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Study on the regulatory mechanism and experimental verification of Ardisia crenata for the treatment of head and neck squamous cell carcinoma

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Article

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Abstract

Objective Head and neck squamous cell carcinoma (HNSCC) is one of the most common squamous epithelial malignancies. *Ardisia crenata*has an effect in the inhibition of tumor cells by regulating the cell cycle and inducing cell apoptosis. This study aimed to investigate the potential mechanism of *A. crenata* anti-HNSCC based on network pharmacology, molecular docking and in vitro experiments.

Methods The active compounds of *A. crenata* and HNSCC related targets were retrieved from SwissTargetPrediction, BATMAN-TCM, and SymMap v2 databases. The protein-protein interaction (PPI) network was constructed and the key targets were screened. GO and KEGG enrichment was conducted in DAVID. Survival analysis and core targets identification were conducted in TISIDB. The main active compounds of *A. crenata* were docked with the corresponding core targets by AutoDockTools and Autodock Vina. The regulatory effect of *A. crenata* on HNSCC was verified in FaDu cells.

Results 163 common target genes were identified as candidate targets of *A. crenata* for the treatment of HNSCC, the top core targets are TP53, GAPDH, AKT1, STAT3, CCND1 and SRC. KEGG pathway enrichment analysis indicated that *A. crenata* exerted anti-HNSCC effects mainly through pathways in cancer, prostate cancer, neuroactive ligand-receptor interaction, PI3K-Akt signaling pathway, p53 signaling pathway, EGFR tyrosine kinase inhibitor resistance and endocrine resistance. It's also confirmed that *A. crenata*could effectively inhibit the proliferation of FaDu cells, and down-regulate the expression of p-PI3K and p-AKT.

Conclusion The study demonstrated the multi-targets and multi-pathways characteristics of *A. crenata* in the treatment of HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC), which includes oral cancer, oropharyngeal cancer, laryngeal cancer and hypopharyngeal cancer, is the eighth most common cancer worldwide. In 2020, there were 878,348 new cases and 444,347 deaths reported, approximately 60% of cases are diagnosed as locally advanced stage [1, 2]. HNSCC poses a significant threat to human health, currently, the main clinical treatment approach is surgical resection combined with radiation and chemotherapy. However, these methods might impose long-term effects on patient health and lead to a range of complications, such as changes in eating, speech, hearing, and breathing [3]. Therefore, it's an urgent clinical need to develop drugs with low side effects has become urgent.

Traditional Chinese Medicine (TCM) has the characteristics of multi-component and multi-target, and some of them (such as taxol extracted from Taxus chinensis) have been proven to have good anti-cancer effects. The dried roots of *Ardisia crenata* (Zingiberaceae) was traditionally used for the treatment of upper respiratory tract infection and rheumatic arthritis in Chinese, recently it has been proven to be effective in anti-tumor, hepato-protection and inhibition of platelet aggregation [4]. In vitro experiments, *A.*

crenata showed cytotoxic to various cancer cells (A549, MCF-7, HepG2 and MDA-MB-231), but the molecular mechanism of its anti-cancer effect has not been clearly defined.

In recent years, network pharmacology has been widely used in TCM research. Based on the biological network of drug components-target-diseases and TCM components, network pharmacology systematically explains the influence and mechanism of drugs on specific diseases, thereby contributing to the development of new drugs [5]. In this study, network pharmacology was used to explore the active ingredients of *A. crenata* and their mechanism of action in HNSCC, which was further verified by molecular docking and cell experiments.

Material and methods

Active components targets of A. crenata and HNSCC-related targets

UPLC-Q-TOF-MS was used to collect the mass spectrum data of *A. crenata* (Shanghai Standard Technology Co., Ltd). SDF file of the active ingredients and the standard SMILES number was downloaded from the Pubchem database (https://pubchem.ncbi.nlm.nih.gov/); The targets of effective components of *A. crenata* were obtained and intersected from the SwissTargetPrediction database (http://new.swisstargetprediction.ch/); BATMAN-TCM database (http://bionet.ncpsb.org.cn/batman-tcm/) and cinnabar SymMap v2 database (www.symmap.org).

Keywords 'Head and neck squamous cell carcinoma' were retrieved in the GeneCards database (https://www.genecards.org/) and OMIM database (https://omim.org/) to obtain HNSCC-related genes and targets, then the duplicate data was excluded to get the potential targets.

