


# Comprehensive Analysis of ESRRA in Endometrial Cancer

Technology in Cancer Research & Treatment  
Volume 20: 1-9  
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DOI: 10.1177/1533033821992083  
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## Abstract

**Background:** Estrogen-related receptor alpha (ESRRA) was reported to play an important role in multiple biological processes of neoplastic diseases. The roles of ESRRA in endometrial cancer have not been fully investigated yet. **Methods:** Expression data and clinicopathological data of patients with uteri corpus endometrial carcinoma (UCEC) were obtained from The Cancer Genome Atlas (TCGA). Comprehensive bioinformatics analysis was performed, including receiver operating characteristics (ROC) curve analysis, Kaplan-Meier survival analysis, gene ontology (GO) enrichment analysis, and Gene Set Enrichment Analysis (GSEA). Immunohistochemistry was used to detect the protein expression level of ESRRA and CCK-8 assay was performed to evaluate the effect of ESRRA on the proliferation ability. **Results:** A total of 552 UCEC tissues and 35 normal tissues were obtained from the TCGA database. The mRNA and protein expression level of ESRRA was highly elevated in UCEC compared with normal tissues, and was closely associated with poor prognosis. ROC analysis indicated a very high diagnostic value of ESRRA for patients with UCEC. GO and GSEA functional analysis showed that ESRRA might be mainly involved in cellular metabolism processes, in turn, tumorigenesis and progression of UCEC. Knockdown of ESRRA inhibited the proliferation of UCEC cells in vitro. Further immune cell infiltration demonstrated that ESRRA enhanced the infiltration level of neutrophil cell and reduced that of T cell (CD4<sup>+</sup> naïve), NK cell, and cancer associated fibroblast (CAF). The alteration of immune microenvironment will greatly help in developing immune checkpoint therapy for UCEC. **Conclusions:** Our study comprehensively analyzed the expression level, clinical value, and possible mechanisms of action of ESRRA in UCEC. These findings showed that ESRRA might be a potential diagnostic and therapeutic target.

## Keywords

ESRRA, endometrial cancer, the cancer genome atlas, prognosis, diagnosis, bioinformatics

## Abbreviations

ESRRA, Estrogen-related receptor alpha; ACS, American Cancer Society; ESRR, estrogen-related receptor; TCGA, The Cancer Genome Atlas; UCEC, uteri corpus endometrial carcinoma; FPKM, Fragments per kilobase million; ROC, Receiver operating characteristics; AUC, area under the ROC curve; GO, gene ontology; BP, biological processes; CC, cell component; MF, molecular function; GSEA, Gene Set Enrichment Analysis; NES, normalized enrichment score; siRNAs, small interfering RNAs; CAF, cancer associated fibroblast; PR, progesterone receptor; AR, androgen receptor.

Received: September 15, 2020; Revised: December 30, 2020; Accepted: January 14, 2021.

## Background

Endometrial cancer is the most commonly diagnosed reproductive cancer in women.<sup>1</sup> Based on the GLOBOCAN,<sup>2</sup> there were an estimated 382,069 new cases of endometrial cancer in 2018 across the globe. According to American Cancer Society (ACS), new cases of endometrial cancer in the United States would reach about 61,880 in 2019.<sup>3</sup> It is classified into 2 histological types, including type I and type II. The former, estrogen-dependent endometrioid adenocarcinomas, is the

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most common endometrial cancer, approximately accounting for 75-90% of cases.<sup>4</sup> Type II is referred to estrogen-independent endometrial cancer, and principally includes clear cell carcinoma and mucinous adenocarcinoma.<sup>5</sup> Generally, endometrial cancer has been reported to have a relatively favorable prognosis.<sup>6</sup> The 5-year survival rate of early-stage endometrial cancer patients is up to 96%.<sup>7</sup> However, delayed diagnosis, recurrence and metastasis will lead to poor clinical outcomes. The 5-year survival rate of patients with stage IV endometrial cancer is only 17%.<sup>7</sup> Thus, more effective therapy options are needed to improve their prognosis.

Estrogen-related receptor alpha (ESRRA) is located at chromosome 11q13 and encodes a nuclear 46 kDa protein.<sup>8</sup> It is a transcription factor that belongs to one of estrogen-related receptor (ESRR) family, and contains 5 domains: a N-terminal domain, a C-terminal domain, a DNA binding domain, a ligand binding domain and a hinge region domain.<sup>9</sup> Studies have found that ESRRA participated in numerous metabolic pathways, including glucose and lipid metabolism.<sup>10</sup> Furthermore, ESRRA also played an important role in multiple biological processes of neoplastic diseases. ESRRA and Gremlin-1 (GREM1) could form a positive feedback loop to promote the growth and invasiveness of breast cancer cells.<sup>11</sup> Yixin et al. showed that ESRRA directly acted on CPT1C to influence cell proliferation and metabolism of breast cancer.<sup>12</sup> In prostate cancer, ESRRA and oncogenic transcription factor ERG cooperatively enhanced the growth of cancer cells.<sup>13</sup> To date, however, few reported the role of ESRRA in endometrial cancer. Recently, a novel risk score system developed by Hongyu et al.,<sup>14</sup> including ESRRA and 13 other hub genes, had been shown to be prognostic in endometrial cancer.

