

Expression of transforming growth factor-beta 1 and connective tissue growth factor in women with pelvic organ prolapse

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ABSTRACT

الأهداف: التحقق من نسبة ظهور كلاً من عامل النمو التحويلي بيتا 1، وعامل نمو الأنسجة الضامة لدى النساء المصابات بهبوط الأعضاء التناسلية (هبوط أعضاء الحوض).

الطريقة: أُجريت هذه الدراسة في قسم النساء والولادة بمستشفى رينمين التابع لجامعة وهان، هيوبي، وهان، الصين وذلك خلال الفترة من مايو إلى ديسمبر 2009م. لقد تم جمع عينات الأنسجة من 50 مشاركة في الدراسة واللاتي خضعن لعملية استئصال الرحم عن طريق المهبل، وقد شملت عينة الدراسة مجموعة المشاركات المصابات بهبوط الأعضاء التناسلية (30 مريضة) حيث تم تقسيمهن اعتماداً على تصنيف الهبوط التناسلي إلى التالي: ج1 (العدد=10)، وج2 (العدد=10)، وج3 (العدد=10)، بالإضافة إلى المشاركات في مجموعة الشاهد (20 مشاركة) واللاتي يشكين من الأمراض التناسلية الحميدة. لقد تمت الاستعانة باختبار تصبغ الأنسجة الكيميائي المناعي، واختبار ويستربن بلوت من أجل الكشف عن ظهور عامل النمو التحويلي بيتا 1، وعامل نمو الأنسجة الضامة.

النتائج: لقد كثر ظهور التصبغ الكيميائي المناعي لعامل النمو التحويلي بيتا 1، وعامل نمو الأنسجة الضامة في سيتوبلازم الخلايا الليفية. أشارت نتائج الدراسة إلى وجود علاقة عكسية واضحة بين ظهور هذين العاملين من جهة ومرحلة تصنيف الهبوط التناسلي من جهة أخرى، فيما كان هناك علاقة طردية فيما بينهما. لقد كانت نسبة ظهور عامل النمو التحويلي بيتا 1 في المجموعات الثلاثة المصابة بالهبوط التناسلي أقل بكثير من نسبة ظهوره في مجموعة الشاهد، فيما لم يكن هناك اختلافاً واضحاً في نسبة هذا العامل بين المجموعات الثلاثة باستثناء الاختلاف الذي ظهر بين ج1 وج3. وكانت نسبة ظهور عامل نمو الأنسجة الضامة في المجموعات الثلاثة المصابة أقل بكثير من مجموعة الشاهد، بالإضافة إلى وجود اختلاف واضح في نسبة هذا العامل بين المجموعات الثلاثة المصابة بالهبوط التناسلي.

خاتمة: أسفرت الدراسة عن مدى ارتباط هبوط الأعضاء التناسلية بنسبة ظهور عامل النمو التحويلي بيتا 1، وعامل نمو الأنسجة الضامة وخصوصاً في مراحل تصنيف الهبوط التناسلي.

Objectives: To investigate the presence of transforming growth factor-beta 1 (TGF-β1) and connective tissue growth factor (CTGF) in women with pelvic organ prolapse (POP).

Methods: This study was conducted from May to December 2009. Fifty patients with POP that underwent vaginal hysterectomy in the Department of Gynecology, Renmin Hospital of Wuhan University, Hubei, Wuhan, China were enrolled in this study. They were divided into: Group 1 (n=10); Group 2 (n=10); and Group 3 (n=10) according to Pelvic Organ Prolapse Quantitation (POP-Q). Meanwhile, 20 cases treated by vaginal hysterectomy for other benign gynecological diseases were selected as the control group. Immunohistochemical staining and Western blot were performed to detect the expression of TGF-β1 and CTGF.

Results: Immunohistochemical staining of TGF-β1 and CTGF were mainly expressed in the cytoplasm of fibroblast cells. The expression of TGF-β1 and CTGF protein was significantly negatively correlated with POP-Q stage. There were significantly positive correlations between the expression of TGF-β1 and CTGF protein. The expression of TGF-β1 protein among the 3 POP groups were all significantly lower than that of the control group, while there was no significant differences in the expression of TGF-β1 protein among the POP groups, excluding the comparison between Groups 1 and 3. The expression of CTGF protein in the 3 POP groups were all significantly lower than that of the control group, and significant differences were also detected among the 3 POP groups.

Conclusion: In this study, we found that the TGF-β1 and CTGF protein expression may be associated with POP, especially in POP-Q stages.

