HOXA 11 and 13 expression in women with pelvic floor disorder

Soo Rim Kim

Department of Medicine

The Graduate School, Yonsei University

HOXA 11 and 13 expression in women with pelvic floor disorder

Soo Rim Kim

Department of Medicine

The Graduate School, Yonsei University

HOXA 11 and 13 expression in women with pelvic floor disorder

Directed by Professor Sang Wook Bai

The Master's Thesis
submitted to the Department of Medicine
the Graduate School of Yonsei University
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

Soo Rim Kim

December 2013

This certified that the Master's Thesis
of Soo Rim Kim is approved.

Thesis Supervisor : Sang Wook Bai

Thesis Committee Member#1: Sei Kwang Kim

Thesis Committee Member#2: Sunghoon Kim

Thesis Committee Member#3: Young Jun Oh

Thesis Committee Member#4: Eun Hee Yoo

The Graduate School
Yonsei University

December 2013

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere gratitude to all those who supported my thesis.
Foremost, I send my deepest gratitude to the members of the thesis committee- Professor Sang Wook Bai, Professor Sei Kwang Kim, Professor Sunghoon Kim, Professor Young Jun Oh and Professor Eun Hee Yoo for their dedicated guidance and support this work. Especially, I would like to thank Professor Sang Wook Bai, my respectable mentor, for his hearty encouragement, support, and sharp supervision of my paper.
I would also like to thank my lab, Young Shin Yim for giving me all of her effort, endless advice and support in this experiment. I could not have completed this thesis without her help.
My love and appreciation go out to my family – especially Jung Ryang Kim and Jung Soon Oh who are the love of my life.

Thank you.

Soo Rim Kim

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

HOXA 11 and 13 expression in women with pelvic floor disorder

Soo Rim Kim
Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Sang Wook Bai)

Pelvic floor dysfunction (PFD), including stress urinary incontinence (SUI) and pelvic organ prolapse (POP), is a major health problem for elderly women. Although PFD is a highly prevalent disease, the underlying mechanism and basic pathophysiology are poorly understood. Uterosacral ligaments (USLs) are the key structures of the uterus and vagina and are fragile in patients with PFD. Endopelvic fascias in the vagina compose the supportive tissue of the pelvic floor and often are diminished in PFD.

Homeobox (*HOX*) genes are transcriptional regulators that orchestrate embryonic development. The *HOXA11* gene controls the development of the lower uterine cervix and vagina. The *HOXA13* gene is also responsible for the development of the vagina and regulates extracellular matrix constituents. We hypothesized that expressions of *HOXA11* in USLs and *HOXA13* in the vagina might be decreased in women with PFD. In addition, we investigated quantitative mRNA expression of matrix metalloproteinases (MMPs), collagen I, and collagen III, from PFD and non-PFD patients.

Sixty-eight subjects were enrolled for the experimental procedure. Biopsy specimens were obtained from the anterior and posterior apex of the vagina

and bilateral USLs from women with no PFD, SUI combined with POP, and POP; from patients with SUI, we took only anterior vaginal specimens by biopsies. Specimens were divided in half. One section was prepared for real-time polymerase chain reaction (RT-PCR), and the other half was saved for western blot analysis. RT-PCR and western blots were used to determine *HOXA11* and *HOXA13* expression and the protein levels. In addition, we performed RT-PCR to compare the expression of MMP2, MMP9, collagen I, and collagen III genes in the vaginal wall and USLs of all enrolled patients. Quantitative data are expressed as mean + SEM and as percentages. The results of relative mRNA expression analysis were compared using the Kruskal–Wallis test for independent 'k' samples, complemented when necessary by Dunnett's multiple comparisons test, to evaluate possible differences between patient groups, for both vaginal wall and USL samples. Statistical analyses were performed with SPSS 19 (SPSS Inc. Chicago, IL, USA) and Graph-Pad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). A P value less than 0.05 was considered statistically significant for all data analyzed.

We found that the relative *HOXA13* mRNA expression in the anterior vaginal wall of SUI patients was 4.3-fold lower compared with that of controls ($P = 0.006$). In addition, the anterior vaginal wall specimens from patients in the SUI combined POP and POP groups had lower expression of *HOXA13* compared with the controls (4.3-fold, $P = 0.007$; 6.3-fold, $P < 0.001$, respectively). In the posterior vaginal wall, the relative mRNA expression of *HOXA13* of SUI combined POP and POP patients was lower (3.1-fold, $P < 0.001$; 2.7-fold, $P < 0.001$, respectively) than that of control patients. There were no differences in vaginal expression of *HOXA13* between SUI and POP specimens or between specimens obtained from patients with SUI combined POP and patients with only POP. Expression of *HOXA11* was 2.3-fold lower in women with POP compared with controls ($P = 0.004$). *HOXA 11*

expression in USLs was also 2.7-fold lower in SUI combined POP patients compared with controls ($P = 0.004$); however, there was no statistically significant difference between POP patients and women with SUI combined POP ($P = 0.08$). Western blot analysis demonstrated the presence of HOXA11 and 13 in the USLs and vagina, although the levels were lower in the POP and/or SUI group compared with controls ($P < 0.001$). Collagen I and collagen III expressions were lower in groups of PFD patients than controls. The expression of MMP 2 mRNA in USLs was elevated in POP patients with/without SUI compared to controls, although this alteration was not statistically significant for women with POP. In addition, the change in expression detected for both MMP2 and MMP9 was not statistically significant in samples from the anterior and posterior vaginal wall.

Expression of *HOXA13* and both collagens in the vagina was reduced in women with SUI, SUI combined POP, and POP compared with control women. Expression of *HOXA11* and both collagens in USLs was also reduced in all PFD patients. These reduced levels may contribute to alterations in the biomechanical strength of the pelvic supportive tissue, leading to prolapse or incontinence. A better understanding of the influence of these genes may prove beneficial in defining the underlying etiologies of PFD development and aid in the development of new treatment options.

Key word: HOXA 11; HOXA 13; pelvic floor disorder; pelvic organ prolapse; stress urinary incontinence ; uterosacral ligament; vagina

HOXA 11 and 13 expression in women with pelvic floor disorder

Soo Rim Kim
Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Sang Wook Bai)

I. Introduction

Pelvic floor dysfunction (PFD), including stress urinary incontinence (SUI) and pelvic organ prolapse (POP), is a major health problem for women. Although the prevalence of PFD is high (reported in up to 30–50% of women[1-3]), the underlying mechanism and basic pathophysiology are poorly understood. The most important factor contributing to the development of SUI is loss of mechanical stability at the urethra and the bladder neck. The mechanical stability of the genitourinary tract depends on intact, functional collagen fibers to support the bladder neck, urethra, and pelvic organs.[4] POP is the result of weakened endopelvic fascia causing herniation of the uterus, bladder, bowel, and/or rectum into the vaginal canal.[5] Clinical risk factors for the development of POP have been classically delineated as parity, increasing age, menopause, and body mass index (BMI).[6–8] In women with POP, biochemical analyses of the pelvic floor supportive structures (endopelvic fascia and uterosacral ligaments (USLs), the key supporting ligaments of the uterus and vagina) have demonstrated alterations in extracellular matrix (ECM) metabolism, including changes in the content and quality of collagen and expression of metalloproteinases (MMPs).[9–13] However, little is known regarding regulation of the ECM proteins and pathophysiological pathways associated with abnormalities of pelvic supportive tissue. An investigation of

PFD pathophysiology could aid in identifying women at risk for developing PFD and improve prevention and treatment strategies for this condition.

