Differential Regulation of HOXA10 Gene in Ovarian Cancer and Endometriosis by *ESR1*

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ABSTRACT

HOXA10 is expressed in development of female reproductive tract and participates in the endometrial differentiation and may contribute to development of endometriosis and epithelial ovarian cancer (EOC). In order to understand its role in differential regulation in these two gynaecological diseases, expressions of *HOXA10*, *ESR1*, *ESR2* and *PGR* were examined. Gene expressions were determined in tissues collected from 140 endometriosis patients, 79 EOC patients and 19 women without endometriosis by real-time PCR. Our data showed that expression of *HOXA10*, *ESR1* and *PGR*, in endometriotic tissues, were 20.1, 1.67 and 3.04 folds lower, respectively, and *ESR2* was higher by 4.52 folds. In EOC, expression levels of *HOXA10*, *ESR1* and *ESR2* were 1.60 to 2.41 folds higher and *PGR* was 2.80 folds lower in comparing with normal tissues. The higher expression of *HOXA10* in EOC and lower in endometriosis were coincidence with *ESR1*. In addition, HOXA10 also plays a critical role in regulating the differentiation of EOC to endometrioid and mucinous subtypes. Our study was the first to establish the relationship between hormone control and the expression of HOXA10 in development of these two gynaecological diseases. These results may provide a new focus on the regulated information for hormone therapy, and provide *HOXA10* as a potential biomarker for diagnosis the two diseases.

Keywords: HOXA10, ESR1, ESR2, PGR, endometriosis, ovarian cancer, real-time PCR

INTRODUCTION

Endometriosis is a major hormone-dependent disorder in gynecological disease, characterized by presence of endometrial tissue outside of normal uterine location.[1-3] Clinical symptoms include chronic pelvic pain, infertility, dysmenorrhea, menorrhagia, and dyspareunia.[3-4] With a substantial wide variation in prevalence across various studies, the disease has been estimated to affect about 2-20% of the reproductive women and is the leading cause for infertility in 30-50% of the patients.[5-7] The causes of the disease are complex, involving multiple immunological, environmental and genetic factors which all have an influence on women's fertility.

HOXA10 has been known for its importance in female reproductive tract development and proper embryo implantation.[8] As a transcription factor, by activating or repressing target genes, it guides development of the uterus, and facilitates growth and differentiation of adult endometrial lining in a cyclical manner during the menstrual cycle.[9] In animal model, *HoxA10* knockout mice exhibited abnormal female reproductive system leading to infertility.[10-11] Clinical studies also identified genetic alterations or mutations in some endometriotic and infertile patients.[12-14] However, linkage analysis studies showed no correlation between *HOXA10* mutations and the endometriosis.[15] DNA alterations that change protein function may disrupt normal biological mechanism, yet direct experimental data to support the role of *HOXA10* in the onset or progression of the disease is lacking. Instead of genetic alterations or polymorphism, studies from whole genome association study (WGAS) implied that changes in gene expression playing an increasingly significant role in complex diseases.[16] *HOXA10* expression is spatial and temporal important for both normal and ectopic growth of the endometrium;[17-19] and it is reduced in tissues of endometriosis and infertile patients.[20] In addition, deregulated *HOXA10* expressions have been found in association with a variety of malignancies such as hematopoietic, breast, gastric, oral, cervical and ovarian cancers.[21-23]

Previous studies demonstrated regulation of *HOXA10* expression in endometrium is primarily by sex hormone through estrogen receptor (ER) and progesterone receptor (PR).[24-26] Two major forms of ER, ERα and ERβ encoded by *ESR1* and *ESR2*, respectively, have distinct tissue expression patterns and exerted multiple actions in reproductive tissues. Levels of these receptors in endometriosis were also different from that of endometrium. Noticeably higher levels of ERβ and lower levels of ERα were found in human endometriotic tissues in comparing with eutopic endometrial tissues and cells.[27-28] Markedly elevated *ESR1* and an increased *ESR1*: *ESR2* ratio was found in association with estrogen-driven epithelial ovarian carcinoma (EOC).[29-30] PR, encoded by *PGR* which is a target gene of ERα, level varies with hormonal status and during carcinogenesis. Expression of *PGR* was also significantly lower in tissues of endometriosis.[31] and the epigenetically silenced *PGR* was observed and correlated with cancer.[32]

