

ERR α inhibitor acts as a potential agonist of PPAR γ to induce cell apoptosis and inhibit cell proliferation in endometrial cancer

Meimei Huang^{1,2}, Lili Chen³, Xiaodan Mao², Guifen Liu^{1,2}, Yuqin Gao^{1,2}, Xiaoqing You⁴, Min Gao⁵, Jalid Sehoul⁶, Pengming Sun^{1,2}

¹Department of Gynecology and Obstetrics, Fujian Maternity and Child Health Hospital, Affiliated Hospital of Fujian Medical University, Fuzhou 350001, China

²Laboratory of Gynecologic Oncology, Fujian Maternity and Child Health Hospital, Affiliated Hospital of Fujian Medical University, Fuzhou 350001, China

³Reproductive Center, Fujian Maternity and Child Health Hospital, Affiliated Hospital of Fujian Medical University, Fuzhou 350001, Fujian, China

⁴Department of Cell Biology and Genetics, School of Basic Medical Sciences, Fujian Medical University, Fuzhou 350108, Fujian, China

⁵Department of Gynecologic Oncology, Peking University Cancer Hospital, Beijing 100046, China

⁶Department of Gynecology, Campus Virchow Clinic, CharitéUniversitätsmedizin Berlin, Humboldt University of Berlin, Berlin 13353, Germany

Correspondence to: Pengming Sun; **email:** sunfemy@hotmail.com, <http://orcid.org/0000-0002-5072-6091>

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ABSTRACT

Two transcriptional factors, peroxisome proliferator-activated receptor- γ (PPAR γ) and estrogen-related receptor- α (ERR α), have been reported to be key regulators of cellular energy metabolism. However, the relationship between ERR α and PPAR γ in the development of endometrial cancer (EC) is still unclear. The expression levels of PPAR γ and ERR α in EC were evaluated by quantitative real-time PCR, western blot, tissue array and immunohistochemistry. A significant negative correlation was identified between PPAR γ and ERR α expression in women with EC ($\rho=-0.509$, $P<0.001$). Bioinformatics analyses showed that PPAR γ and ERR α can activate or inhibit the same genes involved in cell proliferation and apoptosis through a similar ModFit. ERR α activation or PPAR γ inhibition could promote proliferation and inhibit apoptosis through the Bcl-2/Caspase3 pathways. Both PPAR γ and ERR α can serve as serum tumor markers. Surprisingly, as evaluated by receiver operating characteristic (ROC) curves and a logistic model, a PPAR γ /ERR α ratio ≤ 1.86 (area under the ROC curve (AUC)=0.915, Youden index=0.6633, $P<0.001$) was an independent risk factor for endometrial carcinogenesis (OR=14.847, 95% CI= 1.6-137.748, $P=0.018$). EC patients with PPAR γ (-)/ERR α (+) had the worst overall survival and disease-free survival rates (both $P<0.001$). Thus, a dynamic imbalance between PPAR γ and ERR α leads to endometrial carcinogenesis and predicts the EC prognosis.

INTRODUCTION

Endometrial cancer (EC) is the second most common gynecological tumor [1, 2]. In 2019, approximately

61,880 new EC cases and 12,160 deaths due to EC were reported in the United States [1]. Recently, the incidence of EC has markedly increased in China, likely due to the growing obesity epidemic as obesity is a

strong risk factor for EC [3]. Although most ECs can be found in the early stage, the prognosis of patients with high-grade, poorly differentiated, advanced-stage EC is poor. The use of traditional tumor biomarkers, namely, CA125, CA199, CA153 and CEA, to monitor EC has produced unsatisfactory results in clinical practice [4, 5]. Therefore, tumor biomarkers for predicting and monitoring EC are still needed.

Peroxisome proliferator-activated receptor γ (PPAR γ), a steroid hormone receptor, was discovered by Issemann and Green in 1990 and plays an important role in the regulation of glucose and lipid metabolism in some obesity-related cancers [6], such as breast cancer [7] and EC [8]. Early in 2006, Kyoko O et al. suggested that PPAR γ immunoreactivity and mRNA levels were significantly lower in EC than in the normal endometrium [9]. Nickkho-Amiry et al. found that PPAR γ activation reduced endometrial cell proliferation, that nuclear PPAR γ was most abundant in benign endometrial tissue, and that nuclear PPAR γ correspondingly decreased with increasing pathological grades [8]. Thus, PPAR γ seems to act as a protective factor against EC. However, other studies have shown that PPAR γ has protumorigenic effects in pancreatic cancer [10], metastatic prostate cancer [11], and bladder carcinoma [12]. Thus, the role of PPAR γ in cancer remains controversial. Wei W et al. identified a conserved PPAR response element (PPRE) in the estrogen-related receptor α (ERR α) promoter and suggested that ERR α was a direct PPAR γ target gene, specifically in osteoclastogenesis, by ChIP assay [13]. Coincidentally, Fujimoto J et al. demonstrated that ERR α was expressed at higher levels in EC tissues and was correlated with a poor prognosis in EC [14]. Moreover, we found that ERR α reduced EC cell proliferation and promoted apoptosis [15–17]. Thus, ERR α is a potential tumor biomarker and therapeutic target for EC. However, the potential mechanisms of ERR α and PPAR γ in tumorigenesis are still unclear. In PubMed, no report focusing on the interaction between ERR α and PPAR γ in EC is available. Therefore, we hypothesized that some crosstalk might occur between PPAR γ and ERR α to promote EC carcinogenesis and progression. To clarify the possible biological and clinical roles of PPAR γ and/or ERR α in human EC, we focused on the interaction between PPAR γ and ERR α in EC cells *in vitro* and EC tissues *in vivo*. Furthermore, the downstream target pathways of PPAR γ and ERR α in EC were explored based on bioinformatics data-mining analysis and further confirmed in cell lines. We also discuss the potential roles of PPAR γ and ERR α in the EC diagnosis and try to determine the best threshold. Finally, the disease-free survival (DFS) and overall survival (OS) rates of EC patients with different expression patterns of PPAR γ and ERR α were analyzed.

RESULTS

PPAR γ is negatively correlated with ERR α in EC tissue

The expression of PPAR γ mRNA in 77 EC patient tissues and 39 normal control tissues was determined by qRT-PCR. Compared to normal endometrium tissues, lower expression of PPAR γ in EC tissues was noted ($P < 0.05$, Figure 1A). In contrast, EC tissue showed higher expression of ERR α ($P < 0.05$, Figure 1B). No significant differences in either PPAR γ or ERR α expression were found among EC patients with different FIGO stages ($P > 0.05$, Figure 1A, 1B). PPAR γ and ERR α protein expression was also detected by immunohistochemistry. Positive immunoreactivity for PPAR γ was detected in the nuclei of carcinoma cells and normal endometrial gland cells. Immunoreactivity of PPAR γ was significantly lower in endometrial carcinoma tissue than in normal endometrial tissue ($P < 0.001$, Figure 1C, 1E). However, the ERR α levels detected in 70 of 77 (90.1%) EC tissue samples were higher than those detected in 24 of 39 (61.54%) normal endometrial tissue samples ($P < 0.001$, Figure 1C, 1F). Thus, PPAR γ and ERR α presented opposing expression patterns in both normal endometrial and EC tissues. PPAR γ immunoreactivity was lower in type II EC than in type I EC ($P < 0.05$, Figure 1D), but no obvious difference in ERR α immunoreactivity was observed between the two types of EC ($P > 0.05$, Figure 1D). The correlations between the expression of PPAR γ and ERR α and patient clinicopathologic variables, including stage and pathological type and grade, were summarized in Table 1. Based on the data, a negative correlation was identified between PPAR γ immunoreactivity and ERR α immunoreactivity according to Spearman's rank correlation analysis ($\rho = -0.509$, $P < 0.001$).