Herein, we aimed to perform an evaluation of the expression levels, prognostic role, and possible involved mechanism of ESRRA in endometrial cancer using the data from The Cancer Genome Atlas (TCGA). TCGA is one of the largest available gene databases and comprises 33 different human cancer types, which provides comprehensive molecular profiles for each cancer type (e.g. genomics, transcriptomics, and proteomics).<sup>15,16</sup> It is expected to provide novel evidence for clinical diagnosis and treatment of endometrial cancer.

## Methods

### Data Collection and Clinicopathological Characteristics

Expression data and clinicopathological data of patients with uteri corpus endometrial carcinoma (UCEC) were obtained from the TCGA database (<https://portal.gdc.cancer.gov/>). Fragments per kilobase million (FPKM) RNA-seq data was used for the following analysis. Patients were divided into high expression group (top 50%) and low expression group (last 50%) according to the mRNA expression level of ESRRA.

Receiver operating characteristics (ROC) curve analysis was used for assessing the diagnostic ability of ESRRA, and area under the ROC curve (AUC), cut-off value, sensitivity, and specificity were calculated separately. The prognostic

effect of ESRRA was assessed by the Kaplan-Meier plotter (<https://kmplot.com>).

UCEC tissues and normal endometrial tissues from 18 patients were collected and made into 3  $\mu$ m sections for immunohistochemical staining. The sections were incubated with the polyclonal antibody against ESRRA (1:100 dilution, Santa Cruz Biotechnology), then 4 degrees overnight. They were further incubated with the secondary antibody (horseradish peroxidase, HRP). All the sections were observed under the microscope. Immunostaining scores were reported by 2 independent pathologists. The intensity scores were defined as: 0, negative; 1, weak; 2, medium; and 3, strong. The percentage scores of staining were defined as: 0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. The final staining scores were obtained by the sum of the intensity and percentage scores.

Clinicopathological data of UCEC patients included age, grade, clinical stage, and follow-up data. All the factors together with ESRRA expression were further subjected to univariate and multivariate Cox regression analysis. The significant level of p-value was 0.05.

### GEPIA and LinkedOmics

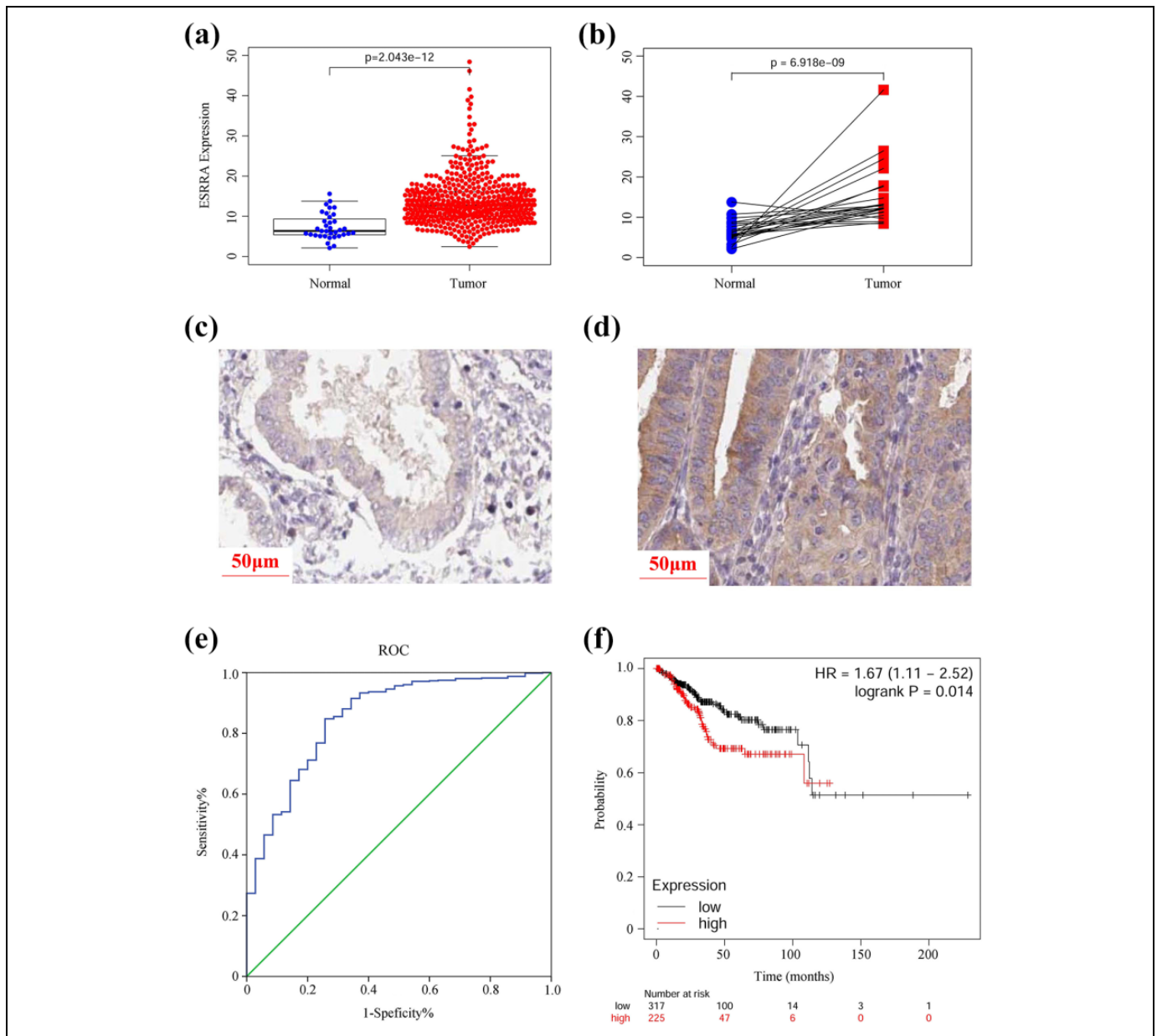
The Gene Expression Profiling Interactive Analysis (GEPIA) database (<http://gepia.cancer-pku.cn/>) is an online website and was used to assess the correlation between clinical stage and the ESRRA expression. The LinkedOmics database (<http://www.linkedomics.org/>) is a publicly available online bio-metrics platform and was used to identify ESRRA-associated genes, including up-regulated genes and down-regulated genes.

