

Expression of estrogen receptor α and β in esophageal squamous cell carcinoma

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Received August 16, 2013; Accepted September 17, 2013

DOI: 10.3892/or.2013.2770

Abstract. Estrogen receptors (ERs) are frequently expressed in human tumor tissues. There have been several studies concerning ER expression in esophageal cancers, yet the results are inconsistent, and the prognostic value of the receptors remains unclear. In the present study, we investigated the expression of ER protein and its correlation with clinical features of esophageal squamous cell carcinoma (ESCC) patients. Immunohistochemical staining for the ERs was carried out on paraffin-embedded primary tumor tissue sections from 89 patients with ESCC. Quantitative analyses were performed to determine the prognostic value of the expression of ERs, and Pearson's correlation was used to examine the relationship between ER α and ER β expression levels. Our results showed that ER α immunoreactivity was significantly lower in ESCC than that in the non-neoplastic epithelium ($P=0.0445$), whereas the ER β status was much stronger in ESCC than that in the non-neoplastic epithelium ($P=0.0243$). A significant inverse correlation was observed between ER α expression and depth of tumor invasion ($P=0.0426$). Correlation analysis revealed a statistically significant inverse correlation between the expression of ER α and ER β in ESCC ($r=-0.2902$, $P=0.0058$). Kaplan-Meier survival analysis showed that the patients with ER α expression (21/89) had a better outcome than patients without ER α expression ($P=0.0280$), whereas patients with high ER β immunoreactivity (44/89) were significantly associated with worse survival ($P=0.0366$). In conclusion, ER α and ER β levels were inversely correlated, and the downregulation

of ER α and upregulation of ER β may indicate unfavorable prognosis of ESCC.

Introduction

Human esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignancies. Prominent gender differences in the prevalence of ESCC have been demonstrated by most epidemiological studies. Esophageal cancer is generally more common among males than females, with a male to female ratio exceeding 3-4:1 (1). The prognosis of ESCC female patients is significantly better than that of male patients (2). While these data indicate the potential involvement of sex hormone-related pathways in the pathogenesis of ESCC, the relevant molecular events remain unclear.

Previous studies suggest a possible correlation between the estrogenic pathway and tumorigenesis and/or progression of ESCC (3,4). Esophageal cancer in females tends to occur postmenopausally, and the incidence rate for females increases with age (5). Sex steroids, such as estrogen, are well-known for their role(s) in the control of cell differentiation and proliferation in estrogen-dependent tissues such as breast and endometrial tissues. However, it is noteworthy that estrogens appear to play a pivotal role in several types of human malignancies that are not considered as classical, estrogen-dependent, neoplasms, such as lung (6,7), urinary bladder (8) and gastrointestinal tract malignancies (9,10). Consequently, estrogenic pathways may be involved in the regulation of the biological behavior of ESCC.

Human tissues contain two isoforms of ER, ER α and ER β , which are generated by genes located on human chromosome 6q25 (11) and chromosome 14q22-24 (12), respectively. While several studies have identified the expression of ERs in ESCC (4,13-19), the distribution patterns of ER α and ER β as well as their clinical implication remain highly controversial (15,16,18,20). Accumulated data indicate a functional interaction between ER α and ER β in human cells (21,22). Comprehensive comparison of the available data on ER α and ER β expression in ESCC is difficult since they are determined separately in different studies using different methods. We considered that simultaneous determination of both ER α and

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Key words: esophageal carcinoma, estrogen receptor, immunohistochemistry, histopathology

ER β expression will allow us to analyze the quantitative relationship between ER α and ER β , which may have prognostic value for ESCC and aid in the better understanding of their involvement in the development of the malignant phenotype of ESCC.

The aim of this study was to determine the expression of ER protein and its correlation with clinical variables of ESCC patients.

Materials and methods

Ethics statement. The study was approved by the Ethics Committee of the Shantou University Medical College (Shantou, China), and only patients who provided written informed consent were included in the study.

