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Diagnostic Availability of Estrogen Receptor Alpha mRNA on Cervical Cancer Tissue

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자궁경부암 조직에서 에스트로겐 수용체 알파 mRNA의 진단적 유용성

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Cervical cancer is the fourth most frequently diagnosed cancer in women worldwide. In lower Human Development Index countries, it has the second highest incidence and mortality among cancer in women. Therefore, better diagnosis and treatment systems are needed. Among them, estrogen receptor alpha (ER-α) mRNA expression has been analyzed with RT-qPCR since several studies reported that ER-α is necessary in the maturation of the uterus and is related to cervical cancer. In this study, ER-α quantitative analysis was performed on various lesions and normal tissue samples. Based on the receiver operating characteristic (ROC) curve, its sensitivity and specificity were 85% and 75%, respectively, showing higher or similar results to those of conventional HPV tests. In addition, its expression level was analyzed with clinical information. With regression analysis, the R square value between the ER-α mRNA expression level and menopause status was 0.5041, indicating a strong correlation. This study was performed as part of a pilot study and suggests that ER-α is related to carcinogenesis. Future studies will examine other hormones and menopausal factors with a larger sample size.

Key words: Age, Cervical cancer, Estrogen receptor alpha, Formalin-fixed paraffin-embedded tissue, Menopause

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INTRODUCTION

Cervical cancer ranks as the fourth most frequently diagnosed cancer with an estimated 570,000 cases in women worldwide [1]. And it also ranks as the fourth cancer leading death with an estimated 311,000 deaths in women worldwide. However, in lower human development index (HDI) countries, it shows the second most incidence and mortality among the women cancer. Therefore, the better diagnosing and treatment systems

are urgently needed. Since human papillomavirus (HPV) is most pivotal cause of cervical cancer, HPV test is generally performed to diagnose cervical cancer with cytological and histological examination [2-4]. Recently, European Society for Medical Oncology and European Cancer Congress issued a guideline recommending HPV test alone as a primary screening test in 2015 [5]. However, several studies have confirmed the association between cervical cancer and hormone, especially estradiol (E2) [6-8]. Sufficient estrogen induces sexual maturation and ovulation to mouse. However, continuous estrogen promotes cervical carcinogenesis regardless of HPV transgenic mouse or not [9-13]. Moreover, some studies show dramatic differences estrogen receptor (ER) expression between cancer and normal [14].

Those results indicate estrogen conditions could be essential to promote cancer and affect risk of cervical cancer more than HPV. A similar example can be found in human. The premenopausal women affected by estrogenic stimulation continuously like oral contraceptives or consequence of pregnancy [15, 16]. Actually, several clinical studies conducted on human shows HPV negative patient could develop into cancer [17-19]. And their estrogen related markers such as Ki-67 and p16 expressed relatively higher than normal [20]. Among them, studies related to estrogen receptor-α (ER-α) were the most common in particular [21-24]. Therefore, our study group analyzed relation between ER-α and cervical cancer carcinogenesis quantitatively. We also conducted with pre-cancer group to certify ER-α as prognosis marker. And the results were analyzed by age of the patients. Cervical cancer occurs most commonly among women in their 40s and 50s [25-28]. It is generally explained by the hypothesis that HPV has developed into cancer by persistent infection [29]. On the other hand, the 40s and 50s are hormone imbalance periods such as the menopause. This fact also suggests the relation between hormone and cancer occurrence. Thus, our research group analyzed ER-α expression and age with patient sample and evaluated its clinical significance.

MATERIALS AND METHODS

1. Study subjects

1) Histological samples

The samples were collected from cervical cancer patients and healthy subjects at Wonju Severance Christian Hospital, Wonju, Republic of Korea, from January 2010 to December 2014. All subjects provided clinical information and this study was approved by the Institutional Ethics Committee of Yonsei University Wonju College of Medicine (approval no. YWMR-12-4-010). The study subjects were formalin fixed paraffin embedded (FFPE) cervical cancer tissue samples, those were composed of 20 squamous cell carcinoma (SCC), 20 cervical intraepithelial neoplasia grade 3 (CIN3), 20 CIN1, and 20 normal subjects (Table 1). The subjects' age ranged from 26 to 79 years, and the median age was 48 years old.

