic organ prolapse-related protein fibulin-5, TGFβ, and Smad2/3 in Uyghur women of Xinjiang

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Objective: The purpose of this study was to investigate the role of fibulin-5 in the transforming growth factor beta (TGF-β)/Smad signaling pathway by measuring the differential expression of fibulin-5, TGF-β, and Smad2/3 in the connective tissue of the vaginal anterior wall of Uyghur women with pelvic organ prolapse (POP). *Methods*: Thirty-six Uyghur patients diagnosed with POP, who were admitted to the First Affiliated Hospital of Xinjiang Medical University from March 2015 to June 2018, were enrolled in the study. In the same period, 15 patients with benign uterine hysterectomy were included in the control group. The relative protein expression levels of fibulin-5, Smad2/3, TGF-β1, TGF-βRI, and TGF-βRII in the anterior vaginal wall of each group were determined by immunohistochemistry, quantitative PCR, and Western blotting. *Results*: Immunohistochemistry showed that the expression levels of Smad2/3, fibulin-5, TGF-β1, TGF-βRI, and TGF-βRII were significantly lower in the POP group than in the control group ($P < 0.05$). RT-PCR showed that the mRNA expression levels of TGF-β1, TGF-βRI, TGF-βRII, and fibulin-5 were significantly lower in the POP group, with statistical significance ($P < 0.05$), whereas the expression of Smad2 and Smad3 decreased, but without statistical significance ($P > 0.05$). Western blot analysis showed that the expression of TGF-β1, TGF-βRI, TGF-βRII, fibulin-5, and phosphorylated Smad2/3 was significantly lower in the POP group than in the control group ($P < 0.05$), but there was no difference in Smad2/3 protein expression compared with the control ($P > 0.05$). *Conclusion*: The amount of functional elastic fibers in the pelvic connective tissue structure of POP patients decreased via involvement of the TGF-β/Smad signaling pathway. Future studies are needed to confirm the pathogenesis of POP.

Keywords

Pelvic organ prolapse (POP); Fibulin-5; TGF-β; Smad2/3; TGF-β/Smad signaling pathway

1. Introduction

Pelvic organ prolapse (POP) severely affects women's health and their quality of life, causing economic and social burden to society, leading to the term "social cancer". An epidemiological survey of more than 5,000 women in Xinjiang found that the incidence of pelvic organ dysfunction was 41.96% [1], which is significantly higher than the domestic and foreign rates of 30.9% and 33%, respectively [2, 3]. Previous research has also shown that the prevalence of POP in Uyghur women in Xinjiang, China is significantly higher than that in the Hans Chinese population. The expression of transforming growth factor beta (TGF-β) and fibulin-5 in the vaginal wall tissues is lower in POP patients than in healthy individuals. There are no racial differences in TGF-β levels [4], but the expression of fibulin-5 in the vaginal wall of POP patients. There are also statistically significant racial differences in fibulin-5 expression [5]. Therefore, we used immunohistochemistry, quantitative PCR (qPCR), and Western blot analysis to detect the expression of TGF-β, TGFR I/II, Smad2/3, and fibulin-5 in the anterior vaginal tissues and to study the related signaling pathways.

2. Materials and methods

2.1 Specimen collection and preservation

Patients undergoing vaginal hysterectomy at first Xinjiang Medical University Hospital due to POP (III–IV) were enrolled from March 2015 to June 2018. The anterior vaginal tissue of resected Uyghur patients was used as the experimental group, and known POP Uyghur patients who underwent vaginal hysterectomy due to other benign gynecological diseases were the control group. Exclusion criteria included: patients with malignant tumors, pelvic endometriosis, and acute pelvic inflammatory disease, and those receiving hormone replacement therapy, who could not tolerate surgery and anesthesia due to other systemic diseases. All patients provided written informed consent before participation in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Xinjiang Medical University (Approval No. 20160218-65; Xinjiang, China).

2.2 Materials and instruments

For specimen collection, tissue from the anterior vaginal wall of approximately 0.5 cm $\times$ 0.5 cm $\times$ 1 cm in size, followed by DAB staining of the elastin fibers.

2.3 Instruments and reagents

The Eppendorf Research Plus manual single-channel pipette was from Eppendorf (Hamburg, Germany). For PCR, the Bio-Rad MyCycler Thermal Cycler was used (Bio-Rad, Hercules, CA, USA). TGF-b, Smad2/3, fibulin-5 antibodies were from Abcam (Cambridge, MA, USA).

