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Original article

Pristimerin induces Noxa-dependent apoptosis by activating the FoxO3a pathway in esophageal squamous cell carcinoma

Mengyuan Feng[a,b,c,Δ], Anjie Zhang[a,c,Δ], Jingyi Wu[a,c,Δ], Xinran Cheng[a], Qingyu Yang[a], Yunlai Gong[a], Xiaohui Hu[a], Wentao Ji[a], Xianjun Yu[a,b,c,*], Qun Zhao[a,c,*]

[a] *School of Basic Medical Sciences, Hubei University of Medicine, Shiyan 442000, China*
[b] *Department of Gastroenterology, Taihe Hospital, Hubei Key Laboratory of Wudang Local Chinese Medicine Research, Hubei University of Medicine, Shiyan 442000, China*
[c] *Inflammation-Cancer Transformation and Wudang Chinese Medicine Research, Hubei Talent Introduction and Innovation Demonstration Base, School of Pharmacy, Hubei University of Medicine, Shiyan 442000, China*

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ABSTRACT

Pristimerin, which is one of the compounds present in *Celastraceae* and *Hippocrateaceae*, has antitumor effects. However, its mechanism of action in esophageal squamous cell carcinoma (ESCC) remains unclear. This study aims to investigate the efficacy and mechanism of pristimerin on ESCC *in vitro* and *in vivo*. The inhibitory effect of pristimerin on cell growth was assessed using trypan blue exclusion and colony formation assays. Cell apoptosis was evaluated by flow cytometry. Gene and protein expressions were analyzed through quantitative reverse transcription-polymerase chain reaction (qRT-PCR), Western blotting, and immunohistochemistry. RNA sequencing (RNA-Seq) was employed to identify significantly differentially expressed genes (DEGs). Cell transfection and RNA interference assays were utilized to examine the role of key proteins in pristimerin's effect. Xenograft models were established to evaluate the antitumor efficiency of pristimerin *in vivo*. Pristimerin inhibited cell growth and induced apoptosis in ESCC cells. Upregulation of Noxa was crucial for pristimerin-induced apoptosis. Pristimerin activated the Forkhead box O3a (FoxO3a) signaling pathway and triggered FoxO3a recruitment to the Noxa promoter, leading to Noxa transcription. Blocking FoxO3a reversed pristimerin-induced Noxa upregulation and cell apoptosis. Pristimerin treatment suppressed xenograft tumors in nude mice, but these effects were largely negated in Noxa-KO tumors. Furthermore, the chemosensitization effects of pristimerin *in vitro* and *in vivo* were mediated by Noxa. This study demonstrates that pristimerin exerts an antitumor effect on ESCC by inducing AKT/FoxO3a-mediated Noxa upregulation. These findings suggest that pristimerin may serve as a potent anticancer agent for ESCC treatment.

1. Introduction

Squamous cell carcinomas include a wide range of malignancies originating from squamous epithelium organs, including the esophagus, skin, head, and neck. Esophageal carcinoma, a type of esophageal cancer, ranks as the 7th most prevalent cancer and the sixth leading cause of cancer-related mortality worldwide, with 604,100 (3.1%) new cases and 544 076 (5.5%) estimated deaths [1]. Esophageal squamous cell carcinomas (ESCC) constitute approximately 90% of esophageal carcinomas, characterized by a poor prognosis with a five-year survival rate of 19% [2,3]. In clinical practice, ESCC patients undergo various treatments, including surgery, chemo/radiotherapy, and endoscopic therapy [4,5]. The majority of patients diagnosed with ESCC present with locally advanced or distant metastases, necessitating radiotherapy and chemotherapy [6,7]. According to the National Comprehensive Cancer Network (NCCN) guidelines, recommended therapeutic approaches for esophageal patients include 5-fluorouracil/oxaliplatin, paclitaxel/cisplatin, and paclitaxel/carboplatin or cisplatin [8]. However, few candidate drugs for ESCC therapy demonstrate long-term benefits due to chemotherapy resistance [9,10]. Thus, there is an urgent need to identify more effective agents and elucidate the molecular mechanisms to prevent or combat ESCC.

Pristimerin, the primary bioactive component extracted from *Celastraceae* and *Hippocrateacea* [11] has been extensively studied for its diverse therapeutic properties. Research has established pristimerin's anti-inflammatory, antioxidant, antibacterial, and insecticidal effects [12-14]. Moreover, pristimerin demonstrates antitumor activity across various human malignancies, including lung, breast, colorectal, glioma, and liver cancers [15-19]. Recent findings indicate that pristimerin inhibits the proliferation and migration of ESCC cells through nuclear factor kappa-B (NF-κB) signaling inactivation [20]. Therefore, elucidating the mechanism of pristimerin-induced apoptosis in ESCC warrants further investig-

* Corresponding author.
E-mail addresses: xjyu@hbmu.edu.cn (X. Yu); zhaoqun@hbmu.edu.cn (Q. Zhao)
Δ These authors contributed equally to this work.

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ation.

During cellular apoptosis, Noxa is induced and translocated to the mitochondria, where it initiates caspase cascades [21]. Knockout of Noxa prevents chemotherapeutic agent-induced apoptosis [22]. Although P53-dependent transcription of Noxa has been extensively studied, p53-independent mechanisms likely contribute to Noxa expression regulation [23]. This study demonstrates that pristimerin upregulates Noxa expression through the protein kinase B (AKT)/Forkhead box O3a (FoxO3a) signaling pathway in ESCC. Furthermore, we demonstrated that Noxa was essential for pristimerin-induced apoptosis and chemosensitization *in vitro* and *in vivo*. These results suggest that pristimerin may serve as a therapeutic agent for ESCC patients.