Common targets of drug- diseases and protein interaction network

The common targets of *A. crenata* and HNSCC were intersected using Venny2.1 and exhibited as a Venn diagram. The protein-protein interaction (PPI) network was constructed by the STRING database (http://string-db.org/), the species selection is 'Homo sapiens', the minimum interaction threshold is 'medium confidence > 0.4', and other parameters kept default Settings; then the tsv file was downloaded and imported into Cytoscape3.9.0 software for visualization, the CytoHubba plug-in of Cytoscape was used to get key targets.

GO and KEGG enrichment analysis

To illustrate the gene functions of *A. crenata* potential targets against HNSCC, the functional annotation and enrichment analysis was conducted according to Kyoto Encyclopedia of genes Kyoto Encyclopedia of Genes and Genomes (KEGG) in the DAVID database (https://david.ncifcrf.gov/) ($P < 0.01$), the most significantly enriched genes were annotated and visualized.

Survival analysis and core genes verification

The TISIDB database (http://cis.hku.hk/TISIDB/) and the TIMER 2.0 database (http://timer.comp-genomics.org/) were used to probe the interaction between tumors and the immune system [6, 7]. The TISIDB database was used to assess the relation of key targets expression and the prognosis of HNSCC patients (core targets selected, $P < 0.05$). The TIMER 2.0 database was used to investigate the expression profiles of core genes in HNSCC tissues and adjacent normal tissues.

Molecular docking

The 3D structure SDF files of bergenin were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov), and the protein structures of the core targets were downloaded from the PDB database (https://www.rcsb.org/). The native ligands and water molecule of proteins were removed in PyMOL software and stored as pdb format. Protein hydrogenation and docking site detection were conducted in AutoDockTools, the generated pdbqt file was used for molecular docking. Finally, the affinity of molecular docking was evaluated in AutoDock Vina. Pymol was used for visualization.

Preparation of A. crenata water extract and FaDu cells

The materials used in this study mainly include human hypopharyngeal squamous cell line FaDu cells (National Collection of Authenticated Cell Cultures, CN); CCK8 kit and BCA protein quantitative kit (Beijing Solarbio Science & Technology Co., Ltd, CN); immune anti-PI3K, rabbit anti-AKT and anti-GAPDH (UK Abcam company).

Bergenin is the quality and quantity index of *A. crenata* root, The HPLC was used to detect the content of bergenin in the water extract of *A. crenata*, that is 1.21% and reached the pharmacopeia standard. FaDu cells were cultured in high sugar 90% DMEM + 10% FBS culture medium at 37°C in a 5% CO2 incubator and subcultured at the log phase.

CCK8 detection

Cells at the logarithmic phase were laid on 96-well plates. After cell adhesion, medium with *A. crenata* roots water extract (0, 5, 10, 20μg/mL) was added, respectively; the control group was added with an equal volume of 0.5% DMSO; and there were no cells in the blank group. After 24h, CCK-8 reagent was added and incubated at 37℃ for 2h. The absorbance (A) value at 450nm of each well was determined by enzyme-linked immunosorbent assay (ELISA) reader, and the cell survival rate was calculated as (ODexperiment -ODblank)/ (ODcontrol -ODblank) ×100%.

Western blot

The cells were collected and added cell lysing reagent on ice for 30 min, then centrifuged 12 000 ×g for 10 min at 4℃. After full lysis, the liquid supernatant was absorbed and the total protein was conducted by BCA protein quantitative kit. The samples were mixed with SDS buffer and then heated to denaturate. Proteins were separated by SDS-PAGE gel electrophoresis. Then transferred to the membrane, the

membrane was blocked with 5% non-fat milk for 2h, then washed by TBST three times, 10 minutes each time, finally incubated with primary antibody at 4℃ overnight. The membrane was washed as the above method, then added second antibody and incubated for 2h at room temperature in the shaker, and the membrane was washed again for exposure and development. The relative expression level of the target protein was analyzed by Image J.

Statistical analysis

Statistical analysis was conducted in SPSS 21.0 and the quantitative data were represented by the mean value of standard deviation. Data comparison among groups was tested by one-way ANOVA, single groups were analyzed by T-test, and $P < 0.05$ was considered as statistical significance.