2) Deparaffinization of FFPE tissue and total RNA extraction

Three pieces of 10-μm-thick sections of cervical FFPE tissues were used for total RNA extraction. Qiagen RNeasy FFPE kits (Qiagen, Hilden, Germany) were used according to the manufacturer's protocol. To remove paraffin from FFPE samples, a 160 μL deparaffinization solution was added. After shaking and vortexing, the tube was heated for 3 min at 56°C for the paraffin to be melted. After centrifugation, 150 μL proteinase K digestion buffer (PKD) was added and mixed by vortexing. The tube was centrifuged at 11,000 × g for 1 min at room temperature. After that, 10 μL proteinase K was added to the lower, clear phase, which was incubated at 56°C for 15 min, then at 80°C for 15 min. The lower, uncolored phase is

Table 1. Clinical information of histological samples

Histological diagnosis	Age (mean)	No. of samples
SCC	35~79 (57.1)	20
CIN3	29~64 (46.0)	20
CIN1	26~69 (45.5)	20
Normal	35~76 (48.2)	20
Total	26~79 (49.2)	80

Abbreviations: SCC, squamous cell carcinoma; CIN, cervical intraepithelial neoplasia.

transferred into a new 2 mL micro-centrifuge tube, which was centrifuged for 15 min at 20,000 × g at room temperature. The supernatant was transferred to a new microcentrifuge tube taking care not to disturb the pellet. After adding 16 μL DNase booster buffer and 10 μL DNase I solution, it was inverted and centrifuged briefly. Through incubating at room temperature for 15 min, 320 μL RBC buffer and 720 μL 100% ethanol were added to the sample. Then, the sample was transferred to RNeasy minelute spin column and centrifuged for 15 seconds at 8,000 × g. The flow-through was discarded. After adding 500 μL buffer RPE, the tube with column was centrifuged for 2 min at 8,000 × g to wash the spin column membrane and the flow-through was discarded. Then, RNeasy MinElute spin column was placed to new 2 mL collection tube. After adding 15 μL RNase-free water directly to the spin column membrane. The tube was centrifuged for 1 min at full speed to elute the RNA. The purity and concentration of the total RNA were determined by measuring the absorbance ratio at 260 and 280 nm using a spectrophotometer (Infinite 200, Tecan, Salzburg, Austria). All preparation and handling procedures were conducted under RNase-free conditions. The isolated total RNA was stored at −70°C until used.

3) cDNA synthesis

By using M-MLV reverse transcriptase kits (Invitrogen, Carlsbad, CA, USA) and random hexamers (Invitrogen, Carlsbad, CA, USA), complementary DNA (cDNA) was synthesized according to the manufacturer's recommendation. In short, 10 μL of total RNA was added to a mixture containing 1 μL of 10 mM dNTP mix at neutral pH, 1 μL of 0.25 μg/μL random hexamers, and 5 μL of DEPC-treated water. The PCR mixures were incubated at 65°C for 5 min and chilled on ice. After adding a mixture of 4 μL of first-strand buffer (5×), 2 μL of 0.1 M dithiothreitol (DTT), and 1 μL of M-MLV reverse transcriptase (at room temperature), cDNA synthesis was performed at 25°C for 10 min, 37°C for 50 min, and 70°C for 15 min. The cDNA was stored at -70°C until used.