3. Experimental methods

3.1 Immunohistochemistry

The fixed tissues were dehydrated in a concentration gradient of 70% ethanol, 80% ethanol, 90% ethanol, and 95% ethanol for 3 h, 2 h, 2 h, and overnight, respectively. The next day, the tissues were soaked in anhydrous ethanol for another 30 min, and then incubated in fresh anhydrous ethanol for 30 min. The dehydrated liver tissue was paraffin-embedded and cut into 5 μm thick slices, and the sections were fixed on a slide. The tissue sections were baked at 65 °C for 1.5–2 h, and then soaked in xylene (I) and xylene (II) for 10 min each, followed by 5 min incubations in anhydrous ethanol (I), anhydrous ethanol (II), 95% ethanol, 90% ethanol, 80% ethanol, 70% ethanol, and DD H2O, respectively. After boiling in 0.01 m citrate buffer (pH 6.0), the slices were immersed in repair solution for 10 min, and then incubated with hydrogen peroxide for 10 min to inactivate endogenous peroxidase activity. After three washes with phosphate-buffered saline (PBS), the sections were incubated with primary antibody at 4 °C overnight. After another three washes with PBS, the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 20 min at room temperature. Color development was performed by incubation with DAB solution for 3–10 min, and staining (brown granule precipitation) was visualized under a microscope. Sections were incubated in tap water for 3 min to terminate color development.

Table 1. PCR reaction components for first-strand cDNA synthesis.

Component	Volume
Total RNA	800 ng, 7 μL
Random Primer (0.1 μg/μL)	1 μL
2× TS Reaction Mix	10 μL
TransScript®RT/RI Enzyme Mix	1 μL
gDNA Remover	1 μL
RNase-free Water	Up to 20 μL

3.2 qPCR experiment

Analysis of fibulin-5, TGF-β1, and Smad2 mRNA expression was done by qPCR. The components of the PCR reaction for first-strand cDNA synthesis are listed in Table 1 and the primers are presented in Table 2. The qPCR components and reaction conditions are shown in Tables 3 and 4, respectively.

Table 2. Primer sequences for qPCR.

Primer name	Sequence, (5' to 3')	Primer size
TGF-β1 F	GGCCAGATCCTGTCCAAGC	201
TGF-β1 R	GTGGGTTTCCACCATTAGCAC	
TGF-βRI F	ACGGCGTTACAGTGTTTCTG	167
TGF-βRI R	GCACATACAAACGGCCTATCTC	
TGF-βRII F	GTAGCTCTGATGAGTGCAATGAC	132
TGF-βRII R	CAGATATGGCAACTCCCAGTG	
smad2 F	CGTCCATCTTGCCATTCACG	182
smad2 R	CTCAAGCTCATCTAATCGTCCTG	
smad3 F	CCATCTCCTACTACGAGCTGAA	149
smad3 R	CACTGCTGCATTCCTGTTGAC	
Fibulin-5 F	TCGCCAGTCAGGACAGTGT	152
Fibulin-5 R	AGTAGGGGTTCGAGTAGGGC	

Table 3. qPCR system reaction components.

Reagent	Volume (μL)
2 × SYBR Green Select Mix	5
Forward Primer	0.7
Reverse Primer	0.7
ROX	0.05
cDNA	1
RNase-free Water	Up to 10

Table 4. qPCR reaction conditions.

Stage (ABI)	Temperature	Time	Cycle
Predegeneration	95 °C	2 min	1
Degeneration	95 °C	5 sec	40
Annealing/extension	60 °C	30 sec	

Table 5. Antibody dilution ratio.

Primary antibody	Dilution	Secondary antibody	Dilution
β-actin	1 : 800		1 : 15000
Fibulin-5	1 : 500		1 : 5000
TGF-β1	1 : 1000	goat anti-rabbit	1 : 5000
TGF-βRI	1 : 1000		1 : 5000
TGF-βRII	1 : 1000	IgG H&L (HRP)	1 : 5000
Smad2/3	1 : 1000		1 : 5000
p-Smad2/3	1 : 1000		1 : 5000

3.3 Western blot analysis incubate at 4 °C overnight

Total protein was extracted and the protein concentration was determined by the BCA method using a BCA protein assay kit. The proteins were resolved by and SDS-PAGE, followed by electrotransfer to nitrocellulose membranes. The membranes were incubated with primary antibody incubate at 4 °C overnight (Table 5), and proteins were visualized by chemiluminescence.