In view of the fact that expression levels of *HOXA10* is associated with both endometriosis and EOC, we hypothesized that HOXA10 may contribute at some level to the etiology of disease progression. The aim of this study is to evaluate *HOXA10* expression in endometriosis and EOC by focusing on its relationship with *ESR1*, *ESR2* and *PGR*. The clinical features were further analyzed to investigate the relationship between *HOXA10* expression and pathological outcomes of patients.

MATERIAL AND METHOD

Samples and ethics statement

The specimens were collected from patients by surgery including endometriosis (n=140), ovarian cancer (n=79) with their normal paired, and 19 control samples from women received laparoscopic surgery for diagnosis or non-endometriosis ovarian cyst. Informed written consent was obtained from all women before enrollment, and human ethics approval for tissue collection and experimental protocol was granted from the Institutional Review Board of Changhua Christian Hospital (approval # 070911). The histopathological types and FIGO staging of these subjects were identified by pathologist. All the tissue specimens were stored at -80˚C as soon as receiving from operation until further use.

RT and real time PCR

Total RNA was prepared from tissues using Trizol (Life Technologies) following the manufacturer's instructions. RNA was converted to cDNA using M-MLV Reverse Transcriptase Kit (Invitrogen). The quantity and integrity of the cDNA were confirmed using a Nanodrop 1000 spectrophotometer (Thermo Scientific). Real-time PCR was performed by LightCycler 2.0 instrument (Roche Diagnostics) using the LightCycler® FastStart DNA Master SYBR Green I kit (Roche Diagnostics). Information for all primers used in this study was listed in Table 1. Cycling conditions were: pre-incubation at 95°C for 5 min, followed by 45 cycles of 10s at 95°C, immediately annealing (Table 1) and then extension at 72˚C for 10s.

Statistical analysis

HOXA10, *ESR1*, *ESR2* and *PGR* gene expression levels were analyzed using SPSS, version 16.0 (SPSS, Chicago, IL, USA). The significance of differences between groups was estimated by Student's t-test, chi-square test, Fisher's exact test and one way analysis of variance (ANOVA) as appropriate. Data were considered significant as the $p \leq 0.05$.

RESULT

A total of 140 patients with endometriosis and 19 women without endometriosis as control were enrolled in this study. Table 2 shows the demographic of study groups and patients' clinical data is summarized in table 3. No statistical difference was found in age, height, weight, body mass index, and serum CA-125 among all the patients. The mean AFS score was 72.9 (range: 20 to 150) and 65.0% (91/140) of the patients had CA-125 value greater than 35 U/ml, 20.7% (29/140) with the recurrent endometriosis, and the percentage of infertility was 26.4% (37/140).

The clinical and pathological characteristics of EOC patients were summarized in table 4. Tissues were collected from 79 patients included serous (28, 35.4%), endometrioid adenocarcinoma (15, 19.0%), mucinous (20, 25.3%), clear cell adenocarcinoma (11, 13.9%) and mixed unclassified (5, 6.4%) histopathology subtypes for quantitative RT-PCR analysis. The post-operative serum CA-125 concentration was significantly lower (p<0.01) than in pre-operative.

HOXA10, *ESR1*, *ESR2* and *PGR* expressions in endometriotic tissues

The mRNA expressions of *HOXA10*, *ESR1*, *ESR2* and *PGR* were compared between endometriosis and normal control tissues (Figure 1). *HOXA10* expression was 20.12 folds significantly lower in the patient's tissues (p<0.001). *ESR1*and *PGR* was 1.68 folds and 3.04 folds (p<0.01) lower, respectively, than control. On the other hand, expression of *ESR2* in

endometriosis patients was 4.52 folds significantly higher than control (p<0.01).