### GO and GSEA

The gene ontology (GO) terms included biological processes (BP), cell component (CC) and molecular function (MF). GO enrichment analysis was conducted in R package gProfileR (version 0.6.434). Gene Set Enrichment Analysis (GSEA) (<http://www.broad.mit.edu/gsea>) was performed for pathway analysis. The criterion for significance was set at a normalized enrichment score (NES)  $\geq 1.0$  and a p-value less than 0.05.

### Cell Transfection and CCK-8 Assay

Small interfering RNAs (siRNAs) targeted at ESRRA (s4829, s4830, and s4831) were purchased from ThermoFisher. Transfections were performed in the endometrial cancer cell line ECC-1 with siRNAs using Lipofectamine 2000 Transfection Reagent (Invitrogen). Transfected cells were harvested at 48 h post transfection. The transfection efficiency was detected by quantitative real-time PCR (qRT-PCR). Western Blot assay was performed according to the previously described method.<sup>17</sup> Briefly, equal amounts of protein from transfected cells were loaded and transferred to a polyvinylidene difluoride membrane. Blots were incubated overnight with primary antibody rabbit anti-ESRRA (1:1000; Invitrogen). Subsequently, goat secondary anti-rabbit IgG antibody (Santa Cruz Biotechnology)



**Figure 1.** Expression and clinical roles of ESRRA in UCEC. (a) The mRNA expression level of ESRRA in entire cohort (552 UCEC tissues and 35 normal tissues) from the TCGA database ( $p < 0.001$ ). (b) The mRNA expression level of ESRRA in 23 paired samples from the TCGA database ( $p < 0.001$ ). (c) Expression of ESRRA protein in normal endometrial tissues. (d) Expression of ESRRA protein in UCEC tissues. (e) ROC curve analysis and corresponding AUC was 0.854 ( $p < 0.001$ ). (f) Kaplan-Meier survival analysis. The black line represents ESRRA-low expression group and red represents ESRRA-high expression group. The survival difference was determined by a log-rank test.

was used. Western Blot results were quantified with ImageJ (National Institutes of Health). The siRNA (s4831) with the optimal transfection efficiency was selected to perform the CCK-8 assay. Transfected cells were adjusted to a density of  $5 \times 10^3$  cells/well and then plated in 96-well plates with 3 replicate wells. Cells were further cultured for 24-96 h at  $37^\circ\text{C}$  according to the corresponding requirements. 10 µl CCK-8 reagent was added and incubated for 2 h at  $37^\circ\text{C}$ . Cell absorbance was assayed using a microplate reader at 490 nm.

