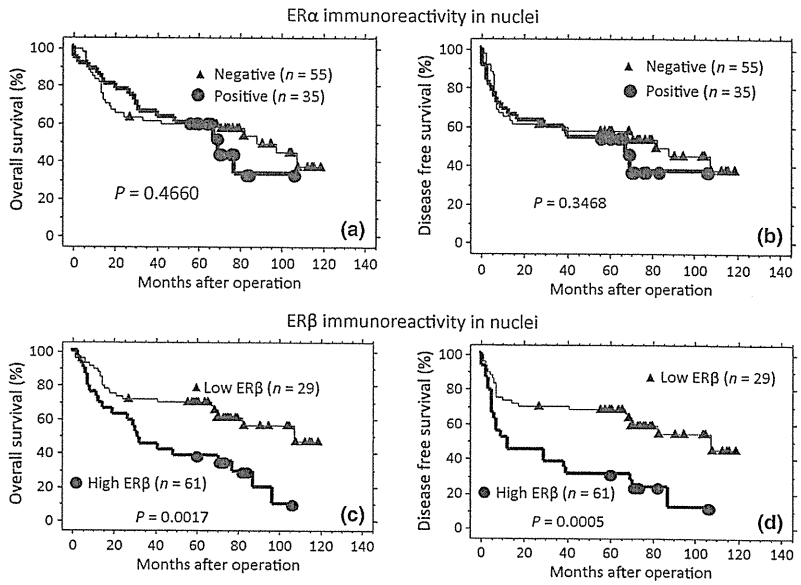

Fig. 2. Overall and disease-free survival (OS and DFS) curves of 90 Japanese patients with esophageal squamous cell carcinoma examined in this study according to the status of estrogen receptor (ER)α and ERβ immunoreactivity (Kaplan–Meier method). Significant difference in survival of the patients was noted between those with high and low ERβ nuclear immunoreactivity (c,d), but significant difference was not detected according to the status of ERα nuclear immunoreactivity (a,b). Cases were tentatively classified into two groups according to results of ERβ H score in the nuclei: high ERβ, >250 H score and low ERβ, <250 H score. High ERβ status in carcinoma cells was significantly associated with unfavorable clinical outcome of the patients examined.

Table 2. Univariate and multivariate analysis of overall survival/disease free survival in 90 esophageal squamous cell carcinoma patients

	Univariate		Multivariate	
	P-value	Relative risk (95% CI)	*P*-value	Relative risk (95% CI)
(A) Overall survival				
Sex (male/female)	0.1337	2.196 (0.785–6.139)		
Age (≥65/<64 years)	0.9419	1.022(0.572–1.824)		
Depth of tumor (pT3,4/pT1,2)	0.0069**	2.418 (1.274–4.589)		
TNM–pN (positive/negative)	0.0105**	2.287 (1.213–4.311)		
TNM–pM (positive/negative)	0.0579	2.191 (0.974–4.927)		
pStage (III,IV/I,II)	0.0003**	3.065 (1.675–5.609)	0.0019**	2.786 (1.459–5.322)
Tumor size (≥50/<50 mm)	0.0485**	1.807 (1.004–3.252)	0.4515	1.279 (0.674–2.427)
Lymphatic invasion (positive/negative)	0.0521	1.866 (0.994–3.503)		
Venous invasion (positive/negative)	0.3368	1.361 (0.726–2.552)		
Growth pattern (infiltrative/expansive + intermediate)	0.0200**	2.428 (1.150–5.126)	0.0185**	2.531 (1.169–5.484)
Histological grade (por/well + mod)	0.3653	1.424 (0.662–3.062)		
Ki67 status†	0.7021	0.997 (0.484–1.540)		
ERα status (positive/negative)	0.4692	1.249 (0.684–2.281)		
ERβ status (high/low)	0.0025**	2.469 (1.374–4.438)	0.0010**	2.754 (1.509–5.027)
Brinkman index (≥400/<400)	0.9389	0.975 (0.504–1.885)		
(B) Disease free survival				
Sex (male/female)	0.1142	2.286 (0.819–6.378)		
Age (≥65/<64 years)	0.9997	1.000 (0.567–1.763)		
Depth of tumor (pT3,4/pT1,2)	0.0124**	2.200 (1.186–4.080)		
TNM–pN (positive/negative)	0.0218**	2.051 (1.110–3.788)		
TNM–pM (positive/negative)	0.1305	1.865 (0.831–4.185)		
pStage (III,IV/I,II)	0.0006**	2.804 (1.560–5.039)	0.0091**	2.304 (1.230–4.314)
Tumor size (≥50/<50 mm)	0.0366**	1.840 (1.038–3.259)	0.3092	1.378 (0.743–2.555)
Lymphatic invasion (positive/negative)	0.1816	1.506 (0.826–2.746)		
Venous invasion (positive/negative)	0.4967	1.236 (0.671–2.276)		
Growth pattern (infiltrative/expansive + intermediate)	0.0416**	2.150 (1.029–4.490)	0.0328**	2.307 (1.071–4.971)
Histological grade (por/well + mod)	0.1013	1.844 (0.887–3.835)		
Ki67LI†	0.6212	0.996 (0.981–1.012)		
ERα status (positive/negative)	0.3563	1.317 (0.734–2.363)		
ERβ status (high/low)	0.0010**	2.627 (0.734–2.363)	0.0007**	2.828 (1.551–5.158)
Brinkman index (≥400/<400)	0.8198	1.079(0.560–2.078)		