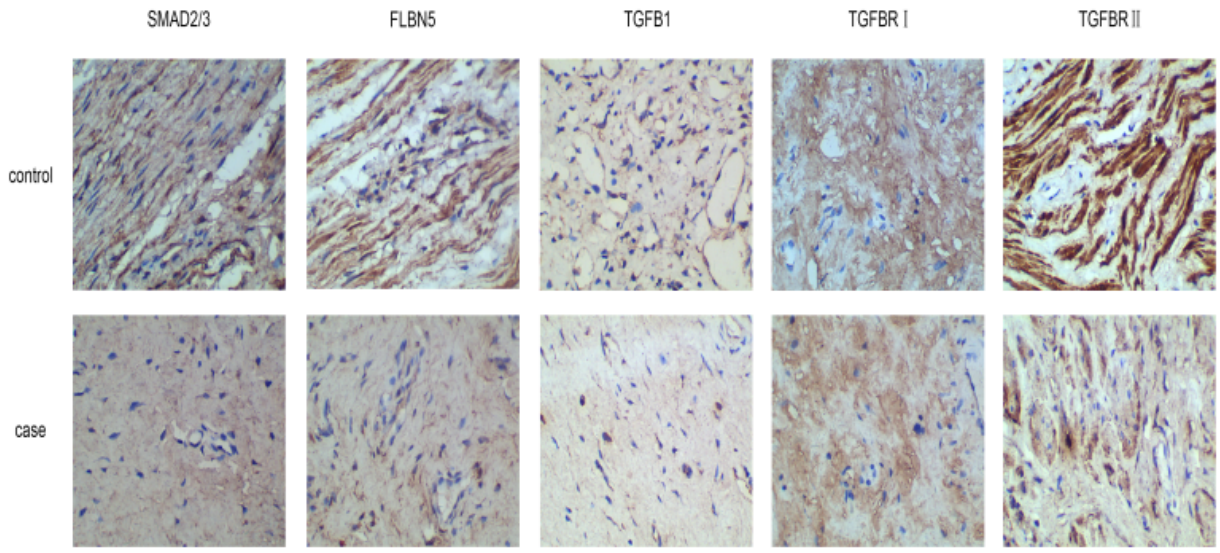

Fig. 1. Immunohistochemistry of two groups of POP-related proteins (DAB 400×).

Table 6. Protein expression in the two groups of women.

Group	SMAD2/3	Fibulin-5	TGFB1	TGFBRI	TGFBRII
Non POP	2.778 ± 0.441	2.444 ± 0.527	1.778 ± 0.441	3.000 ± 0.000	2.667 ± 0.500
POP	1.636 ± 0.809	1.091 ± 0.831	1.091 ± 0.302	2.636 ± 0.505	1.364 ± 0.505
T/Z	4.008	4.228	-3.040	-1.971	5.769
P	0.001	0.001	0.002	0.049	0.000

3.4 Statistics

All data are expressed as the mean ± standard deviation. SPSS 19.0 software was used for statistical analyses. If the data conformed to normal distribution, the independent samples *t*-test was used; otherwise, the rank-sum test was used. $P < 0.05$ was considered statistically significant.

4. Results

4.1 Immunohistochemistry results

Immunohistochemistry showed that were no statistically significant differences in age, menopause age, birth history, obesity, diabetes, or other factors between the two groups (Patients undergoing vaginal hysterectomy at first Xinjiang Medical University Hospital due to POP (III–IV) were enrolled from March 2015 to June 2018. The anterior vaginal tissue of resected Uyghur patients was used as the experimental group, and known POP Uyghur patients who underwent vaginal hysterectomy due to other benign gynecological diseases were the control group). The protein expression of SMAD2/3, fibulin-5, TGF-βl, TGF-βRI, and TGF-βRII in the anterior vaginal wall tissue was significantly lower in Uyghur women with POP than in the control group ($P < 0.05$; Table 6 and Fig. 1).

4.2 qPCR experiment results

The qPCR results showed that in the Uyghur POP case group, the mRNA expression of TGF-β1, TGF-βRI, TGF-βRII, and fibulin-5 was significantly decreased ($P < 0.05$). Although the levels of Smad2/3 decreased, there was no statistical difference compared with the control group ($P > 0.05$; Table 7 and Fig. 2).