2. Material and methods

2.1. Reagents and antibodies

Pristimerin (purity ≥ 98%) was obtained from Yuanye Biological Technology Co., Ltd. (Shanghai, China). Actinomycin D and 5-fluorouracil (5-FU) were obtained from MedChemExpress (MCE, Shanghai, China). DMEM, penicillin, streptomycin, and trypsin were supplied by Thermo Fisher Scientific (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from QmSuero/Tsingmu Biotechnology, Wuhan (Wuhan, China). A nuclear and cytoplasmic extraction kit was obtained from Beyotime Biotechnology (Shanghai, China). Poly (ADP-ribose) polymerase (PARP), cleaved caspase-3, Noxa, B-cell lymphoma protein 2 (Bcl-2)-associated X (Bax), Bim, Bcl-2, phosphorylated AKT (p-AKT), and AKT antibodies were obtained from Cell Signaling Technology (CST, Danvers, MA, USA). p-FoxO3a and FoxO3a were obtained from ABclonal (Wuhan, China). Flag antibody was obtained from Sigma (St Louis, MO. USA). GAPDH antibody was purchased from Wuhan Servicebio Technology Co., Ltd. (Wuhan, China). The secondary antibodies were supplied by Jackson Labs (Wes Grove, PA, USA).

2.2. Cell culture

Eca-109 and KYSE-150 cells were obtained from the Cell Bank of the Chinese Academy of Sciences and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were incubated at 37 °C in an atmosphere containing 5% CO_2.

2.3. Trypan blue exclusion assay

Eca-109 and KYSE-150 cells were cultured in 12-well plates and subsequently exposed to varying concentrations of pristimerin for specified time periods. Cell viability was assessed using trypan blue exclusion assays.

2.4. Flow cytometry analysis

Eca-109 and KYSE-150 cells were treated with pristimerin, subsequently harvested, and double-stained with Annexin V-FITC and propidium iodide (PI) in a binding buffer. The rate of cell apoptosis was detected by flow cytometry (Beckman Flow Cytometer, USA).

2.5. Western blotting analysis

The total protein concentration was determined using the BCA protein quantification method. Protein samples were denatured in SDS buffer, separated *via* sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes. These membranes were incubated in 5% nonfat milk, followed by incubation with primary and secondary antibodies. Finally, the proteins were visualized using an enhanced chemiluminescence substrate (Tanon, China) on a chemiluminescent imaging system (Bio-Rad, USA).

2.6. RNA sequencing (RNA-Seq)

Total RNA was extracted from Eca-109 cells following pristimerin treatment. To minimize inter-group variation, RNA from three replicates was pooled for each group. Gene expression levels were analyzed using a software package. The differentially expressed genes (DEGs) were then subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses to identify significant signaling pathways. RNA-Seq was performed by GeneRead Biotechnology (Wuhan, China).

2.7. Cell transfection and RNA interference assay

AKT, FoxO3a, Noxa, and vector plasmids were transfected with Lipofectamine 3000 (Thermo Fisher Scientific). siRNA knockdown was performed using FoxO3a, p53, and scramble siRNA (Tsingke Biotechnology, Beijing, China).

2.8. Nuclear translocation assessment

Nuclear and cytoplasmic proteins were extracted using a nuclear/cytoplasmic extraction kit and then analyzed *via* Western blotting. Lamin B1 was used as a loading control for nuclear proteins, and α-tubulin was used as a loading control for cytosolic proteins.

2.9. Mouse xenograft model

The animal experiments in this study were approved by the Ethics Committee of the Hubei University of Medicine (No. 2022-055). To establish a xenograft model, Eca-109 cells (2×10^6 cells) were subcutaneously implanted into the flank of BALB/c nude mice. When the tumor volume reached approximately 100 mm^3, the mice were administered the indicated drugs or vehicle via intraperitoneal injection every two days. The tumor volumes were calculated using the formula: $1/2 \times \text{length} \times \text{width}^2$.

2.10. Immunohistochemical staining

The tumors were fixed in paraformaldehyde, embedded in paraffin and subsequently cut into 5-μm thick sections. After antigen retrieval, the slides were blocked with 5% bovine serum albumin and then incubated with primary and secondary antibodies. Finally, the slides were stained with diaminobenzidine solution and counterstained with hematoxylin.

2.11. Statistical analysis

SPSS version v23.0 (IBM corporation) and Prism 6 (GraphPad Software) were used for statistical analysis. Data are presented as the mean ± standard error of the mean (SEM) values. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Pristimerin induces growth inhibition and apoptosis in ESCC cells

To assess the antiproliferative effects of pristimerin (Supple-

mentary Fig. 1A) in ESCC cells, Eca-109 and KYSE-150 cells were treated with increasing concentrations of pristimerin. Cell viability assays revealed a significant, dose-dependent reduction in cell growth in both cell lines(Fig. 1A). Additionally, colony formation assays demonstrated that pristimerin markedly suppressed the clonogenic potential of ESCC cells, further indicating its long-term inhibitory effects on proliferation (Fig. 1B, Supplementary Fig. 1B). To determine whether pristimerin induces apoptosis, Eca-109 and KYSE-150 cells were treated with pristimerin, and apoptotic cell populations were quantified. Flow cytometry analysis showed a concentration-dependent increase in apoptotic cells (Fig. 1C). Moreover, Western blotting analysis revealed that pristimerin treatment significantly upregulated the levels of cleaved PARP and cleaved caspase-3, further confirming its pro-apoptotic activity (Fig. 1D). Collectively, these findings indicate that pristimerin exerts potent growth-inhibitory and pro-apoptotic effects in ESCC cells in a dose-dependent manner.

3.2. Noxa is required for pristimerin-induced apoptosis in ESCC cells

To investigate the molecular mechanism underlying pristimerin-induced apoptosis, we examined the involvement of Bcl-2 family proteins, which are key regulators of apoptotic cell death [24, 25]. Western blotting analysis revealed that pristimerin specifically upregulated Noxa protein levels without affecting the expression of other Bcl-2 family members under the same conditions (Fig. 2A). Moreover, quantitative polymerase chain reaction (PCR) analysis showed a dose-dependent increase in Noxa mRNA expression following pristimerin treatment (Fig. 2B). However, pretreatment with the transcription inhibitor actinomycin D abolished this upregulation, indicating that pristimerin regulates Noxa expression at the transcriptional level (Fig. 2C). Since p53 is a well-established transcriptional regulator of Noxa [26], we investigated its role in pristimerin-induced Noxa upregulation. p53 knockdown did not affect Noxa induction by pristimerin (Supplementary Fig. 2A-2B), suggesting that pristimerin enhances Noxa expression through a p53-independent mechanism in ESCC cells.