Functional annotation and pathway enrichment for PPAR γ and ERR α

PPAR γ and ERR α gene expression and EC were selected and queried on the <http://ualcan.path.uab.edu> website. UALCAN lists queried genes with links to gene expression analyses and survival data. Analysis of TCGA samples showed that PPAR γ expression was higher in normal samples ($n = 35$), and that ERR α expression was obviously increased in EC samples ($n = 546$, $P < 0.001$, Figure 2A). Moreover, PPAR γ expression was higher in endometrioid adenocarcinoma ($n = 409$) than in serous carcinoma ($n = 115$, $P < 0.001$, Figure 2B). However, no difference in ERR α expression was found between endometrioid adenocarcinoma and serous carcinoma ($P > 0.05$, Figure 2B). These data were all consistent with our results described above. Although the STRING analysis revealed no research

overlap between PPAR γ and ERR α , the same cofactors, namely, PPAR γ coactivator-1 α (PGC-1 α), nuclear receptor coactivator 1 (NCOA1), and cyclic adenosine monophosphate response element-binding protein (CREBBP), were reported to be shared between them.

CREBBP can transcribe, coactivate and increase the expression of its target genes. PPAR γ and ERR α can compete with the cofactor CBP to increase or decrease Bcl2 expression (Figure 2C). As shown in Figure 2D, the microarray data revealed 41,739 target genes of

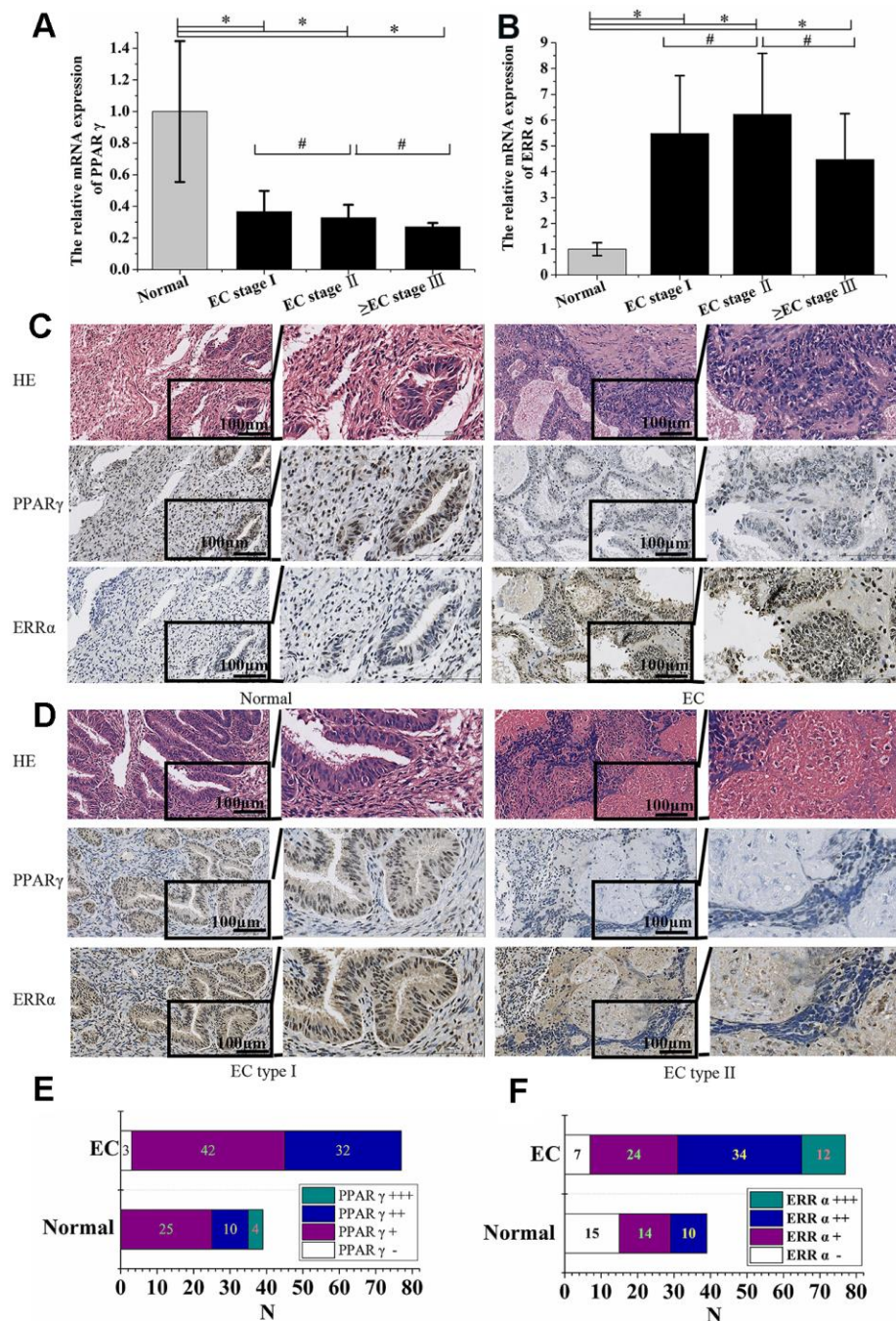


Figure 1. PPAR γ was negatively correlated with ERR α in EC tissue. (A) The relative mRNA expression of PPAR γ and (B) the relative mRNA expression of ERR α in EC patients with different FIGO stages. (C) Immunohistochemical expression of PPAR γ and ERR α in the normal endometrium and EC ($\times 200$ & $\times 400$). (D) Immunohistochemical expression of PPAR γ and ERR α in EC type I and EC type II ($\times 200$ & $\times 400$). (E) Immunohistochemical expression of PPAR γ in the normal endometrium and EC. (F) Immunohistochemical expression of ERR α in the normal endometrium and EC. CON: normal endometrium. EC, endometrial cancer. *, $P < 0.05$; #, $P > 0.05$.

Table 1. Correlation between PPAR γ , ERR α expression immunoreactivity in EC.

	NO. of cases	PPARγ-positive	%	P^a	ERRα-positive	%	P^a
FIGO stage							
I	48	40	83.33	0.001	41	85.42	0.08
II-IV	29	14	48.28		29	1	
Type							
I	63	48	76.19	0.014	56	88.89	0.427
II	14	6	42.86		14	1	
Grade							
G1	27	23	85.19	0.014	24	88.89	0.276
G2	29	23	79.31		27	93.1	
G3	7	2	28.57		5	71.43	

^aThe results were evaluated using χ -square test.

PPAR γ and 30,718 target genes of ERR α . A total of 28,434 identical target genes, including Bcl2 and Caspase3, were found between PPAR γ and ERR α (Figure 2D). Figure 2E–2G graphically depicts the most common GO codes for the three major GO categories. Further searches revealed a repeating sequence in both the PPAR γ and ERR α genes (Figure 2I, 2J). These data suggested that a significant number of protein-coding genes with important biological functions, especially in the apoptosis pathway, may be regulated by PPAR γ and ERR α . By KEGG analysis, we found that a large number of genes coregulated by PPAR γ and ERR α are involved in the apoptosis pathway (Figure 2K). Further confirming our hypothesis, PPAR γ and ERR α compete for the same cofactors and increase or decrease the same genes, including Bcl2 and Caspase3, involved in the apoptosis pathway (Figure 2L).

Reciprocal inhibition between ERR α and PPAR γ in EC cells

PPAR γ and ERR α were both expressed in EC cell lines, including RL952, ECC-1, HEC-1A, and HEC-1B. Interestingly, the mRNA expression of PPAR γ and ERR α was higher in the ER α -positive cell lines RL952 and ECC-1 than in the ER α -negative cells HEC-1A and HEC-1B ($P < 0.05$, Figure 3A, 3B). The proteins were also expressed at high levels in the ER α -positive lines RL952 and ECC-1 and at low levels in the ER α -negative lines HEC-1A and HEC-1B ($P < 0.05$, Figure 3C). A similar tendency was observed for PPAR γ and ERR α protein expression in these EC cells. The semiquantitative PPAR γ protein expression levels in RL952, ECC-1 HEC-1A, and HEC-1B cells were 1 ± 0.0611 , 0.6313 ± 0.0696 , 0.1566 ± 0.0069 , and 0.2027 ± 0.0274 , respectively, and the semiquantitative ERR α protein expression levels in RL952, ECC-1 HEC-1A, and HEC-1B cells were 1 ± 0.0699 , 1.0429 ± 0.0378 , 0.7135 ± 0.0374 , and 0.6514 ± 0.0948 , respectively.