Collagen constructs the matrix of the pelvic connective tissue and is synthesized by complex post-translational processing, which ultimately requires cross-linking of fascial collagen chains by pyridinoline to mature into a high tensile strength support structure.[4] Twenty-eight types of collagen have been identified in the vaginal epithelium and endopelvic fascia. Among them, type I and type III collagen are the main structural components of epithelial tissue in the pelvic floor structure.[14,15] Collagen I is ubiquitous, flexible, and offers strong resistance to tension. Collagen III is predominant in tissues that require increased flexibility and distension and that are subject to periodic stress.[16] Collagen III is the primary collagen subtype in the vagina and supportive tissues, and it is the major collagen in skin at birth before it is replaced by collagen I later in life. Both type I and type III are found in granulation tissue during wound repair.[17,18] Collagen I co-polymerizes with collagen III to form fibrils with controlled diameters, and these fibrils influence the biomechanical characteristics of tissues.[19] An increase in collagen III decreases the mechanical strength of connective tissue by decreasing fiber size.[20] It is generally agreed that a higher I/III ratio in the ligament is indicative of greater strength, whereas a lower ratio may result in tissue laxity. Therefore, the balance between synthesis and degradation of collagen is important for maintaining tissue integrity and tensile strength during continuous tissue remodeling.[21]

Degradation of collagen depends on the activity of MMPs that compose a family of structurally related proteins, which degrade the ECM and basement membrane components.[22] Type I, II, and III interstitial collagens, the most abundant connective tissue proteins, are cleaved by the interstitial collagenases (MMP 1, MMP 8, and MMP 13), which specifically cleave native triple helical collagen at a single peptide bond in each chain, yielding

two fragments. This cleavage produces three-quarter and one-quarter length collagen fragments, which are very susceptible to rapid gelatinase (MMP 2 and MMP 9) degradation to amino acids. [21] Previous reports indicate that MMP 2 and MMP 9 might be critically important to the loss of fibrous collagen strength and subsequent loss of tissue integrity even inconclusive results.[21, 23, 24]

Homeobox (*HOX*) genes are evolutionarily conserved genes encoding transcription factors that regulate mammalian embryonic growth and development of the urogenital tract. The *HOXA* cluster genes mediate segmental differentiation of the paramesonephric duct into morphologically distinct organs of the female reproductive tract. Their collinear expression occurs in a temporal, spatial, and tissue-specific fashion.[25, 26] Only three studies have shown that *HOX* genes are related to the development of POP.[26-28] However, to date, there has been no report about the expression of *HOXA* genes in patients with SUI. *HOXA11* is responsible for the development of the USL and is decreased in women with POP.[26, 27] Decreased expressions of *HOXA11* and both collagens have been reported, while MMP 2 increased in patients with POP.[26] *HOXA13* is responsible for the development of the upper vagina and is decreased in women with POP; however, previous studies have not revealed the signaling pathway downstream of HOXA13 activity, which might include MMPs or collagens.[25, 28]

In women with PFD, the USL or endopelvic fascia is often weakened. Because *HOXA13* is a regulatory gene of the vagina, and *HOXA11* is a regulatory gene of the USL and cervix, we hypothesized that expression of *HOXA11 or HOXA13* would be altered in women with PFD. Therefore, we sought to evaluate the role of these transcription factors on the homeostasis of the ECM in the pelvic supportive structure. In addition, we investigated the *HOXA13* downstream signaling pathway, including MMP 2, MMP 9, collagen I, and collagen III in patients with PFD.

II. Material and method

1. Acquisition of human tissue

All experiments were performed with approval of the hospital's Institutional Review Board, and all patients provided informed consent to participate in the study. All specimens were collected between August 2011 and August 2012 from 68 women undergoing a transobturator tape, hysterectomy, and/or anterior and posterior vaginal repair at our institution. Before their surgery, a pelvic examination was performed to determine the presence of SUI or POP. All enrolled patients underwent a urodynamic study (UDS) for the diagnosis of SUI. Vaginal prolapse was graded according to the POP quantification system advocated by the International Continence Society.[29] Women with stage II prolapse or greater were assigned to the POP group. The control group was chosen from a group of women undergoing hysterectomy for benign gynecological disorders with prolapse no greater than stage I. Data regarding age, menopause status, BMI, and parity were recorded. Women with previous pelvic inflammatory disease, endometriosis, gynecological cancer, previous pelvic surgery, neurological disease, hormone use, pessary use before surgery, and >75 years old were excluded from the study. Eight women undergoing abdominal hysterectomy due to myomas were enrolled as controls. Sixty patients with PFD were divided into 3 groups, consisting of women with SUI (n = 20), SUI combined POP (n = 20), and POP (n = 20).

At the time of surgery, 6 samples were obtained from 3 biopsy sites of PFD and control patients, except patients with SUI. For consistency, 5-mm biopsies were taken from the midline anterior and posterior vaginal cuff; in patients with SUI, we biopsied only anterior vaginal specimens. In addition, 5-mm samples of the USL were taken from the proximal ligament at its

insertion into the cervix, where the ligament is consistently identifiable. Specimens were divided in half, and both sections were promptly placed in RNAlater (Ambion, Inc, CA, USA.) and stored at -80℃ until extraction of RNA was performed. One section was prepared for real-time quantitative polymerase chain reaction (RT-PCR), and the other half was preserved for western blot analysis.

2. RT-PCR analysis

A. RNA preparation and complementary DNA (cDNA) conversion

A total of 66 vaginal and 48 USL specimens were evaluated by RT-PCR: 20 anterior vaginal specimens obtained from women with SUI, 20 anterior and posterior vaginal specimens from women with SUI combined POP, 20 from women with POP, and 6 from controls; 20 USL specimens obtained from women with SUI combined POP, 20 from women with POP, and 8 from control women. For RNA preparation (RNeasy mini kit; Qiagen, Valencia, CA, USA), specimens in RNAlater were thawed on ice, and 30 μg of tissue sample was obtained. The tissue was disrupted and homogenized in 600 μL of RLT buffer (Qiagen, Valencia, CA, USA). Then, the lysate was centrifuged for 3 min to eliminate everything but the supernatant. After adding 1 volume of 70% ethanol to the cleared supernatant, the sample was transferred to the RNeasy spin column and centrifuged at 8000 × g for 15 s. The RNeasy spin column was centrifuged two more times at 8000 × g for 15 s—once after adding 700 μL of RW1 buffer and again after adding 500 μL of RPE buffer. Then finally, 30 μL of RNase-free water was added directly to the spin column membrane for RNA elution. cDNA was synthesized from 200 ng total RNA (total reaction volume of 20 μL) by reverse transcription using SuperScript III (Invitrogen, CA, USA) according to the manufacturer's instructions.