HOXA10, ESR1, ESR2 and *PGR* expressions in EOC

Comparing gene expressions between EOC and normal control, *HOXA10*, *ESR1*and *ESR2* were 1.99, 1.60 and 2.42 folds higher in EOC, respectively (Figure 1); however, *PGR* was significantly 2.80 folds lower in EOC than in normal paired (p<0.05).

Relationship between *HOXA10* expression and clinicopathological variables in endometriosis and EOC

There was no significant correlation between *HOXA10* expression and most clinical data except AFS score in endometriosis (p=0.04; Table 5). The expression of *HOXA10* was negatively correlated with FIGO stage of EOC (p=0.009; Table 6) and was significantly higher in endometrioid (p=0.015; Figure 2) and mucinous subtypes (p=0.041).

DISCUSSION

HOXA10 expression is known regulated by sex steroid including estrogen and progesterone.[24,26] Yet, abnormal expression are involved in a variety of gynecological diseases, including EOC and endometriosis.[19,21,23] In this study, after screening 140 endometriosis patients, the expression of *HOXA10* was 20.12 folds lower in patients' tissue which is in agreement with results reported by previous studies.[12,19] On the other hand, by examining 79 patients with EOC, 46.8% of the EOC patients were found to have higher expression of *HOXA10* and the expression of *HOXA10* was 1.99 folds higher in EOC patients' tissues than control. The higher expression of *HOXA10* in the EOC patients' tissue is comparable with a previous study in which overexpression of *HOXA10* gene were found in ovarian cancer cell lines by real-time PCR.[33] Study by Yamashita *et al.* also indicated that overexpression *HOX* genes can promote the invasion ability of ovarian cancer cells.[33]

HOXA10 also differentially expressed in different EOC subtypes where expression levels were higher in endometrioid and mucinous EOC. Stable *HOXA10* overexpression in cancerous cells may lead to cell cycle arrest for succeeding cell differentiation by acting on p21 promoter.[34] Several cell adhesion molecules taking part in cancer progression, such as integrin and cadherin, also have known as targets for HOXA10.[35,36] Modulating target gene's promoter activity may alter interactions and adhesions of cell-cell and the surrounding extracellular matrix that promotes tumor growth and progression. These data may suggest that HOXA10 may be an important regulator in the process of ovarian carcinogenesis.

Steroid receptors also play important roles in the pathogenesis of endometriosis by regulating target genes whose products are known to affect endometriotic cellular activities. In this study, expression of *ESR1* was 1.60 folds higher in EOC and was 1.68 folds lower in endometriosis. The expression pattern is coincidence with *HOXA10*: higher expression level of *HOXA10* in EOC and lower in endometriosis. Since both EOC and endometriosis are estrogen-disorder diseases and there are two EREs in *HOXA10* promoter,[37] the result supports the fact that *HOXA10 expression is estrogen-responsive*. Additionally, we also observed higher *ESR2* and lower *ESR1* expressions in endometriotic tissues which is in agreement with other reports.[38,39] Defect in methylation-dependent mechanism was proposed for the high *ESR2* expression in endometriotic cells.[40] Previous studies in s association between endometriosis and *PR* gene expression were controversial; due to

possible influence by progesterone resistance in the disease or aberrant expression of the *PR*.[41-43] We observed significantly lower level of PR in both endometriosis and EOC. The results were agreeing with studies of Godbole *et al.* and Attia *et al.*[31,43]

In this study, we demonstrated the relationship between *HOXA10* and hormone receptors, *ESR1*, *ESR2*, and *PR* in endometriosis and EOC. We would thus suggest a possible role of HOXA10 in regulating disease cells' proliferation and differentiation. These results may provide a new focus on the regulated information for hormone therapy, and provide *HOXA10* as a potential biomarker for diagnosis the two diseases.

