Mechanisms of *Sophora flavescens* in the treatment of cervical squamous cell carcinoma based on comprehensive biological analysis, network pharmacology, and experimental verification

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**Abstract**

**Objective:** This study used comprehensive bioinformatics analysis and network pharmacology analysis to investigate the potentially relevant mechanisms of *Sophora flavescens* against cervical squamous cell carcinoma. **Methods:** Consistently altered genes involved in cervical squamous cell cancerization were analyzed in the GEO database. The chemical ingredients and target genes of *Sophora flavescens* were explored using the TCMS database. We obtained the potential therapeutic targets of *Sophora flavescens* by intersecting the above genesets and validated them in the GEPIA database. The interaction between *Sophora flavescens* and target genes was predicted by molecular docking. RT-qPCR was used to verify the changes of target genes in HeLa cells treated with *Sophora flavescens*. Single-gene GSEA functional analysis were performed to determine the molecular mechanisms. **Results:** Fifteen genes related to the transformation of cervical squamous cell carcinoma were identified, among which AR and ESR1 were confirmed as targets for kaempferol, wighteone, formononetin, and phaseolamin. These compounds are the active ingredients in *Sophora flavescens*. Low expressions of AR and ESR1 correlate with a poor prognosis, while *Sophora flavescens* treatment increases the expression of AR and ESR1 in HeLa. GSEA analysis showed that AR and ESR1 mainly participate in the epithelial-mesenchymal transition in cervical squamous cell carcinoma. **Conclusion:** *Sophora flavescens* exert anti-tumor effects by targeting AR and ESR1, which may regulate cancer metastasis.

**Keywords:** cervical squamous cell carcinoma; biological analysis; network pharmacology; *Sophora flavescens*
Introduction

Cervical cancer (CC) is women's fourth most frequent malignancy worldwide [1]. It takes one year to a decade for cervical intraepithelial neoplasia (CIN) to ultimately evolve into cervical squamous cell carcinoma (CSCC) for most cases [2]. Despite a significant decline in the global incidence of cervical cancer due to regular cervical screening and HPV vaccination, metastasis and recurrence of CC remain the leading cause of death in patients [3]. Targeted therapy and immunotherapy have shown promising effects for metastatic or recurrent cervical cancer patients but may be limited by severe side effects, such as gastrointestinal perforations and cytokine release syndrome [4, 5]. Therefore, we must explore more suitable therapeutic targets and potential agents.

Traditional Chinese medicine (TCM) is therapeutic and regulatory in various cancers [6]. Whether used alone or in conjunction with chemotherapy, TCMs can improve clinical efficacy and enhance immunity in treating CC [7, 8]. *Sophora flavescens* is an important TCM with many active ingredients, presenting wide-ranging anti-tumor, antimicrobial, antipretic, antiinflammatory, and anti-inflammatory pharmacological abilities [9]. Numerous studies have indicated that certain active components in *Sophora flavescens* exhibit anti-cervical cancer properties, although the underlying mechanism remains unclear [10-15]. Therefore, we searched for CSCC-related genes by comprehensive biological analysis and targets of *Sophora flavescens* by network pharmacology analysis. We also investigated the potential molecular mechanisms to identify new therapeutic targets and establish a framework for the adjuvant use of *Sophora flavescens* in treating CSCC.

Materials and methods

Data sources

Three datasets (GSE7803, GSE63514, and GSE138080) were extracted from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/?term = ), including samples of normal cervical, CIN, and CSCC tissues that had not undergone preoperative radiotherapy. We also downloaded the target gene mRNA expressions and clinical information of 253 patients with CSCC from the TCGA database (http://cancer genome.nih.gov/) for validation.

Differentially expressed gene screening

We screened differentially expressed genes (DEGs) between normal cervical tissue and CSCC and between CIN and CSCC using the GEO2R website tool (https://www.ncbi.nlm.nih.gov/geo/geo2r/), with the criteria of P-value < 0.05 and |log2(FC)| > 1. A volcano map was drawn using GraphPad Prism (version 8.0.2). The intersections of the DEGs obtained from these datasets were determined using the Venn 2.2.1.0: https://bioinfoogg.cn.bsc.es/tools/venny/index.html.