PPAR γ expression was significantly increased, while ERR α expression was significantly reduced by lentivirus-mediated PPAR γ overexpression in HEC-1A and HEC-1B cells (Figure 3D). PPAR γ expression was decreased, while ERR α expression was increased when the same cells were treated with GW9662 (Figure 3E), a potent antagonist of PPAR γ [18]. When PPAR γ was overexpressed, Bcl2 protein expression was decreased, and Caspase3 protein expression was increased (Figure 3F). After treatment with G9662 for 48 h, Bcl2 protein expression was increased, and Caspase3 protein expression was decreased (Figure 3G). When ERR α was overexpressed in cells, PPAR γ was downregulated (Figure 3H). Subsequently, ERR α was upregulated, and PPAR γ was downregulated when ERR α was overexpressed by lentivirus in HEC-1A and HEC-1B cells (Figure 3H). ERR α was decreased, but PPAR γ was increased in the same cells after treatment with XCT790, a specific ERR α antagonist (Figure 3I). When ERR α was upregulated, Bcl2 protein expression was increased, and Caspase3 protein expression was decreased (Figure 3J). After treatment with XCT790 for 48 h, Bcl2 protein expression was decreased, and Caspase3 protein expression was increased (Figure 3K). Taken together, these data indicate that PPAR γ and ERR α participate in a negative feedback loop in EC. This regulatory pattern was also true for the Bcl2 and Caspase3 proteins (Figure 3F–3G and Figure 3J–3K).

PPAR γ and ERR α compete to activate or inhibit cell proliferation and apoptosis through Bcl2/Caspase3 signals

Our results showed that PPAR γ overexpression slowed proliferation (Figure 4A), but that GW9662 treatment accelerated the proliferation of HEC-1A and HEC-1B cells (Figure 4A). Moreover, HEC-1A and HEC-1B cell proliferation was inhibited by XCT790 treatment (Figure 4A, $P < 0.05$). The cellular proliferation rate was significantly increased when ERR α was overexpressed

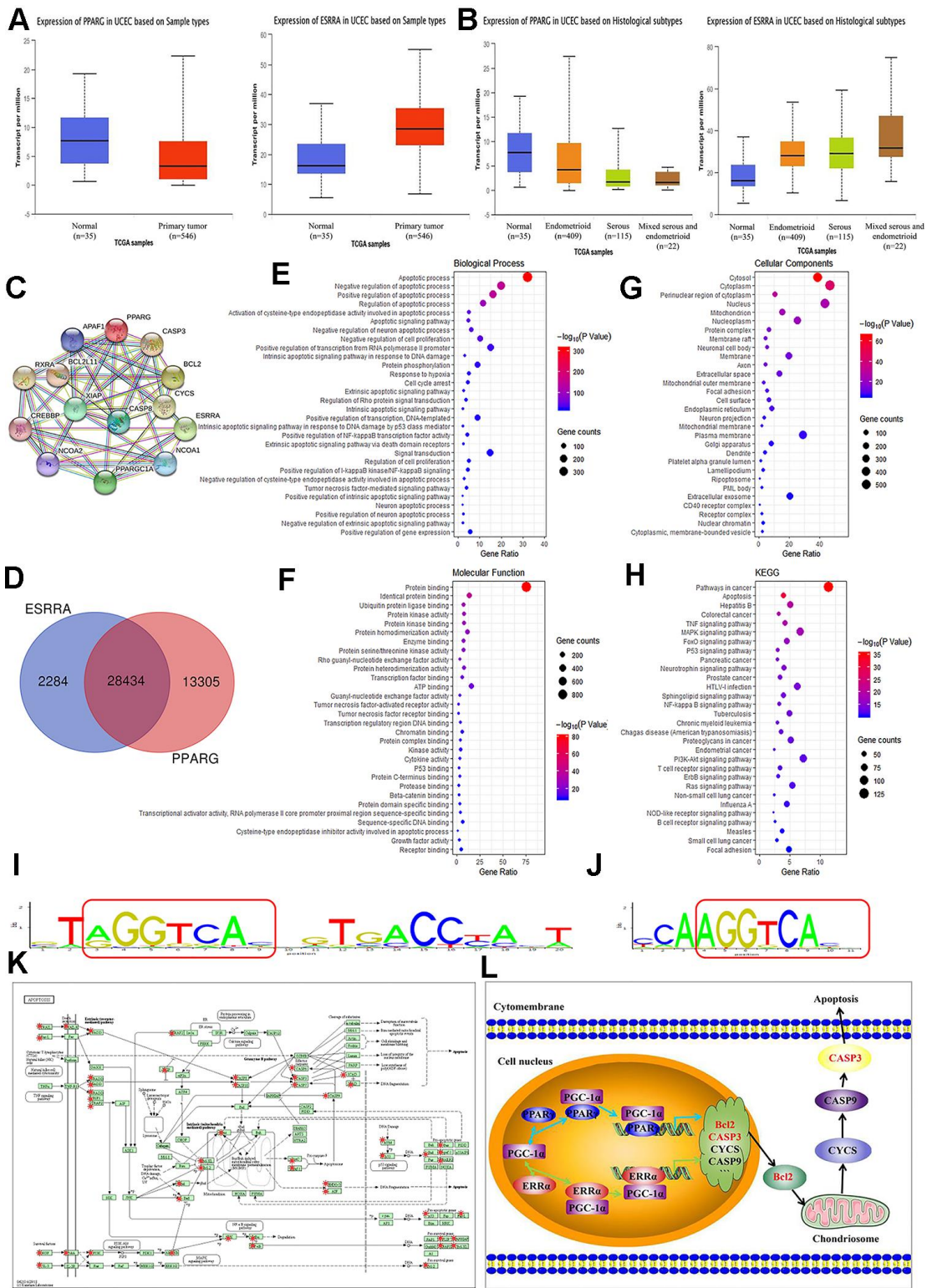


Figure 2. Summary of functional annotation and pathway enrichment for PPAR γ and ERR α . (A) Boxplot showing the relative expression of PPAR γ and ERR α in normal and EC samples from the UALCAN database. (B) Boxplot showing the relative expression of PPAR γ and ERR α in EC based on histological subtypes from the UALCAN database. (C) Protein mapping for PPAR γ and ERR α based on string data. (D) Venn diagram showing the common target genes of PPAR γ and ERR α . (E–G) GO enrichment of the common target genes of PPAR γ and ERR α . (H) KEGG enrichment of the target genes regulated by PPAR γ and ERR α . (I) The ModFit of PPAR γ based on the JASPAR database. (J) The ModFit of ERR α based on the JASPAR database. (K) KEGG pathway maps for apoptosis; The red dots represent target genes of PPAR γ and ERR α . (L) Possible mechanism of interaction between PPAR γ and ERR α . EC, endometrial cancer. *, $P < 0.05$; #, $P > 0.05$.