To determine whether Noxa is required for pristimerin-induced apoptosis, we generated Noxa-knockout (Noxa-KO) Eca-109 and KYSE-150 cells and assessed apoptotic cell death. Flow cytometry analysis demonstrated a significant reduction in pristimerin-induced apoptosis in Noxa-KO cells compared to parental controls (Fig. 2D). Furthermore, Western blotting analysis revealed that cleaved PARP and cleaved caspase-3 levels were markedly reduced in Noxa-KO cells, indicating impaired apoptotic signaling (Fig. 2E). In colony formation assays, Noxa-KO cells exhibited enhanced survival following pristimerin treatment (Fig. 2F). To confirm the requirement of Noxa in pristimerin-induced apoptosis, we reintroduced Noxa into Noxa-KO cells. Restoration of Noxa expression significantly increased apoptotic cell death, validating its essential role in pristimerin-mediated apoptosis (Fig. 2G). The abovementioned results suggest that Noxa is a critical mediator of pristimerin-induced apoptosis in ESCC cells.

3.3. Pristimerin induces FoxO signaling activation in ESCC cells

To elucidate the molecular mechanisms underlying pristimerin-induced apoptosis in ESCC cells, RNA-seq was performed on Eca-109 cells treated with or without pristimerin (Supplementary Fig. 3). Bioinformatic analysis identified 1,015 upregulated and 906 downregulated DEGs (Fig. 3A). GO analysis revealed that these DEGs were primarily enriched in biological processes related to cell migration, apoptosis, proliferation, and invasion (Fig. 3B). KEGG pathway analysis identified the phosphatidylinositol 3-kinase (PI3K)/AKT pathway as the most significantly enriched signaling pathway (Fig. 3C). Moreover, downstream pathways of PI3K/AKT, including FoxO and mTOR signaling, were also significantly affected (Fig. 3C). To validate these findings, we assessed the phosphorylation status of AKT, FoxO3a, and mTOR in Eca-109 and KYSE-150 cells following pristimerin treatment. Western blotting analysis demonstrated that pristimerin markedly inhibited AKT and FoxO3a phosphorylation in a dose-dependent manner, whereas mTOR phosphorylation remained largely unaffected under the same conditions (Fig. 3D). Given these results, we further investigated whether the AKT/FoxO3a axis mediates pristimerin-induced Noxa upregulation and apoptosis in ESCC cells.

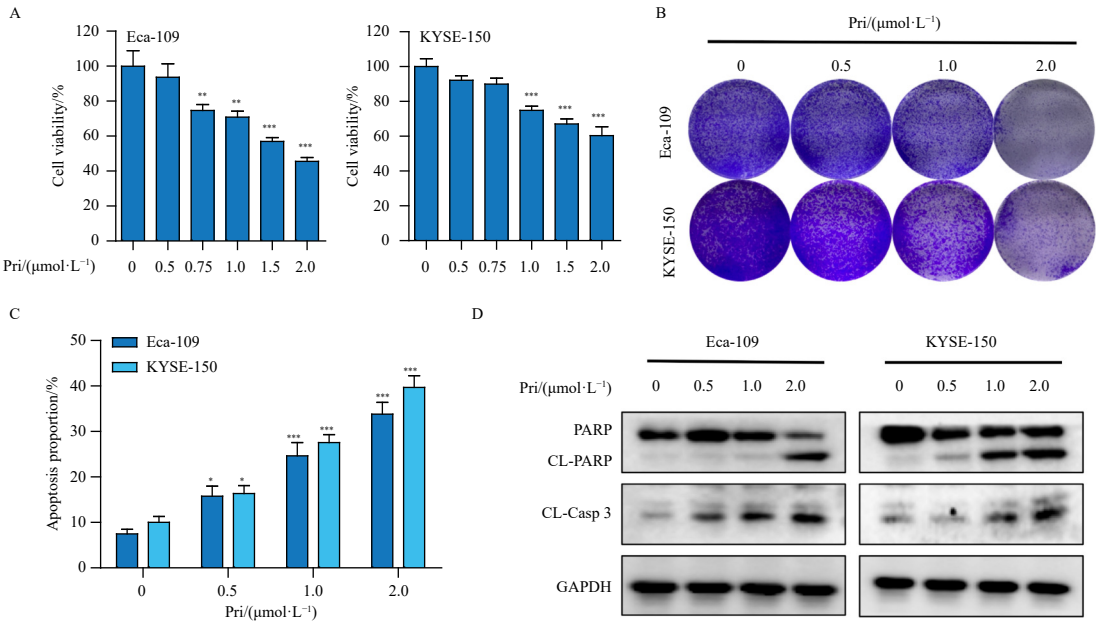


Fig. 1 Pristimerin induces growth inhibition and apoptosis in ESCC cells (A) Eca-109 and KYSE-150 cells were incubated with various concentrations of pristimerin for 24 h, and cell viability was assessed by trypan blue exclusion assay. (B) Colony formation assays of Eca-109 and KYSE-150 cells treated with pristimerin. (C) Flow cytometry analysis of the percentage of apoptotic cells in Eca-109 and KYSE-150 cells after pristimerin treatment for 24 h. (D) Western blotting analysis of cells treated with pristimerin for 24 h. The data were shown as the mean± SEM. In A and C, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