**Data considered significant ($P < 0.05$) in the univariate analysis were examined in the multivariate analysis. CI, confidence interval; ER, estrogen receptor; LI, labeling index; mod, moderate; por, poor. ERα status (positive; LI ≥ 10%/negative; LI < 10%). ERβ status (high; H score ≥ 250/low; H score < 250). †Data were evaluated as continuous values and displayed mean ± SEM (range).

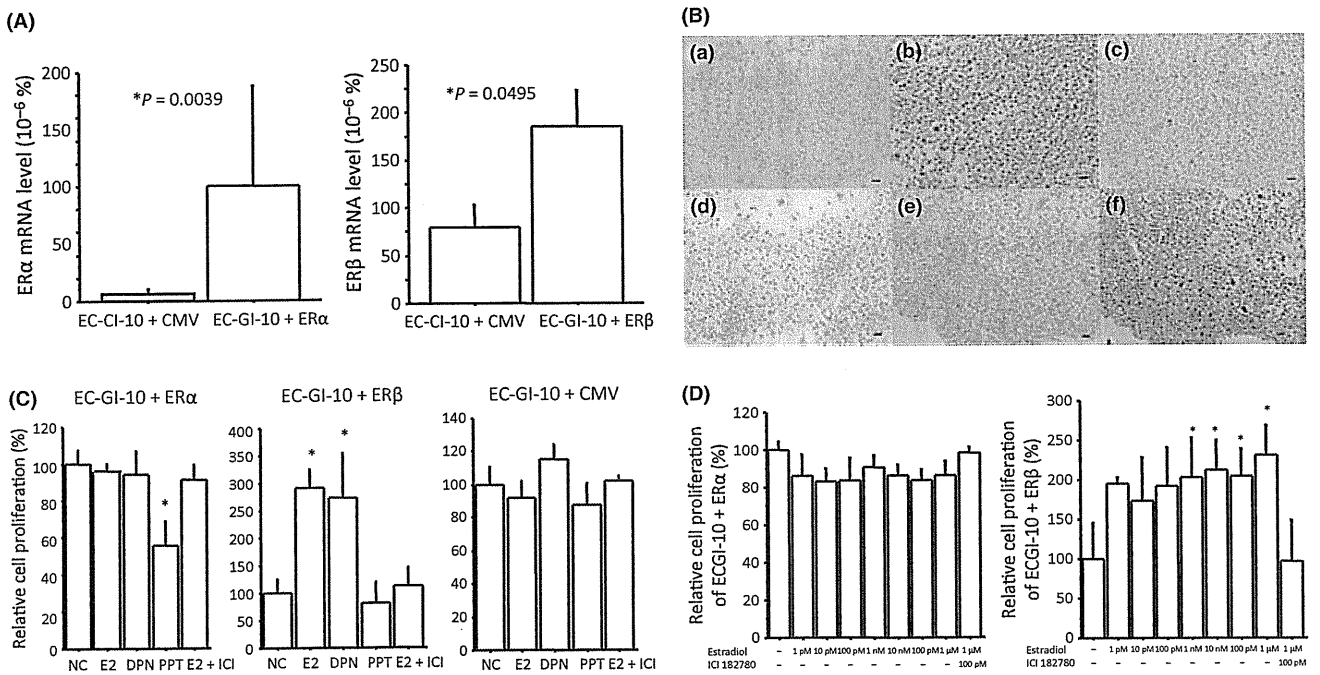

Fig. 3. (A) mRNA level of EC-GI-10 + CMV versus EC-GI-10 + ERα(left), mRNA level of EC-GI-10 + CMV versus EC-GI-10 + ERβ(right), mean ± SD (n = 6). (B) Immunostaining for estrogen receptor (ER) isoforms in the EC-GI-10 transformants. (a,d) EC-GI-10 + CMV; (b,e) EC-GI-10 + ERα; (c,f) EC-GI-10 + ERβ. ERα immunoreactivity was detected in the nuclei of EC-GI-10 + ERα cells (b), ERβ immunoreactivity was detected in the nuclei of EC-GI-10 + ERβ cells (f). No significant immunoreactivity for ER isoforms was detected in EC-GI-10 + CMV cells (a,d). Immunoreactivity was evaluated in the cell blocks specimens. Bar represents 100 μm. (C) Left, EC-GI-10 + ERα cells; center, EC-GI-10 + ERβ cells; right, EC-GI-10 + CMV. Estradiol (1 μmol/L) with or without ER antagonist ICI 182780 (100 pmol/L), ERα agonist PPT (1 μmol/L), ERβ agonist DPN (1 μmol/L) were added to these cells. They were then cultured for 72 h. NC, no changes. The cell proliferation activity was evaluated as a ratio (%) compared with that of controls (no treatment with either estradiol, PPT, DPN or ICI 182780 for 72 h). Mean ± SD (n = 6). *P < 0.05 versus controls (Kruskal–Wallis test and Scheffe test). (D) Effects of the estrogen-mediated proliferation of EC-GI-10 + ERα cells, EC-GI-10 + ERβ cells. The proliferative activity was evaluated as a ratio (%) compared to that of controls (no treatment with either estradiol or ICI 182780 for 72 h). Mean ± SD (n = 6). *P < 0.05 versus controls (Kruskal–Wallis test and Scheffe test).

Discussion

Freedman *et al.* (2010) report that menopausal hormone therapy is significantly associated with lower risk of head, neck and ESCC in the National Institutes of Health-American Association of Retired Persons (NIH-AARP) Diet and Health Study.[27] Bodelon *et al.* also report the significant association between hormone replacement therapy and the risks of developing ESCC in postmenopausal women enrolled in a Women's Health Initiative (WHI) and observational study.[28] They both demonstrate that the women who took both estrogen and progestin had lower risk of developing ESCC than those who took a placebo, but this association was not detected when women took estrogen alone.[28] Further investigations regarding hormone receptor status, including progesterone receptor in ESCC, are certainly required for clarification, but it has become important to examine the details of estrogenic actions, especially the status of ER in development and behavior of ESCC patients.