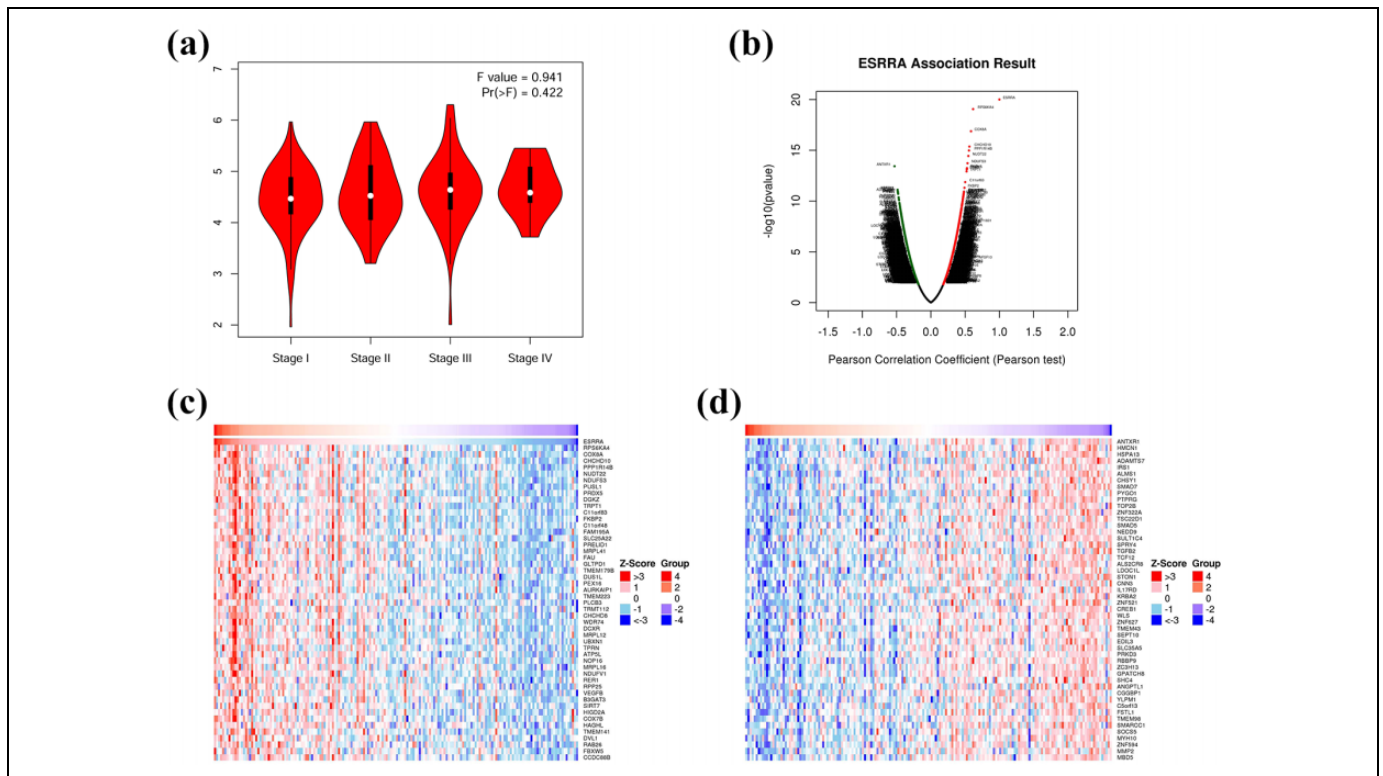
## TIMER 2.0

The online tool TIMER 2.0 (<http://timer.cistrome.org/>) is a comprehensive analysis platform, including XCELL, TIMER, EPIC, TIDE, and etc. It was used to analyze the immune infiltration levels across diverse cancer types. Seven common immune cell subtypes associated with ESRRA were analyzed, including neutrophil cell, T cell ( $\text{CD4}^+$  naïve), T cell ( $\text{CD8}^+$ ), NK cell, cancer associated fibroblast (CAF), B cell, and macrophage.

**Table 1.** Univariate and Multivariate COX Analysis of OS in Patients With UCEC.

| Characteristics | Univariate            |         | Multivariate         |         |
|-----------------|-----------------------|---------|----------------------|---------|
|                 | HR (95% CI*)          | P value | HR (95% CI*)         | P value |
| Age, yrs        | 1.033 (1.012-1.054)   | 0.002   | 1.032 (1.011-1.054)  | 0.003   |
| Grade           |                       |         |                      |         |
| G1              | Reference             |         | Reference            |         |
| G2              | 7.104 (1.614-31.269)  | 0.010   | 5.664 (1.277-25.111) | 0.022   |
| G3              | 13.073 (3.203-53.351) | <0.001  | 7.289 (1.755-30.270) | 0.006   |
| Stage           |                       |         |                      |         |
| I               | Reference             |         | Reference            |         |
| II              | 1.993 (0.943-4.211)   | 0.071   | 1.646 (0.775-3.493)  | 0.195   |
| III             | 3.618 (2.183-5.995)   | <0.001  | 3.308 (1.970-5.552)  | <0.001  |
| IV              | 8.898 (4.753-16.657)  | <0.001  | 5.982 (3.142-11.388) | <0.001  |
| ESRRA           | 1.037 (1.006-1.069)   | 0.020   | 1.033 (1.003-1.065)  | 0.033   |

\* CI, confidence interval.