*Sophora flavescens* network pharmacology

We searched for active components and target genes of *Sophora flavescens* using the TCM Systems Pharmacology Database (TCMSP, http://tcmspw.com/tcmsp.php) with the criteria of oral utilization ≥ 30% and drug-like characteristics ≥ 0.18. Network diagrams between the active components and target genes of *Sophora flavescens* were created using Cytoscape 3.8.0. *Sophora flavescens* target genes intersected with the DEGs in CSCC to determine the therapeutic targets for CSCC by Venny 2.2.1.0.

Expression and prognostic validation of drug target genes

The expression of therapeutic target genes in normal cervical tissues and CC was validated using the GEPIA (Gene Expression Profiling Interactive Analysis) database (http://geopia.cancer-pku.cn/). The survival curves of the CC patient with different expressions of target genes were drawn using the Kaplan-Meier plotter (https://kmplot.com/analysis/).

Molecular docking

After searching the PDB database (http://www.rcsb.org) and TCMSM, the structural files of the target proteins and ligand molecules were downloaded and saved in PDBQT format. The binding energy, represented as the docking score, was obtained using the molecular docking software AutoDockTools 1.5.6 to evaluate the binding abilities of ligands and receptors. A smaller value indicates a higher binding activity and less than −7.0 represents intense interaction. The results were visualized using VMD software.

Cell culture and drug intervention

We purchased the human cervical cancer cell line HeLa from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum(FBS, Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin. *Sophora flavescens* intervention in HeLa cells were cultured using 10% DMEM with *Sophora flavescens* (50 μg/mL) provided by the School of Pharmacology, Jiangnan University.

RNA extraction and real-time quantitative PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). RNA purity and concentration were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). cDNA synthesis was performed using a Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA). Quantitative real-time PCR (qRT-PCR) was conducted with a 7500 Real-Time PCR System (Applied Biosystems, USA) using SYBR Green I Master Mix (Invitrogen, Carlsbad, CA) to measure the levels of AR and ESR1, which were normalized to GAPDH. Expression levels were quantified using the 2^-ΔΔCT method. The specific PCR primers were as follows: Human AR forward, 5′-GCTTACTGACGACCTGATG-3′ and reverse, 5′-GAAGATC TCTCCAACAGGCTTGC-3′; Human ESR1 gene forward, 5′-CCACA CACAACACGCTTGC-3′ and reverse, 5′-GTCTATTTCCTGAAATTTGACCCGTG-3′, Human GAPDH gene forward, 5′-TCGACCA CACAACACTGCTTACGG-3′ and reverse, 5′-GGCTGACTCTGCTGACTGAG-3′.

Gene Set Enrichment Analysis (GSEA)

We performed GSEA in the expression matrix through the MSigDB Collection (h.all.v7.0symbols.gmt/c5.bp.v7.0 symbols.gmt) to detect significantly different pathways in the CC samples that express high- and low-target genes. Absolute values of NES > 1.0, NOM P-value < 0.05, and FDR q-value < 0.25 are generally considered meaningful gene collections.

Statistical analysis

GraphPad Prism (version 8.0.2) was used for statistical analysis and visualization. Differences between treatment groups were analyzed using ordinary one-way ANOVA. Data are presented as mean ± s.e.m. P-values ≤ 0.05 were considered statistically significant. All differences highlighted by asterisks were statistically significant (P < 0.05; *P < 0.01; **P < 0.001).