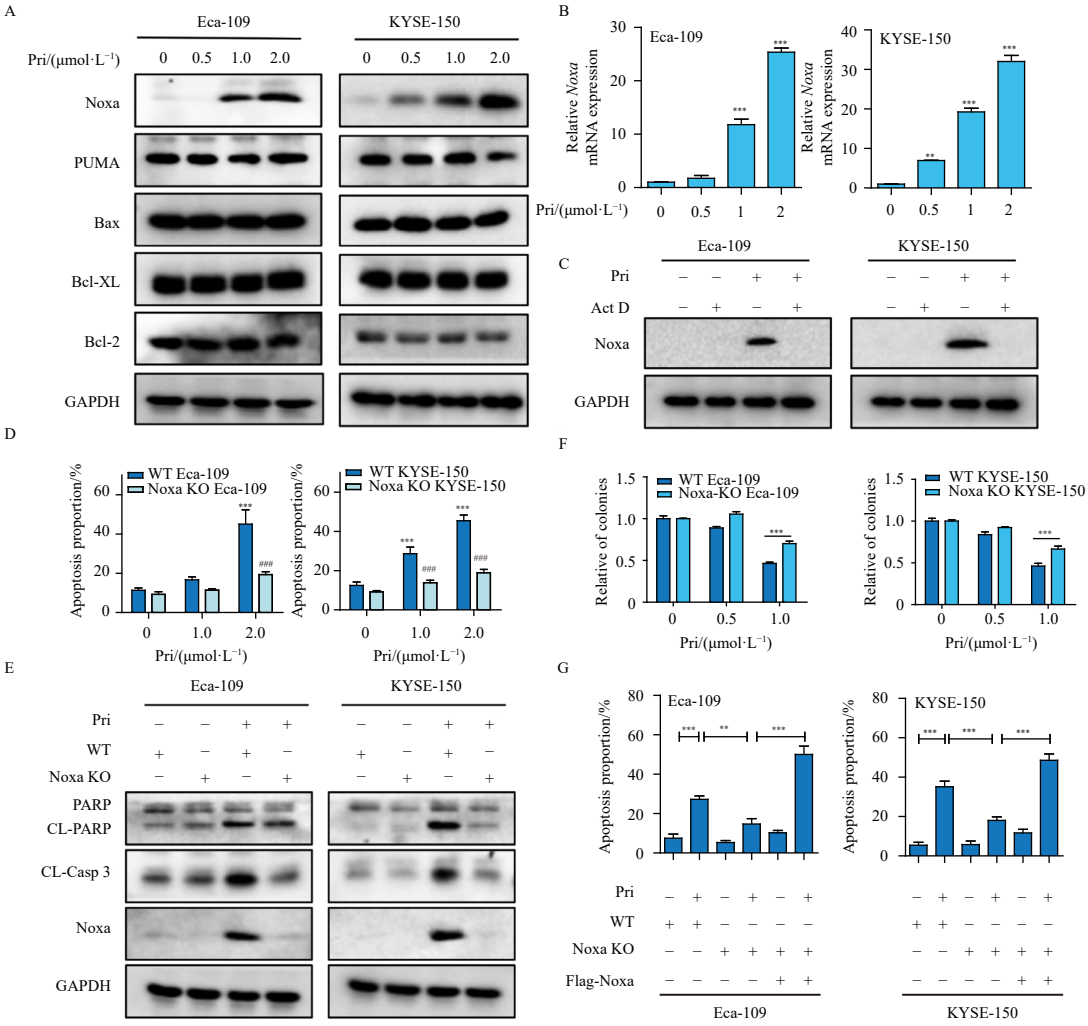


Fig. 2 Noxa is required for pristimerin-induced apoptosis in ESCC cells (A) Western blotting analysis of Eca-109 and KYSE-150 cells treated with the indicated concentrations of pristimerin for 24 h. (B) qRT-PCR analysis of Noxa expression in Eca-109 and KYSE-150 cells treated with pristimerin. (C) Western blotting of Eca-109 and KYSE-150 cells treated with pristimerin for 24 h with or without actinomycin D. (D) Flow cytometry analysis of apoptosis in WT and Noxa-KO Eca-109 and KYSE-150 cells treated with pristimerin for 24 h. (E) Western blotting analysis of WT and Noxa-KO Eca-109 and KYSE-150 cells treated with pristimerin for 24 h. (F) WT and Noxa-KO Eca-109 and KYSE-150 cells were treated with pristimerin, and colony numbers were recorded. (G) WT and Noxa-KO Eca-109 and KYSE-150 cells were transfected with NOXA or control empty vector for 24 h and subsequently treated with pristimerin for 24 h. Cell apoptosis was analyzed by flow cytometry. The data were shown as the mean± SEM. In B, D, F, and G, $^{**}P < 0.01$ and $^{***}P < 0.001$. In D, WT cells *vs* Noxa-KO cells after pristimerin treatment. $^{###}P < 0.001$.

3.4. Pristimerin-induced Noxa expression is mediated by AKT/FoxO3a

To determine whether FoxO3a regulates Noxa expression, we modulated FoxO3a levels in Eca-109 and KYSE-150 cells. FoxO3a overexpression significantly enhanced Noxa expression, whereas FoxO3a knockdown abolished pristimerin-induced Noxa upregulation (Figs. 4A–4B). Furthermore, western blotting analyses demonstrated that pristimerin treatment promoted FoxO3a nuclear translocation in both cell lines (Fig. 4C). To confirm that FoxO3a directly regulates Noxa transcription, chromatin immunoprecipitation (ChIP) assays were performed. The results showed that pristimerin significantly enhanced FoxO3a binding to the Noxa promoter, supporting a direct transcriptional regulatory mechanism (Fig. 4D). Since AKT regulates FoxO3a phosphorylation and subcellular localization [27], we further examined whether pristimerin-induced Noxa expression is mediated by AKT/FoxO3a signaling. AKT overexpression markedly increased FoxO3a phosphorylation, thereby inhibiting its nuclear translocation following pristimerin treatment (Figs. 4F-4G). More importantly, FoxO3a knockdown significantly attenuated pristimerin-induced apoptosis, as evidenced by reduced levels of cleaved PARP and cleaved caspase-3 (Fig. 4G). Furthermore, FoxO3a silencing and AKT overexpression more effectively suppressed Noxa protein levels (Fig. 4H). These results establish the AKT/FoxO3a/Noxa axis as a critical mediator of pristimerin-induced apoptosis.