Results

The active components and drug targets of A. crenata

The samples of *A. crenata* were analyzed by UPLC-Q-TOF/MS. According to the mass spectrum information of the samples, combined with the high-resolution mass database of natural products and related literature, 17 compounds were identified from *A. crenata* root samples. Nine of the main components (Bergenin, Ardipusilloside I, Ardisicrenoside B, Ardisicrenoside A, Ardisiacrispin A, Ardisiacrispin B, Lauryldiethanolamine, Rapanone, Embinin, Embelin and Norbergenin) were imported into SwissTargetPrediction, BATMAN-TCM and SymMap v2 databases. After deleting duplicates, 273 targets of *A. crenata* were screened (Figure. 1).

The core targets of A. crenata anti-HNSCC

The key words ‘Head and neck squamous cell carcinoma’ were retrieved in Gene Cards and OMIM database, after removing the duplicate targets, 5,536 HNSCC-related targets were obtained. The targets of *A. crenata* and diseases were intersected in Venny2.1 (Figure. 2A), and the result showed that there were 163 effective targets of *A. crenata* anti-HNSCC.

Based on the interaction relationship of the intersected proteins obtained from the STRING database, there are 162 nodes and 2,018 edges in the interaction network diagram, and the average node degree is 24.9. The data was visualized in Cytoscape, The nodes in the PPI network (Figure. 2B) represent all proteins produced by a single protein-coding gene locus, and the edges represent the protein-protein interaction relationship. The size of the node means the Degree value and is proportionate to its pharmacological effect. According to the Degree algorithm of CytoHubba, finally 10 key targets were obtained, including AKT1, GAPDH, TP53, SRC, IL6, CASP3, EGFR, CCND1, STAT3, and ESR1 (Figure. 2C).

GO and KEGG enrichment analysis

GO and KEGG enrichment analysis was conducted in the DAVID database, and GO enrichment obtained a total of 213 items ($FDR < 0.01$), including 196 biological process (BP), 44 cell composition (CC), and 61

molecular function (MF) items. In the 10 most significant potential targets anti-HNSCC, five targets were related to biological processes, including response to drug, response to xenobiotic stimulus, positive regulation of MAP kinase activity, excitatory postsynaptic potential, positive regulation of cell proliferation; three targets related to cell composition, including integral component of plasma membrane, postsynaptic membrane, plasma membrane; two of them related to molecular functions, which are neurotransmitter receptor activity and identical protein binding (Figure. 3A).

A total of 109 KEGG pathways were enriched ($P < 0.01$), and the top 20 signal pathways were screened out (Figure. 3B). The results showed that the potential targets of *A. crenata* anti-HNSCC were enriched in pathways in cancer, prostate cancer, neuroactive ligand-receptor interaction, PI3K-AKT signaling pathway, p53 signaling pathway, EGFR tyrosine kinase inhibitor resistance and endocrine resistance.

Survival analysis and verification of core genes

The significance of expression levels of the key genes in the clinical survival of HNSCC patients was verified in the TISIDB database. After screening (Log-rank $P < 0.05$), a total of five genes, *GAPDH*, *AKT1*, *CCND1*, *SRC*, and *ESR1*, were proved to affect the prognosis and survival of HNSCC patients (Figure. 4A). The protein expression levels of core genes were obtained from TIMER database and compared between normal and HNSCC tissues. The results showed the expressions of *GAPDH*, *AKT1* and *SRC* were significantly different between normal and HNSCC tissues (Figure. 4B).

Molecular docking analysis

Bergenin, the main active component of *A. crenata* was selected for molecular docking with the target proteins that affected the prognosis of HNSCC. Molecular docking showed (Figure. 5) that the binding energy of bergenin with GAPDH (5jy6), AKT1 (4ejn), CCND1 (2w96), SRC (4u5j) and ESR1 (6psj) were − 7.7, -8.7, -6.9, -7.7 and − 7.8 kcal/mol, respectively. The results indicated the binding activity of bergenin and the core target was good and the screening results were reliable.