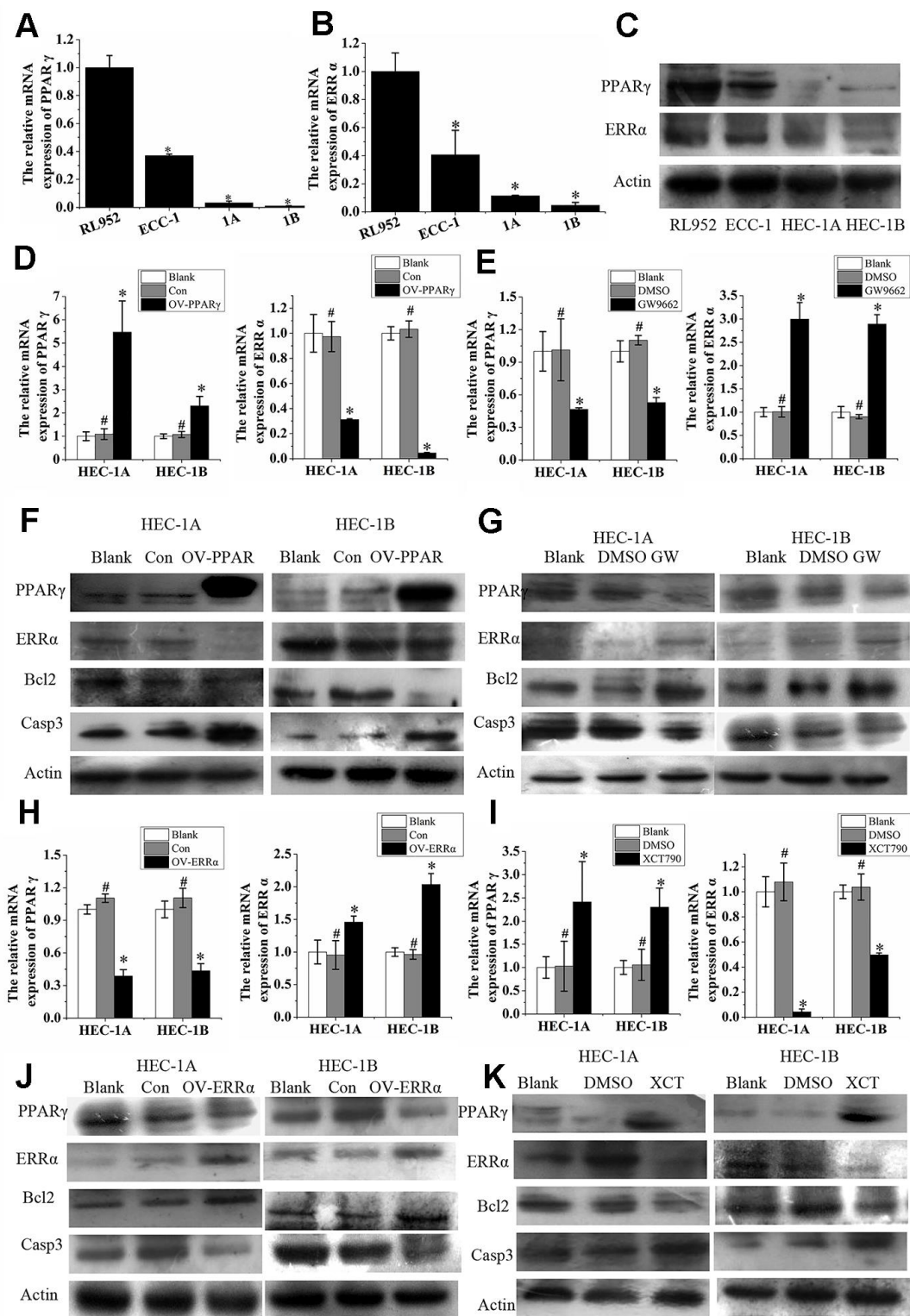


Figure 3. ERR α and PPAR γ negatively regulate each other in EC cells. (A) The relative mRNA expression of PPAR γ and (B) the relative mRNA expression of ERR α in RL952, ECC-1, HEC-1A, and HEC-1B cells. (C) Protein expression of PPAR γ and ERR α in RL952, ECC-1, HEC-1A, and HEC-1B cells. (D) Upregulated PPAR γ and (E) suppressed PPAR γ by GW9662: the relative mRNA expression of PPAR γ and ERR α in EC cells. (F) Upregulated PPAR γ and (G) suppressed PPAR γ by GW9662: the protein expression of PPAR γ and ERR α in EC cells. (H) Upregulated ERR α and (I) suppressed ERR α by XCT790: the relative mRNA expression of ERR α and PPAR γ in EC cells. (J) Upregulated ERR α and (K) suppressed ERR α by XCT790: the protein expression of PPAR γ and ERR α in EC cells. OV-PPAR γ , overexpression of PPAR γ . OV-ERR α , overexpression of ERR α . EC, endometrial cancer. *, $P < 0.05$; #, $P > 0.05$.

by a lentivirus (Figure 4A, $P<0.05$). The HEC-1A and HEC-1B cell cycles were analyzed after PPAR γ and ERR α modulation. The data suggested that PPAR γ downregulation by GW9662 led to G₀-G₁ phase shortening and S phase lengthening, while ERR α downregulation by XCT790 resulted in S phase shortening but G₂-M phase lengthening in HEC-1A cells (Figure 4B-C). The same trend for the S phase and M phase was found in HEC-1B cells (Figure 4B, 4C). Apoptosis of HEC-1A cells and HEC-1B cells increased when PPAR γ was upregulated (Figure 4D). In contrast, apoptosis of HEC-1A cells and HEC-1B cells decreased when PPAR γ was downregulated (Figure 4E). Apoptosis of HEC-1A cells and HEC-1B cells decreased when ERR α was upregulated by a lentivirus (Figure 4F). Treatment with XCT790 significantly increased cell apoptosis (Figure 4G).

The PPAR γ /ERR α combination as a biomarker for EC diagnosis

A receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of PPAR γ and ERR α mRNA expression to further determine whether PPAR γ and ERR α could serve as tumor markers. Classical serum biomarkers, including CA125, CA153, CA199, AFP and CEA, were also assessed in terms of their abilities to distinguish EC from benign lesions. At the highest area under the ROC curve (AUC, by best-point analysis), which was 0.681, PPAR γ could distinguish women with benign lesions from those with EC tumors (Figure 5A). The AUC of ERR α was 0.763 ($P<0.001$) (Figure 5A). However, even though both AUCs were statistically significant, their sensitivity and specificity for predicting EC were poor. As a diagnostic predictor, ERR α seemed to be slightly better than PPAR γ for EC. Surprisingly, when PPAR γ and ERR α were combined to predict the genesis of EC, the AUC of PPAR γ /ERR α was 0.915, with an optimal threshold of 1.86 (Youden index=0.6633, $P<0.001$); the predictive performance of this combination was substantially improved compared to that of each as a single indicator (Figure 5A). Other common clinical indicators, including CA125, CA153, CA199, AFP and CEA, were also analyzed by ROC curves (Figure 5A). The predictive abilities of these indicators were obviously lower than that of PPAR γ /ERR α . We also determined whether PPAR γ /ERR α could serve as an independent risk factor for EC. As shown in Table 2, we obtained meaningful indicators derived from clinical characteristics. After adjusting the data, we found that the risk of EC development in cases with PPAR γ /ERR α ≤ 1.86 was higher than that in cases with PPAR γ /ERR α > 1.86 (OR = 14.847, 95% CI = 1.6-137.748, $P=0.018$).

Prognostic value of the PPAR γ /ERR α expression pattern

The 5-year OS rates were significantly higher for patients with PPAR γ -positive disease than for those with PPAR γ -negative disease (100.00% vs. 88.24%; $p=0.001$). The difference between the 5-year DFS rates for PPAR γ -positive and PPAR γ -negative patients was also significant (100.00% vs. 82.35%; $P<0.001$) (Figure 5B). However, no difference in OS or DFS was found between ERR α -positive and ERR α -negative patients. When using PPAR γ /ERR α as a predictor of prognosis, we found that OS and DFS were lowest for PPAR γ -negative and ERR α -positive patients (OS: 100.00% vs. 85.19%, $P<0.001$; DFS: 100.00% vs. 77.78%, $P<0.001$, Figure 5B).

DISCUSSION

We found that the expression of PPAR γ , whether at the transcriptional (mRNA) or posttranscriptional (protein) level, was reduced in EC, which is consistent with other reports [9, 19–23]. However, several studies also showed that PPAR γ expression was more marked in carcinoma tissues of several types of human malignancies than in normal tissues [10–12]. PPAR γ has been associated with clinicopathological variables, such as EC stage, which is in agreement with previous studies [19]. The results of our study are also consistent with these results, which support the hypothesis that the PPAR γ gene is a tumor suppressor gene, and that dysfunction of PPAR γ contributes to tumorigenesis [7, 24, 25]. In our present study, strong ERR α immunoreactivity was detected in endometrial carcinoma tissues. ERR α mRNA expression was also higher in carcinoma tissues than in normal tissues. Similar results have also been reported in other cancers [26–28]. No significant associations between ERR α and clinicopathological variables were identified, although the number of cases involved in our research was limited, and this finding is inconsistent with those of some studies [10, 12, 28]. Fujimoto J found that the expression of ERR α was upregulated with tumor progression involving dedifferentiation and myometrial invasion [14]. Our analysis of PPAR γ and ERR α expression highlighted differences between benign and malignant tissues. We further confirmed that a significant negative correlation exists between PPAR γ expression and ERR α expression in women with EC. Therefore, we believe that crosstalk may occur between PPAR γ and ERR α in EC. To the best of our knowledge, this is the first study to analyze the correlation between PPAR γ and ERR α in EC patients, and negative correlations between PPAR γ and ERR α were observed. Thus, PPAR γ may be a protective factor against EC, while ERR α may be an adverse factor for endometrial carcinogenesis.