3.5. Pristimerin suppresses xenograft tumor growth in a Noxa-dependent manner

The antitumor effect of pristimerin was evaluated *in vivo* in an Eca-109 cell xenograft mouse model. The study revealed that tumor growth and weight were significantly reduced in the pristimerin-treated group compared to the vehicle group (Figs. 5A–5C). To verify pristimerin's ability to suppress proliferation and induce apoptosis *in vivo*, immunohistochemistry was employed to assess the expression levels of Ki-67 and cleaved caspase-3. The results demonstrated that pristimerin treatment decreased Ki-67 expression and increased cleaved caspase-3 levels (Fig. 5D). Moreover, the phosphorylation levels of AKT and Foxo3a were reduced in pristimerin-treated tumors (Fig. 5E). Consistent with these findings, Western blotting analysis indicated that pristimerin treatment increased Noxa and cleaved caspase-3 levels while decreasing AKT and Foxo3a phosphorylation (Supplementary Fig. 4).

To further evaluate the role of Noxa in pristimerin-induced

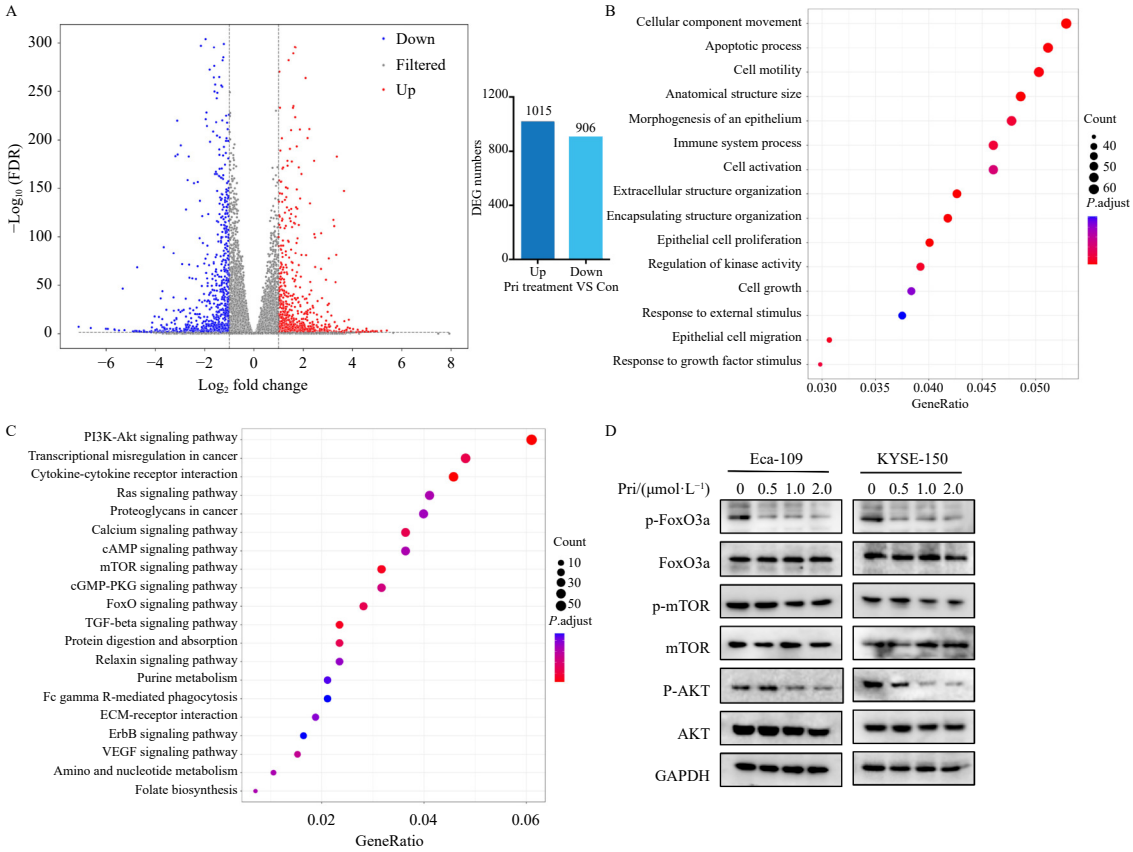
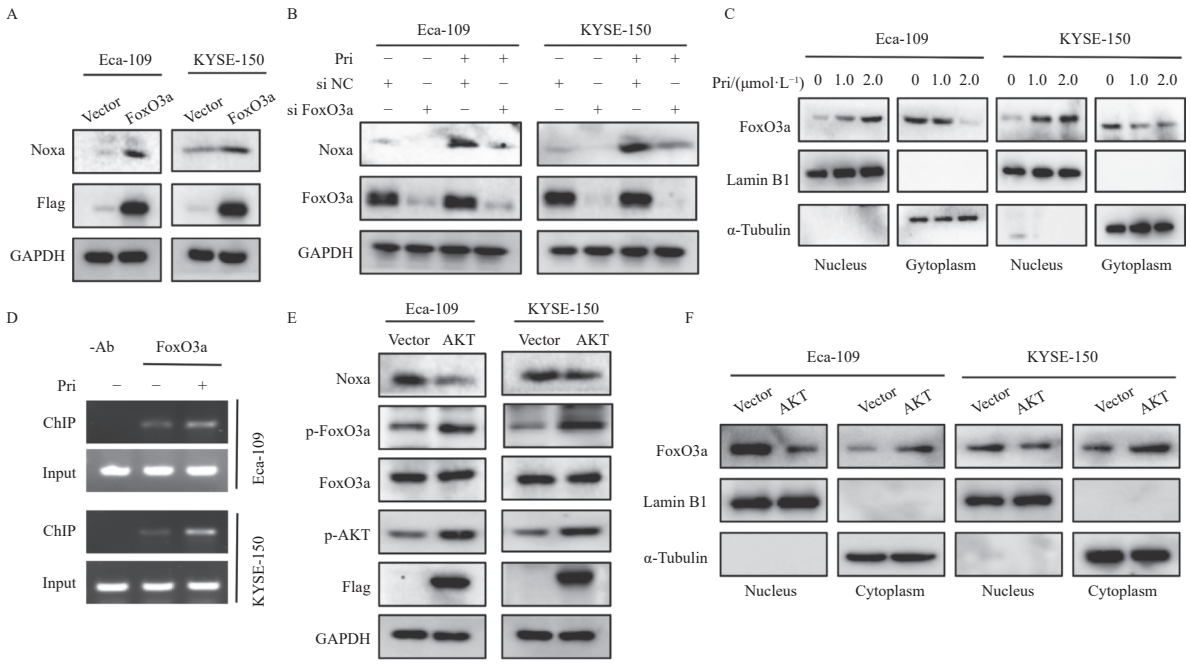