The effect of A. crenata on FaDu cells and PI3K/AKT pathway

The effect of *A. crenata* water extract on the activity of FaDu cells was detected by CCK-8. compared with the control group, FaDu cells treated with different concentrations of *A. crenata* water extract (0, 5, 10, 20μg/mL) showed lower cell survival rate, and the effect of *A. crenata* water extract had concentration and time dependence (Figure. 6A). Western blot analysis showed that compared with the blank control group, different concentrations of *A. crenata* water extract (0, 5, 10, 20μg/mL) treated FaDu cells could inhibit the protein expression of P-PI3K and P-AKT ($P < 0.05$), and the inhibitory effect was proportionate to the concentration (Figure. 6B).

Discussion

HNSCC is a common aggressive cancer with a high death rate, its carcinogenic factors include long-term smoking, betel nut chewing, alcohol consumption, occupational exposure and high-risk HPV infection. HNSCC has high genetic heterogeneity, most HNSCC patients are diagnosed with advanced cancer at their initial visit. Despite combined therapy for HNSCC has made some progress, its poor prognosis and serious complications still had a significant adverse impact on a patient's quality life. Therefore, there is an urgent need to develop effective treatment strategies for patients with HNSCC [8–10]. At present, traditional Chinese medicine has been gradually accepted as an alternative therapy for cancer for its low toxicity, high efficacy and safety [11].

In this study, we preliminarily explored the relevant targets and signal pathways of *A. crenata* anti-HNSCC based and obtained 163 related targets and 109 pathways by network pharmacology. The core targets were selected by PPI network topology analysis in combination with CytoHubba; the expression of core targets and their Clinical significance in the survival of HNSCC patients was investigated through TISIDB and TIMER database, and we got finally five important targets, GAPDH, AKT1, CCND1, SRC and ESR1 ($P < 0.05$). GAPDH is one of the enzymes involved in glycolysis, it has various activities in different subcellular locations. GAPDH plays an important role in tumor progression, including tumor cell survival, tumor angiogenesis, regulation of gene expression and mRNA transcription of tumor cells [12]. In larynx and lower hypopharynx cancer, it's reported that the expression of GAPDH in the patients' plasma could be clinically meaningful [13]. AKT (also known as protein kinase B or PKB) has three subtypes, AKT1, AKT2, and AKT3, it's a key intracellular kinase in the PI3K/AKT signaling pathway and also a key regulator in the canceration of normal cells. The overexpression of AKT1 and AKT2 is the hallmark of oral squamous cell carcinoma (OSCC); while the silencing of AKT1 and AKT2 could lead to cell cycle arrest in the G2/M phase, and ultimately lead to apoptosis of cancer cells [14, 15]. In HNSCC, active AKT and AKT1 mutation could be detected, indicating that the AKT signaling pathway can be used as the target for the development of anti-tumor drugs [16, 17]. In fact, inhibitors targeting AKT1 have been used in OSCC studies.15 CCND1 belongs to the highly conserved cyclin family. Overexpression of CCND1 may cause imbalance of cyclin-dependent kinases (CDK) and lead to limited signal transmission in mitotic, thus helping the tumor cells avoid cell cycle checkpoints and promoting their growth and metastasis [18]. Meanwhile, in vitro studies have shown that the disorder of CCND1 expression is significantly correlated with HNSCC resistance to EGFR inhibitors, and CCND1 may be a key downstream gene in EGFR-driven tumorigenesis [19]. SRC is a group of intracellular non-receptor tyrosine kinase, it could promote the proliferation and angiogenesis of tumor cells, inhibit cell apoptosis and affect the invasion and metastasis of tumors [20]. In addition, SRC is a common target for cancer treatment, and its inhibitors have shown potent antitumor activity in preclinical studies. Matrine plays an efficient role against cancer, it could down-regulate the phosphorylation of MAPK/ERK, JAK2/STAT3 and PI3K/Akt signal pathways by targeting SRC [21]. Syntenin-1 (SDCBP) expression is associated with SRC activation, histologic grade, advanced tumor stage and short survival rate, it can also regulate the chemical resistance of HNSCC cells [22]. Researchers have synthesized the small molecule prodrug of SRC inhibitor dasatinib and oxaliplatin and loaded it on amphiphilic diblock copolymer iPDPA, the pH-responsive nanoprodrugs(PDONPs) has significant immunological effect in HNSCC chemotherapy [23]. ESR1 encodes estrogen receptors, and

ESR1 gene mutations may lead to epigenetic reprogramming, increased expression of keratin in basal cells and the activation of anti-tumor immunity, it has been considered to be an emerging clinical biomarker of breast cancer [24, 25]. In addition, ESR1 can be used as a potential marker for prognostic estimation of ovarian cancer [26].