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Pelvic floor dysfunction (PFD) that results from pelvic floor defects, or loss of pelvic floor support tissue consisted of a group of gynecological diseases, such as stress urinary incontinence (SUI), pelvic organ prolapse (POP [including uterine prolapse, vaginal anterior bulging, and vaginal posterior wall bulging]), and chronic pelvic pain. It is a common and frequently occurring disease in older women, with an incidence rate of approximately 30-40%.¹ Although it is not a life-threatening disease for patients, it leads a serious impact on older women's health and life quality, also an enormous economic burden on individuals and society. So far, the pathogenesis of PFD remains poorly understood. Epidemiological studies have showed that the development of PFD is multifactorial, and factors involved in the development of PFD includes pregnancy, vaginal childbirth, genetic factors, obesity, ageing, and chronic conditions, such as asthma and constipation. One of the most prevalent pelvic floor disorders is POP, a condition in which the pelvic organs (bladder, vagina, cervix, and uterus) herniated through the vaginal opening. Support of the pelvic viscera is maintained by fibromuscular connective tissues of the female pelvic floor and a group of skeletal muscles, known as the levator ani. Collagen constitutes the main component of ligaments and fascia. The change of collagen in the pelvic floor connective tissue leads to the relaxation of ligaments, fascia, and other support structure, and POP finally occur. A large number of studies²⁻⁶ found a reduction of collagen synthesis and collagen cross-linking, while an increase of its decomposition, with the addition of abnormal collagen morphology in the pelvic tissues of POP patients indicates that collagen plays an important role in the occurrence and development of POP. However, the mechanism of collagen change in the pelvic floor support tissue of women with POP is not known at present. The transforming growth factor-beta 1 (TGF- β 1) and connective tissue growth factor (CTGF) are key factors to regulate collagen protein. In this study, we investigate the expression of TGF- β 1 and CTGF in the pubocervical fascia tissue of POP patients by immunohistochemistry and Western blot in order to explore their role for POP occurrence and development.

Methods. An oral consent was obtained from each patient, although we did not take extra blood or tissues from any patient. All we reviewed were the medical records. Ethical approval was obtained from

the Research Ethics Committee of our hospital. After obtaining informed consent, samples of the cervical fascia tissue were collected from 50 women undergoing vaginal hysterectomy at the Department of Obstetrics and Gynecology, Renmin Hospital of Wuhan University, Hubei, Wuhan, China from May to December 2009. Thirty of the patients with POP studied were placed into Group 1 (n=10), Group 2 (n=10), and Group 3 (n=10) according to Pelvic Organ Prolapse Quantitation (POP-Q).⁷ Group 1 represents POP II, Group 2 - POP III, and Group 3 - POP IV. Twenty cases with other benign gynecological diseases were selected as the control group. None of the patients had immunological and connective tissue diseases, endometriosis, history of acute or chronic pelvic inflammatory disease, hormone replacement, pelvic surgery history, and prior pessary use. All patients were matched to exclude possible influences such as age, parity, body mass index, and other POP-related medical history (constipation, chronic cough, and so forth). The paired patient data are showed in Table 1.

Samples. Approximately 100 mg of tissue sample was obtained with a scalpel intraoperatively from the pubocervical fascia tissue for each patient.

Immunohistochemistry. Immunohistochemical staining for TGF- β 1 and CTGF was performed to determine the presence and distribution of these proteins in the pubocervical fascia tissue of POP patients. Semiquantitative staining intensity score was determined on a scale from 0-3 (0-none, 1-weak, 2-intermediate, 3-strong). Two investigators who had no idea of the patients' clinical information assess the staining intensity independently. Cases rated differently were re-evaluated, and score assessment was carried out by a joint decision. Using optical microscope (Leica, Wetzlar, Germany), a 10 high power field in each slice were randomly selected, and observed immunohistochemistry staining of TGF- β 1 and CTGF protein. All of the staining results were categorized as follows: weak staining (+), middle staining (++), strong staining (+++). Preimmune sera were used as a negative control. Positive cells according to their number and the color intensity is divided into 4: no positive cells (-); expression of positive cell number no more than 10%, staining intensity was weak-positive, or only individual cells were positive staining in Xeon, that is, weak positive (+); positive cell count for more than 60% positive for Xeon, a few cells can be a weak positive, that is, the expression of strong positive expression (+++); number and intensity of positive cell-mediated between the 2 above those for the positive (++). The total positive rates of TGF- β 1 and CTGF in each group are calculated by the number of TGF- β 1 and CTGF which were stained (+),(++), and (+++) divided by the total number of each group.