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Conflict of interest: None

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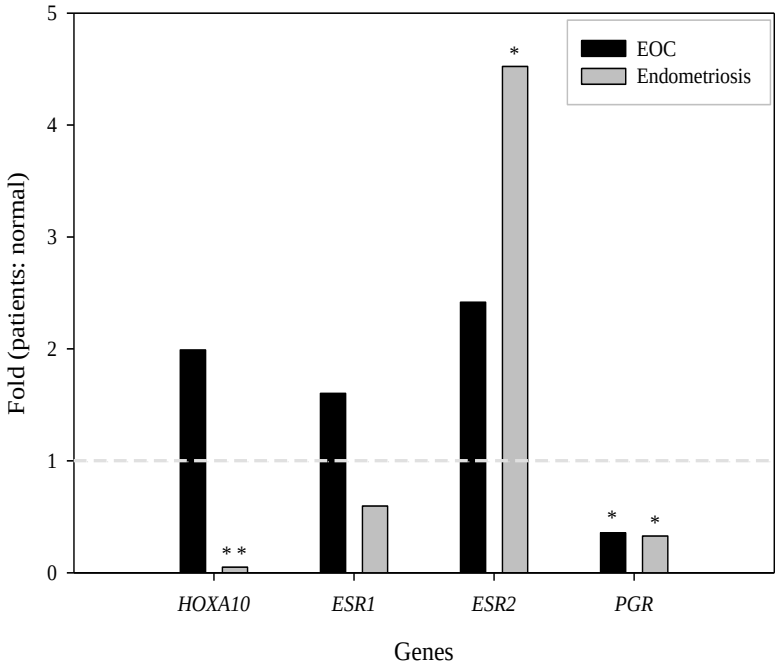

Figure 1: Expression patterns of *HOXA10, ESR1, ESR2* and *PGR* in endometriotic and EOC tissues. The mean relative quantitation (RQ) value of *HOXA10* in EOC (1.99) *vs.* the value in endometriosis (0.05) was 39.8. The mean RQ value of *ESR1* in EOC (1.60) *vs.* the value in normal endometriosis (0.60) was 2.67. The mean RQ value of *ESR2* in EOC (2.42) *vs.* the value in normal sample (4.52) was 0.535. The mean RQ value of *PGR* in EOC (0.36) *vs.* the value in normal sample (0.33) was 1.90. Fold changed was calculated using $2^{-(\Delta\Delta Ct)}$ by comparing with the control. * $p<0.05$; ** $p<0.001$.

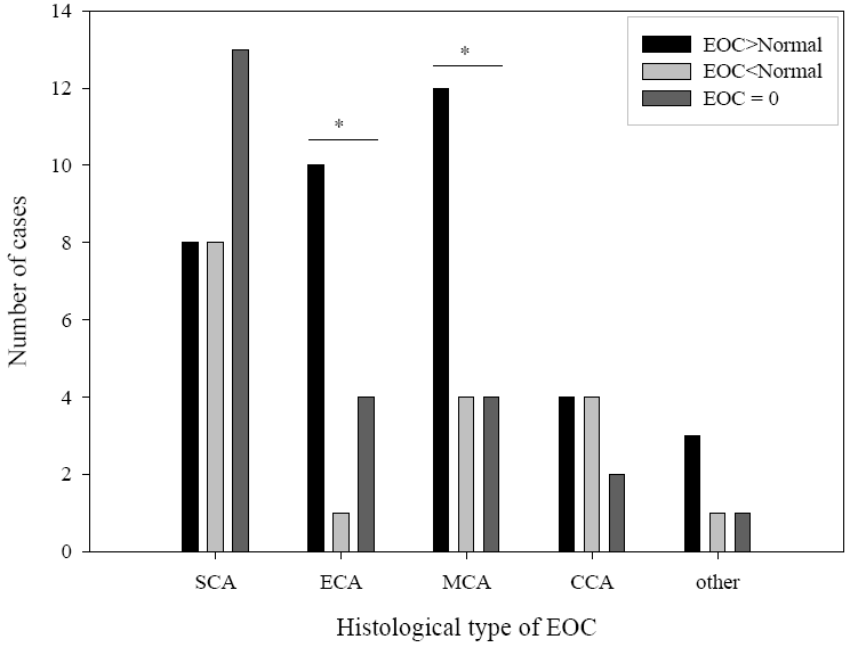

Figure 2: HOXA10 Expressions in each EOC subtype. The quantification of HOXA10 expression can be classified into three groups: EOC>normal, EOC<normal and EOC = 0, according to the higher or lower expression in EOC tissues than normal tissues or no expression in EOC tissues. The expression of HOXA10 was statistically different in ECA and MCA subtype of EOC. SCA, serous subtype; ECA, endometrioid subtype; MCA, mucinous subtype; CCA, clear cell subtype. * $p<0.05$.