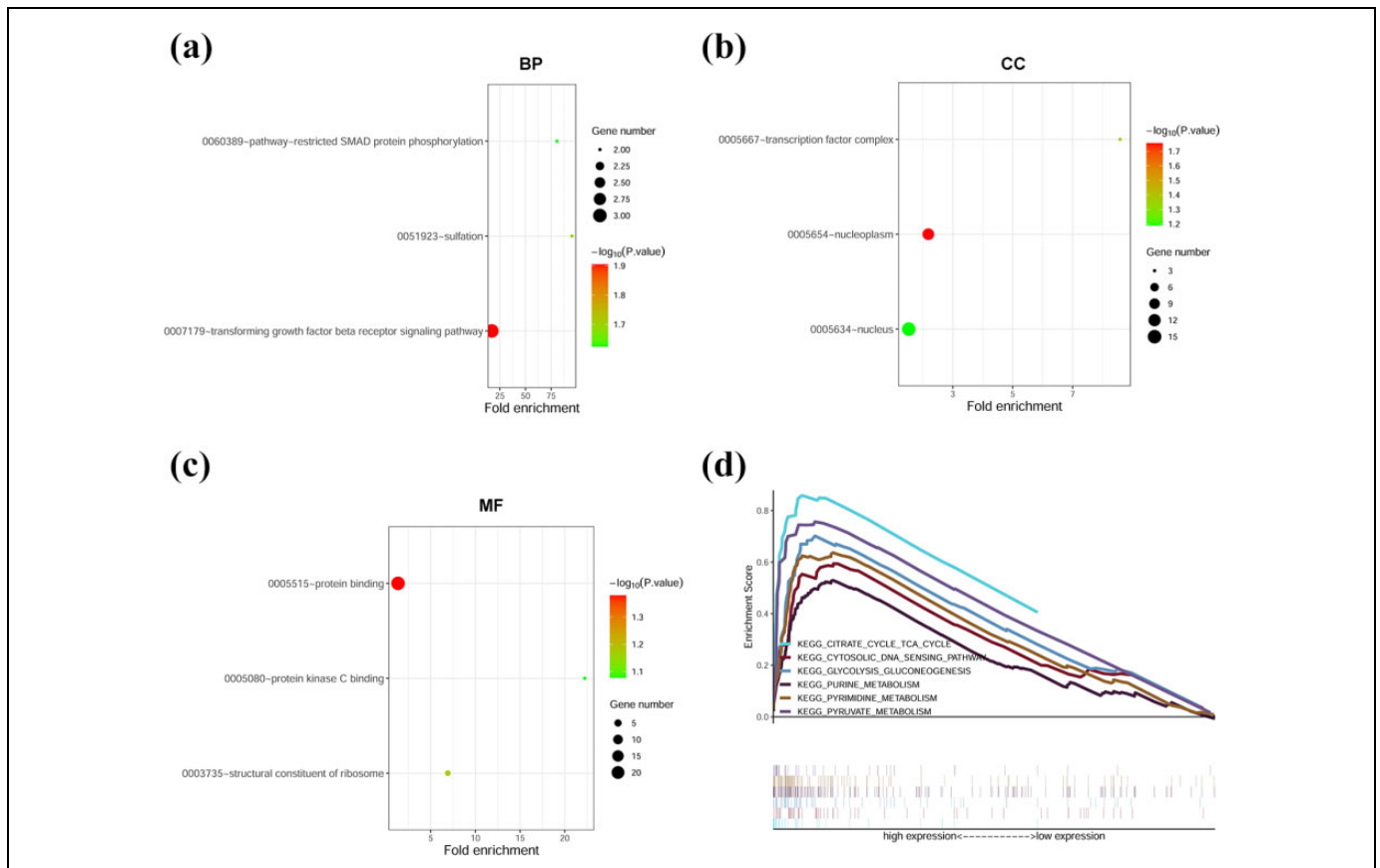
**Figure 2.** Clinical correlation analysis and co-expressed genes. (a) Correlation between ESRRA and clinical stage ( $p = 0.422$ ). (b) Volcanic map of co-expressed genes. (c) Heatmap displaying genes positively correlated with ESRRA (Top 50 genes). (d) Heatmap displaying genes negatively correlated with ESRRA (Top 50 genes).

## Results

### Expression and Clinical Roles of ESRRA

A total of 552 UCEC tissues and 35 normal tissues were obtained from the TCGA database. The mRNA expression level of ESRRA in UCEC tissues was significantly higher than those in normal tissues (Figure 1A,  $p < 0.001$ ). The mRNA expression level of ESRRA in 23 paired samples was further analyzed and the result was similar (Figure 1B,  $p < 0.001$ ).

Immunohistochemical experiments confirmed the increase in the protein expression level of ESRRA in UCEC tissues (Figure 1C and 1D). Univariate and multivariate Cox regression analysis indicated that ESRRA was an independent prognostic factor for UCEC (Table 1,  $p < 0.05$ ). ROC curve analysis demonstrated that the AUC of ESRRA was 0.854 (Figure 1E,  $p < 0.001$ ), which indicated ESRRA was a strong predictive indicator to distinguish UCEC and normal group. Kaplan-Meier survival analysis revealed that high ESRRA expression



**Figure 3.** GO and GSEA analysis. (a) Top 3 GO BP terms. (b) Top 3 GO CC terms. (c) Top 3 GO MF terms. (d) GSEA enrichment plot. Circle size represents gene number, and the smaller the p-value is, the more red the color is.

was significantly associated with poor prognosis of UCEC (Figure 1F,  $p < 0.05$ ).

### Clinical Correlation Analysis and Co-Expressed Genes

The correlation between ESRRA and clinical stage was analyzed and there was no significant correlation between them (Figure 2A,  $p = 0.422$ ). We used the LinkedOmics database (<http://www.linkedomics.org/>) to identify ESRRA-associated genes (Figure 2B). Top 10 upregulated genes were RPS6KA4, COX8A, CHCHD10, PPP1R14B, NUDT22, NDUFS3, PUSL1, PRDX5, DGKZ, and TRPT1 (Figure 2C). It is noteworthy that VEGF- $\beta$  was also upregulated (TOP 50). Top 10 downregulated genes were ANTXR1, HMCN1, HSPA13, ADAMTS7, IRS1, ALMS1, CHSY1, SMAD7, PYGO1, and PTPRG (Figure 2D).

### GO and GSEA Analysis

The aforementioned genes were entered into the GO functional analysis. The top 3 GO terms were shown in the Figure 3. The top 3 GO BP terms included pathway-restricted SMAD protein phosphorylation, sulfation, and transforming growth factor beta (TGF- $\beta$ ) receptor signaling pathway (Figure 3A). The top 3 GO CC terms included transcription factor complex, nucleoplasm, and nucleus (Figure 3B). The top 3 GO MF terms included

protein binding, protein kinase C binding, and structural constituent of ribosome (Figure 3C).