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Western blot. The samples were lysed using radio-immunoprecipitation assay buffer with protease inhibitors (Sigma, Silicon Valley, America) to recuperate the whole cell proteins on ice. The protein content was measured by the Bicinchoninic acid protein assay kit (Bioss, Beijing, China). Sample protein were run on a precast 12% acrylamide gel for 2 hours, and transferred onto a polyvinylidene fluoride membrane for 1.25 hours, then the membrane were blocked with 1% (bovine serum albumin) in Tris-buffered saline with 0.1% Tween-20 (TBST) for 2 hours, and incubated with a 1:200 dilution of primary rabbit antibody to TGF- β 1 or CTGF at 4°C overnight. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G were applied at a dilution of 1:4000 for 1.5 hours at room temperature (25°C). Immunoreactivity was detected with enhanced chemiluminescence detection kit (ECL

kit [Amersham Pharmacia Biotech, Rockford, IL, USA]), and visualized by autoradiography. Beta actin protein was used as a loading control.

Data processing was conducted using the Statistical Package for Social Sciences version 13.0 (SPSS Inc, Chicago, IL, USA). Continuous data were compared using the Student's t test. Differences were considered significant when $p < 0.05$.

Results. Immunohistochemistry. Immunohistochemical analysis of TGF- β 1 and CTGF demonstrated that TGF- β 1 and CTGF protein were both stained brown, of granules or flakes, and were mainly located in the fibroblast cytoplasm (Figure 1). There was a reduction of TGF-1 and CTGF staining in the fibroblast cytoplasm of patients with POP compared with the control group (Figure 1). Semi-quantitative H-

Table 1 - Paired data of patients with pelvic organ prolapse that underwent vaginal hysterectomy.

Groups	n	Age (year)	Parity (time)	Body mass index	Constipation	Chronic cough
1	10	48.3 ± 9.4	2.7 ± 1.6	25.1 ± 3.3	0	1
2	10	50.1 ± 8.7	3.2 ± 2.5	25.4 ± 2.8	1	0
3	10	54.6 ± 10.5	3.8 ± 1.9	23.6 ± 3.1	2	1
Control	20	49.4 ± 11.2	2.5 ± 2.0	24.0 ± 2.7	2	1
Test value		F=0.430	F=1.671	F=0.677	X ² =1.870	X ² =1.753
P-value		0.733	0.186	0.570	0.600	0.625

F - Levene's Test for equality of variances, comparison was carried out between each pelvic organ prolapse group and control group ($p > 0.05$).