Table 1: Oligonucleotide primer pairs of *HOXA10*, *ESR1*, *ESR2*, *PGR* and *RPS18* for real-time PCR analysis

Gene	Primer sequence (5'→3')	Amplicon Length (bp)	Tm (˚C)	PCR Efficiency (%)[a]
HOXA10[b]	F: 5'-GCCCCTTCCGAGAGCAGCAAAG-3'	212	64	100
	R:5'-AGGTGGACGCTGCGGCTAATCTCTA-3'			
ESR1[c]	F: 5'-CATTCATTTGCTTGCTCAGT-3'	129	63	100
	R: 5'-ATCCTCACGCTTAGTAACATAG-3'			
ESR2[d]	F: 5'-GGTCCATCGCCAGTTATC-3'	179	63	99.1
	R: 5'-CGCAGAAGTGAGCATCC-3'			
PGR[e]	F: 5'-CACAGCGTTTCTATCAACTTAC-3'	179	60	100
	R: 5'-TTTCACCATCCCTGCCA-3'			
RPS18[f]	F: 5'-TCTTGAACAGACAGAAGGATGTAA-3'	127	63	98.8
	R: 5'-TGACGCAGCCCTCTATG-3'			

F = forward primer; R = reverse primer; bp = base pair; Tm = annealing temperature.

[a] The formula of PCR efficiency: $E = [10^{(-1/slope)}-1] \times 100\%$.

[b] GenBank accession number NM_018951.

[c] GenBank accession number NM_000125.

[d] GenBank accession number NM_001437.

[e] GenBank accession number NM_000926.

[f] GenBank accession number NM_022551.

Table 2: Demographics of patients with endometriosis and controls

Characteristic	Patients (n = 140)	Controls (n = 19)	*p*-value[a]
Age (year-old)	32.7 ± 6.9; 20-50	34.5 ± 10.4; 18-53	NS
Weight (kg)	53.7 ± 8.2; 35-78	63.6 ± 15.1; 44-102	NS
Height (cm)	159.1 ± 5.5; 140.5-170.0	156.1 ± 5.2; 148.0-163.0	NS
Body mass index	21.2 ± 3.0; 14.2-28.8	26.1 ± 6.1; 19.6-38.9	NS
CA-125 (U/ml)	156.8 ± 607.33; 9.0-6270.1	89.7 ±148.9; 9.1-354.0	NS

Values are means ± SD; range. NS = not statistically significant.

[a] Student's *t* test; $p<0.05$ = statistically significant.

Table 3: Clinical and pathological characteristics of endometriosis patients (n = 140)

Characteristic		
AFS score (mean ± SD; range)	72.9 ± 35.2; 20-150	
Stage		
III	28	(20.0%)
IV	110	(78.6%)
NA	2	(1.4%)
CA-125		
>35 (U/ml)	91	(65.0%)
≤35 (U/ml)	36	(25.7%)
NA	13	(9.3%)
Adhesion		
(+)	127	(90.7%)
(−)	13	(9.3%)
Infertility		
(+)	37	(26.4%)
(−)	103	(73.6%)
Recurrent		
(+)	29	(20.7%)
(−)	111	(79.3%)
Pain		
(+)	94	(67.1%)
(−)	46	(32.9%)
Dysmenorrhea		
(+)	87	(62.1%)
(−)	53	(37.9%)

Values are numbers of patients unless otherwise stated.
(+) or (−) mean that patients with or without the symptom.
AFS = American Fertility Society. NA = not available.