4) Histopathological diagnosis

The histopathological diagnosis was classified as normal, cervical intraepithelial neoplasia 1 (CIN 1), cervical intraepithelial neoplasia 2 (CIN 2), cervical intraepithelial neoplasia 3 (CIN 3), adenocarcinoma (ADC), and squamous cell carcinoma (SCC). CIN1 is the least risky type and represents only mild dysplasia or abnormal cell growth. It is confined to basal 1/3 of the epithelium. This usually corresponds to HPV infection, though it can take several years to clear. CIN 2 is moderate dysplasia confined to the basal 2/3 of the epithelium. CIN 3 is severe dysplasia that spans more than 2/3 of the epithelium and may involve the full thickness. This lesion may sometimes also be referred to as cervical carcinoma *in situ*.

5) RT-qPCR TaqMan assays of ER-α mRNA

The ER-α mRNA expression was detected by RT-qPCR quantitatively. RT-qPCR was performed in a final volume of 20 μL, containing 10 μL of 2× Thunderbird probe qPCR mix (Toyobo, Osaka, Japan), 5 μL of primer, 5 μL of template cDNA, 2 μL of distilled water, and 3 μL of TaqMan probe mixture. TaqMan probe mixture is composed with 1 μL of each primer (10 pmol), and 1 μL of probe (10 pmol). The primers and probe were designed using the ABI Primer Express 3.0 Software (Applied Biosystems, Foster City, CA, USA) and Primer-BLAST (NCBI; National Center for Biotechnology Information, Bethesda, MD, USA). The sequences of the primers used were 5′-GTGCCAGGCTTTGTGGATTT-3′ and 5′-GATCTCTAGCCAGGCACATTCT-3′ for ER-α and 5′-FAM-ACCCTCCATGATCAGGTCCACCTT-BHQ1-3′ for the probe.

The positive and negative controls were included in every procedure with the samples simultaneously. The conditions of PCR were 3 min at 95°C, followed by 50 cycles of 3 min at 95°C for 3 sec and at 60°C for 30 sec using a CFX-96 real-time PCR system (Bio-Rad, Hercules, CA, USA).

It is very difficult to extract a high quality of RNA from clinical specimens such as FFPE tissues. And relative mRNA levels need to be determined based on the reference housekeeping genes. Thus, to determine RNA

quality and avoid false negatives, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

In order to confirm how the levels of ER-α were expressed, FFPE cervical cancer tissues and noncancerous FFPE normal tissues were performed by RT-qPCR. The gene expression was calculated using the delta Ct (△Ct).

△Ct=[△Ct, Target (Ct target gene−Ct housekeeping gene]

Relative gene expression was assessed using the comparative Ct method (△△Ct method) [30]. The amount of target, normalized to an internal housekeeping gene, GAPDH, and relative to a calibrator, is given by 2- △△Ct which was the normalized according to the following equation:

△△Ct=[△Ct, Target (Ct target gene−Ct housekeeping gene on target sample)−△Ct, Control (Ct target gene−Ct housekeeping gene on reference sample)].

Then, data were calculated the expression ratio by 2-△△Ct that represents gene expression.

6) Statistical analysis

Statistical analysis was conducted using GraphPad Prism software (Version 5.02, La Jolla, CA, USA) and Statistical Package for the Social Sciences (SPSS) software v18.0 (SPSS Inc., Chicago, IL, USA).

The Kolmogorov-Smirnov test and Shapiro-Wilk test were used to determine whether or not characteristic of data was parametric. A Student's t-test and Mann-Whitney U test were used to determine the statistical significance as parametric and non-parametric statistics, respectively. To apprehend the relationship between variables, Spearman's rho and Kendall's tau-b were used for the correlation analysis. Furthermore, to estimate the result, simple linear regression and multiple linear regression were used for conducting the regression analysis. For all tests, $P<0.05$ was considered statistically significant. The differences were considered statistically significant when $^{*}P<0.05$, $^{**}P<0.01$, or $^{***}P<0.001$. The cut-off value for distinguishing between positive and negative results is determined from the receiver operating characteristic (ROC) curve.