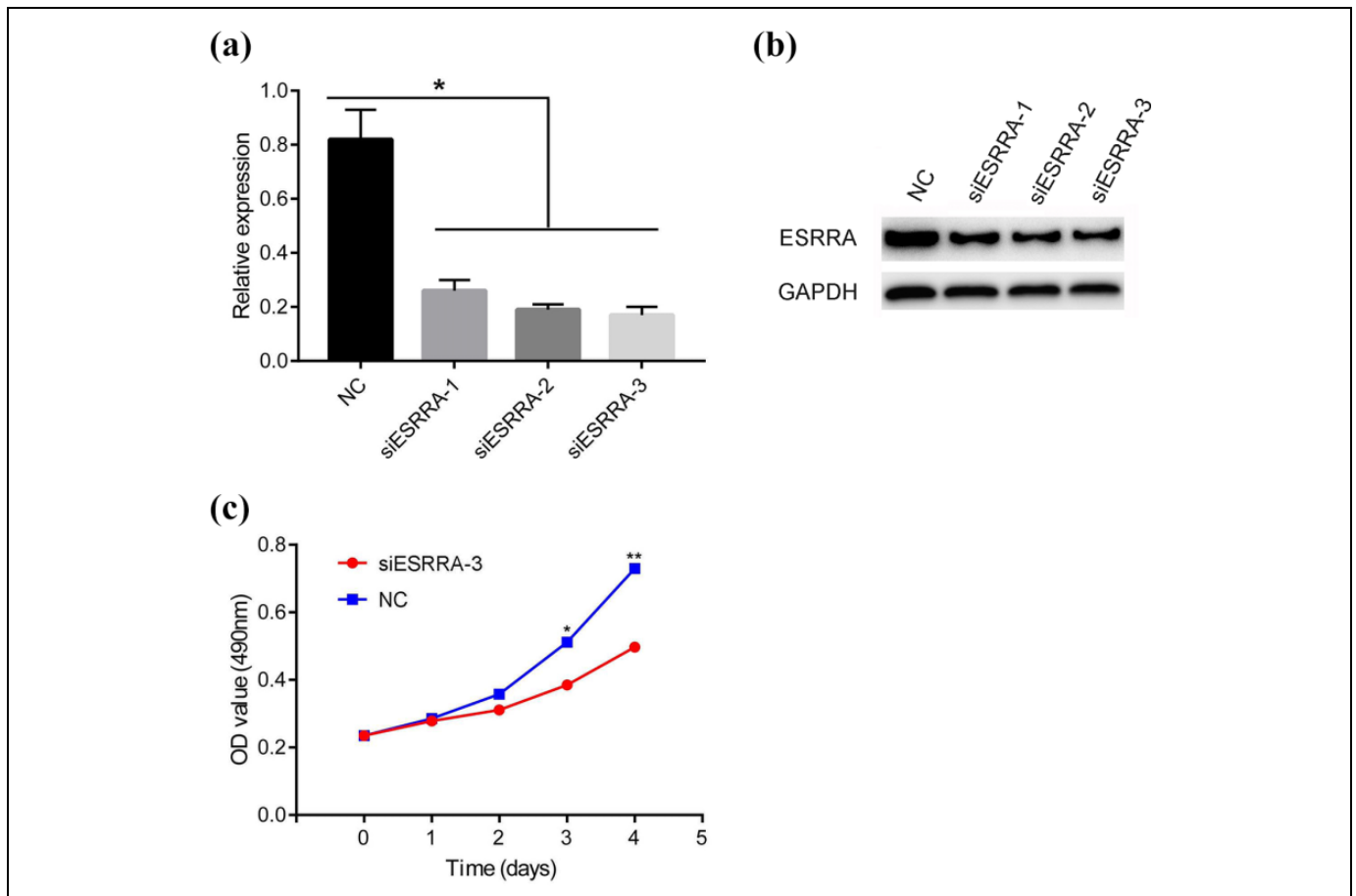
GSEA analysis indicated that 43 signaling pathways were significantly upregulated and no pathways were significantly downregulated. The top 6 upregulated pathways were shown in Figure 3D, including citrate cycle TCA cycle, cytosolic DNA sensing pathway, glycolysis gluconeogenesis, purine metabolism, pyrimidine metabolism, and pyruvate metabolism.

### Effect of ESRRA on Cell Proliferation

The transfection efficiency was 69%, 81%, and 83% for siESRRA-1, siESRRA-2, and siESRRA-3, respectively (Figure 4A). Western Blot assay also showed siESRRA-3 exhibited the most significant inhibition of ESRRA protein expression (Figure 4B). Therefore, siESRRA-3 was selected to do next experiment. The results of CCK-8 assay showed that interfering with the expression of ESRRA may inhibit the proliferation of UCEC cells (Figure 4C).

### Correlation Analysis Between ESRRA and Immune Cell Infiltration

Immune infiltration status was assessed using the TIMER 2.0. As shown in Figure 5, infiltration level of neutrophil cell was



**Figure 4.** CCK-8 assay. (a) The transfection efficiency of 3 siRNAs was checked by qRT-PCR, and siESRRA-3 exhibits the most significant inhibition of ESRRA mRNA expression ( $p < 0.05$ ). (b) The transfection efficiency of 3 siRNAs was checked by Western Blot assay, and siESRRA-3 exhibits the most significant inhibition of ESRRA protein expression ( $p < 0.05$ ). (c) The effect of ESRRA on cell proliferation. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