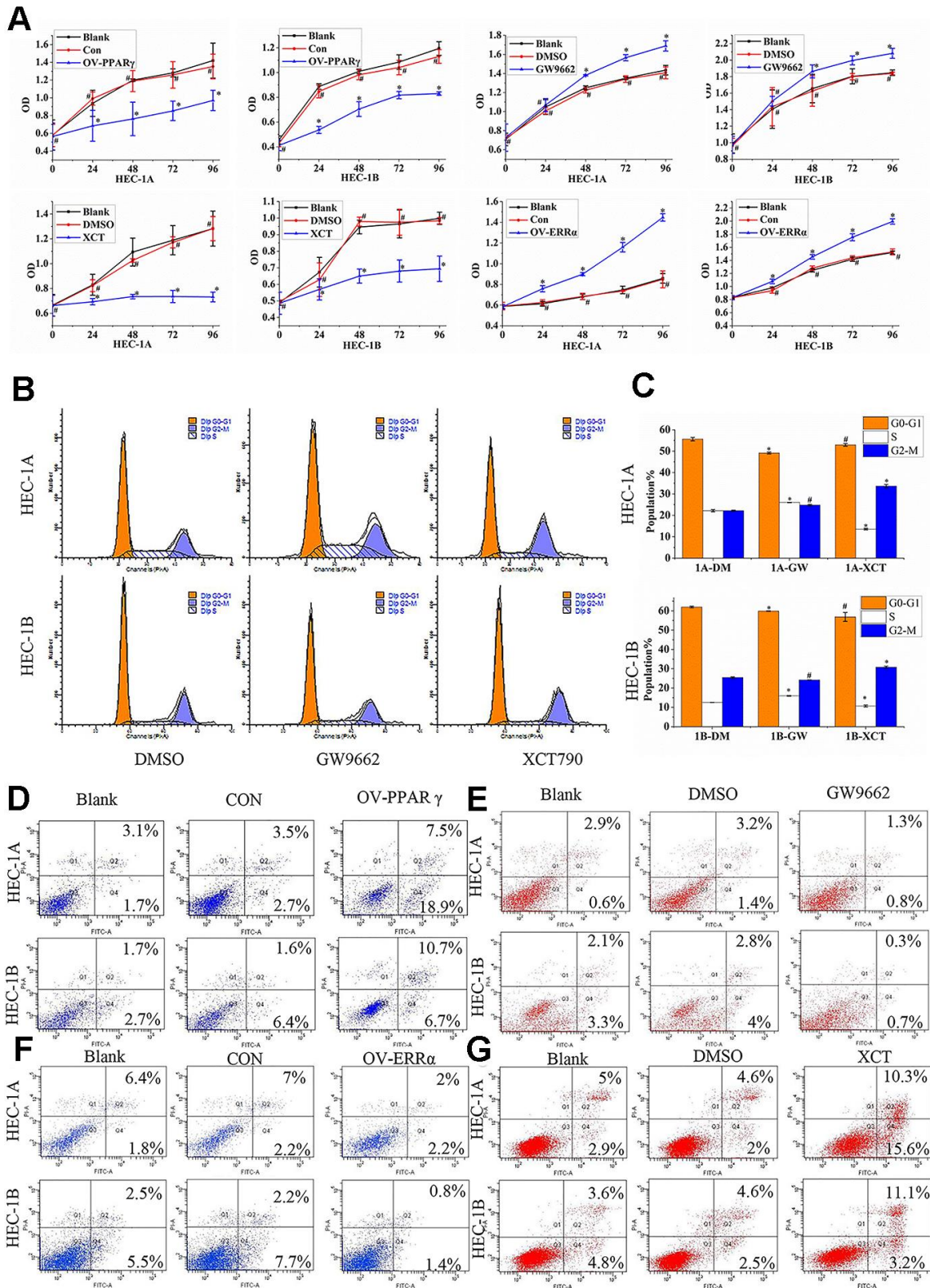


Figure 4. PPAR γ and ERR α compete to control cell proliferation and promote apoptosis in EC cells. (A) The effect of OV-PPAR γ , OV-ERR α , GW9662 or XCT790 on proliferation. The OD values of HCE-1A and HEC-1B cells were detected at 0, 24, 48, 72, and 96 h after transfection with lentivirus or treatment with ERR α and PPAR γ antagonists. (B, C) The effect of GW9662 or XCT790 on the cell cycle. HEC-1A and HEC-1B cells were treated with DMSO, GW9662 (5 μ M) or XCT790 (10 μ M) for 72 h. (D, E) The effect of OV-PPAR γ or GW9662 on apoptosis. (F, G) The effect of OV-ERR α or XCT790 on apoptosis. OV-PPAR γ : overexpression of PPAR γ ; OV-ERR α : overexpression of ERR α . EC, endometrial cancer. *, P < 0.05; #, P > 0.05.