RESULTS

1. Distribution of relative expression of ER−α mRNA in normal and cancer group

The relative expressions of ER-α mRNA were detected with RT-qPCR in normal and cancer group. As shown in Figure 1, normal and cancer group was distinguished by ER-α gene expression showing a statistically significant difference ($P<0.05$). In order to determine the optimal cut-off value for clinical use, ROC curve analysis was also performed. The area under the curve (AUC) obtained using samples was 0.7588. The cut-off value was determined by the likelihood ratio. The highest likelihood ratio was a relative gene expression over 0.66 that had the highest sensitivity and specificity.

2. ER−α gene expressions analysis according to the lesions with histologic samples

ER-α gene expression of each lesion was compared to that of normal group. The normal and CIN3 group

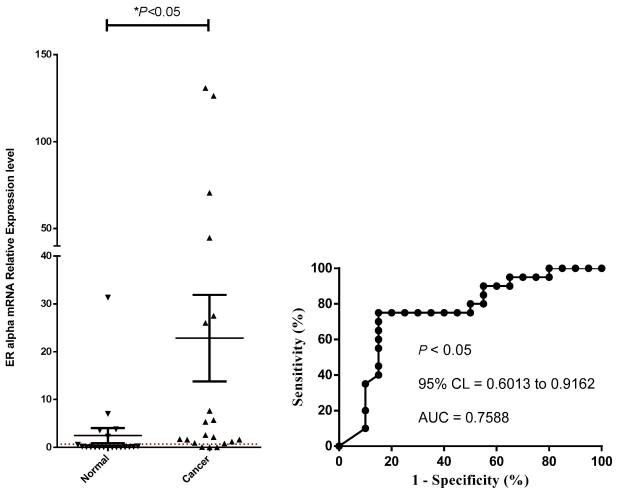

Figure 1. Relative distribution of ER−α mRNA expression in normal and cancer group. The gene expressions of ER−α were analyzed in normal and cancer groups by RT−qPCR.

compared to cancer group showed $P<0.05$, respectively (Figure 2). As previously mentioned, the gene expressions greater than 0.66 were determined as positive. The positive rate of normal, CIN1, CIN3, and cancer group shows 25.0, 55.0, 40.0, and 85.0, respectively (Table 2). The normal and CIN3 group discriminated from cancer group statistically showing $P<0.05$.

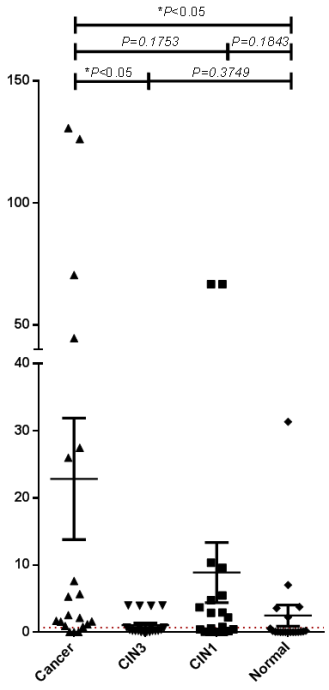

Figure 2. ER-α gene expression distribution according to the lesions. The ER-α gene expressions in each lesion are detected. The normal and CIN3 group compared to cancer group showed $P<0.05$, respectively.

3. Age-dependent ER-α expression with histologic samples

The correlation between ER-α expression and age was examined with regression analysis (R-squared, R^2). The analysis was conducted in normal and cancer group. As shown in Figure 3A, R^2 value of cancer was 0.2261 while that of normal was 0.0019. To examine the correlation between menopause status and ER-α expression, pre-menopausal patient and post-menopausal patient are categorized to '0' and '1'. Its R^2 value is 0.5041 (Figure 3B).

Table 2. Comparison of ER-α mRNA RT-qPCR results with histological diagnosis

Groups	ER-α mRNA RT-qPCR	
	Positives*	Negatives
Cancer patients	15 (85.0)	5 (25.0)
CIN3 patients	8 (40.0)	12 (60.0)
CIN1 patients	11 (55.0)	9 (45.0)
Normal subjects	5 (25.0)	15 (75.0)
Total	39	41

Values are presented as N (%).
*The positive rates of cervical cancer and normal groups were calculated based on cut-off of ER-α mRNA levels.
Abbreviations: ER-α, estrogen receptor alpha; CIN, cervical intraepithelial neoplasia.