Patients and tissues. Eighty-nine ESCC tissues were obtained from patients who underwent potentially curative esophagectomy from 2000 to 2006 at the Shantou Central Hospital (Shantou, China). These patients had received neither chemotherapy nor irradiation therapy prior to surgery. From these 89 cases, a total of 7 specimens of non-neoplastic epithelium were obtained for evaluating the expression of ER α , and 9 specimens were obtained for ER β evaluation. These specimens had undergone tissue microarray (TMA) construction before immunohistochemical staining. Relevant clinical data were retrieved from careful review of the medical records. All of the tumors were confirmed as ESCC by pathologists in the Clinical Pathology Department of the Shantou Central Hospital. The pathological stage of each cancer was determined according to the TNM system, and each lesion was graded histologically according to World Health Organization classification. The median follow-up time was 30.8 months (range, 1.4-54.4 months).

Immunohistochemistry. Mouse monoclonal antibodies for ER α (F-10; dilution, 1:50) and ER β (14C8; dilution, 1:100) were purchased from Santa Cruz Biotechnology, Inc. and GeneTex Inc., respectively. Immunohistochemical staining was performed using the biotin streptavidin-peroxidase procedure as described previously (23).

Image analysis. Images were acquired using a Nikon microscope coupled to a Nikon DS-Fi1 digital camera (Nikon, Tokyo, Japan). Ten randomly selected discontinuous fields (x200) per slide were evaluated for each specimen. The positive area was stained yellow, and Image-Pro Plus software was used to quantify the integrated optical density (IOD). When evaluating the possible correlation between ER status and clinical outcome among individual patients, the cases were tentatively classified into two groups according to their ER IOD. A value of ER α IOD (range, 84-14,665) \geq 5,200 was considered as positive expression and a value $<$ 5,200 was considered as negative expression. A value of ER β IOD (range, 3,877-31,923) \geq 18,000 was considered as high expression and a value $<$ 18,000 was considered as low expression.

Statistical analysis. Results are expressed as means \pm SD. The statistical analyses between ER IOD and clinicopathological parameters of individual patients were carried out with the

Table I. Comparison of the clinicopathologic features between male and female esophageal squamous cell carcinoma patients.

Clinical parameters	Gender, n (%)		P-value
	Male	Female	
Age (years) ^a	56.5 \pm 8.010	63.8 \pm 7.229	0.0005
Tumor size (cm) ^b			0.129
\leq 3	37 (53)	15 (79)	
$>$ 3 - \leq 5	25 (36)	3 (16)	
$>$ 5	8 (11)	1 (5)	
Histologic grade ^b			0.398
G1	24 (34)	4 (21)	
G2	40 (57)	12 (63)	
G3	6 (9)	3 (16)	
Invasive depth ^b			0.289
T2	3 (4)	2 (11)	
T3+T4	67 (96)	17 (89)	
LN metastasis ^b			0.797
N0	37 (53)	9 (47)	
N1+N2+N3	33 (47)	10 (53)	
TNM classification ^b			0.615
IB+IIA+IIB	38 (54)	9 (47)	
IIIA+IIIB+IIIC+IV	32 (46)	10 (53)	

^aStudent's t-test; ^bFisher's exact test. Each P-value is two-tailed and the significant level was set at P=0.05. Data are presented as means \pm SD. LN, lymph node.

use of the Student's t-test, one-way ANOVA test, the Mann-Whitney U test, the Kruskal-Wallis test and paired t-test. Statistical analyses between gender and other clinicopathological characteristics were assessed with the Fisher's exact test. Correlation of ER α with ER β was performed using Pearson's correlation. Overall survival (OS) curves of the patients were generated according to the Kaplan-Meier method, and statistical significance was calculated using the Breslow test. Statistical differences were examined using SPSS software. Each P-value is two-tailed, and the significance level was set at P=0.05.

Results

Association between gender and clinicopathological parameters of the ESCC patients. The relationship between the gender of the patients and clinicopathological parameters are summarized in Table I. Females had a significantly older age at the onset of ESCC than that of males (P=0.0005). No significant association was detected between gender and tumor size, differentiation, depth of invasion, presence of lymph node metastasis or TNM classification of tumors.