Bergenin is the main active component of *A. crenata*, and its obvious anti-tumor properties has been confirmed in a variety of tumors, including bladder cancer and cervical cancer [27, 28]. Therefore, we conducted molecular docking with bergenin and the above five key targets, the results showed that bergenin had good binding interaction with the key target proteins related to the survival of HNSCC patients, and their binding energy was less than − 5 kcal/mol. Enrichment analysis showed that the 163 potential targets of *A. crenata* anti-HNSCC were significantly enriched in pathways in cancer, prostate cancer, neuroactive ligand-receptor interaction, PI3K-Akt signaling pathway, p53 signaling pathway, EGFR tyrosine kinase inhibitor resistance and endocrine resistance. The PI3K-AKT signaling pathway plays a key role in cancer progression, it has an influence on the proliferation, survival, metabolism of tumor cells [29]. Many studies have shown that the dysfunction of the PI3K-AKT pathway can affect the pathogenesis of squamous cell carcinoma, therapies targeting this pathway effectively inhibit tumors in both animal models and clinical research [30]. BKM120 is a highly specific PI3K inhibitor, which has a strong anti-proliferation effect and cooperativity in the radiosensitization of tumor cells. The combination of PI3K/AKT/mTOR inhibitor and radiation significantly increases the radiation-induced degeneration of OSCC [31]. Due to the importance of the PI3K-AKT pathway, we verified the effect of *A. crenata* on this pathway in HNSCC.

In vitro cell experiments were conducted to verify the predictions of network pharmacology. The results showed that *A. crenata* effectively inhibited the proliferation of Fadu cells in a dose and time-dependent manner. Western blot showed that *A. crenata* could inhibit the expression of p-PI3K and p-AKT. These results are consistent with the anti-HNSCC mechanism of *A. crenata* analyzed by network pharmacology, which further confirms the accuracy and reliability of the network pharmacology technology.

Conclusion

The study primarily investigated the effective anti-HNSCC mechanism of *A. crenata* based on network pharmacology, molecular docking and in vitro experiments. The results verified that *A. crenata* played an anti-HNSCC role by inhibiting the PI3K/AKT pathway, which provided a theoretical basis for the application of *A. crenata* in clinical treatment. However, this study still has some limitations, it mainly based on protein level, further studies should be conducted in animal experiments and transcriptional mechanisms.

Declarations

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The author declares that there is no confict of interest.

Authors' contributions

Zhongjia Tian, Faming Wu, and Qian Wang had the idea for the article. Literature search was performed by Zhongjia Tian, Lin Zhu, Huan Hu, Qin Lin, Qian Luo and Huaqian Liu. The manuscript was written by Zhongjia Tian and Faming Wu critically revised the work. Zhongjia Tian and Qian Wang reviewed and edited the manuscript. All authors read and approved the final manuscript.

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32. Statement
33. Ardisia crenata Sims root used in this study complied with the IUCN Policy Statement on Research Involving Endangered Species and the Convention on Trade in Endangered Species of Wild Fauna and Flora. The raw materials were provided by Sanli Pharmaceutical Company of Guizhou Province, identified by Professor Wu Faming of Zunyi Medical University, and stored in the School of Pharmacy for management.
34. Bergenin is an indicator component in A. crenata. In view of this, qualitative and quantitative detection of A. crenata aqueous extract was carried out. Reference was made to the chromatographic conditions in the 2020 edition of the "Chinese Pharmacopoeia" to prepare the reference substance and test solution, and finally the HPLC chart was obtained (Fig. 7). It can be seen from the figure that both bergenin can be detected at a wavelength of 275 nm, and the mass fraction of bergenin in the *A. crenata* aqueous extract is 1.21%, which meets the pharmacopoeia standard.