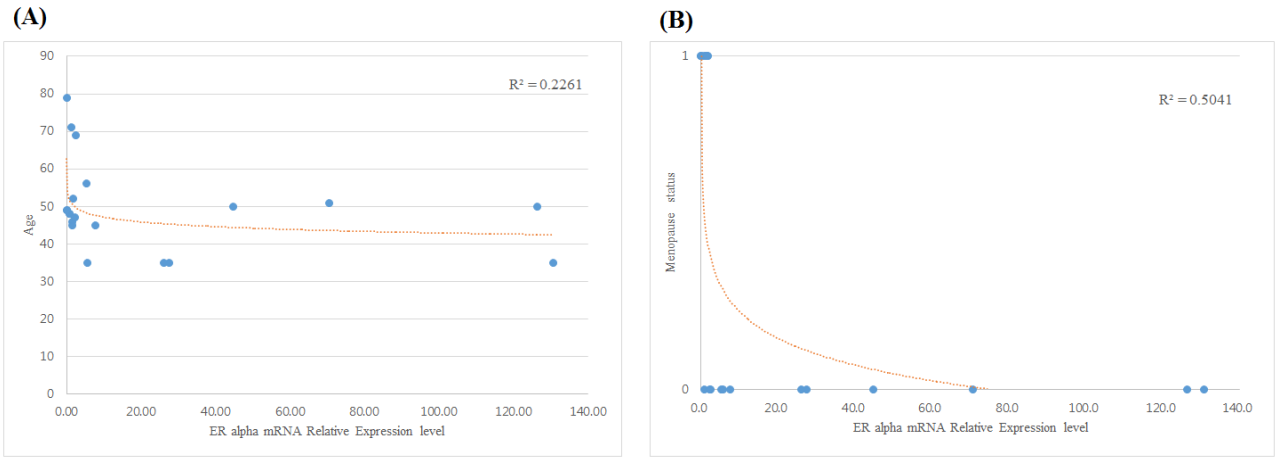

Figure 3. ER-α gene expression in cancer group. The gene expression analysis was performed using regression analysis in cancer group. (A) It was performed between ER-α expression and patient ages. The R^2 value of cervical cancer was 0.2261 (B) It was performed between ER-α expression and menopause status. The menopause status 0 and 1 stands for pre-menopausal patient and post-menopausal patient, respectively. The R^2 value of cervical cancer was 0.5041.

DISCUSSION

The study was investigated to understand the relevance between cervical cancer and hormone. Especially, relative expression of ER-α was analyzed among the biomarkers related to the inflow of estrogen. In fact, there are a number of articles about ER-α with clinical samples [31, 32]. However, this study was performed with quantitative analysis. And FFPE tissue was used in this study while cervical exfoliated cell is used for the study commonly. In case of cytology sample, normal sample could be brought into cancer sample in process of extraction [33]. Whereas FFPE tissue prevents normal sample flowing freely to the cancer samples through microscopic inspection and immunohistochemistry staining assay differentiating the normal tissue and cancer tissue correctly. Based on the ROC curve, positive rate of normal and cancer tissues was 25% and 85% respectively. Also, it interpreted specificity of 75% and sensitivity of 85%. While the specificity was similar to existing diagnostic marker of cervical cancer, the sensitivity was much higher [34, 35]. And the HPV-negative sample in cancer tissue was shown as the positive result in ER-α implying HPV-related marker and ER-α marker could diagnose cervical cancer complementarily. And the relative risk was analyzed to investigate the effect of ER-α on the cervical cancer occurrence. The odds ratio was 17, the higher value than that of HPV E6/E7 (13.4).

To analyze expression levels depends on lesion severity, relative expression was also analyzed in CIN1 and CIN3, precancerous stage. The positive rate was estimated to 55% and 40%, respectively. With regression analysis, R square was estimated as 0.062.