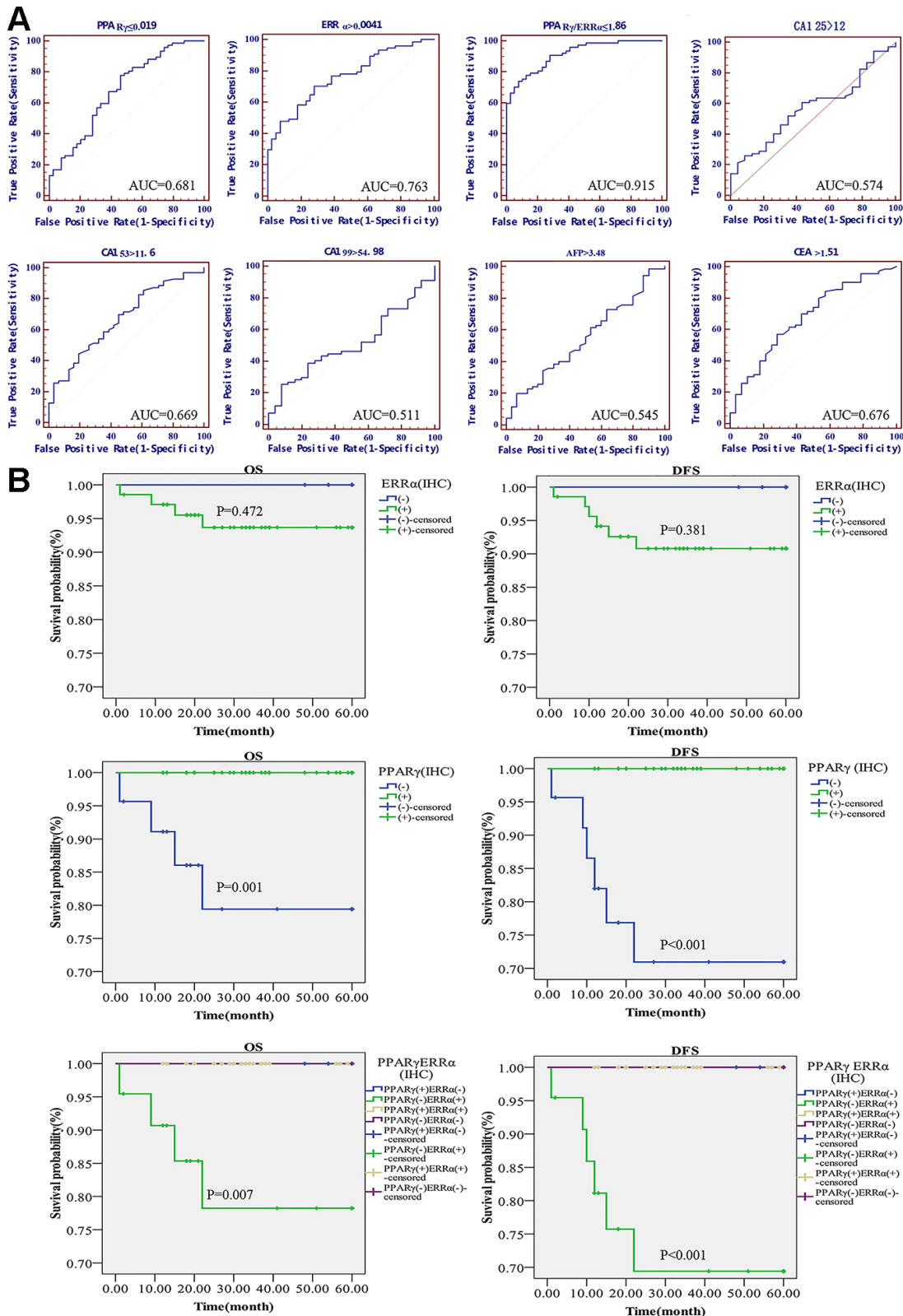


Figure 5. Diagnostic and prognostic value of the PPAR γ /ERR α ratio for EC. (A) ROC curves of the mRNA expression of PPAR γ , the mRNA expression of ERR α , the PPAR γ /ERR α ratio, CA125, CA199, CA153, CEA, and AFP. (B) DFS and OS in EC patients with different expression patterns of PPAR γ /ERR α . Patients with PPAR γ (+)/ERR α (-) had longer DFS and OS. The mRNA expression of PPAR γ and ERR α was quantified using the 2^{- Δ CT} method. PPAR γ /ERR α was quantified as the ratio of the mRNA expression of PPAR γ to the mRNA expression of ERR α . ROC, receiver operating characteristic; DFS, disease-free survival. OS, overall survival.

Table 2. Logistic regression model: risk factors for endometrial cancer.

Variables	OR	95%CI	P ^a	OR	95%CI	P ^b
Age						
≤55	1(R)	-		1(R)	-	
>55	33.366	4.358-255.432	0.001	4.49	0.126-160.285	0.41
BMI						
<25	1(R)	-		1(R)	-	
≥25	6.48	1.825-23.014	0.004	11.363	0.354-365.035	0.17
Parity						
≤1	1(R)	-		1(R)	-	
>1	3.777	1.642-8.688	0.002	0.22	0.028-1.712	0.148
Gravidity						
≤2		-			-	
>2	13.667	3.877-48.181	<0.001	1.571	0.069-35.596	0.777
Pausimenia						
No	1(R)	-		1(R)	-	
Yes	11.1	3.149-39.124	<0.001	4.703	0.362-61.067	0.237
Hypertension						
No	1(R)	-		1(R)	-	
Yes	9.99	2.234-44.677	0.003	2.93	0.07-122.652	0.573
Hyperlipaemia						
No	1(R)	-		1(R)	-	
Yes	9.99	2.234-44.677	0.003	0.218	0.003-13.557	0.47
Diabetes mellitus						
No	1(R)	-		1(R)	-	
Yes	13.333	1.717-103.56	0.013	0.603	0.015-24.505	0.789
CA153						
≤31.3	1(R)	-		1(R)	-	
>31.3	1.122	1.021-1.233	0.017	1.007	0.131-6.682	0.835
CEA						
≤5	1(R)	-		1(R)	-	
>5	2.055	1.12-3.771	0.02	0.955	0.944-1.073	0.925
CA125						
≤35	1(R)	-		-	-	
>35	1.001	0.997-1.005	0.74	-	-	-
CA199						
≤37	1(R)	-		-	-	
>37	1.004	0.996-1.012	0.347	-	-	-
AFP						
≤8.78	1(R)	-		-	-	
>8.78	1.237	0.821-1.864	0.309	-	-	-
PPAR γ^c						
>0.019	1(R)	-		1(R)	-	
≤0.019	0.243	0.106-0.556	0.001	0.034	0.001-2.075	0.107
ERR α^d						

≤0.0041	1(R)	-	<0.001	1(R)	-	0.14
>0.0041	5.976	2.551-13.999		16.66	0.397-699.901	
PPAR γ /ERR α ^e						
>1.86	1(R)	-	<0.001	1(R)	-	0.018
≤1.86	32	8.896-115.107		14.847	1.6-137.748	

^aThe results were evaluated using univariate analysis. ^bThe results were evaluated using logistic regression analysis. ^cThe mRNA expression of PPAR γ was quantified using 2^{- Δ CT} method. ^dThe mRNA expression of ERR α was quantified using 2^{- Δ CT} method. ^ePPAR γ /ERR α was quantified the ratio of mRNA expression of PPAR γ and ERR α . FBG, fasting blood glucose.

To date, little is known about the crosstalk between PPAR γ and ERR α in EC. We found a significant negative correlation between PPAR γ and ERR α expression in women with EC. Janice M. Huss et al. found that ERR α activated PPAR α gene expression by directly binding to the PPAR α gene promoter to control energy metabolism in cardiac and skeletal muscles [29]. PPARs belong to the nuclear receptor (NR) superfamily. Different subtypes of PPARs, including PPAR α , PPAR β/δ , and PPAR γ , have similar structures. To the best of our knowledge, no studies have proven beyond question that ERR α can activate PPAR α gene expression. Wei W et al. found conserved PPREs in the ERR α promoter [13]. When activated by ligand binding, PPAR γ forms a heterodimer with another NR, retinoid X receptor (RXR), and then activates gene expression by binding to PPRE. Therefore, PPAR γ may inhibit the expression of ERR α by combining with PPRE. Wei W et al. also highlighted a functional link between PPAR γ and ERR α pathways, namely, their convergence at the transcriptional coactivator PGC-1 β [13]. Through a STRING protein interaction analysis, we found a large number of cofactors shared by PPAR γ and ERR α , including PGC-1 α , NCOA1, NCOA2, and CREBBP. PGC-1 α protein was originally shown to be a coregulator of PPAR γ [30], and ERR α has also recently been shown to serve as a functional partner for the PGC-1 family [31, 32], the members of which have emerged as key regulators of mitochondrial metabolism and biogenesis [33, 34]. PGC-1 α proteins likely act as surrogate protein ligands for ERRs in regulating the expression of mitochondrial genes and other gene sets involved in maintaining energy homeostasis, as well as a region of retinoic acid receptor α (RAR α) [35]. They also activate the activity of a large number of RXR chaperones involved in glucose and lipid metabolism [36]. PPAR γ plays important roles in the regulation of several metabolic pathways, including lipid biosynthesis and glucose metabolism. RXR acts as a mandatory partner for PPAR γ [35]. PPAR γ forms heterodimers with RXR and develops into a complex intact PPAR γ -RXR α structure on DNA [37]. Therefore, a tendency toward mutual repression can be speculated to exist between PPAR γ and ERR α . PPAR γ and ERR α

may compete for the same coactivators, including RXR or PGC-1 α , to perform their transcriptional functions.

To further explore the possible interaction between PPAR γ and ERR α , we also explored the relationship between PPAR γ and ERR α in EC cells. Interestingly, when the expression of PPAR γ was upregulated by lentiviral-mediated overexpression constructs, the expression of ERR α was decreased. In contrast, when the expression of PPAR γ was inhibited by the specific inhibitor GW9662, the expression of ERR α was increased. Moreover, we found that PPAR γ expression could also be inhibited by activating ERR α expression. Upregulating ERR α expression could inhibit PPAR γ expression in EC cells. After treatment with XCT790, which is an ERR α -specific antagonist [38], PPAR γ expression was enhanced in EC cells. Our work is the first to report that PPAR γ and ERR α can inhibit one another through a negative feedback loop in EC cells. Our previous study showed that XCT790 can inhibit proliferation and promote apoptosis of EC cells by targeting ERR α [15]. However, we did not discuss the detailed mechanism in our previous work. After data mining based on bioinformatics analysis, we found that the levels of both Bcl2 and Caspase3, which are associated with cellular proliferation and apoptosis, were increased or decreased by PPAR γ and ERR α . Furthermore, we confirmed that Bcl2 expression was inhibited, and that Caspase3 expression was promoted after PPAR γ activation or ERR α inhibition *in vivo*. Bcl2 expression was promoted, and Caspase3 expression was inhibited after PPAR γ was downregulated or after ERR α was upregulated in EC cells. This result was also partly proven by Woo CC et al. [39]. They found that thymoquinone can increase PPAR γ activity and downregulate the expression of Bcl2 and Bcl-xL in breast cancer [39]. Weng JR et al. also found that 3 β , 7 β -dihydroxy-25-methoxycucurbita-5,23-diene-19-al (DMC) can induce PPAR γ activation and suppress some PPAR γ -targeted signals, including cyclin D1, CDK6, Bcl-2, XIAP, cyclooxygenase-2, NF- κ B, and estrogen receptor α , in breast cancer cells [40]. PPAR γ activation inhibits Bcl-2 expression, which promotes the mitochondrial release of cytochrome C and initiates