Expression of ER protein in human esophageal non-neoplastic epithelium and carcinoma. Both ER α and ER β were expressed

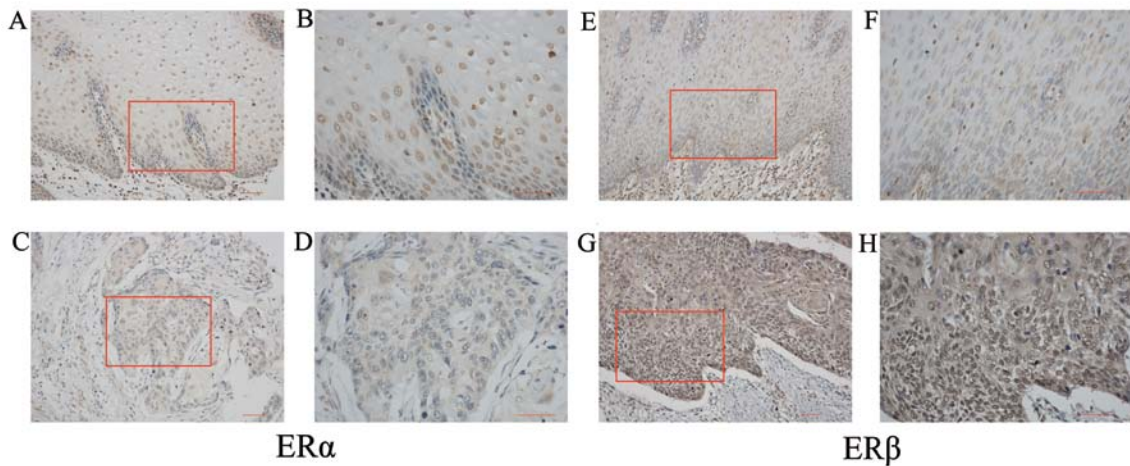


Figure 1. Expression of ER α and ER β in human non-neoplastic epithelium and ESCC. (A and B) Moderate ER α immunoreactivity was detected in the nuclei of non-neoplastic epithelial cells. (C and D) Weak ER α immunoreactivity was detected in the cytoplasm as well as in the nuclei of ESCC cells. (E and F) Negative to weak expression of ER β was found in the nuclei of non-neoplastic epithelial cells. (G and H) Strong ER β immunoreactivity was detected in both the nuclei and in the cytoplasm of ESCC cells. (B, D, F and H) Magnified areas of the corresponding embedded micrographs at the left. Scale bars, 50 μ m.