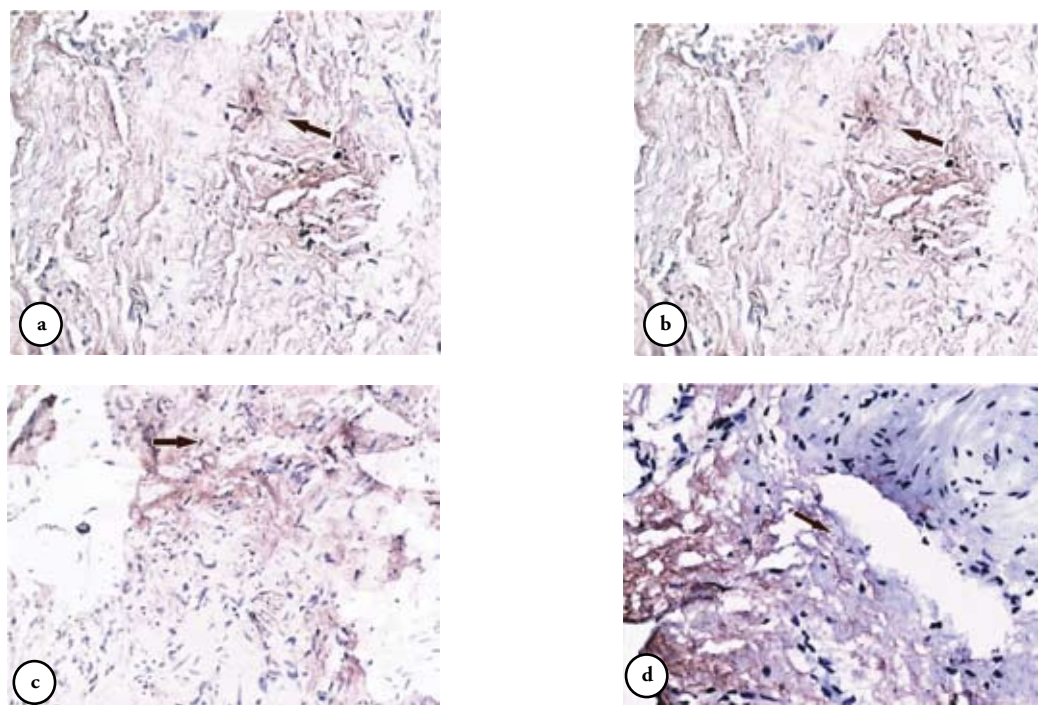


Figure 1 - The transforming growth factor-beta 1 (TGF- β 1) and connective tissue growth factor (CTGF) were detected mainly in the fibroblast cytoplasm of pubocervical fascia tissue. Immunohistological staining in the case group and control group showing: a) TGF- β 1 in cases; b) TGF- β 1 in the control; c) CTGF in cases; and d) CTGF in the control, 200X. The pattern of the distribution of TGF- β 1 and CTGF is similar. Significant differences of expression of these proteins were found between the case and control groups.

Table 2 - Data of transforming growth factor-beta 1 protein expression in the 4 groups.

Groups	Transforming growth factor-beta 1				Total positive rate (%)
	-	+	++	+++	
1	3	7	0	0	70.0
2	5	5	0	0	50.0
3	8	2	0	0	20.0
POP	16	14	0	0	46.7*
Control	2	5	9	4	90.0

(-) - no positive cells, (+) - expression of positive cell number no more than 10%, staining intensity was weak-positive, or only individual cells were positive staining in Xeon, that is, weak positive, (+++) - positive cell count for more than 60% positive for Xeon, a few cells can be a weak positive, that is, the expression of strong positive expression, (++) - number and intensity of positive cell-mediated between the 2 above those for the positive. *comparison of total positive rate between group pelvic organ prolapse (POP) and control group, $p=0.005$; correlation factor of connective tissue growth factor and POP-Quantitation stage is -0.409 , $p=0.002$

Table 3 - Data of connective tissue growth factor (CTGF) protein expression in the 4 groups.

Groups	CTGF				Total positive rate, %
	-	+	++	+++	
1	2	8	0	0	80.0
2	4	6	0	0	60.0
3	9	1	0	0	10.0
POP	15	15	0	0	50.0*
Control	3	5	7	5	85.0

(-) - no positive cells, (+) - expression of positive cell number no more than 10%, staining intensity was weak-positive, or only individual cells were positive staining in Xeon, that is, weak positive, (+++) - positive cell count for more than 60% positive for Xeon, a few cells can be a weak positive, that is, the expression of strong positive expression, (++) - number and intensity of positive cell-mediated between the 2 above those for the positive. *comparison of total positive rate between pelvic organ prolapse (POP) group and control group ($p=0.026$); correlation factor of CTGF and POP-Quantitation stage is -0.572 ($p=0.001$)

Table 4 - Data of the correlation between the expressions of transforming growth factor-beta (TGF-β1) and connective tissue growth factor (CTGF) protein.

TGF-β1	CTGF				Total number
	-	+	++	+++	
-	11	5	0	0	16
+	4	10	0	0	14
++	0	0	0	0	0
+++	0	0	0	0	0
Total	15	15	0	0	30

(-) - no positive cells, (+) - expression of positive cell number no more than 10%, staining intensity was weak-positive, or only individual cells were positive staining in Xeon, that is, weak positive, (+++) - positive cell count for more than 60% positive for Xeon, a few cells can be a weak positive, that is, the expression of strong positive expression, (++) - number and intensity of positive cell-mediated between the 2 above those for the positive. Correlation factor: $r=0.401$, $p=0.002$

score analysis of TGF-β1 and CTGF immunostaining confirmed a statistically significant reduction in patients with POP compared with the controls (Tables 2 & 3). The positive rates of TGF-β1 and CTGF in the POP group were 46.7% and 50%, with 70% and 80% in Group 1, 50% and 60% in Group 2, and 20% and 10% in group 3, which was significantly lower than 90% and 85% in the control group ($p=0.005$) (Tables 2 & 3). The expression of TGF-β1 ($r=-0.409$) and CTGF protein was significantly negatively correlated with the POP-Q stage ($r=-0.572$, $p=0.001$, Tables 2 & 3), and there are significantly positive correlations between the expression TGF-β1 and CTGF protein ($r=0.401$, $p=0.002$, Table 4).