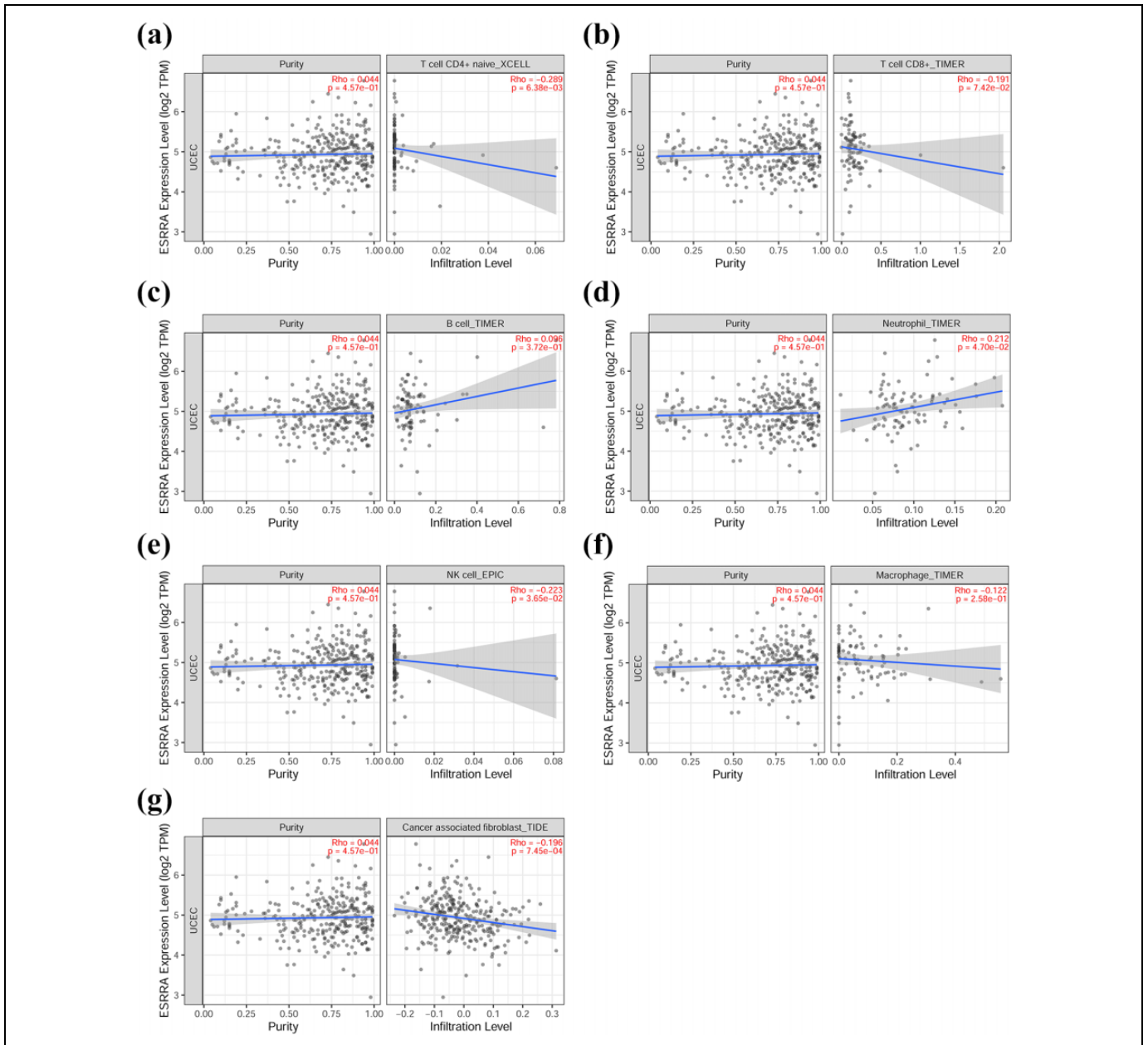
found to be positively correlated with the ESRRA expression. Infiltration level of T cell ( $CD4^+$  naïve), NK cell, and CAF had a negative correlation with the ESRRA expression. No significant correlation was observed between T cell ( $CD8^+$ ), B cell, macrophage and ESRRA.

## Discussion

In the present study, we comprehensively analyzed the expression level, clinical value, and possible mechanisms of action of ESRRA in UCEC. Small samples experimental data showed that ESRRA was highly expressed in UCEC.<sup>18</sup> Data from the TCGA confirmed the findings. The mRNA and protein expression level of ESRRA was highly elevated in UCEC compared with normal tissues, and was closely associated with poor prognosis. ROC analysis indicated a very high diagnostic value of ESRRA for patients with UCEC. GO and GSEA functional analysis showed that ESRRA might be mainly involved in cellular metabolism processes, in turn, tumorigenesis and progression of UCEC. In vitro results showed that ESRRA could enhance the proliferation ability of UCEC cells. Further immune cell infiltration demonstrated that ESRRA enhanced

the infiltration level of neutrophil cell and reduced that of T cell ( $CD4^+$  naïve), NK cell, and CAF. The alteration of immune microenvironment will greatly help in developing immune checkpoint therapy for UCEC.