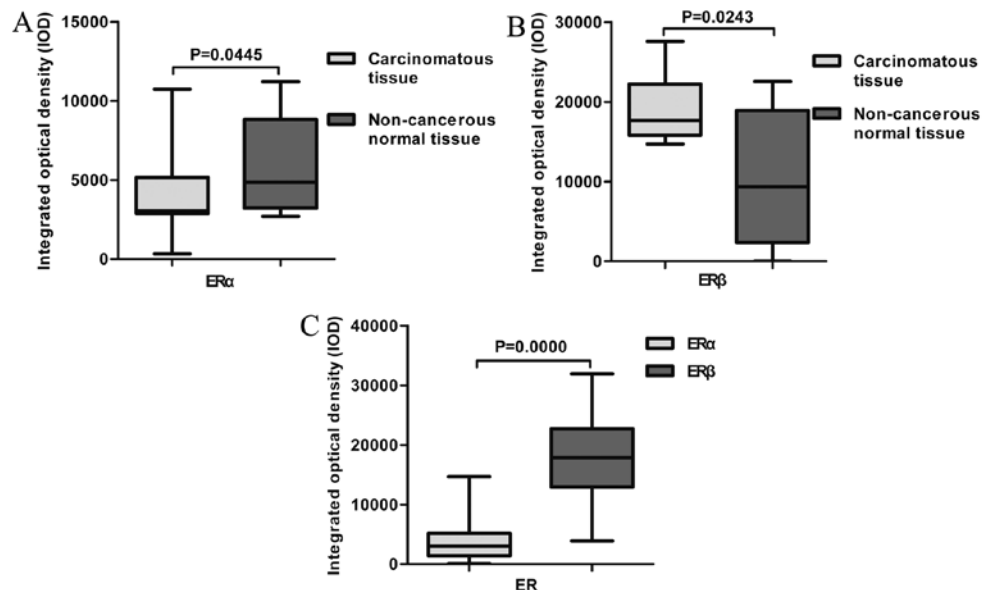


Figure 2. (A and B) Comparison of ER α and ER β staining between the non-neoplastic epithelial and ESCC tissue samples. The expression of ER α was significantly weaker in ESCC than that in non-neoplastic epithelium ($P=0.0445$). However, the ER β staining was significantly stronger in ESCC than that in the non-neoplastic epithelium ($P=0.0243$). (C) Relationship between ER α and ER β expression in ESCC. There was a significantly inverse correlation between ER α and ER β expression ($P=0.0000$).

in human esophageal non-neoplastic epithelium (Fig. 1A, B, E and F) with ratios of 3/7 and 6/9, respectively. ER α immunoreactivity was detected in the nuclei of carcinoma cells in 21/89 ESCC tissue samples. The mean value of ER α IOD in the 89 ESCC tissue samples was $3,741.0 \pm 2,978.6$. ER β immunoreactivity was detected in the nuclei of carcinoma cells in 87/89 ESCC tissue samples. The mean value of ER β IOD in 89 ESCC tissue samples was $17,998.7 \pm 6,664.5$.

Based on the staining intensity, ER α and ER β displayed opposite immunostaining patterns. For ER α , most of the cells showed moderate immunostaining in non-neoplastic epithelium (Fig. 1A and B), whereas negative to weak signals were observed in ESCC (Fig. 1C and D). However, ER β immunoreactivity was detected to be much weaker in the non-neoplastic

epithelium (Fig. 1E and F) than in ESCC (Fig. 1G and H). The expression difference between non-neoplastic epithelium and ESCC was statistically significant for both ER α ($P=0.0445$) and ER β ($P=0.0243$; Fig. 2A and B).

Association between the expression levels of the ERs and clinicopathological parameters of the ESCC patients. Associations between ER expression levels and clinicopathological parameters of the patients are summarized in Table II. There was a statistically significant inverse correlation between ER α IOD and invasive depth of the tumor ($P=0.0426$). No significant association was detected between ER α status and age, gender, tumor size, differentiation, presence of lymph node metastasis or TNM classification of tumors.

Table II. Association between ER α and ER β and clinicopathological variables in 89 esophageal squamous cell carcinoma patients.

Clinical parameters	N	IOD			
		ER α		ER β	
		Mean \pm SD	P-value ^a	Mean \pm SD	P-value ^b
Age (years)			0.5823		0.7437
≤ 58	43	3,671.2 \pm 2,432.4		17,757.8 \pm 6,303.0	
> 58	46	3,806.3 \pm 3,437.9		18,223.9 \pm 7,047.7	
Gender			0.7264		0.7158
Male	70	3,557.7 \pm 2,702.0		17,863.6 \pm 6,842.6	
Female	19	3,831.9 \pm 3,004.4		18,496.5 \pm 6,109.7	
Tumor size (cm)			0.3405		0.9545
≤ 3	52	3,573.8 \pm 3,068.8		17,899.4 \pm 6,768.9	
$> 3 - \leq 5$	28	3,388.4 \pm 2,183.5		18,299.6 \pm 6,599.9	
> 5	9	4,551.4 \pm 2,408.9		17,636.0 \pm 6,995.5	
Differentiation			0.4880		0.7710
G1	28	3,249.8 \pm 2,501.5		18,034.6 \pm 6,696.4	
G2	52	3,729.6 \pm 2,735.5		18,259.8 \pm 6,509.5	
G3	9	4,480.7 \pm 3,646.0		16,495.1 \pm 8,010.6	
Invasive depth			0.0426		0.2677
T2	5	5,373.8 \pm 1,291.0		14,773.2 \pm 7,422.8	
T3+T4	84	3,511.1 \pm 2,787.2		18,190.7 \pm 6,616.0	
LN metastasis			0.9368		0.2964
N0	46	3,593.9 \pm 2,508.5		17,281.6 \pm 6,602.0	
N1+N2+N3	43	3,640.9 \pm 3,020.3		18,765.8 \pm 6,722.8	
TNM classification			0.6823		0.1914
I+II	47	3,613.9 \pm 2,484.1		17,123.0 \pm 6,619.7	
III+IV	42	3,620.2 \pm 3,053.8		18,978.6 \pm 6,656.1	