Results

Identification of differentially expressed genes

Three datasets, GSE7803, GSE63514, and GSE138080, were selected to include 44 normal cervical epithelial samples, 62 CIN samples, and 59 CSCC samples (Figure 1A–B, Supplementary Table S1). A total of 301 genes were significantly differentially expressed between CSCC and normal cervical tissue samples according to the screening criteria (Figure 1D), and 21 genes were significantly differentially expressed between CSCC and CIN (Figure 1F). Fifteen significant DEGs were obtained by taking the intersection of 301 genes and 21 genes, including nine upregulated genes, POLQ, PLOD2, ECT2, PLSCR1, RFC4, CENPN, APOC1, and SLC16A1, and six downregulated genes, AR, ESR1, CFD, THSD4, TPS313, and TST (Figure 1E). There were 301 (Figure 1G) significantly differential genes in CSCCSvsNC and 21 (Figure 1I).
significantly differential genes in CSCC vs CIN, and 15 (Figure 1H) significantly differentially expressed genes in the progression of cervical cancer were obtained from the intersection of the above two datasets.

**Sophora flavescens** target screening
According to the screening criteria, 13 active ingredients of *Sophora flavescens* and the corresponding 206 molecular targets were obtained from TCMSP (Figure 2A). AR and ESR1, as potential therapeutic targets of *Sophora flavescens* for cervical squamous cell carcinoma, were obtained by the intersections with 15 DEGs and *Sophora flavescens* target genes (Figure 2B).

**Expression and prognostic validation of drug target genes**
GEPIA showed that AR and ESR1 were lowly expressed in cervical cancer. High expression of AR and ESR1 was related to a better prognosis (Figure 3). In addition, a positive correlation was found between AR and ESR1 in ovarian cancer in the GEPIA (R = 0.56) (Figure S1).

**Molecular docking**

We predicted molecular docking results for the active constituents with AR and ESR1 protein. AR exhibited satisfactory binding activities with the 8-isopentenyl-kaempferol; wighteo; formononetin; luteolin; norartocarpetin; phaseolin; quercetin and (2R)-5, 7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one (Table 1). The binding activities between ESR1 and the following compounds were evaluated: (2R)-5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one; 8-isopentenyl-kaempferol; wighteo; formononetin; (2R)-7-hydroxy-2-(4-hydroxyphenyl)chroman-4-one; glyceollin; (2S)-7-hydroxy-2-(4-hydroxyphenyl)-5-methoxy-8-(3-methylbut-2-eny l)chroman-4-one; kushenin; leachianone G; and phaseolin. The respective binding energies were recorded as −7.82 kJ/mol, −7.34 kJ/mol, −7.00 kJ/mol, −6.79 kJ/mol, −7.62 kJ/mol, −7.38 kJ/mol, −6.08 kJ/mol, −6.75 kJ/mol, −6.61 kJ/mol, and −6.24 kJ/mol (Table 1). The two proteins with the highest binding energies were visualized. The results showed that AR and ESR1 combined with the active constituents primarily through hydrogen bonding. The maximum binding energy was visualized in Figure 4A–B separately.

![Figure 1](https://www.tmrjournals.com/cancer)

**Figure 1** Identification of differentially expressed genes among cervical cancer, cervical intraepithelial neoplasia and normal. Volcano plot of differentially expressed genes between CSCC and Normal in GSE7803. (A) GSE63514. (B) GSE138080. (C) dataset. Volcano plot of differentially expressed genes between CSCC and CIN in GSE7803. (D) GSE63514. (E) GSE138080. (F) Dataset. Genes (depicted in red) show upregulated expression with adjusted P < 0.05, genes (depicted in blue) show downregulated expression with adjusted P < 0.05. (G) Intersection of differentially expressed genes between CSCC and Normal in GSE7803, GSE63514, and GSE138080 dataset. (H) Intersection of differentially expressed genes between CSCC vs NC and CSCC vs CIN. (I) Intersection of differentially expressed genes between CSCC and CIN in GSE7803, GSE63514, and GSE138080 datasets.
Figure 2 Compound-target network of *Sophora flavescens* and the target for treating CSCC. (A) Potential constituents-target network of kushen in the treatment of cervical cancer. (B) Intersection of between 15 differentially expressed genes and target gene of *Sophora flavescens*.