Western blot. The TGF-β1 and CTGF protein expression is presented by calculating the level of TGF-β1 gray bands/actin band gray-scale value, and the gray value of CTGF bands/actin band gray value x100%. The Western blot showed the expression of TGF-β1 and CTGF on POP-Q at different stages: 0.4344 ± 0.097 (control group), 0.2441 ± 0.060 (POP-Q II), 0.1844 ± 0.041 (POP-Q III), 0.1345 ± 0.033 (POP-Q IV) and 1.2732 ± 0.18 (control group), 0.2132 ± 0.034 (POP-Q II), 0.1045 ± 0.028 (POP-Q III), 0.094 ± 0.015 (POP-Q IV) (Figure 2). The expression of TGF-β1 protein among 3 groups of POP were all significantly lower than that of the control group ($p=0.000$), while there were no significant differences in the expression of TGF-β1 protein among POP groups ($p=0.068$ between group 1 and 2, 0.126 between group 2 and 3) excluding the comparison between Groups 1 and 3 ($p=0.000$). The expression of CTGF protein in 3 POP groups were all significantly lower than that of the control group ($p=0.000$), and significant differences was also detected among the 3 POP groups ($p=0.000$).

Discussion. Collagen synthesis and metabolism are complex processes, which are related to many cytokines and growth factors. The TGF-β1, a multifunctional

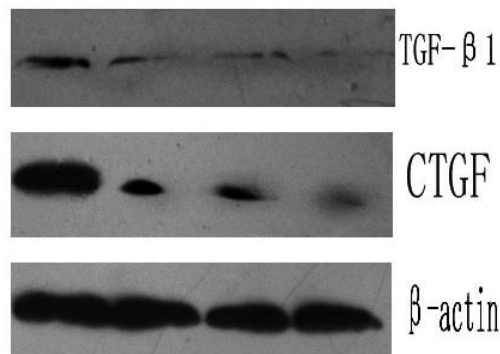


Figure 2 - Western blot of TGF-β1 and CTGF in the fibroblast cytoplasm of pubocervical fascia tissue. TGF-β1 - transforming growth factor-beta 1, CTGF - connective tissue growth factor

cytokine is TGF- β superfamily including TGF- β s, bone morphogenetic proteins (BMPs), activins inhibins, and myostatin. It participates in a wide range of processes, from tissue differentiation through to regulation of mesenchymal and immune cell functions. A line of studies have suggested that TGF- β 1 was master switch for the induction of the collagen synthesis program and induced fibroblasts to synthesize collagen.⁸ It regulated collagen mainly by promoting collagen and metalloproteinase inhibitor-1 gene expression and inhibiting matrix metalloproteinase-1 gene expression.⁹⁻¹² The CTGF as a downstream of TGF- β 1 also plays an influential role^{13,14} in the collagen metabolism process. The study has demonstrated that CTGF might be induced by TGF- β , and was a key downstream mediator of TGF- β effects on fibroblasts. This study has shown that pubocervical fascia of women with POP do express TGF- β 1 and CTGF protein in the fibroblast cytoplasm, and the control group expressed significantly more TGF- β 1 and CTGF protein compared to the POP group and the expression of TGF- β 1 and CTGF protein was significantly negatively correlated with POP-Q stage. This suggests that obvious reduction of TGF- β 1 and CTGF protein may be associated with the POP and accelerate its development. Wen et al¹⁵ demonstrated an obvious reduction of TGF- β 1 mRNA and protein in the SUI patient vagina tissue compared to the asymptomatic control in the proliferative phase.¹⁵ It was found recently¹⁶ that relaxin reduced the TGF- β 1 overall level and activity in fibroblasts of SUI patients, thus someone infers that the SUI in pregnant women may be due to a reduction of the TGF- β 1 and its activity in fibroblasts caused by a large number of relaxin. The explanation underlying the phenomenon may be that TGF- β 1 mediate many biologic process especially inflammatory processes, and with the development of POP pelvic floor tissue may be to the extent inflammatory. Meanwhile, this study has shown that there are significant positive correlation between the expression of TGF- β 1 and CTGF protein among the POP groups, and these data indicate that TGF- β 1 may be involved in the occurrence and development of POP by down regulating CTGF protein expression. Some studies demonstrated that by blocking CTGF, the role of TGF- β 1 to promote collagen synthesis was attenuated significantly, while its role to promote inflammation remained unaffected. But to confirm the exact relation between TGF- β 1 and CTGF and POP further larger sample sizes, and further proteomics experiments are recommended. The number of the cases in our study is not enough, and the molecular mechanisms of POP are needed to be further investigated.

In conclusion, the expression of TGF- β 1 affected by various risk factors may be the POP generant mechanism. The TGF- β 1 leads to a reduction in the transcription of CTGF, which causes a reduction of collagen secretion by fibroblast, which can enhance the activity of some

metalloproteinase such as collagenase, stromelysin and elastase, and so forth. All of the above lead to the reduction of collagen content and the occurrence and development of POP at last. We may be able to treat this as a target for the POP as a new method, but its feasibility remains to be studied further.

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