apoptosis. Cytochrome C activates Caspase9, which activates downstream Caspase3 and induces apoptosis [41]. Zhang Y et al. found that PGC-1 α overexpression in the human epithelial ovarian cancer cell line HO-8910 induced cell apoptosis through coordinated regulation of Bcl-2 and Bax expression in a PPAR γ -dependent pathway [42]. Wang M et al. found that PGC-1 α can increase the expression of Bcl-2 and promote the survival of mesenchymal stem cells via the PGC-1 α /ERR α interaction [43]. Based on the above studies and our experimental results, increasing the expression of PPAR γ and decreasing the expression of ERR α can be concluded to have the same effect on downstream signals, such as Bcl2 and Caspase3. PPAR γ and ERR α may compete to control these signals by suppressing the function of one another. Apoptosis induced by PPAR γ activation or ERR α inhibition still requires further verification. GO and KEGG enrichment bioinformatics analyses indicated that many mutual downstream target genes of PPAR γ and ERR α participate in various biological functions, including exocytosis, gene silencing, lipid transport, protein transport, cell proliferation and apoptosis. Whether these two transcription factors compete for the same downstream cofactors requires further study. Simultaneous PPAR γ activation and ERR α inhibition may amplify this pro-apoptotic effect and improve the prognosis of EC.

Although most ECs can be detected in the early stage by means of abnormal uterine bleeding after menopause, some patients have unusual patterns of regional and systemic recurrence [44]. Moreover, EC-specific tumor markers are still lacking in clinical practice. Our study confirmed that PPAR γ is a protective factor against EC, and that ERR α is a poor prognostic factor for EC in the same population. We further discuss their potential roles as EC tumor biomarkers. Although the ROC curves indicated that ERR α was superior to PPAR γ for diagnosing EC, PPAR γ was more suitable than ERR α for predicting the survival of EC patients. Both PPAR γ and ERR α induce or inhibit the occurrence of EC and showed greater accuracy and effectiveness as markers of EC than other known serum biomarkers, such as CA125 and CA199. However, neither PPAR γ nor ERR α alone can serve as an independent risk biomarker to predict the occurrence of EC. Encouragingly, regarding the prediction of EC occurrence, the PPAR γ /ERR α ratio is superior not only to PPAR γ or ERR α alone but also to currently used clinical serum tumor markers, namely, CA125, CA199, CA153, AFP and CEA. Moreover, surprisingly, the AUC of PPAR γ /ERR α for diagnosing EC reached 0.915, with an optimal threshold ratio less than 1.86. In addition, the expression patterns of PPAR γ and ERR α may be used to predict the prognosis of patients with EC. Both OS and DFS were lowest for EC patients with

PPAR γ (-)/ERR α (+) disease. Due to limitations in our study scale, this result should be further confirmed by more studies. However, the combination of PPAR γ and ERR α can be used not only to diagnose but also to predict the prognosis of EC.

In conclusion, the expression and immunoreactivity of PPAR γ were found to be negatively correlated with the expression and immunoreactivity of ERR α in EC. PPAR γ and ERR α compete to control downstream Bcl2/Caspase3 signals to activate or inhibit EC cell proliferation and apoptosis. A dynamic imbalance in PPAR γ /ERR α leads to endometrial carcinogenesis. A PPAR γ /ERR α ratio \leq 1.86 is an independent risk factor for endometrial carcinogenesis. Moreover, PPAR γ (+) EC patients have a favorable prognosis, but PPAR γ (-)/ERR α (+) patients have the worst prognosis in terms of both OS and DFS. Simultaneous PPAR γ activation and ERR α inhibition may be beneficial in terms of therapeutic efficacy against EC and improve the disease prognosis.

MATERIALS AND METHODS

Study population

This study was approved by the ethics committee of Fujian Maternity and Child Health Hospital (FMCH2018-13), Affiliated Hospital of Fujian Medical University. All specimens were collected from 2011.9 to 2017.9, and the patients provided informed consent. The exclusion criteria were as follows: 1. patients with a history of other malignancies; 2. patients with nonepithelial cancers of the uterus, such as carcinosarcoma; 3. patients treated with chemotherapy, radiotherapy or hormone therapy before surgery; 4. patients missing clinical pathology data or with an unclear diagnosis; 5. patients who did not agree to further analysis of their pathological tissue; and 6. patients with a pathological follow-up inconsistent with the original pathological results. The clinical stage of each cancer case was reviewed and diagnosed again according to the FIGO Stage 2014 classification (48 cases of stage I; 13 cases of stage II; 15 cases of stage III; 1 case of stage IV). Thirty cases of well-differentiated endometrioid adenocarcinoma of the endometrium (G1), 27 cases of moderately differentiated endometrioid adenocarcinoma (G2), and 6 cases of poorly differentiated endometrioid adenocarcinoma (G3) were identified. In the same period, 39 samples of endometrial tissue were collected from patients with other benign gynecological diseases. Clinical characteristics, including age, BMI, gravidity, parity, menopause status, and complicated disease cases, including EC with a history of diabetes, hypertension or hyperlipemia, were obtained. Serum tumor markers, including CA125, CA153, CA199, CEA, and AFP, were measured two days before surgery.

Tissue microarray

Morphological observation to identify typical lesions and markers was performed using hematoxylin and eosin (H&E)-stained sections. Tissue microarray (TMA) sections measuring 1.5 mm in diameter were subsequently dewaxed with xylene/ethanol for immunohistochemical staining.

Immunohistochemistry

Immunohistochemical staining was carried out on 4- μ m-thick sections. The sections were washed with a gradient ethanol/water mixture and then with distilled water. Next, the samples were immersed in citrate buffer (pH 9), boiled for 10 min and treated with 3% hydrogen peroxidase to block endogenous peroxidase activity. A 5% BSA blocking solution was added after antigen retrieval, followed by incubation for 35 min at room temperature. The primary antibody dilutions used in the present study were as follows: 1:25 for PPAR γ (ab45036) and 1:100 for ERR α (ab87980) (both from Abcam, Cambridge, MA, USA). Primary antibody incubation was performed overnight at 4°C in a humidified chamber. The results were evaluated with a semiquantitative integration method and scored according to the staining range and staining intensity. The slides were evaluated by 2 of the authors who were blinded to the patient characteristics and outcomes using a standard light microscope. A semiquantitative grading system incorporating staining intensity (score, 0–3) and tumor area with positive staining (0, 0%; 1, 1%–25%; 2, 26%–50%; 3, 51%–75%; and 4, 76%–100% of tumor cells) was applied. The staining index (SI) was calculated as the product of staining intensity and area and ranged from 0 to 12, as described in several publications [45–47]. Immunohistochemical activity was stratified by the SI (-, 1–2; +, 3–4; ++, 5–8; and +++, 9–12). Immunological activities were classified as positive (+, ++, +++) and negative (-).

Cell culture

The human endometrial carcinoma cell lines RL-952, ECC-1, HEC-1A, and HEC-1B were purchased from the American Type Culture Collection (ATCC) and maintained in our laboratory. All cell lines were identified by BIONG Biotechnology, Shanghai, China. All cells used were the most common *in vitro* EC cell models. The RL-952 and ECC-1 cell lines were positive for ER α expression, but the HEC-1A and HEC-1B cell lines were negative for ER α expression. These model differences created conditions for exploring the relevance of PPAR γ and ERR α . RL-952 cells were cultured in DMEM/F12 medium supplemented with 0.005 mg/mL insulin and 10% fetal bovine serum (FBS), and ECC-1 cells were

cultured in F12 medium supplemented with 10% FBS. HEC-1A and HEC-1B cells were cultured in high-glucose DMEM supplemented with 10% FBS at 37°C in a 5% CO₂ atmosphere. All culture media were purchased from Thermo Fisher Scientific.