Statement

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Figures

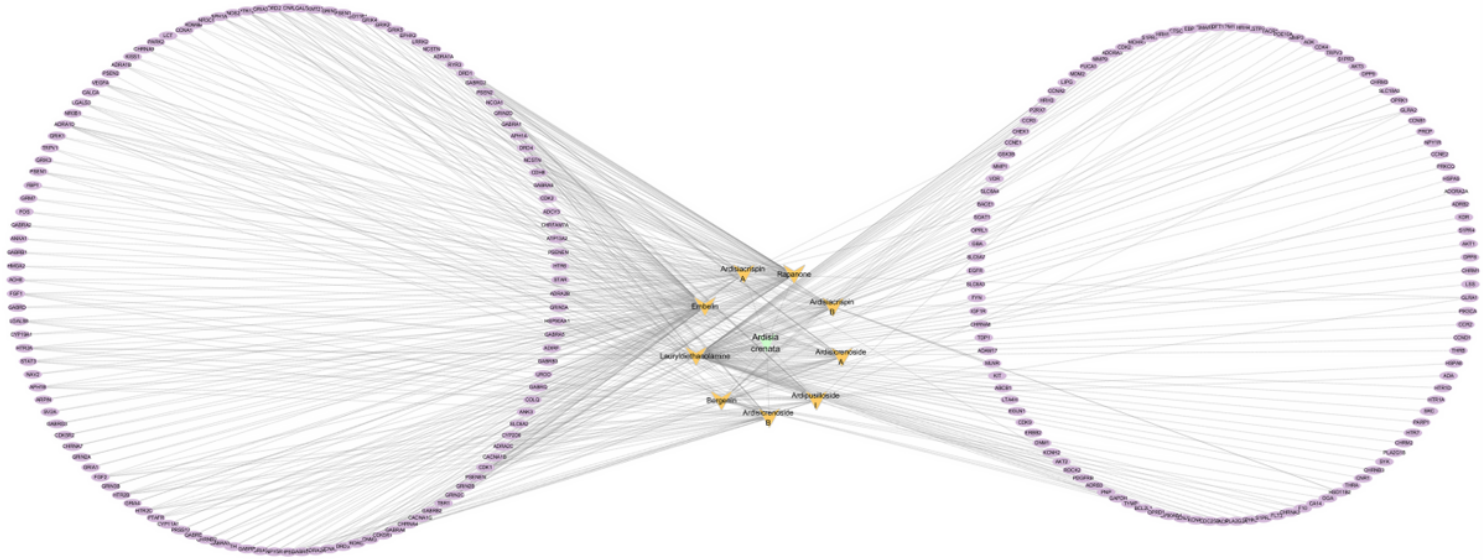


Figure 1

The Drug - component - target diagram of *A. crenata*.