Enmark *et al.* report the presence of two different isoforms of ER, ERα and β, in many types of human tissues.[5] Subsequent studies confirm that ERα and ERβ are not only expressed in classical estrogen target tissues but are also rather widely distributed in humans. Taylor *et al.* report the presence of ERα and ERβ in normal esophagus squamous epithelium.[29] Nozoe *et al.* report the presence of ERα and ERβ in ESCC patients.[13] Kalayarasan *et al.* report that the status of ERβ is correlated with aggressive behavior in ESCC patients,[15] but different results have been also reported.[17] Therefore, the clinical and biological significance of ERα and ERβ in ESCC has not been clarified.

In the present study, we examined the nuclear immunoreactivity of ERα and ERβ in both squamous cell carcinoma and non-neoplastic squamous cell epithelium of the esophagus. A relatively high level of ERα immunoreactivity was detected in the nuclei of non-neoplastic basal layer cells in normal esophageal mucous. In mammary glands, Khan *et al.* report increased ERα immunoreactivity in normal epithelium obtained from tumor-bearing breasts compared to non-tumor bearing breast.[30] Lawson *et al.* also report that ERα expression is higher in the breast tissue of women from a population at high risk of breast cancer compared with that in the tissue of women associated with a relatively low risk of the disease development.[31] It is also interesting to note that Zhai *et al.* report the loss of ERα expression in the advanced stages of cervical squamous cell carcinoma progression.[32] In the present study, a relatively high level of ERα immunoreactivity was also detected in non-neoplastic esophageal mucosa bearing ESCC compared to that in concomitant or adjacent carcinoma cells. Therefore, in human squamous mucosa, estrogen may help to maintain normal cell cycle or exert a protective effect upon epithelial cells through the ERα. Further investigation is necessary to clarifying this interesting hypothesis, for example through comparison of ERα status of morphologically normal esophageal mucosa in tumor bearing and non-tumor bearing subjects. However, it is also true that the status of ERα immunoreactivity in ESCC was by no means associated with any of the clinicopathological variables of the

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patients examined in this study, including their clinical outcome. In contrast, ERβ status of carcinoma cells was significantly associated with unfavorable clinical outcome of the patients and ERβ status in carcinoma cells also turned out to be an independent unfavorable prognostic factor for the patients (determined using multivariate analysis). Therefore, it has become important to examine the effects of estrogen signals mediated through ERβ in these patients to clarify the possible involvement of estrogens in the biological behavior of ESCC.