Fig. 3 Pristimerin induces FOXO signaling activation in ESCC cells (A) Differentially expressed genes (DEGs) were shown in Eca-109 cells after 2.0 μmol·L^{-1} pristimerin for 24 hours. (B–C) Pathway enrichment analysis of the DEGs. (D) Eca-109 and KYSE-150 cells were treated with the indicated concentrations of pristimerin for 24 h. The indicated protein levels were analyzed by Western blotting.

antitumor efficacy *in vivo*, wild-type (WT) and Noxa-KO Eca-109 cells were utilized to establish xenograft mouse models. As shown in Fig. 5F and 5G, Noxa-KO tumors exhibited reduced sensitivity to pristimerin treatment. Furthermore, a significant decrease in Ki-67 expression and an increase in cleaved caspase-3 were observed in the WT tumors, while these effects were attenuated in Noxa-KO tumors (Figs.5H-5I). Collectively, these data suggest that Noxa is an important regulator involved in the antitumor effects of pristimerin *in vivo*.

3.6. The chemosensitization effects of pristimerin are mediated by Noxa

Pristimerin is often combined with conventional cytotoxic



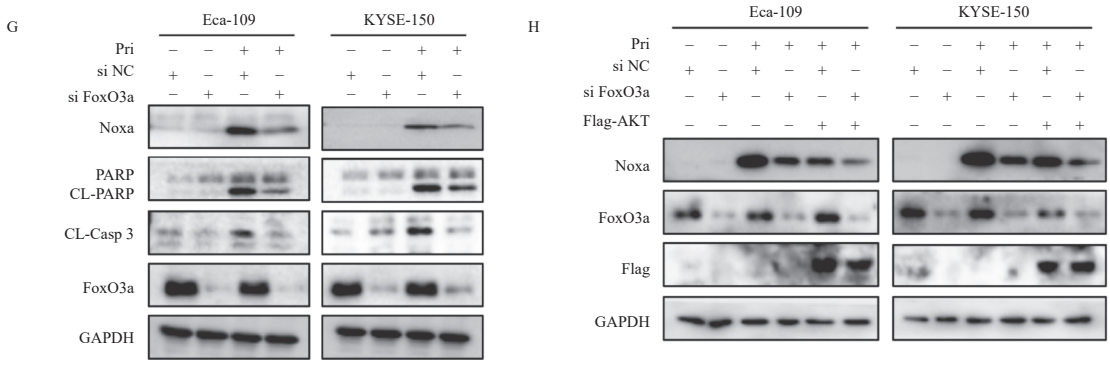


Fig. 4 Pristimerin-induced Noxa expression is mediated by AKT/FoxO3a (A) Western blotting of Eca-109 and KYSE-150 cells transfected with the FoxO3a or empty vector plasmid. (B) Western blotting of NC siRNA- or FoxO3a siRNA-transfected cells treated with pristimerin (2.0 μmol·L^{-1}). (C) Western blotting of FoxO3a expression in the nuclear and cytoplasmic fractions after pristimerin treatment. (D) ChIP analysis of FoxO3a on the promoter of Noxa after pristimerin (2.0 μmol·L^{-1}) treatment. (E–F) After Eca-109 and KYSE-150 cells were transfected with AKT or empty vector plasmids for 24 h and then treated with 2.0 μmol·L^{-1} pristimerin for 24 h. The indicated protein levels were analyzed and the expression of FoxO3a in the nuclear and cytoplasmic fractions were analyzed by Western blotting. (G) Western blotting of NC siRNA- or FoxO3a siRNA-transfected cells treated with 2.0 μmol·L^{-1} pristimerin. (H) Eca-109 and KYSE-150 cells were transfected with AKT plasmid and FoxO3a siRNAs for 24 h and subsequently treated with 2.0 μmol·L^{-1} pristimerin for 24 h. The indicated protein levels were analyzed by Western blotting.