As mentioned in introduction, the study has analyzed the relevance between age and relative expression. Many articles and researcher explain why the cervical cancer occurrence is the highest in forty and fifty by persistent infection of HPV [36-38]. Considering that most of cervical cancer patients are of the forties and fifties regardless of standards in health and vaccine policy, another point of view is necessary [25-28]. The researcher also noted the average age of menopausal Korean women was 49.7 years old [39]. This period is hormonally unbalanced, so it could affect cancer occurrence. So, at first, regression analysis was performed between the relative expression of ER-α and age. Its R square value was 0.2261 in cancer group. In addition, the analysis was also performed in normal group to examine the association of ER-α in normal subject and its value was 0.0019. This time, instead of numerical statistics analysis, categorical statistics analysis by menopause status was applied. R square value was 0.5041 in cancer patient and 0.0361 in normal group, respectively. In general, R square value greater than 0.5 is statistically significant [40-42]. In sum, these results suggest ER-α could affect carcinogenesis strongly related with menopause status. Therefore, this study show ER-α is involved in the development of cancer with clinical tissue samples and it is necessary to study with menopause status.

This result has been conflicting. Some studies reported the number or expression of ER-α markers decreases and disappears in cervical cancer [14, 43] However, these reports did not consider subject's age and menstruation condition. Those things considered, another consequence could be drawn.

The hormone including ER-α would have strong connection with cervical cancer carcinogenesis especially adenocarcinoma or adeno-squamous carcinoma; adenocarcinoma is originated from organ producing hormone [4, 44]. Moreover, the portion of adenocarcinoma in cervical cancer is getting higher [46, 47]. Therefore, ER-based diagnostics applying to adeno-squamous carcinoma and adenocarcinoma is also rational.

This study was performed as part of the pilot study because ER-α mRNA expression hasn't be analyzed quantitatively with clinical tissue sample before. Further study would be conducted with increasing the number of samples. And it will cover other hormone and menopausal factor.

요 약

자궁경부암은 세계적으로 여성에서 네번째로 많이 진단되는 암이다. 개발 지수가 낮은 개발도상국이나 후진국에서는 자궁경부암의 발생률과 사망률이 훨씬 심각하다. 따라서 더 나은 진단법과 치료법이 시급하다. 인유두종바이러스가 자궁경부암 발생의 주요 원인으로 알려진 이후로 바이러스에 대한 검사가 세포검사·조직검사와 함께 자궁경부암을 진단하는데 일상적으로 쓰이고 있다. 하지만, HPV 검사에서 음성을 보이는 환자가 자궁경부암으로 발전하는 사례가 계속해서 발견되고 있다. 본 연구에서는 HPV 외에 자궁경부암의 원인으로 생각되는 인자들에 대해 분석하였다. 그 중에서도 에스트로겐 수용체 알파의 mRNA 발현양을 실시간 역전사 중합효소 연쇄반응을 통해 분석하였다. 에스트로겐 수용체 알파에 대해서는 예전부터 자궁내막을 성숙시키면서 동시에 자궁경부암의 발암 기전에 연관이 있을 것이라는 추측이 있었는데, 본 연구에서는 이에 대해 임상검체를 통해 양적 분석을 진행하였다. ROC 곡선을 바탕으로, 85%의 민감도와 75%의 특이도를 확인할 수 있었는데, 이 값은 기존의 HPV 검사법보다 유사하거나 더 높은 값이었다. 나이, 병변의 심한 정도 등을 포함한 임상 정보를 바탕으로 회귀 분석한 결과 폐경 여부와 에스트로겐 수용체 알파의 발현양이 높은 연관성을 확인하였다. 본 연구는 예비 연구의 일종으로, 추후 연구에서 가능성이 확인된 호르몬과 폐경에 관련된 유전자를 대상으로 더 많은 검체로 분석해야 할 것이다.

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Conflict of interest: None

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