B. Real-Time PCR

We used RT-PCR to detect the mRNA levels of HOXA 11, HOXA 13, MMP 2, MMP 9, pro- collagen type 1A, procollagen type 3A, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; internal control). The RT-PCR was performed with a 7500 RT-PCR system (Applied Biosystems, CA, USA), using fluorescent SYBR Green technology (Applied Biosystems, CA, USA). The melting peak of each sample was routinely determined by melting curve analysis to confirm that only the expected products had been generated. Negative controls for each target gene were run in the absence of reverse transcription. Gene expression levels of target genes were standardized by calculating mRNA ratios relative to the internal control. The fold change in the target gene relative to the *GAPDH* internal control gene was determined by the following formula for $2^{-\Delta(\Delta Ct)}$: Fold change = $2^{-\Delta(\Delta Ct)}$, $\Delta Ct = Ct_{(\text{target gene})} - Ct_{(GAPDH)}$, $\Delta(\Delta Ct) = \Delta Ct_{(\text{disease group})} - \Delta Ct_{(\text{control group})}$. RT-PCR was performed with 2 μL of cDNA with the following cycling conditions: pre-incubation at 95℃ for 10 min, followed by 40 cycles of denaturation at 95℃ for 15 s, and annealing and extension at 60℃ for 1 min. The detailed nucleotide sequences are shown in Table 1.

Table 1. Oligo sequences used in Real-Time PCR Primer sequences used for real-time PCR reactions evaluating expression of HOXA 11, HOXA 13, procollagen type I and III, GAPDH, MMP 2 , and MMP 9 in human.

Gene		sequence (5'-3')
HOXA 11	Forward	GTACTTACTACGTCTCGGGTCCAG
	Reverse	AGTCTCTGTGCACGAGCTCCT
HOXA 13	Forward	ATGCCTGGCTACCTGGATATGC
	Reverse	GGGCAGAGTGGACTTCCAGAGGT
MMP 2	Forward	CGTCTGTCCCAGGATGACATC
	Reverse	ATGTCAGGAGAGGCCCCATA
MMP 9	Forward	CGCCAGTCCACCCTTGTG
	Reverse	CAGCTGCCTGTCGGTGAGA
procollagen type 1A	Forward	AGGGCCAAGACGAAGACATC
	Reverse	AGATCACGTCATCGCACAACA
procollagen type 3A	Forward	TGGCTACTTCTCGCTCTGCTT
	Reverse	CGGATCCTGAGTCACAGACACA
GAPDH	Forward	ATGGAAATCCCATCACCATCTT
	Reverse	CGCCCCACTTGATTTTGG

3. Western blot

In the control group, 6 vaginal specimens and 8 USL specimens were processed for western blot. In the PFD groups, 15 specimens from the vaginal wall and USLs were prepared for western blot. The specimens was weighed and homogenized, using a glass homogenizer in homogenizing buffer (0.32 M sucrose, 100 mM HEPES, pH 7.4). Homogenate tissue lysates were then centrifuged. The resulting supernatants were resuspended in a lysis buffer (1% Triton X-100, 100 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% Na-deoxycholate), incubated for 30 min in a 4℃ rotator, and then centrifuged. Protein concentrations of the samples were determined by a protein assay using bicinchoninic acid (Pierce, CA, USA). Fifty micrograms of protein were separated by SDS-PAGE (polyacrylamide gel electrophoresis) and blotted onto polyvinylidene fluoride membranes in a methanol-based Tris-glycine buffer. Membranes were then blocked with 5% non-fat milk (Bio-Rad). Each of the primary polyclonal antibodies (anti-HOXA11, anti-HOXA13, and anti-actin; Abcam, Cambridge, UK) were suspended in 1% non-fat milk and incubated over the membrane with continuous mixing overnight at 4℃. Membranes were rinsed at least three times with Tris-buffered saline and Tween 20 (TBS-T) for 5 min each and then incubated in 1% non-fat milk, which contained a horseradish peroxidase-conjugated secondary antibody, for 1 h at room temperature. After a final TBS-T rinse, the membranes were suspended in an enhanced chemiluminescence solution (Pierce, CA, USA) and exposed to Kodak film. Then, the films were scanned and quantified by densitometry using Scion Image Analysis Software (National Institutes of Health, Bethesda, MD, USA).

4. Statistical Analysis

Quantitative data are expressed as mean + SEM (standard error mean) and percentages. The results of relative mRNA expression were compared using the Kruskal–Wallis test for independent ‘k’ samples and complemented, when necessary, by *Dunnett’s* multiple comparisons test to evaluate possible differences between patients in all groups, for both the vaginal wall and USL samples. In all cases, the rejection level for null hypothesis was set at a value less than or equal to 0.05 (5%). Statistical analysis was performed with SPSS 19 (SPSS Inc. Chicago, IL, USA) and Graph-Pad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). A P value less than 0.05 was considered statistically significant for all data analyzed.

III. Result

1. Demographic characteristics of enrolled women.

We evaluated the demographic characteristics of our enrolled PFD and control patients. In this study, 20 women with SUI, 20 women with SUI combined POP, 20 women with POP, and 8 control women were included. Differences in age, parity, menopausal status, and BMI between PFD and control patients are shown in Table 2. The PFD group patients were significantly older than the control patients similar to menopausal status. Both BMI and parity were also higher in PFD patients than controls.

2. Vaginal HOXA13 expression is lower in women with PFD compared to controls, but there is no significantly statistical difference within women with PFD.

To quantitatively evaluate the vaginal expression of *HOXA13* in each group of women, we used RT-PCR. We found that the relative mRNA expression of *HOXA13* in the anterior vaginal wall for SUI patients was 4.3-fold lower compared with that of controls ($P = 0.006$). In addition, the specimens of both the SUI combined POP and the POP groups had lower expression of *HOXA13* in the anterior vaginal wall compared with the controls (4.3-fold, $P = 0.007$; 6.3-fold, $P < 0.001$, respectively) (Fig. 1A). In the posterior vaginal wall, there were lower levels of the relative mRNA expression of *HOXA13* for POP patients with/without SUI (3.1-fold, $P < 0.001$; 2.7-fold, $P < 0.001$, respectively) compared to controls (Fig. 1C). There were no differences in vaginal expression of *HOXA13* between SUI specimens and POP specimens or between SUI combined POP specimens and POP specimens). Western blot results demonstrated the presence of *HOXA13*

in the vagina of control and PFD patients; furthermore, *HOXA13* levels were consistently lower in the PFD patient groups compared with control group (Fig. 2A, C). The band expressions of western blot for the SUI, POP, and SUI combined POP specimens were similar to each other, as was the level of proteins in patients with PFD ($P < 0.001$).

3. HOXA11 is decreased in the USL of women with POP and/or SUI.

To determine whether a relationship between *HOXA11* expression and the integrity of USLs exists, we used RT-PCR and western blot to characterize the expression of *HOXA11* and levels of *HOXA11* in surgical USL specimens of control and POP patients with/without SUI. Evaluation of the relative expression of *HOXA11* mRNA for POP patients with/without SUI showed similar results when compared to controls. *HOXA11* expression was 2.3-fold lower in women with POP compared with controls ($P = 0.004$), and *HOXA 11* expression was also 2.7-fold lower in SUI combined POP patients compared with controls ($P = 0.004$) (Fig. 1B). However, there was no statistically significant difference between POP patients and SUI combined POP patients ($P = 0.08$). Western blot demonstrated the presence of HOXA11 in the USLs from all groups, although the protein levels were lower in the POP and/or SUI groups compared with controls ($P < 0.001$) (Fig. 2B).