Table 4: Clinical and pathological characteristics of epithelial ovarian carcinoma patients (n = 79)

Characteristic		
Age (year-old; means ± SD; range)	51.2 ± 13.5; 21-86	
FIGO stage		
I	32	(40.5%)
II	4	(5.1%)
III	35	(44.3%)
IV	3	(3.8%)
NA	5	(6.3%)
Histological type		
Serous	28	(35.4%)
Endometrioid	15	(19.0%)
Mucinous	20	(25.3%)
Clear cell	11	(13.9%)
Other[a]	5	(6.4%)
Mean pre-operative CA-125 (U/ml; range)	674.0; 3.3-10000.0	
>35 (U/ml)	45	(57.0%)
≤35 (U/ml)	17	(21.5%)
NA	17	(21.5%)
Mean post-operative CA-125 (U/ml; range)	101.6; 1.7-2671.4	
>35 (U/ml)	27	(34.6%)
≤35 (U/ml)	43	(55.1%)
NA	8	(10.3%)

Values are numbers of patients unless otherwise stated.
NA = not available.
[a] Includes mixed epithelial ovarian carcinoma and unclassified EOC.

Table 5: Relationship between *HOXA10* expression and the clinicopathologic characteristics in endometriosis

Characteristic	Expression of *HOXA10*			*p*-value
	EM>N[a]	EM<N[b]	EM = 0[c]	
Mean age (years)	35.8	33.2	31.9	NS[d]
Mean weight (kg)	50.6	54.5	52.9	NS[d]
Mean height (cm)	158.0	158.7	159.7	NS[d]
Mean body mass index	20.2	21.6	20.8	NS[d]
Mean CA-125 (U/ml)	176.0	116.3	206.1	NS[d]
Mean AFS score	95.8	66.5	78.9	0.04[d]
Pain/no pain	4/1	50/26	40/19	NS[e]
Dysmenorrhea/no dysmenorrhea	4/1	48/28	35/24	NS[e]
Adhesion/no adhesion	5/0	68/8	54/5	NS[e]
Infertility/no infertility	1/4	20/56	16/43	NS[e]
Recurrent/no recurrent	1/4	11/65	17/42	NS[e]
Stage				
III	0	17	11	NS[e]
IV	5	58	47	
NA	0	1	1	
Endometriosis				
On right ovary	1	21	12	NS[e]
On lest ovary	2	26	17	
On both ovaries	2	28	30	
NA	0	1	0	
Cul-de-sac obliteration				
None	0	37	19	NS[e]
Partial	0	13	11	
Complete	3	26	29	

Values are numbers unless otherwise stated. AFS = American Fertility Society.
NA = not available. NS = not statistically significant.
[a] EM>N means the gene expression in endometriosis tissue was higher than normal.
[b] EM<N means the gene expression in endometriosis tissue was lower than normal.
[c] EM = 0 means the gene was no expression in endometriosis tissue.
[d] One-way ANOVA analysis;
[e] Pearson's *chi-squared* test and Fisher's *exact* test; $p<0.05$ = statistically significant.

Table 6: Relationship between *HOXA10* expression and the clinicopathologic characteristics in EOC

Characteristic	Expression of *HOXA10*			*p*-value
	EOC>N[b]	EOC<N[c]	EOC = 0[d]	
Mean age (years)	49.8	54.3	51.2	NS[e]
FIGO stage				
I	21	2	9	0.009[f]
II	1	0	3	
III	11	14	10	
IV	1	1	1	
NA	3	1	1	
Histological type				
Serous	8	8	13	NS[f]
Endometrioid	10	1	4	
Mucinous	12	4	4	
Clear cell	4	4	2	
Other[a]	3	1	1	
Mean pre-operative CA-125 (U/ml)	465.7	390.1	1246.3	NS[e]
Mean post-operative CA-125 (U/ml)	51.2	49.7	205.8	NS[e]

Values are numbers unless otherwise stated.
NA = not available. NS = not statistically significant.
[a] Includes mixed epithelial ovarian carcinoma and unclassified epithelial ovarian carcinoma.
[b] EOC>N means the gene expression in EOC tissue was higher than normal.
[c] EOC<N means the gene expression in EOC tissue was lower than normal.
[d] EOC = 0 means the gene was no expression in EOC tissue.
[e] One-way ANOVA analysis;
[f] Pearson's *chi-squared* test and Fisher's *exact* test; $p<0.05$ = statistically significant.