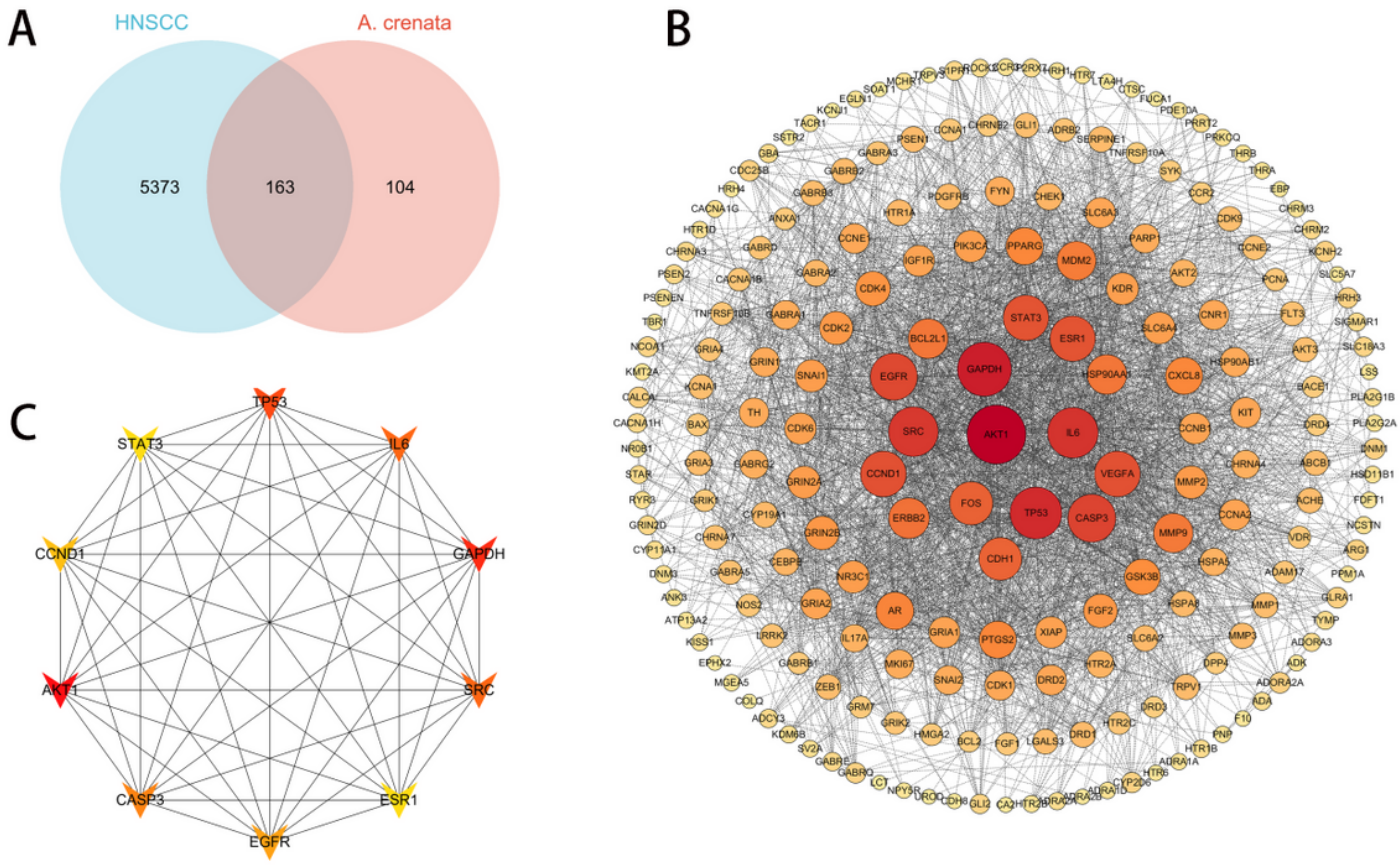


Figure 2

Selection of core targets of *A. crenata*anti-HNSCC. (A) The potential targets and intersection targets of *A. crenata*and HNSCC; (B) PPI network of *A. crenata* anti-HNSCC; (C) The key targets of *A. crenata* anti-HNSCC.