Transcription factors could switch genes on or off and then impact cell behavior. A growing body of evidence suggested that transcription factors played critical roles in malignant tumors. Sara et al.<sup>19</sup> revealed several known and novel transcriptional regulatory modules and networks based on 6 types of cancer, including UCEC. A recent pan-cancer analysis provided new insights into transcription factor MYC network,<sup>20</sup> which opened new avenues for developing novel transcriptional factor markers. ESRRA, a transcriptional factor, also functioned as an oncogene to predict poor prognosis in various tumors, including breast cancer, prostate cancer, and renal cell carcinoma.<sup>11,21,22</sup> Similar results were obtained in our study. High expression of ESRRA predicted worse patient outcome. Meanwhile, both diagnostic specificity (74.3%) and sensitivity (84.8%) of ESRRA were acceptable. These results indicated that ESRRA was expected to be an important target for diagnosis and therapeutic intervention.



**Figure 5.** Immune infiltration levels evaluated using TIMER 2.0. (a) Association between ESRRR and infiltration level of T cell (CD4<sup>+</sup> naïve) ( $p < 0.01$ ). (b) Association between ESRRR and infiltration level of T cell (CD8<sup>+</sup>) ( $p > 0.05$ ). (c) Association between ESRRR and infiltration level of B cell ( $p > 0.05$ ). (d) Association between ESRRR and infiltration level of neutrophil cell ( $p < 0.05$ ). (e) Association between ESRRR and infiltration level of NK cell ( $p < 0.05$ ). (f) Association between ESRRR and infiltration level of macrophage ( $p > 0.05$ ). (g) Association between ESRRR and infiltration level of CFA ( $p < 0.01$ ).

Remarkably, we found that VEGF- $\beta$ , one of the VEGF family, was co-expressed with ESRRR. There are 4 ESRRR binding sites in the promoter region of VEGF.<sup>23</sup> The VEGF family played a crucial role in regulating vasculogenesis and angiogenesis in a variety of physiological and pathological processes.<sup>24</sup> Anti-VEGF biologics combined with traditional chemotherapy drug had also been shown to be effective in improving the outcome of patients with cancer.<sup>25</sup> Hiroshi et al.<sup>18</sup> demonstrated that knockdown of ESRRR could inhibit the expression of VEGF in UCEC and the ESRRR-dependent

regulation of VEGF is critically important for tumor angiogenesis. These provided a theoretical rationale for the development of anti-VEGF biologics applied to UCEC.

The mutual influence between transcription factors and micro-RNA had been reported in numerous studies. Pinho et al.<sup>26</sup> showed that estrogen receptor alpha (ER $\alpha$ ), as a nuclear receptor like ESRRR, could inhibited the cell proliferation of breast cancer cell through targeting miR-515-5p. ER $\alpha$  could also lead to reduced miR-221-222 expression in breast cancer.<sup>27</sup> Meanwhile, miR-221-222 could increase the proliferation of

ERA(+) cancer cell. The interactions between ER $\alpha$  and miR-221-222 formed a negative regulatory loop to enhance tumor malignancy. In addition to ER $\alpha$ , other transcription factors such as progesterone receptor (PR) and androgen receptor (AR) could also impact the miRNA expression, including miR-135a, miR-141, miR-141, miR-23, and miR-320.<sup>28-32</sup> On the other hand, micro-RNA could be functioning through regulating the expression of transcription factors. ESRRA was identified as a prominent target of miR-1291 in pancreatic cancer and breast cancer.<sup>12</sup> In breast cancer, ESRRA and miR-135a formed a negative feedback loop to influence tumor progression.<sup>33</sup> However, the analysis of ESRRA-micro-RNA axis in UCEC has not yet been reported. Further in-depth research in this field might yield the discovery of novel pathogenic mechanisms.

In our study, we found that ESRRA was closely correlated with immune cell infiltration, especially T cell. This suggested ESRRA might be involved in immune function. Harmit et al.<sup>34</sup> reported that ESRRA was a key mediator in the modulation of the immune response. Ryan et al.<sup>35</sup> showed that ESRRA promoted the proliferation of CD4<sup>+</sup> T lymphocytes and the generation of effector subsets. In UCEC, ESRRA was proved to be a critical transcription factor for immune-related genes and have prognostic significance.<sup>14</sup> These results were consistent with ours.

There are several limitations in our research. First, primary limitation of the present study was lacking sufficient independent external validation. Second, the high-throughput RNA-seq data from the TCGA database was reflective of average mRNA expression level of all cell types within the tumor. Single-cell RNA sequencing data was needed to eliminate the bias caused by intra-tumoral heterogeneity. Third, the sample size was small, especially the number of normal tissues. Fourth, some molecular events (e.g. copy number variation, microsatellite instability, methylation) were not recorded in TCGA database, which was important for better understanding the role of ESRRA in UCEC.

## Conclusion

To sum up, our study comprehensively analyzed the expression level, clinical value, and possible mechanisms of action of ESRRA in UCEC. These findings showed that ESRRA might be a potential diagnostic and therapeutic target.

## Author Contributions

SW designed the study, carried out the analysis, and interpreted the data. XH contributed to the review of previous literature, data discussion and critically commented on the manuscript for scientific content. All authors have read and approved the manuscript.

## Availability of Data and Materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.


## Ethics Approval and Consent to Participate

The study protocol was approved by the Ethics Committee of Maternal and Child Health Care Hospital of Qinhuangdao. All the clinical samples for immunohistochemistry were collected from Maternal and Child Health Care Hospital of Qinhuangdao. The written informed consent was obtained. All patients from the TCGA database gave consent to participate in any scientific researches.

## Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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## Summary Statement

This paper identified ESRRA as a potential diagnostic and therapeutic target in endometrial cancer.

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