4.3 Western blot analysis

Western blot analysis showed that the protein expression of TGF-β1, TGF-βRI, TGF-βRII, fibulin-5, and phosphorylated Smad2/3. was significantly lower in the POP case group than the control group ($P < 0.05$). However, the expression of total Smad2/3 protein was not significantly different between groups ($P > 0.05$; Table 8 and Fig. 3).

5. Discussion

The occurrence of pelvic floor dysfunction is due to anatomical structure and functional changes of pelvic organs, caused by damage to the pelvic floor. In the pelvic floor, elastic fiber defects are closely related to the incidence of POP [6]. In recent years, some scholars [7] have proposed the "elastic fiber imbalance theory", in which the metabolic imbalance of elastic fibers may be the basis of POP lesions; related studies have confirmed this view. Fibroblasts are thought to be the main cellular component of elastin and play a key role in maintaining the normal anatomy of pelvic organs [8]. To date, more than 34 proteins are associated with elastic fibers, but only a few play vital roles in the process of elastic fiber formation [9] including fibulin-1, fibulin-2 [10], lysyl oxidase [11], fibulin-3 [12], fibulin-4 [13, 14], and fibulin-5 [15, 16].

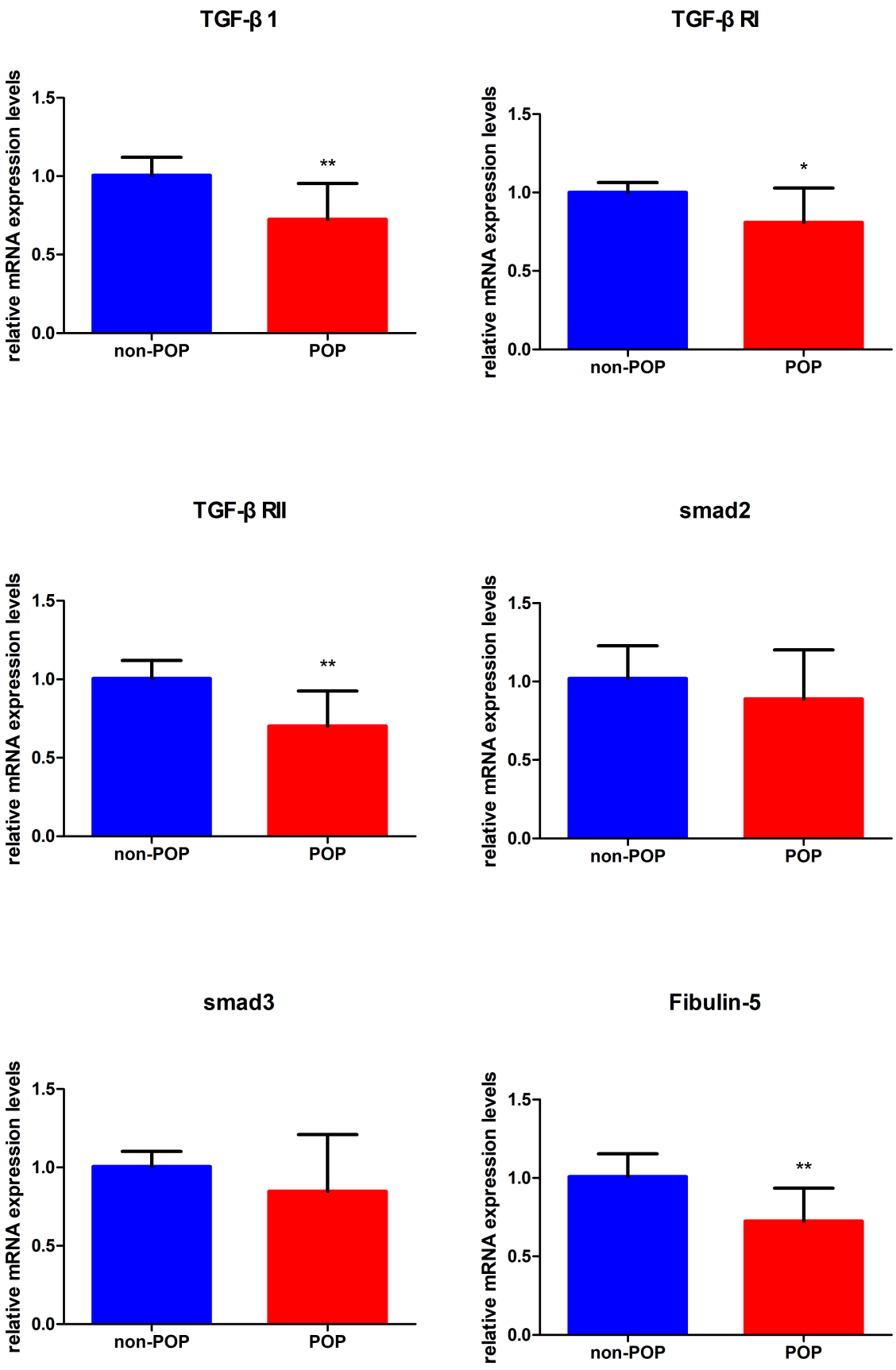

Fig. 2. Relative gene expression in anterior vaginal tissue of different patients.

Table 7. Analysis of relative gene expression levels in anterior vaginal tissue of different patients ($x \pm s$, n = 36).

Group	TGF-β1	TGF-βRI	TGF-βRII	Smad2	Smad3	Fibulin-5
Non POP	1.006 ± 0.115	1.002 ± 0.063	1.005 ± 0.115	1.018 ± 0.209	1.004 ± 0.098	1.009 ± 0.145
POP	0.725 ± 0.228	0.809 ± 0.219	0.702 ± 0.222	0.889 ± 0.312	0.847 ± 0.362	0.726 ± 0.211
T	2.920	2.118	4.888	0.962	2.035	3.128
P	0.006	0.042	0.000	0.343	0.051	0.004

Table 8. Analysis of protein expression levels in anterior vaginal tissue of different patients ($x \pm s$, n = 15).

Group	TGF-β1	TGF-βRI	TGF-βRII	Smad2/3	p-Smad2/3	Fibulin-5
Non POP	0.208 ± 0.045	0.270 ± 0.088	0.328 ± 0.051	0.528 ± 0.207	0.630 ± 0.204	0.255 ± 0.089
POP	0.102 ± 0.029	0.119 ± 0.037	0.240 ± 0.047	0.377 ± 0.109	0.416 ± 0.081	0.157 ± 0.054
T	5.589	3.946	3.366	1.868	2.451	2.408
P	0.000	0.007	0.007	0.084	0.049	0.045

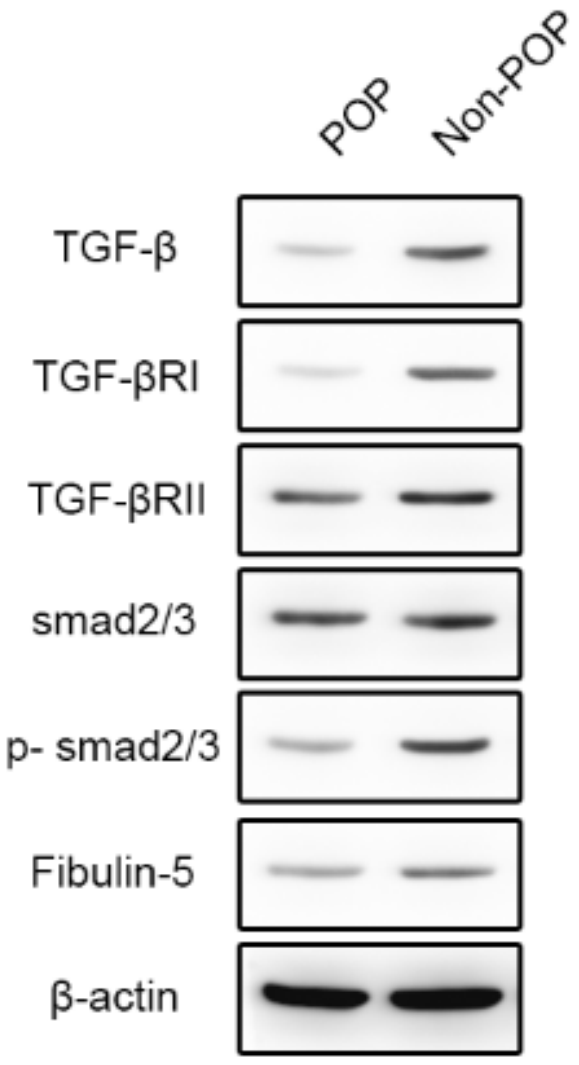

Fig. 3. Difference in protein expression in anterior vaginal tissue between groups.