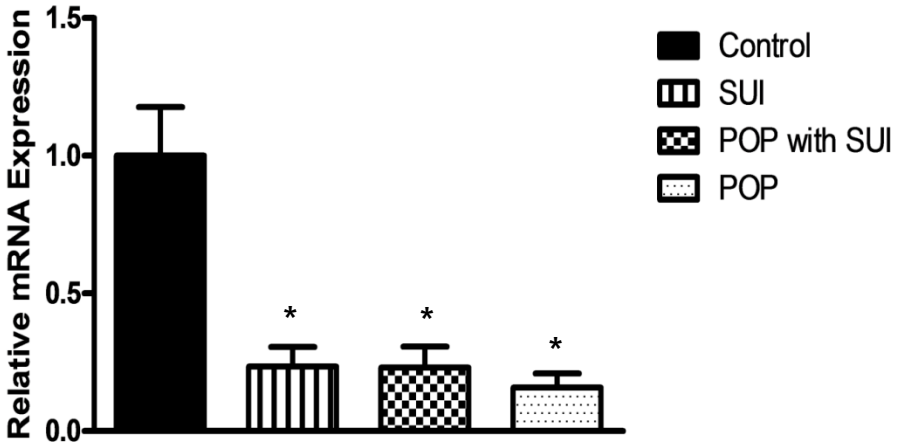
Table 2. Clinical characteristics of control and patients with SUI, POP with SUI and POP

	Control (n=8)	SUI (n=20)	POP combined with SUI (n=20)	POP (n=20)
Age (yr) [a]	49.7±4.6	55.2±10.4	64.2±10.6	62.4±10.0
Parity[a]	1.8±0.4	2.2±0.7	2.7±1.4	2.9±1.2
Menopause	38%	60%	80%	80%
BMI (kg/m^2)[a]	21.3±1.6	25.4±3.2	24.6±2.6	24.1±2.6

[a]Value are presented as mean±SD

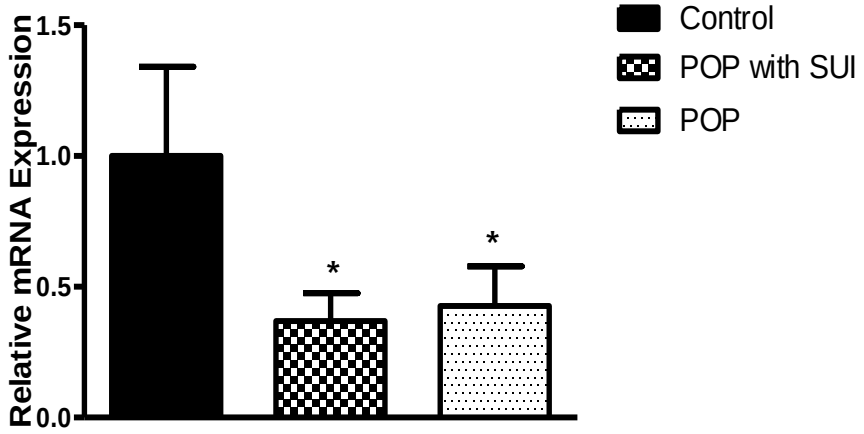
(SUI, stress urinary incontinence; POP, pelvic organ prolapse; BMI, body mass index)

(a)



HOXA13

(b)



HOXA 11

(c)

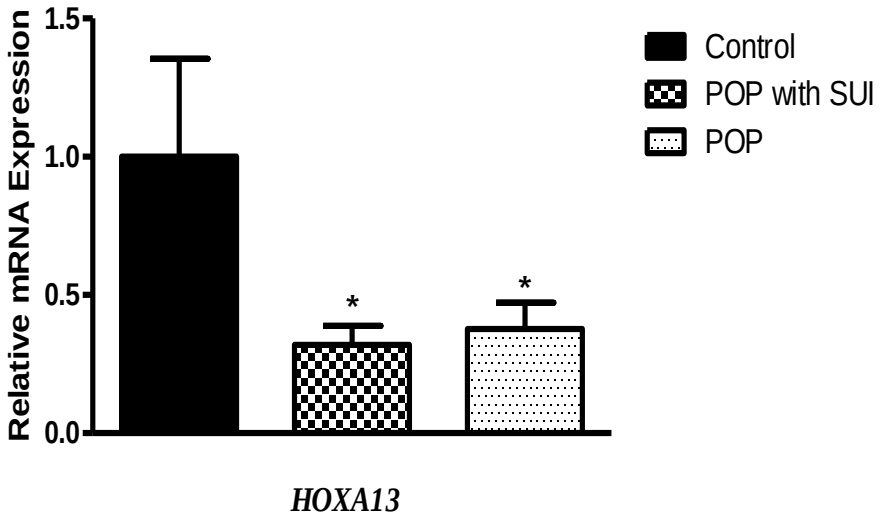


Figure. 1. HOXA 11 and 13 expression in women with and without PFD .

(a) Comparison of *HOXA13* mRNA expression in anterior vaginal specimens. (b) *HOXA11* mRNA expression in the USLs of controls and prolapse women with and without SUI. (**c**) Comparison of mRNA expression of *HOXA13* in posterior vaginal walls in women with and without POP; Control, n = 8 (Vaginal specimen, n=6; USL, n=8); SUI, n = 20; POP combined SUI, n = 20; POP, n = 20. Comparisons were made after normalization with GAPDH. Expression of *HOXA 11* and *HOXA13* was dramatically reduced in women with PFD versus controls (**P* < 0.05). SUI, stress urinary incontinence; POP, pelvic organ prolapse.