Figure 3 Expression and prognostic validation of drug target genes. (A) AR was highly expressed in cervical cancer. (B) Survival curves of AR. (C) ESR1 was highly expressed in cervical cancer. (D) Survival curves of ESR1. *P* < 0.05.
### Table 1 Sophora flavescens compound-target molecular docking analysis

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Crystal structure</th>
<th>Target</th>
<th>Binding energy/(kcal·mol⁻¹)</th>
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<td>8-Isopentenyl-kaempferol</td>
<td>1E3G</td>
<td>AR</td>
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</tr>
<tr>
<td>Wighteone</td>
<td></td>
<td></td>
<td>-6.93</td>
</tr>
<tr>
<td>formononetin</td>
<td></td>
<td></td>
<td>-7.89</td>
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<td>luteolin</td>
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<td></td>
<td>-8.53</td>
</tr>
<tr>
<td>Norartocarpetin</td>
<td></td>
<td></td>
<td>-9.52</td>
</tr>
<tr>
<td>Phaseolin</td>
<td></td>
<td></td>
<td>-6.06</td>
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<tr>
<td>quercetin</td>
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<td>ER</td>
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</tr>
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<td>-7</td>
</tr>
<tr>
<td>formononetin</td>
<td></td>
<td></td>
<td>-6.79</td>
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<td>Glyceollin</td>
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<tr>
<td>Phaseolin</td>
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<td>-6.24</td>
</tr>
</tbody>
</table>

**Figure 4** Visualize the compound-target binding of AR and ESR1. (A) MOL06630-AR. (B) MOL00456-ESR1.
Validation that *Sophora flavescens* affects the expression of mRNA level of target genes

RT-qPCR results showed that AR and ESR1 were highly expressed in HeLa cells after intervention by *Sophora flavescens*, indicating that *Sophora flavescens* could increase the mRNA levels of AR and ESR1 (Figure 5).

Molecular mechanism of involvement of *Sophora flavescens* in anti-CSCC

GSEA showed that 41 out of 50 gene sets were downregulated in the AR high expression phenotype, and 13 gene sets were significantly enriched. The significantly downregulated hallmark gene sets involved in the immune response were allograft rejection, KRAS signaling up, apical surface, apical junction, inflammatory response, IL6_JAK_STAT3_signaling, and complement. The genesets involved in tumorigenesis and metastasis were epithelial-mesenchymal transition (Figure 6A). Among the AR low-expression phenotypes, nine genesets were upregulated, and one geneset was significantly upregulated. The significantly enriched geneset was “MYC targets v2” (Supplementary Table S2). 34 out of 50 genesets were downregulated in the ESR1 high-expression phenotype, while five were significantly enriched, which are epithelial-mesenchymal transition, UV response DN, BILE acid metabolism, myogenesis, apical surface, KRAS signaling DN, and estrogen response early (Figure 6B). In the ESR1 low-expression phenotype, five genesets, MYC targets v2, DNA repair, unfolded protein response, IFN-α response, and MTORC1 signaling, were upregulated (Supplementary Table S3). Thus, *Sophora flavescens* treatment may regulate AR and ESR1 consistently and inhibit the signaling pathway of epithelial-mesenchymal transitions to prevent CCSC metastasis.

![Figure 5 Experimental validation of *Sophora flavescens* docking targets.](image)

**Figure 5** Experimental validation of *Sophora flavescens* docking targets. (A) Flow chart of cell manipulation to validate *Sophora flavescens* docking targets. (B) Relative expression of AR mRNA level. (C) Relative expression of ESR1 mRNA level. The Control group was cultured in 10% DMEM, *Sophora flavescens* intervention group was incubated in 10% DMEM with *Sophora flavescens* (50 μg/ml) for 24 hours. ***P < 0.0001.