Quantitative real-time polymerase chain reaction (qRT-PCR)

All RNA was extracted according to the TRIzol reagent protocol (Invitrogen, Carlsbad, CA, USA). Briefly, 1 μ g of DNase I-treated RNA was reverse transcribed into complementary DNA (cDNA) using a reverse transcription kit (Promega). The PCR primer sets used were as follows: ERR α , sense 5'-ACC GAG AGA TTG TGG TCA CCA-3', antisense 5'-CAT CCA CAC GCT CTG CAG TACT-3' (101 bp); glyceraldehyde 3-phosphate dehydrogenase (GAPDH, control), sense 5'-GCA CCG TCA AGG CTG AGA AC-3', antisense 5'-TGG TGA AGA CGC CAG TGGA-3' (138 bp); and PPAR γ , sense 5'-AATGGAAGACCACTCCCCT-3', antisense 5'-GGTACTCTTGAAGTTTCAGGTC-3' (152 bp). Experiments were carried out in triplicate and repeated twice. The relative level of ERR α mRNA was quantified by the image-intensity ratio of the target gene mRNA to GAPDH mRNA. The target gene mRNA level was quantified using a comparative method ($2^{-\Delta\Delta CT}$ method) and normalized to GAPDH expression.

Western blot analysis

Cell lysates were prepared with a lysis/extraction reagent (Sigma). Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce). Protein samples (30 μ g) were resolved by 12% SDS-polyacrylamide gel electrophoresis, blotted onto PVDF membranes and blocked with TBS-Tween 0.1% containing 5% nonfat dry milk at room temperature for 1 h. Blotted membranes were incubated with anti-human PPAR γ rabbit monoclonal antibody (1:500 dilution, Abcam, Cambridge, MA, USA) or anti-human ERR α rabbit monoclonal antibody (1:300 dilution, Abcam) overnight at 4°C. β -Actin rabbit monoclonal antibody (1:1000 dilution; Beyotime, Shanghai, China) was used as the control. An enhanced chemiluminescence (ECL) detection system (Thermo) was used to visualize the bands.

Lentivirus-mediated vector construction and infection

Full-length cDNA plasmids for PPAR γ (PPARG, NM_015869) and ERR α (ESRRA, NM_004451) were purchased from Genechem (Shanghai, People's Republic of China) and cloned into a lentivirus-based vector carrying the green fluorescent protein (GFP)

gene (GV358, Genechem, Shanghai, China) with the following component sequence: Ubi-MCS-3FLAG-SV40-EGFP-IRES-puromycin. EC cell lines transfected with the lentiviral vector were used as negative control (NC) groups, and cell lines without lentivirus treatment were used as blank controls. The lentiviral vector constructs carrying PPAR γ , ERR α and NC were used to infect EC cells at a multiplicity of infection (MOI) of 100. After 72 h of infection, GFP expression was detected to calculate the infection efficiency. Cells were harvested at an infection rate >90%.

Data mining based on bioinformatics analysis

The expression of PPAR γ and ERR α in EC was determined using the TCGA database from UALCAN (<http://ualcan.path.uab.edu/>). The interaction between PPAR γ and ERR α was analyzed based on the search tool for the retrieval of interacting genes/proteins (STRING, <https://string-db.org/cgi/input.pl>) database. Downstream target genes common to PPAR γ and ERR α were screened in the Gene Transcription Regulation Database (<http://gtrd.biouml.org/>). The ModFit of PPAR γ and ERR α was based on the JASPAR database, which provides the most comprehensive public data on transcription factors and DNA binding site motifs (<http://jaspar.binf.ku.dk/>). The selected genes were subjected to gene function annotation based on the GO database to analyze the significant functions exhibited by the coexpressed genes. Pathway analysis was based on the KEGG database. GO and KEGG analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, <https://david.ncifcrf.gov/home.jsp>). Significant screening criteria were set at $P < 0.05$.

Drug treatment and cell proliferation assay

Cells were cultured in 96-well plates, and XCT790 and GW9662 purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) were used for the experiments. XCT790 is a specific inhibitor of ERR α , and GW9662 is a PPAR γ antagonist. Previous studies have found that the optimal concentration of XCT790 in EC cells is 10 μ M. Through preliminary experiments, the optimal drug concentration of GW9662 was determined to be 5 μ M. XCT790 and GW9662 were dissolved in dimethyl sulfoxide (DMSO) according to the manufacturer's instructions. Drug-treated cells were used as the experimental groups, drug-free DMSO-treated cells were used as the NC groups, and untreated cells were used as the blank control groups. The cells in each group were treated with 10 μ l of CCK-8 solution (MedChemExpress, China) for 0 h, 24 h, 48 h, 72 h, and 96 h, and after 1 h in an incubator, the absorbance values at 450 nm were measured by a microplate reader.

Apoptosis analysis

For flow cytometric analysis, all cells treated with GW9662, XCT790 or lentivirus were seeded into 6-well plates. When the cells reached 80% confluence, trypsin with no EDTA was added, and the cells were harvested. After centrifugation, the cell pellets were washed twice with precooled phosphate-buffered saline (PBS). Cells were resuspended in buffer at 10^6 cells/mL. Then, cells were stained with an Annexin-V-FLUOS or 7-AAD staining kit (BD, USA) according to the manufacturer's instructions. Apoptotic cells were analyzed using the FACS Canto II flow cytometer (BD, USA) and Diva software (BD, USA). All experiments were performed in triplicate. Data were analyzed using the ModFit LT (Verity Software, Topsham, ME, USA) and Cell Quest (BD Biosciences) software packages.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 or MedCalc software for Windows. A P value less than 0.05 was considered statistically significant. The results were evaluated using the T-test, Mann-Whitney U test, or Wilcoxon test. Categories were compared using the Pearson χ^2 or Fisher's exact test where appropriate. ROC curves were used to evaluate the diagnostic value of PPAR γ and ERR α mRNA expression. A logistic model was used to determine the risk of EC. Univariate survival analyses of the time to death due to endometrial carcinoma (OS and DFS) were conducted using the Kaplan-Meier method. The entry date was the date of primary surgery. Patients who died from other causes were censored at their date of death. Differences in survival between groups were estimated by 2-sided log-rank (Mantel-Cox) tests. The results were considered significant when $P < 0.05$.

Abbreviations

BCA: bichinchonic acid; CCK-8: Cell Counting Kit-8; CREBBP: cyclic adenosine monophosphate (cAMP) response element-binding protein; DFS: disease-free survival; EC: endometrial cancer; ECL: enhanced chemiluminescence; ERR α : oestrogen-related receptor α ; FBG: fasting blood glucose; GFP: green fluorescent protein; HDL: high-density lipoprotein; H&E: haematoxylin and eosin; LDL: low-density lipoprotein; MOI: multiplicity of infection; NC: negative control; NCOA1: Nuclear receptor coactivator 1; OS: overall survival; PBS: phosphate-buffered saline; PGC-1 α : PPAR γ co-activator-1 α ; PPAR γ : peroxisome proliferator-activated receptor γ ; PPRE: PPAR response element; qRT-PCR: quantitative real-time polymerase chain reaction; RAR α : retinoic acid receptor α ; ROC: receiver operating characteristic; RXR: retinoid X

receptor; TC: total cholesterol; TG: triglyceride; TMA: Tissue microarray.

AUTHOR CONTRIBUTIONS

Meimei Huang contributed toward performing experiments, drafting and revising the paper, images typesetting, data analysis and experimental design. Lili Chen performed experiments and participated in data collection and follow-up. Xiaodan Mao was responsible for supervising, guiding the experiment and modifying the article. Guifen Liu participated in bioinformatics analysis. Yuqin Gao were responsible for data collection and follow-up. Xiaoqing You and Min Gao were responsible for guiding statistical analysis. Jalid sehouli participated in reviewing the article. Pengming Sun applied and got fund support, reviewed and modified the article and was responsible for experimental design.

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CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest.

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