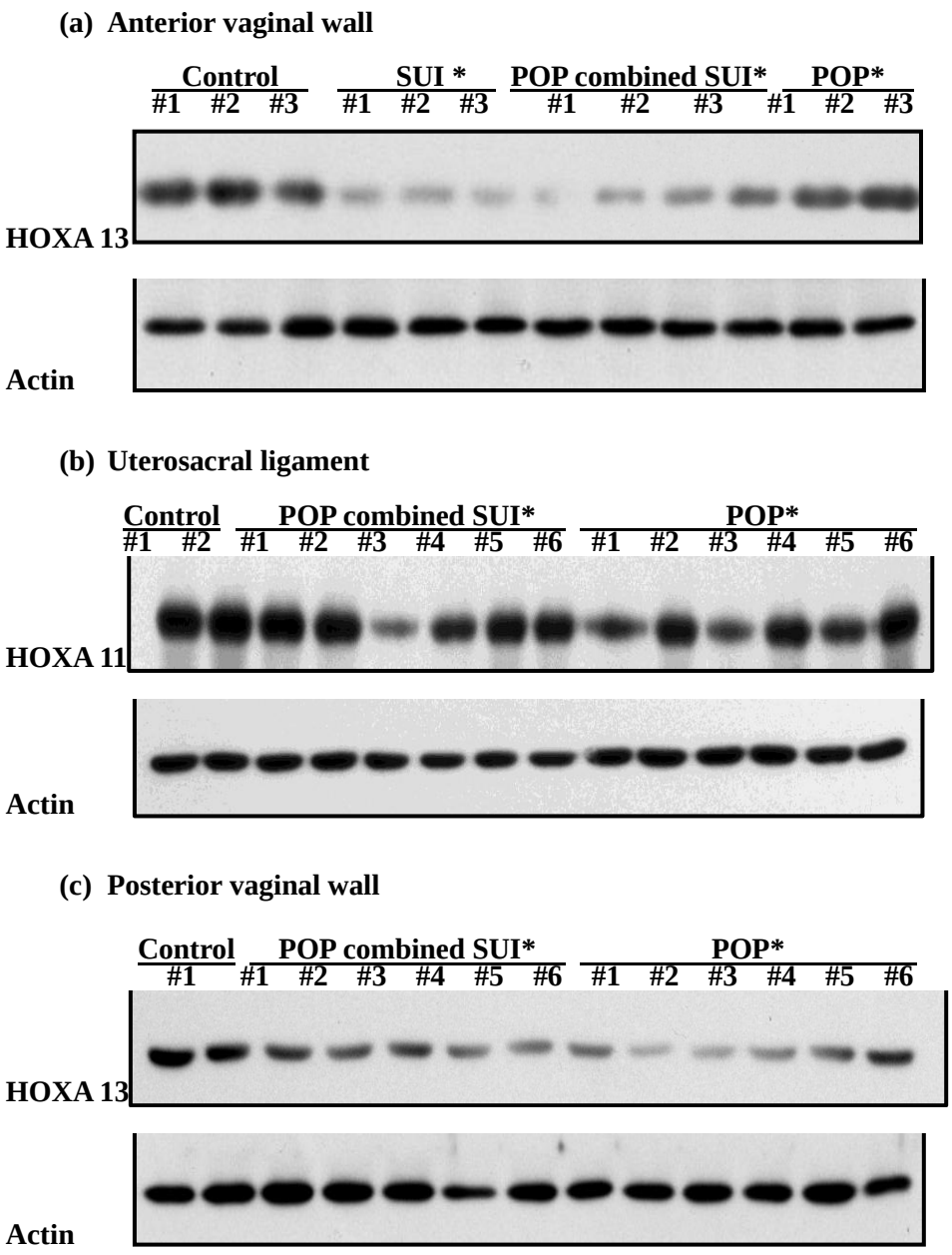


Figure 2. Western blot analysis on vaginal wall tissue and uterosacral ligament: protein from SUI, POP combined SUI, POP patients and controls. (* $P < 0.05$; versus Controls, SUI, stress urinary incontinence; POP, pelvic organ prolapse.)

4. Expression of ECM proteins is also altered in the USLs and vagina of women with PFD.

We compared the mRNA expression of *HOX* genes in the USLs and vaginal walls of women with and without PFD using RT-PCR. Based on western blot analysis, we found that the protein expression of *HOX* genes with the specimens were confirmative among our subjects. In accordance with these results, we also found that the levels of both collagen type I and collagen type III were significantly reduced, 1.5-fold and 8.8-fold, respectively, in the USLs and vaginal walls of women with PDF compared with controls (Tables 3–5). In USL and vaginal wall specimens, collagen type III was decreased in POP patients with/without SUI. The levels of collagen type I was lower in the anterior vaginal walls and USLs of POP patients compared to control patients. Both collagen type I and collagen type III were reduced in women with SUI compared with controls; however, these differences were not statistically significant. Evaluation of the expression of MMP2 and MMP9 revealed that levels of these catabolic enzymes were increased by 1- and 2.5-fold for MMP 2 respectively, in women with PFD. Interestingly, the expression of MMP2 mRNA was elevated in POP women with/without SUI, although there was not statistical significance for women with POP. In addition, the changes in expression of both MMP 2 and MMP 9 were not statistically significant in the anterior and posterior vaginal walls, when compared to controls (Table 3–5).

Table 3. mRNA expression of collagens and MMPs in anterior vaginal wall from women with SUI, POP combined SUI and POP

	SUI			POP combined SUI			POP		
	Change	Fold	P	Change	Fold	P	Change	Fold	P
Collagen type I	↓	2.1	0.23	↓	2.2	0.16	↓	2.9	0.02*
Collagen type III	↓	2.0	0.70	↓	5.1	0.02*	↓	8.8	0.01*
MMP 2	↑	1.7	>0.99	↑	1.4	0.96	-	1.0	0.94
MMP 9	↑	1.2	>0.99	↑	1.2	>0.99	-	1.0	>0.99

↓, decrease; ↑, increase; -, sustain. Fold change relative to control subjects is shown. (*p < 0.05, Bonferroni correction with *Mann-Whitney U analysis* , *df=3*; SUI, stress urinary incontinence; POP, pelvic organ prolapse; MMP, matrix metalloproteionases)

Table 4. mRNA expression of collagens and MMPs in USLs from women with POP and/or SUI

	POP combined SUI			POP		
	Change	Fold	P	Change	Fold	P
Collagen type I	↓	1.8	0.15	↓	4.8	0.002*
Collagen type III	↓	4.3	0.003*	↓	5.5	0.001*
MMP 2	↑	2.2	0.04*	↑	1.8	0.2
MMP 9	↑	1.3	>0.99	↑	1.3	>0.99

↓, decrease; ↑, increase. Fold change relative to control subjects is shown. (* $P < 0.05$, Bonferroni correction with *Dunnett multiple analysis, df=2*; SUI, stress urinary incontinence; POP, pelvic organ prolapse; MMP, matrix metalloproteionases)

Table 5. mRNA expression of collagens and MMPs in posterior vaginal wall from women with POP and/or SUI

	POP combined SUI			POP		
	Change	Fold	P	Change	Fold	P
Collagen type I	↓	1.5	>0.99	↓	2.5	>0.99
Collagen type III	↓	2.4	0.003*	↓	2.9	<0.001*
MMP 2	↑	2.1	0.6	↑	2.5	0.06
MMP 9	-	1.0	>0.99	-	1.0	>0.99

↓, decrease; ↑, increase; -, sustain. Fold change relative to control subjects is shown. (* $P < 0.05$, Bonferroni correction with *Dunnett multiple analysis, df=2*; SUI, stress urinary incontinence; POP, pelvic organ prolapse; MMP, matrix metalloproteionases)

IV. Discussion

Although much is known about the growth and differentiation of the female reproductive tract, the molecular pathways involved in the development and maintenance of both USLs and vaginal walls are not well understood. One possible explanation for variable clinical presentation of POP is the existence of several pathways regulating the urogenital ECM. Currently, there have been no longitudinal studies evaluating the effects of *HOX* genes on the quality of the ECM and its relationship with incontinence and/or prolapse. The present study provides insight into the role of *HOXA11* and *HOXA13* in the embryological development of the USLs and vaginal wall. We found that *HOX* gene expression was decreased in the vaginal tissues and USLs of women with PFD and that expression of *HOXA11* and *HOXA13* gene did not differ between patients with SUI and POP. Our results demonstrated a decrease in the expression of *HOXA11* in women with POP, a finding that is consistent with prior serial studies.[26, 27]