![Figure 6 GSEA analysis of ESR1.](image)

**Figure 6** GSEA analysis of ESR1. (A) the gene sets were significantly enriched in the AR high expression group. (B) the gene sets were significantly enriched in the ESR1 high expression group.
Discussion

Although the incidence and mortality have decreased significantly over the past 30 years, cervical cancer remains a significant threat to women’s lives, especially in developing countries. Treatment for cervical cancer is mainly surgery and/or chemoradiotherapy, depending on the stage of the disease. However, for patients with metastasis and/or recurrence, the treatment is more limited. It is sometimes limited due to a poor prognosis. Despite the boom in targeted therapy and immunotherapy, there remains an urgent need for new agents, particularly for challenging metastasis and recurrent CC.

Sophora flavescens is a traditional Chinese medicine containing many anti-tumor active ingredients. The network pharmacology results identified 13 main compounds in Sophora flavescens for treating CSCC. A lectin extracted from Sophora flavescens was found to induce apoptosis in HeLa cells. Previous experiments confirmed that luteolin and quercetin inhibit CC invasion by reducing UBE2S through epithelial-mesenchymal transition signaling. Sophora flavescens alkaloid gel, a compound of traditional Chinese medicine, inhibited CC proliferation and metastasis by suppressing the Akt/mTOR pathway. Matrine, a quinazoline alkaloid extracted from Sophora flavescens, also induced CC cell autophagy by inhibiting the Akt/mTOR pathway. Naringenin and kaempferol were natural products with the potential for treating different types of cancer. Formononetin inhibited AKT phosphorylation and induced HeLa apoptosis in a dose-dependent manner. However, the anticancer ability of the rest three main compounds, wighteone, (2R)-7-hydroxy-2-(4-hydroxyphenyl)chroman-4-one, and (1)(2S)-7-hydroxy-2-(4-hydroxyphenyl)-5-methoxy-8-(3-methylbut-2-enyl)chroman-4-one, are still unknown. Our molecular docking results showed excellent docking activities between the targets and compounds, indicating that those compounds could make a difference.

In this study, we identify 15 essential genes in the progress of CSCC transformation. It is interesting to show that they were mainly involved in the response to steroid hormones. The therapeutic targets of Sophora flavescens, AR and ESR1, are also steroid hormone receptors. Sex steroids are necessary in CC and regulate cell differentiation, proliferation, secretion, apoptosis, and other functions.

The presence of estrogen (ER) and progesterone receptors (PR) in cervical tumors has attracted widespread attention, while AR has received little attention. Downregulation of ER levels is considered an early event in transforming normal epithelium to CC. ERα is usually expressed in healthy cervical tissue, but its expression is reduced or absent in invasive CC, suggesting that loss of ERα expression plays a significant role in mediating CC invasion and progression. A previous study demonstrated that the absence of AR expression is a common event in high-grade squamous intraepithelial lesions and invasive squamous cell carcinomas due to a complex interaction between high-risk HPV and AR, and this expression decreases with increasing cervical tumor stage. A sequential decrease in ERα and AR expressions from healthy cervical tissue to CIN tissue and then to CC cancer tissues suggests that the absence of ERα and AR plays a vital role in mediating CC progression. ESR1 and AR were expressed at low levels in CSCC and were correlated with prognoses, consistent with previous studies. We further confirmed that Sophora flavescens could promote the expression of AR and ESR1. Molecular pathway analysis revealed that upregulated AR and ESR1 mainly enriched in the EMT signaling pathway, suggests that Sophora flavescens may inhibit CC metastasis.

This study emphasizes the importance of key genes in cancer progression and identifies potential therapeutic targets. However, there are several limitations to our research. Firstly, the phenotypic outcomes and underlying molecular mechanisms were not verified through in vitro and in vivo studies. Additionally, while Sophora flavescens shows promise as a therapeutic agent, its potential for adverse reactions necessitates further investigation to ascertain its safety and efficacy.

Conclusions

The main components of Sophora flavescens for treating CSCC consist of kaempferol, wighteone, formononetin, and phaseolizin, which act on targets such as AR and ESR1, and regulate EMT signaling pathway to inhibit metastasis and recurrence.

References

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