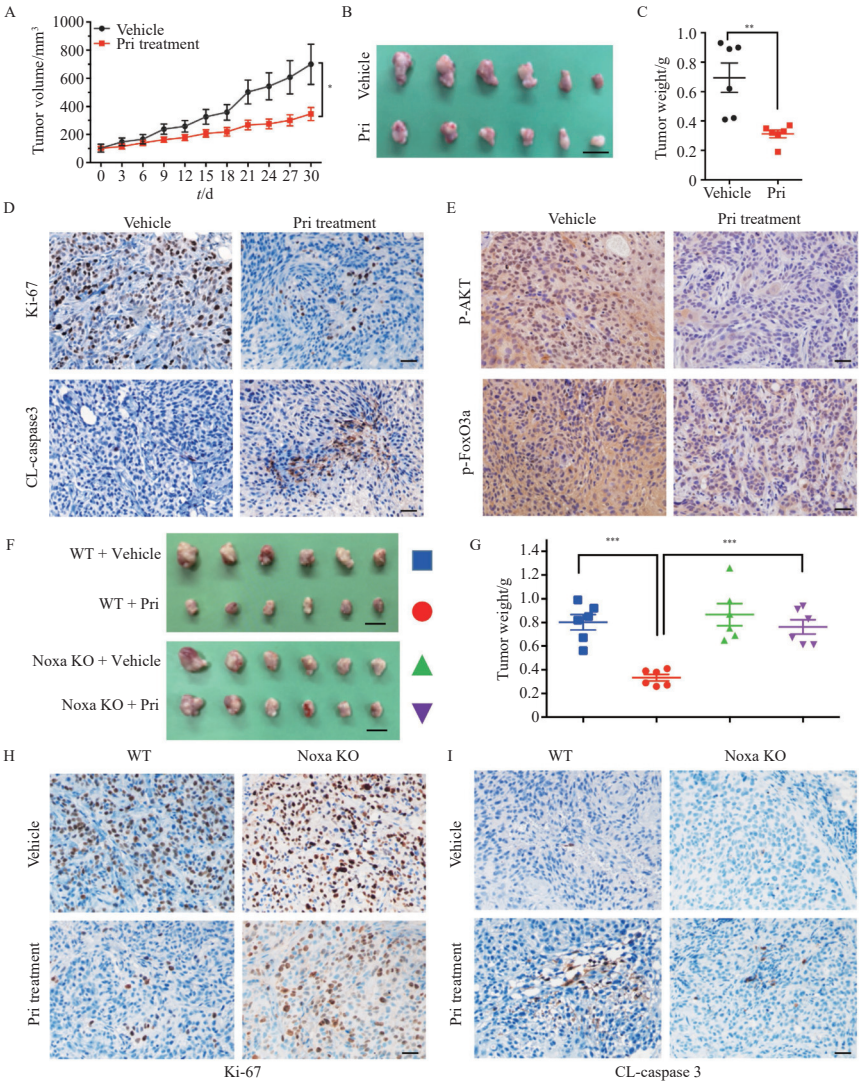


Fig. 5 Pristimerin suppresses xenograft tumor growth in a Noxa-dependent manner (A) Nude mice were subcutaneously injected with Eca-109 cells. When the tumor volume reached 100 mm^3, the mice were injected with pristimerin (0.25 mg·kg^{-1}, intraperitoneally) or vehicle. The tumor volume was recorded, calculated, and plotted. (B–C) After 30 days, the tumors were removed. The images of the xenograft tumors were shown, and the tumor weights were recorded. (D–E) IHC analysis of Ki-67, cleaved caspase-3, p-AKT, p-FoxO3a in tumor sections. Scale bar, 50 μm. (F–G) Nude mice were subcutaneously injected with WT or Noxa-KO Eca-109 cells and then injected with pristimerin (0.25 mg·kg^{-1}) or vehicle. The images of the xenograft tumors and tumor weights were shown. Scale bar, 1 cm. (H–I) IHC analysis of Ki-67 and cleaved caspase-3 in tumor sections. Scale bar, 50 μm. The data were shown as the mean ± SEM. In A, C and G, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

therapies to enhance anticancer efficacy [28, 29]. To assess its chemosensitization potential, Eca-109 and KYSE-150 cells were treated with pristimerin and 5-FU, alone or in combination. The results demonstrated that the combination treatment significantly inhibited cell growth compared to either pristimerin or 5-FU alone (Fig. 6A). In addition, Western blotting analysis revealed that combination treatment further upregulated Noxa expression (Fig. 6B). Furthermore, flow cytometry and colony formation assays showed that combination treatment induced a significantly higher degree of apoptosis and greater inhibition of clonogenic survival in WT cells (Figs. 6C–6D, Supplementary Fig. 5A), but these effects were attenuated in Noxa-KO cells (Figs. 6E–6F, Supplementary Fig. 5B). Nude mice bearing WT or Noxa-KO Eca-109 xenograft tumors were treated with pristimerin, 5-FU, or the combination. As shown in Fig 6G, the combination treatment significantly suppressed WT tumor progression compared to monotherapy. However, the enhanced tumor suppression observed in WT tumors was markedly diminished in Noxa-KO tumors (Fig. 6G). Additionally, tumor weight analysis further confirmed these findings (Fig. 6H). These results establish Noxa as a key mediator of pristimerin-induced chemosensitization both *in vitro* and *in vivo*.

4. Discussion

Despite advancements in surgery, chemotherapy, and radiotherapy, the prognosis for ESCC patients remains poor, with limited treatment efficacy and high recurrence rates [30, 31]. Plant-derived natural compounds have emerged as promising therapeutic alternatives due to their high safety profile and low toxicity [32-34]. Previous studies have demonstrated that pristimerin exerts anticancer effects through multiple signaling pathways, including AKT, NF-κB, Shh/Gli1, IGF-1R, and MAPK [15, 18, 35, 36]. However, the precise mechanisms underlying pristimerin-induced apoptosis in ESCC remain largely unexplored. In this study, we demonstrated that pristimerin exhibits potent antitumor activity in ESCC cells and murine tumor models by modulating the AKT/FoxO3a signaling pathway, which leads to Noxa upregulation and apoptotic cell death.

Noxa, a pro-apoptotic member of the Bcl-2 family, plays a pivotal role in cellular apoptosis. Under normal physiological conditions, Noxa expression remains low but is transcriptionally activated in response to apoptotic stimuli [37]. In addition, Noxa has been implicated in tumor suppression by facilitating apoptotic signaling in cancer cells. Our findings revealed that pristimerin significantly upregulated Noxa expression at the transcriptional level. Furthermore, both *in vitro* and *in vivo* results confirmed that Noxa is essential for pristimerin-induced apoptosis and tumor suppression, highlighting its potential as a therapeutic target in ESCC.