The expression of *HOXA11* was decreased in women diagnosed with SUI combined POP. Williams et al. demonstrate that *HOXA13* regulates many target genes involved in the ECM, including some that have been shown to be altered in women with POP. Their data reveal that expression of *HOXA13* led to the upregulation of several collagen types, which confer both the tensile strength of the vagina and basement membrane constituents.[30] In another study, the expression of *HOXA13* was lower in the vaginal tissues of women with POP, and expression of this gene was not affected by menopause or treatment with leuprolide acetate.[28] Our results, similar to those of Connell et al., show decreased expression of *HOXA13* in the vaginal walls of women with POP compared to controls, suggesting that decreased proliferation may be occurring in these deficient supportive tissues of the pelvic floor.[26-28] In addition, we observed lower expression of *HOXA13* in SUI patients

with/without POP compared with controls. Our findings of decreased *HOXA11* expression in USLs and decreased *HOXA13* in the vagina indicate that the pathophysiology of incontinence involves a pathway controlled by these genes. Decreased *HOXA13* expression in the vagina of PFD patients demonstrates that *HOXA13* regulates many different structural components of the ECM. In addition, this result strongly suggests that *HOXA13* is a candidate gene for further investigation in determining the molecular mechanisms involved in development of both SUI and POP. These results are also consistent with our finding that the *HOXA11* gene was involved in the development of the USLs, and *HOXA11* was dramatically decreased in women with POP with/without SUI.

We recognize the difference in average age between the control and study groups, as most of the postmenopausal women underwent surgery for SUI, POP, or malignancy. In a previous study, both *HOXA11* and *HOXA13* changes were independent of estrogen, age, parity, and BMI.[26-28] Taken together with our findings, these results suggest that *HOX* genes may be key regulators in the maintenance of the ECM in the uterine support structures and vagina.

The stability of the supportive pelvic structures is dependent on the quantity and quality of the collagen fibers. A delicate balance between matrix synthesis and breakdown is necessary to maintain the integrity of the connective tissue. Several previous studies reveal the role of a strength deficit in pelvic connective tissues, which is attributed to changes in synthesis and degradation of different types of collagen in PFD pathophyiology.[31-36] However, data on the changes in collagen metabolism in patients with PFD are controversial. Based on results from several reports, the differences might be due to the analysis of different target tissues and different analysis methods in patients with PFD.[9, 12, 32-37] There are not yet standards for an analysis method or target tissue. The pelvic connective tissue, which supports the

vagina and pelvic organs, can be divided into the suspensory system part (Delancey's level I and II) and the supportive part (level III).[38, 39] The quantity, quality, type, and organization of collagen vary within the different tissues.[36] Thus far, tissues from all three levels have been used to study the possible defects in collagen metabolism in patients with PFD without defining and relating them to the type and stage of the PFD. Besides the variety in biopsy sites, the majority of the studies do not define tissue histology, making it difficult to determine exactly which portion (e.g., epithelium versus subepithelium) was analyzed. Histology of the parametrium and uterosacral and cardinal ligaments demonstrates that these structures have a different composition compared to the vaginal tissue. In addition, variation in biochemical tests that are used for the analysis of vaginal/ pelvic tissues in women with PFD and the heterogeneity in the populations studied contribute to the inconsistency of the results in the literature regarding connective tissue components of vaginal/pelvic tissue in relation to PFD.[40] In this study, type I and III collagen levels were significantly decreased in POP patients with/without SUI. Degradation of type I and III collagens is dependent on the activity of the interstitial MMPs, which ultimately receive signals from MMP 2 and MMP 9.[23, 41, 42]

The majority of previous studies on MMPs investigated synthesis (pro-MMP or MMP mRNA), [12,24,41,42] and they have examined the relationship regarding the activity of MMPs and quantity of collagen in the USLs or vagina in women with POP. The function of MMPs in the vagina and the supportive tissue of patients with POP remains unclear. Some authors report no difference in active MMP 2 and MMP 9 in the USLs.[12] Yet, other authors suggest an increase in metabolic turnover of collagen, since MMP 2 and MMP 9 were significantly higher in prolapsed tissue than in normal tissue.[24] We show here that MMP 2 mRNA in USLs increased 2-fold in POP patients with/without SUI, although this result was not statistically significant in

prolapse patients. MMP 9 mRNA levels did not significantly differ in PFD patients. This result is consistent with findings from other authors who report that pro- and activated MMP 9 proteins were not changed in the USLs or vagina of women with POP.[24, 42]

The total collagen content in tissue from women with SUI was lower than in control samples, and MMP 2 mRNA levels were higher than those in controls, although with no statistical significance.

We found significant decreases in *HOXA11* and collagen expression, accompanied by increased expression of MMP 2, in the USLs of patients with SUI combined POP compared to control patients. Our present results suggest that MMP 2 plays a central role in collagen reorganization and may be a critical factor in USL attenuation associated with SUI combined POP. The significant decrease in collagen levels may reflect the result of sustained upregulation of several pathways responsible for collagen degradation, including MMP 2 activity. And other catalytic pathway of collagen may be existed to development of POP. Thus far, it is not possible to determine whether women with PFD have weakened pelvic connective tissue as a result of changes in a signaling pathway involving *HOXA11 and HOXA13* regulation targeting collagen type III and MMP 2 or if these findings are the result of PFD. However, our study expands our understanding of the molecular mechanisms that regulate the maintenance of the pelvic support system in women with SUI or POP. This study is the first to identify and detect quantitative differences in *HOXA11* and *HOXA13* expression for SUI and POP patients compared with control patients. In addition, this is the first report of *HOXA 11* and *HOXA13* expression in 3 biopsy sites from patients with PFD. Although we did not determine the most appropriate site for biopsy or the most accurate analytical method, we propose that a better comparison in further studies will require standardization of biopsy sites with histological confirmation. Our study suggests that standardization of complementary and

confirmatory methods of protein quantification is necessary.

V. Conclusion

In this study, we determined that USL *HOXA11* and vaginal *HOXA13* expression levels were decreased in women with SUI, POP, and SUI combined POP. Furthermore, we showed that, in women with POP and/or SUI, decreased expression of *HOXA11* in the USL was not significantly different from controls. We also established that expression of *HOXA13* was not different among the PFD groups. These findings suggest that *HOXA11* and *HOXA13* gene products function in some common metabolic pathways associated with PFD.

Deficient *HOXA11 or HOXA13* signaling may limit functional development of the USLs and vagina in susceptible women and contribute to alterations in the biomechanical strength of the pelvic supportive tissue, leading to POP or SUI. Understanding *HOXA11* and *HOXA13* regulatory activities on downstream signaling pathways involved in the homeostasis of the pelvic supportive tissue may offer new therapeutic strategies for women with PFD and improve the quality of life for patients with this condition.

.

Reference

1. Yarnell JWG, Voyle GJ, Richards CJ, Stephenson TP. The prevalence and severity of urinary incontinence in women. J Epidemiol Commun Health 1981;35:71–4.