^aMann-Whitney U test and Kruskal-Wallis test were used; ^bStudent's t-test and one-way ANOVA test were used. Each P-value is two-tailed and significant level was set at P=0.05. Data are presented as means \pm SD. ER, estrogen receptor; IOD, integrated optical density. LN, lymph node.

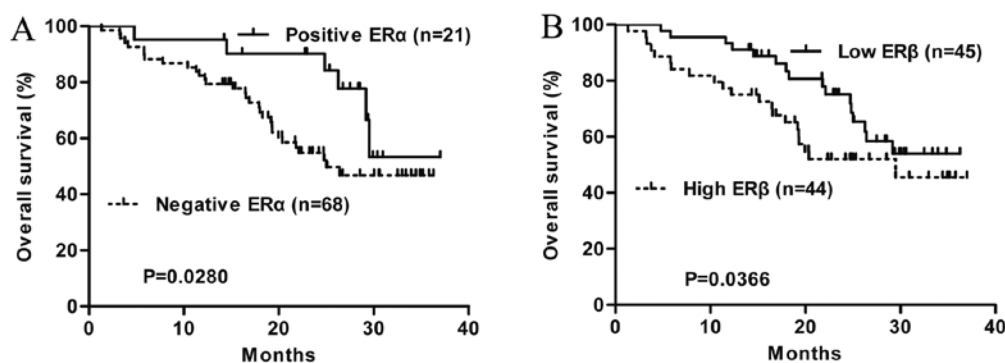


Figure 3. Overall survival (OS) of patients with ESCC stratified by the status of ER α and ER β immunoreactivity. (A) The outcome of patients with ER α expression was significantly more favorable than that of patients without ER α expression (P=0.0280). (B) The patients with high ER β immunoreactivity were significantly associated with worse survival than the patients with low ER β expression (P=0.0366).

No significant association was detected between ER β status and age, gender, tumor size, differentiation, invasive depth, presence of lymph node metastasis or TNM classification of tumors.

ER α expression is inversely correlated with ER β expression in ESCC. Paired t-test showed that the expression of ER β was much stronger than that of ER α (P=0.0000; Fig. 2C). There was also a statistically significant inverse correlation

between the expression of ER α and ER β in ESCC ($r=-0.2902$, $P=0.0058$).

ER α and ER β expression is of prognostic value for ESCC patients. Patients with ER α expression had a more favorable outcome than that of patients without ER α expression ($P=0.0280$; Fig. 3A). However, high ER β levels were found to be associated with unfavorable outcome ($P=0.0366$; Fig. 3B).