Ivanova *et al.* report on the difference between men and women in the modes of actions of ERβ in lung cancer.[33] Therefore, we initially postulated that variations in expression of ER were due to the differences in the prevalence of gender in ESCC. However, in the present study there were no differences in the status of ER according to the gender of the patients. Therefore, the gender differences in the prevalence of ESCC might be due to differences in the lifestyles of male and female patients (e.g. drinking and smoking), but further investigations are required for clarification.

In the present study, ERβ was detected in the nuclei of ESCC, as in Nozoe *et al.* The differences between the results of the present study and those of Nozoe *et al.* could be due to the ERβ antibodies used and the evaluation method of ERβ immunoreactivity. Nozoe *et al.* determined that ERβ nuclear staining in at least 50% of tumor cells were scored as positive for overexpression, based upon a study of lung cancer by Wu *et al.*[13,34] However, we defined ERβ nuclear positive immunoreactivity or overexpression as the cases with an H score of more than 250. The validity of this definition is discussed in the Materials and Methods section. In breast carcinoma, the difference of the cut-off points of the ER status is well-known to be related to the prognosis of individual patients.[35,36] The results of survival analyses of breast carcinoma patients are also known to be different depending on the evaluation method of the ER (e.g. proportion score and intensity score).[35] Further examinations using different ERβ antibodies and evaluation methods are obviously required to clarify the ERβ and prognosis in ESCC.

According to Matsuoka *et al.* and Ueo *et al.*, estrogen prevents cell proliferation of primary ESCC cells, which are reported to be associated with the presence of ER.[3,4] ERβ was not identified in the 1980s and, therefore, results from these previous reports[3,4] could not clarify whether estrogenic signals were mediated in ESCC through ERα, ERβ or both. When both ERα and ERβ are present in the cells and bound to estrogen, estrogen signals through ERβ are generally considered to inhibit ERα-dependent transcription.[37] For instance, estrogen-dependent cell proliferation is reported to be inhibited by transfection of ERβ in breast carcinoma cells.[38] However, several studies state that estrogenic action through ERβ signaling induces rather than suppresses the cell proliferation of carcinoma cells.[8,39,40] For instance, Teng *et al.* demonstrate that the treatment of ERβ specific agonist DPN significantly increased cell proliferation in primary urothelial cells, which predominantly expressed ERβ.[10] They also report that estrogenic signals mediated through ERβ predominantly induce G1/S transition in primary urothelial cells.[41] Hershberger *et al.* report ERβ-dependent cell proliferation through both genomic and non-genomic pathways in lung carcinoma cell line.[42] In the present study, the treatment of PPT, the specific ERα agonist, significantly decreased the number of EC-GI-10 + ERα cells and that of estradiol, and DPN, the specific ERβ agonist, significantly increased the number of EC-GI-10 + ERβ cells. Results of the present study clearly indicate that estrogenic actions through ERβ were predominant in ESCC cells compared to those through the ERα. Therefore, estrogen might also play a pivotal role in the cells in which estrogenic signals are mediated predominantly through ERβ, such as ESCC cells. Interestingly, TGFα, amphiregulin or HB-EGF, all of which are considered EGFR activators, are induced by estradiol treatment in ERα negative/ERβ positive breast carcinoma cell line[43] or ERβ dominantly expressed HNSCC cells.[44] EGFR is also reported to be detected in many ESCC cells,[45,46] and is even considered as a target molecule for a potential target specific therapy in ESCC patients.[47] Therefore, estrogenic effects, including an induction of its target genes, might facilitate the proliferation of ESCC cells through the activation of EGFR signals in ESCC, but further investigations are required for clarification.

In summary, we demonstrated ERα and ERβ expression using immunohistochemistry in human ESCC. The status of nuclear ERβ immunoreactivity in carcinoma cells turned out to be an unfavorable independent prognostic factor in ESCC patients. Results of our immunohistochemical and *in vitro* studies clearly demonstrate that ESCC is an estrogen-dependent human malignancy, as in other human cancers. ERβ might serve as a novel target molecule for ESCC patient therapy.

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Disclosure Statement

The authors have no conflict of interest.

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