2. Iosif CS, Bekassy Z, Rydhstrom H. Prevalence of urinary incontinence in middle-aged women. Int J Gynecol Obstet 1988;26:255–9.

3. Samuelsson EC, Victor FT, Tibblin G, Svardsudd K. Signs of genital prolapse in a Swedish population of women 20-59 years of age and possible related factors. Am J Obstet Gynecol 1999;180:299–305.

4. Eyre DR. The specificity of collagen cross-links as markers of bone and connective tissue degradation. Acta Orthop Scand 1995;266(Suppl):166–70.

5. Jelovsek JE, Maher C, Barber MD. Pelvic organ prolapse. Lancet 2007;369:1027-38.

6. Bradley CS, Zimmerman MB, Qi Y, Nygaard IE. Natural history of pelvic organ prolapse in postmenopausal women. Obstet Gynecol 2007;109:848–54.

7. Kim CM, Jeon MJ, Chung DJ, Kim SK, Kim JW, Bai SW. Risk factors for pelvic organ prolapse. Int J Gynaecol Obstet 2007; 98:248–51.

8. Rortveit G, Brown JS, Thom DH, Van Den Eeden SK, Creasman JM, Subak LL. Symptomatic pelvic organ prolapse:risk factors in a population-based, racially diverse cohort. Obstet Gynecol 2007;109:1396–403.

9. Gabriel B, Denschlag D, Gobel H, Fittkow C, Werner M, Gitsch G, et al. Uterosacral ligament in postmenopausal women with or without pelvic organ prolapse. Int Urogynecol J Pelvic
Floor Dysfunct 2005;16:475–9.

10. Cole EE, Leu PB, Gomelsky A, Revelo P, Shappell H, Scarpero HM, et al. Histopathological evaluation of the uterosacral ligament: is this a dependable structure for pelvic reconstruction? BJU Int 2006;97:345–8.

11. Gabriel B, Watermann D, Hancke K, Gitsch G, Werner M, Tenpfer C, et al. Increased expression of metalloproteinase 2 in uterosacral ligaments

associated with pelvic organ prolapse. Int Urogynecol J Pelvic Floor Dysfunct 2006; 17:478–82.
12. Phillips CH, Anthony F, Benyon C, Monga AK. Collagen metabolism in the uterosacral ligaments and vaginal skin of women with uterine prolapse. Br J Obstet Gynaec 2006;113:39–46.
13. Suzme R, Yalcin O, Gurdol F, Gungor F, Bilir A. Connective tissue alterations in women with pelvic organ prolapse and urinary incontinence. Acta Obstet Gynecol Scand 2007; 86:882-8.
14. Nimni ME. Collagen: structure, function, and metabolism in normal and fibrotic tissues. Semin Arthritis Rheum 1983;13:1–86.
15. Bergman A, Elia G, Cheung D, Perelman N, Nimni ME. Biochemical composition of collagen in continent and stress urinary incontinent women. Gynecol Obstet Invest 1994;37:48–51.
16. Bailey AJ. Molecular mechanisms of ageing in connective tissues. Mech Ageing Dev 2001;122:735–55.
17. Haukipuro K. Synthesis of collagen types I and III in reincised wounds in humans. Br J Surg 1991;78:708–12.
18. Hibbs MS, Postlethwaite AE, Mainardi CL, Seyer JM, Kang AH. Alterations in collagen production in mixed mononuclear leukocyte-fibroblast cultures. J Exp Med 1983;157:47–59.
19. Birk DE. Type V collagen: heterotypic type I/V collagen interactions in the regulation of fibril assembly. Micron 2001;32:223– 37.
20. Birk DE, Fitch JM, Babiarz JP, Doane KJ, Linsenmayer TF. Collagen fibrillogenesis in vitro: interaction of types I and V collagen regulates fibril diameter. J Cell Sci 1990;95:649–57.
21. Chen BH, Wen Y, Li H, Polan ML. Collagen metabolism and turnover in women with stress urinary incontinence and pelvic prolapse. Int Urogynecol J Pelvic Floor Dysfunct 2002;13:80–7.
22. Chung HW, Wen Y, Nezhat C, Woo BH, Lake Polan M. Matrix

metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-3 (TIMP-3) m-RNA expression in endometriosis: MMP-9/TIMP-3 ratio in ectopic and eutopic endometrium. Fertil Steril 2001;75:152–9.
23. Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol 1997;74:111–22.
24. Jackson SR, Avery NC, Tarlton JF, Eckford SD, Abrams P, Bailey AJ. Changes in metabolism of collagen in genitourinary prolapse. Lancet 1996;347:1658–61.
25. Taylor HS, Vanden Heuvel GB, Igarashi P. A conserved Hox axis in the mouse and human female reproductive system: late establishment and persistent adult expression of the HOXA cluster genes. Biol Reprod 1997;57:1338-45.
26. Connell KA, Guess MK, Chen H, Andikyan V, Bercik R, Taylor HS. HOXA11 is critical for development and maintenance of uterosacral ligaments and deficient in pelvic prolapse. J Clin Invest 2008;118: 1050-5.
27. Connell KA, Guess MK, Chen HW, Lynch T, Bercik R, Taylor HS. HOXA11 Promotes Fibroblast Proliferation and Regulates p53 in Uterosacral Ligaments. Reprod Sci 2009;16:694-700.
28. Connell KA, Guess MK, Tate A, Andikyan V, Bercik R, Taylor HS. Diminished vaginal HOXA13 expression in women with pelvic organ prolapse. Menopause 2009; 16: 529-33.
29. Bump RC, Mattiasson A, Bo K, Brubaker LP, DeLancey JL, Klarskov P, et al. The standardization of terminology of female pelvic organ prolapse and pelvic floor dysfunction. Am J Obstet Gynecol 1996;175:10–7.
30. Williams TM, Williams ME, Kuick R, Misek D, McDonagh K, Hanash S, et al. Candidate downstream regulated genes of HOX group 13 transcription factors with and without monomeric DNA binding capability. Dev Biol

2005;279:462-80.
31. Chen B, Wen Y, Zhang ZM, Wang HB, Warrington JA, Polan ML. Menstrual phase-dependent gene expression differences in periurethral vaginal tissue from women with stress incontinence. Am J Obstet Gynecol 2003;189:89–97.
32. Zong W, Zyczynski HM, Meyn LA, Gordy SC, Moalli PA. Regulation of MMP-1 by sex steroid hormones in fibroblasts derived from the female pelvic floor. Am J Obstet Gynecol 2007;
196:349.e1-11.
33. Takano CC, Girao MJ, Sartori MG, Castro RA, Arruda RM, Simoes MJ, et al. Analysis of collagen in parametrium and vaginal apex of women with and without uterine prolapse. Int
Urogynecol J Pelvic Floor Dysfunct 2002;13:342–5.
34. Wong MY, Harmanli OH, Agar M, Dandolu V, Grody MH. Collagen content of nonsupport tissue in pelvic organ prolapse and stress urinary incontinence. Am J Obstet Gynecol 2003;189:1597– 9.
35. Moalli PA, Talarico LC, Sung VW, Klingensmith WL, Shand SH, Meyn LA, et al. Impact of menopause on collagen subtypes in the arcus tendineous fasciae pelvis. Am J Obstet Gynecol
2004;190:620–7.
36. Moalli PA, Shand SH, Zyczynski HM, Gordy SC, Meyn LA. Remodeling of vaginal connective tissue in patients with prolapse. Obstet Gynecol 2005;106:953–63.
37. Buchanan S, Robertson GW, Hocking PM. The relationships between vaginal collagen, plasma oestradiol and uterine prolapse in turkeys. Research in Veterinary Science 1999;67:153– 7.
38. Delancey JO. The anatomy of the pelvic floor. Curr Opin Obstet Gynecol 1994;6:313–6.
39. Delancey JO. Anatomic aspects of vaginal eversion after hysterectomy.