Discussion

In the present study, a significantly lower level of ER α immunoreactivity was detected in ESCC tissues than that in the normal esophageal tissues, which was consistent with the finding of Zuguchi *et al* (18). ER signaling via the PI3K/AKT signaling pathway phosphorylates EZH2 at S21, reducing levels of H3K27me3 in uterine myometrial cells (24). Meanwhile, the expression frequency and expression levels of H3K27me3 were significantly higher in ESCCs than in normal tissues, and high expression of EZH2 was correlated with tumor aggressiveness and adverse patient outcome in ESCC (25,26). Therefore, estrogen might exert a protective effect through ER α in esophageal squamous tissue. As for ER β , Wang *et al* (20) reported that ER β expression was correlated with lower malignant potential of ESCC. Nevertheless, we found a high frequency of ER β expression in carcinomatous tissues than that in non-cancerous normal tissues. This is consistent with the results of Kalayarasan *et al* (16) showing that ER β was overexpressed in poorly differentiated SCC and adenocarcinoma when compared to normal esophageal mucosa. In lung adenocarcinoma, ER β -mediated estradiol was found to enhance epithelial mesenchymal transition through increased transcription of midkine (27). Thus, ER β might also promote the process of ESCC by inducing epithelial mesenchymal transition. Meanwhile, several studies have demonstrated that the estrogenic action through ER signaling promotes the proliferation of carcinoma cells (6,28). For example, treatment with ER β -specific agonist DPN led to a significant increase in the cell proliferation in primary urothelial cells, which predominantly expressed ER β (8). From this point of view, our findings concerning the altered expression of ER α and ER β may carry clinical implication for the treatment of ESCC. Reagents that upregulate the expression of ER α and/or downregulate the expression of ER β may be useful for the chemotherapy of ESCC patients.

In this study, carcinomatous and non-cancerous normal tissues of the esophagus were used, and all of the ESCC tissues were obtained from patients who underwent esophagectomy. The majority of cases were at a relatively high pathological stage (T3). Therefore, T1 cases were lacked, and only 5 cases were classified as T2. The dynamic changes in ER expression throughout the progression of ESCC could not be monitored. Future investigations should include normal esophageal tissues, low-grade dysplasia and high-grade dysplastic tissues, particularly T1 and T2 cases, to elucidate the potential function of ERs in the progression of ESCC.

We addressed the prognostic value of ER protein expression for patients with ESCC in the present study. A significant inverse correlation between the status of ER α and the invasive depth of tumor was detected. The patients with ER α -positive

expression had a more favorable outcome than patients without ER α expression. As for ER β , Kaplan-Meier survival analysis showed that high expression of ER β in ESCC was significantly associated with unfavorable clinical outcome of patients. These results indicate that negativity for ER α and high expression of ER β may be an unfavorable prognostic factor for ESCC patients. Since only immunohistochemistry was used in this study, further research using quantitative molecular methods are obviously required to clarify the prognostic value of ERs in ESCC.

McDonnell and Norris (21) reported that estrogenic signals through ER β can inhibit ER α -dependent transcription when both ER α and ER β are present in cells and are bound to estrogen. An inverse correlation between expression of ER α and that of ER β was also demonstrated in lung cancer (22). In this study, we found that the expression of ER α was inversely correlated with that of ER β , which indicates that there may be some functional interactions between ER α and ER β in ESCC. To the best of our knowledge, this represents a novel observation that has not been previously reported.

Prominent gender differences in the prevalence of ESCC should not be ignored. In the present study, the age of onset for female ESCC patients was significantly older than that of male patients. Zuguchi *et al* (18) postulated that the gender differences in the prevalence of ESCC might be due to lifestyle differences between male and female patients. However, the difference in the onset age and the fact that all of the 19 female ESCC patients were older than the average postmenopausal age could not be explained by lifestyle differences. Rather, the estrogen levels as well as differential distribution of ER α and ER β may contribute to the age- and menopause-related changes in risk for the female population.

In conclusion, our study revealed that ER α was downregulated and ER β was upregulated, and the expression of ER α was negatively associated with that of ER β in ESCC. The absence of ER α and high expression of ER β indicate a poorer prognosis of ESCC. The use of selective ER modulators may be clinically effective for the treatment of ESCC patients. Future investigations are needed to delineate the mechanisms that contribute to the loss of ER α expression and the overexpression of ER β during the progression of ESCC.

Acknowledgements

We thank Dr Sima Sarvari for assistance with the manuscript preparation.

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