Li *et al.* [17], Sderberg *et al.* [18], and Jung *et al.* [8] demonstrated that fibulin-5 was reduced in anterior vaginal wall tissue, paraurethral tissue, and uterine-condylar ligament in patients with POP, consistent with the experimental results of our research group. Our previous research also confirmed that fibulin-5 is not only significantly reduced in tissues of female patients with POP but also has racial differences. TGF-β is a multifunctional cytokine with three subtypes (TGF-β1, β2 and β3). The TGF-β/Smad receptor signaling pathway regulates fibroblast proliferation, differentiation, and apoptosis, collagen metabolism, and other pathophysiological processes. The relationship between TGF-β1 and the extracellular matrix (ECM) is closely related to metabolism [19]. A previous study also showed that TGF-β was highly expressed in the vaginal wall of females with POP [20].

We mainly studied the expression of fibulin-5 and TGF-β/Smad signaling pathway components in the anterior vaginal wall tissue of POP patients through immunohistochemistry, qPCR, and Western blotting to study the pathogenesis of POP. Immunohistochemistry and qPCR showed that the protein and mRNA expression, respectively, of Smad2/3, fibulin-5, TGF-β1, TGF-βI, TGF-βRII was significantly lower in the POP case group than in the control group ($P < 0.05$). However, the difference in Smad2/3 mRNA expression was not statistically significant ($P > 0.05$). These results were confirmed by Western blot analysis. Differences in Smad2/3 protein expression were only seen by immunohistochemistry.

We considered whether there were other signaling pathways in addition to TGF-β involved in POP in Uyghur female patients. For example, [21, 22] studies have found that POP is related to abnormality of the Wnt signaling pathway, which has a series of interrelated and interacting protein components and plays an important role in cell proliferation, differentiation, and body development. The regulatory role is involved in the development of human reproductive organs. This hypothesis needs to be confirmed by studies with increased sample size and verified by *in vitro* cell and *in vivo* animal experiments. Trap-1-like protein selectively interferes with Smad3 signaling, thereby changing the relative stability of Smad2 and Smad3 [23]. Second, the majority of the experimental data were obtained by immunohistochemistry. This method is subject to biopsy site and sample size restrictions.

In particular, biopsy sites in prolapsed tissues also increase variability, because differences in stress load can upregulate different protein expression levels. In addition, pathological diagnosis is susceptible to the subjective judgment of pathologists. To a certain extent, qPCR data are more objective. Thus, to obtain more objective experimental data, in addition to immunohistochemistry, we also performed qPCR, and Western blot analysis as a supplement. In the three experimental methods results obtained, fibulin-5, TGF-β1, TGF-βRI, TGF-βRII expression was significantly reduced, indicating that these proteins are involved in the pathophysiology of POP. We found that TGF-β1 levels in POP were significantly reduced in pelvic tissue, consistent with the results of Liu [24]. Studies on the role of Smad protein in the pathogenesis of POP are rare. Only one study has shown that the expression of Smad2/3 in the anterior vaginal tissue of POP patients is upregulated [25]. In summary, the number of functional elastic fibers in the connective tissue structure of the pelvic floor of patients was decreased. Among them, TGF-β1 and fibulin-5 are fibrogenic cytokines, and their expression is significantly reduced in POP patients. In POP patients, TGF-β1, fibulin-5, TGF-βRI, TGF-βRII, and phosphorylated Smad2/3 expression was significantly decreased. This shows that the TGF-β signaling pathway is involved in the pathological process of POP. However, future studies are needed to determine the exact mechanism by which the TGF-β signaling pathway regulates the metabolic process of fibulin-5.

TGF-β1 and fibulin-5 are profibrogenic cytokines; and TGF-β signaling pathway showed that TGF-β1 can promote TGF-βRI and TGF-βRII expression, which in turn activates smad2/3 activity, so TGF-β1 and Fibulin-5 expression are significantly decreased in POP patients, and TGF-βRI, TGF-βRII, and p-Smad2/3 expression are decreased. This indicates that the TGF-β/Smad Signaling pathway is involved in the process of the POP lesions. However, cytokines and genes which are involved in regulation of the TGF-β. The β/Smad signaling pathway in the require journal cell further experiments and *in vivo* the animal experiments to the investigate further mechanism of pop pathogenesis.

Author contributions

GA and SW conceived and designed the experiments. BK performed the experiments. AM analyzed the data. WXH contributed reagents and materials. SW wrote the paper.

Ethics approval and consent to participate

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Xinjiang Medical University (approval number: 20160218-65).

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Conflict of interest

The authors declare no conflict of interest.

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