FoxO transcription factors are crucial regulators of cellular homeostasis, controlling processes such as apoptosis, proliferation, and stress responses [38]. Among them, FoxO3a plays a key role in apoptosis by translocating to the nucleus and activating target gene transcription [39, 40]. Our RNA-Seq analysis identified FoxO signaling as a key pathway modulated by pristimerin, suggesting a regulatory link between FoxO3a and Noxa. Further experiments confirmed that pristimerin promotes FoxO3a nuclear translocation, where it directly binds to the Noxa promoter, leading to its transcriptional activation. Moreover, FoxO3a knockdown significantly suppressed pristimerin-induced Noxa expression and apoptosis, confirming the FoxO3a/Noxa axis as a crucial mediator of pristimerin's pro-apoptotic effects. FoxO3a activity is negatively regulated by PI3K/AKT signaling [41, 42]. Our results demonstrated that pristimerin significantly inhibited AKT phosphorylation. However, AKT overexpression reversed pristimerin's effects by increasing FoxO3a phosphorylation, preventing its nuclear translocation, and subsequently inhibiting Noxa expres-

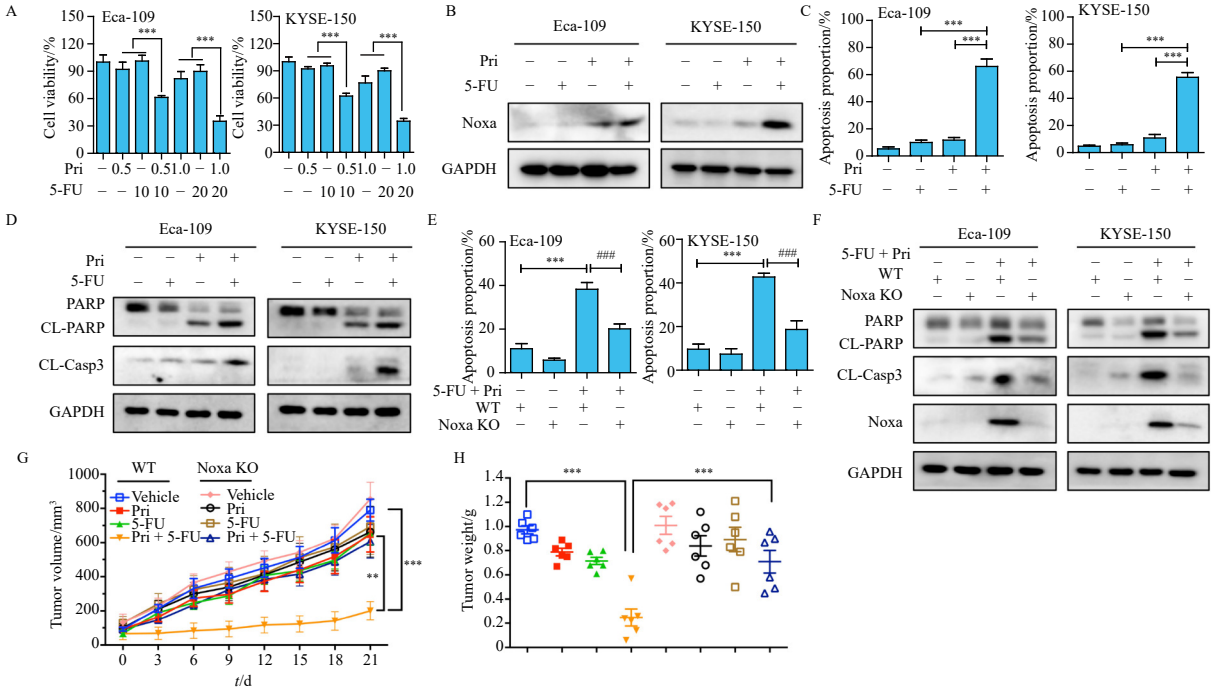


Fig. 6 The chemosensitization effects of pristimerin are mediated by Noxa in ESCC cells (A) Eca-109 and KYSE-150 cells were treated with pristimerin, 5-FU, or their combination at the indicated concentrations for 24 h. The cell viability was evaluated by trypan blue exclusion assay. (B) Western blotting of Eca-109 and KYSE-150 cells treated with pristimerin with or without 5-FU for 24 h. (C) Flow cytometry analysis of apoptosis in Eca-109 and KYSE-150 cells treated with pristimerin with or without 5-FU for 24 h. (D) Western blotting of cells treated with pristimerin with or without 5-FU for 24 h. (E) Flow cytometry analysis of apoptosis in WT and Noxa-KO Eca-109 and KYSE-150 cells treated with pristimerin with or without 5-FU for 24 h. (F) Western blotting of WT and Noxa-KO cells treated with pristimerin with or without 5-FU for 24 h. (G) Nude mice were subcutaneously injected with WT or Noxa-KO Eca-109 cells. When the tumor volume reached 100 mm^3, the mice were injected with pristimerin (0.1 $mg \cdot kg^{-1}$), 5-FU (10 $mg \cdot kg^{-1}$) or their combination. The tumor volume was recorded, calculated, and plotted. (H) After 21 days, the tumors were removed. The weights of xenograft tumors were recorded. The data were shown as the mean ± SEM. In A, C, E, G, and H, *** $P < 0.001$. In E, WT cells *vs* Noxa-KO cells after pristimerin and 5-FU treatment. $^{###}$ $P < 0.001$.

sion. These findings demonstrate that pristimerin exerts its pro-apoptotic effects in ESCC by suppressing AKT phosphorylation and activating FoxO3a, leading to Noxa upregulation and apoptosis.

5. Conclusion

In conclusion, this study demonstrates that pristimerin induces apoptotic cell death in ESCC cells by upregulating Noxa expression both *in vitro* and *in vivo*. Furthermore, our findings identify the AKT/FoxO3a signaling axis as a critical regulator of pristimerin-mediated Noxa upregulation, providing novel insights into its pro-apoptotic mechanism (Supplementary Fig. 5C).

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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