Am J Obstet Gynecol 1992;166:1717–24.
40. Karam JA, Vazquez DV, Lin VK, Zimmern PE. Elastin expression and elastic fibre width in the anterior vaginal wall of postmenopausal women with and without prolapse. BJU Int 2007; 100:346–50.
41. Aimes RT, Quigley JP. Matrix metalloproteinase- 2 is an interstitial collagenase. J Biol Chem 1995;270:5872–6.
42. Overall CM, Lopez-Otin C. Strategies for MMP inhibition in cancer: innovations for the post trial era. Nat Rev Cancer 2002; 2:657–72.

ABSTRACT (IN KOREAN)

골반저 질환을 가진 환자군에서 HOXA 11 과 HOXA 13 유전자의 발현 차이

<지도교수 배상욱>

연세대학교 대학원 의학과

김수림

골반저 질환은 요실금과 골반 장기 탈출증을 포함하는 광범위한 질환군으로 중년 이상의 고령의 여성들에게 삶의 질에 영향을 미치는 중요한 질환군이다. 골반저 질환은 매우 높은 유병율을 가지고 있지만 아직까지 그들의 병태 생리에 대해서는 거의 밝혀지지 않았다. HOX 유전자는 전사인자를 조절하는 조절체로서 개체의 발생 단계 및 분화과정에 관여한다. HOXA 11 은 자궁 체부, HOXA 13 은 질의 분화 과정에 영향을 미치며 이들 유전자에 의해 분화된 기관의 세포 외 기질의 구성 성분에 영향을 미치게 된다. 그러므로 골반저 질환 환자군에서 자궁 천골 인대의 분화에 관여하는 HOXA 11 과 질의 분화에 관여하는 HOXA 13 의 발현이 정상군과 차이 유무가 있다면, 골반저 질환 환자군 사이에서 요실금 환자와 골반 장기 탈출증을 가진 환자군 사이에 발현 차이가 있는지에 관해 검증하고, 이들과 관련된 MMP 2, MMP 9, collagen I 및 collagen III 의 발현차이에 관해 검증하고자 한다.

총 68명의 환자를 대상으로 앞, 뒤 질벽 및 양쪽 자궁 천골 에서 조직을 생검하였다. 단, 복압성 요실금만을 가진 환자는 앞 질벽만을 채취하였다. 채취한 조직 절반은 real -time polymerase chain reaction (RT-PCR)을 시행하였고 나머지 절반은 western blot 을 시행 하여 HOXA 11 과 13 의 발현 정도 차이를 비교 하였다. 또한 MMP 2, MMP 9, collagen I 및 collagen III 의 발현 정도 차이를 비교하기 위해 RT-PCR 을 시행 하였다. 그룹간의 차이를 분석하기 위해 independent 'k' sample 을 이용한 Kruskal-Wallis 분석방법과, Dunnett 의 다중 회귀 분석을 이용하였다. 앞 질벽에서 HOXA 13의 mRNA 의 발현 정도 차이를 보면, 복압성 요실금 환자에서 정상군에 비해 4.3 배 정도 발현 저하를 보였다 (P = 0.006). 또한 복압성 요실금과 골반 장기 탈출증을 동반한 환자군 및 골반 장기 탈출증만을 가진 환자군 역시 정상군에 비해 HOXA 13 의 발현이 현저하게 저하되어 있음을 알 수 있었다(4.3 배, P =0.007; 6.3 배, P < 0.001). 후 질벽에서 역시 복압성 요실금과 골반 장기 탈출증을 동반한 환자군 및 골반 장기 탈출증만을 가진 환자군에서 정상군에 비해 HOXA 13 의 발현이 현저하게 저하되어 있음을 알 수 있었다(3.1 배, P < 0.001; 2.7 배, P < 0.001). 그러나 전, 후 질벽 모두 복압성 요실금, 골반 장기 탈출증, 복압성 요실금과 골반 장기 탈출증을 동반환 환자군 사이에 발현 저하의 차이가 통계학적으로 유의하게 나타나지는 않았다. 골반 장기 탈출증을 가진 환자군에서 자궁 천골 인대의 HOXA 11의 mRNA 발현이 정상군에 비해 2.3배의 발현 저하를 보였다 (P = 0.004). 동일한 부위에서 복압성 요실금과 골반 장기 탈출증을 가진 환자군에서 역시 HOXA 11의 mRNA 발현이 정상군에 비해 2.7배의 발현 저하를 보였다 (P = 0.004). 또한 이들 질환군 사이의

발현 저하의 차이는 통계학적으로 유의하지 않았다 (p=0.08). 동시에 시행한 Western blot 의 결과 역시 정상군에 비해 골반 저 질환의 단백 발현이 현저하게 저하되었던 것을 알 수 있었다 (P< 0.001). 이들의 종물인 collagen I 및 collagen III 역시 정상군보다 골반저 질환 환자군에서 발현 저하를 보였다. 특히 골반 장기 탈출증 환자에서 collagen I 및 collagen III 가 유의하게 감소하였으며, 복압성 요실금을 동반한 환자군에서는 collagen III 가 통계학적으로 유의하게 감소하였다. MMP 2와 MMP 9 의 발현은 정상군과 비슷하거나 그 활성도가 증가하여 나타났으나, 골반 장기 탈출증과 복압성 요실금을 동반한 환자군에서 자궁 천골 인대에서만 MMP 2 의 유의한 활성도 증가를 보였다.

결론적으로 골반저 질환을 가진 여성들은 자궁 천골 인대 및 질 전반에 걸쳐 HOXA 11 및 HOXA 13의 발현 저하를 보였으며, 이들이 관장하는 대사 경로를 통해 골반 지지 조직의 생화학적인 강도의 변화에 영향을 미치며, 이들에 의해 골반 저 질환이 발생하는 것으로 추측해 볼 수 있겠다. 또한 골반저 질환은 HOXA 11 및 HOXA 13 에 의해 통제되는 공통적인 분자 생물학적 메커니즘에 의해 발생 한다는 추측을 해 볼 수 있었다. 본 연구를 통하여, HOXA 유전자가 골반 저질환의 원인의 하나로써 의의가 있음을 밝히고, 더 나아가 이 유전자를 표적으로 골반 저질환의 새로운 치료법이나 예방법을 개발하기 위한 계기가 될 수 있을 것이다.

핵심되는 말 : HOXA 11; HOXA 13; 골반 저질환; 골반 장기 탈출증; 요실금; 자궁 천골 인대; 질