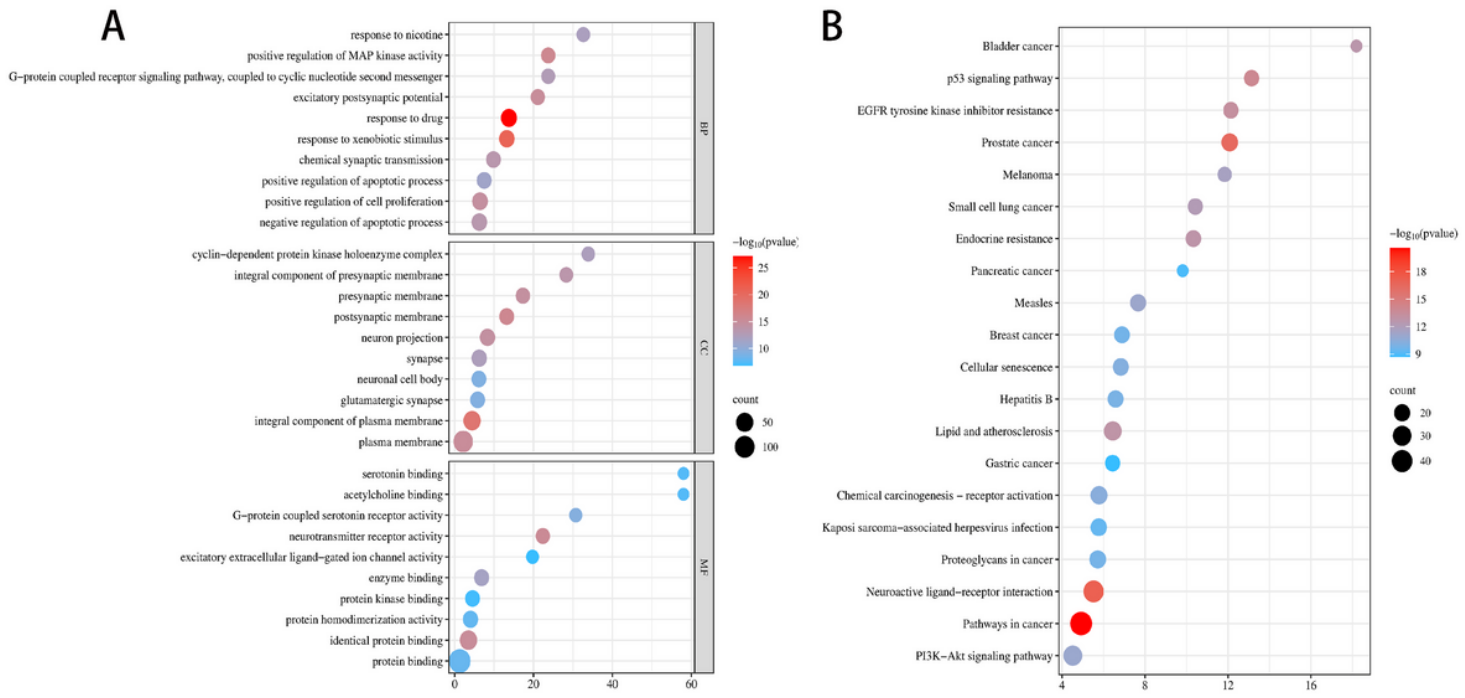


Figure 3

GO and KEGG enrichment analysis of the effective targets of *A. crenata*anti-HNSCC. (A) GO analysis; (B) KEGG enrichment analysis.

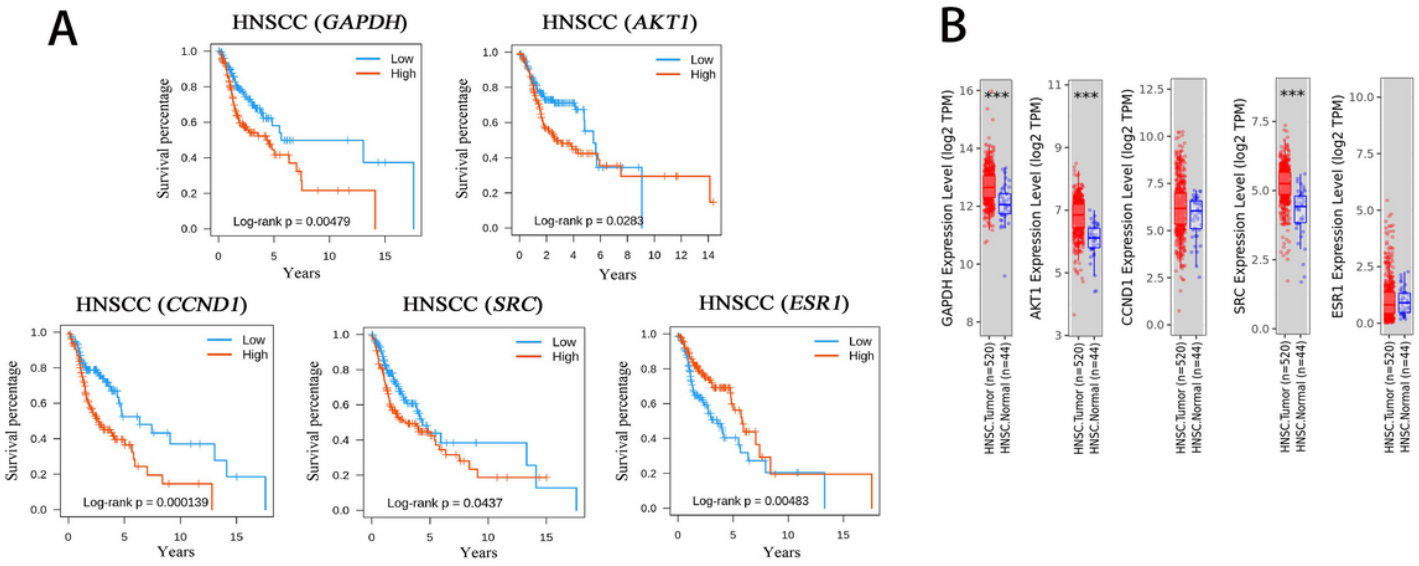


Figure 4

Survival analysis and verification of core genes. (A) The relationship between core genes expression level and the survival rate of HNSCC patients; (B) The expression levels of the core genes in normal tissues and HNSCC tissues.

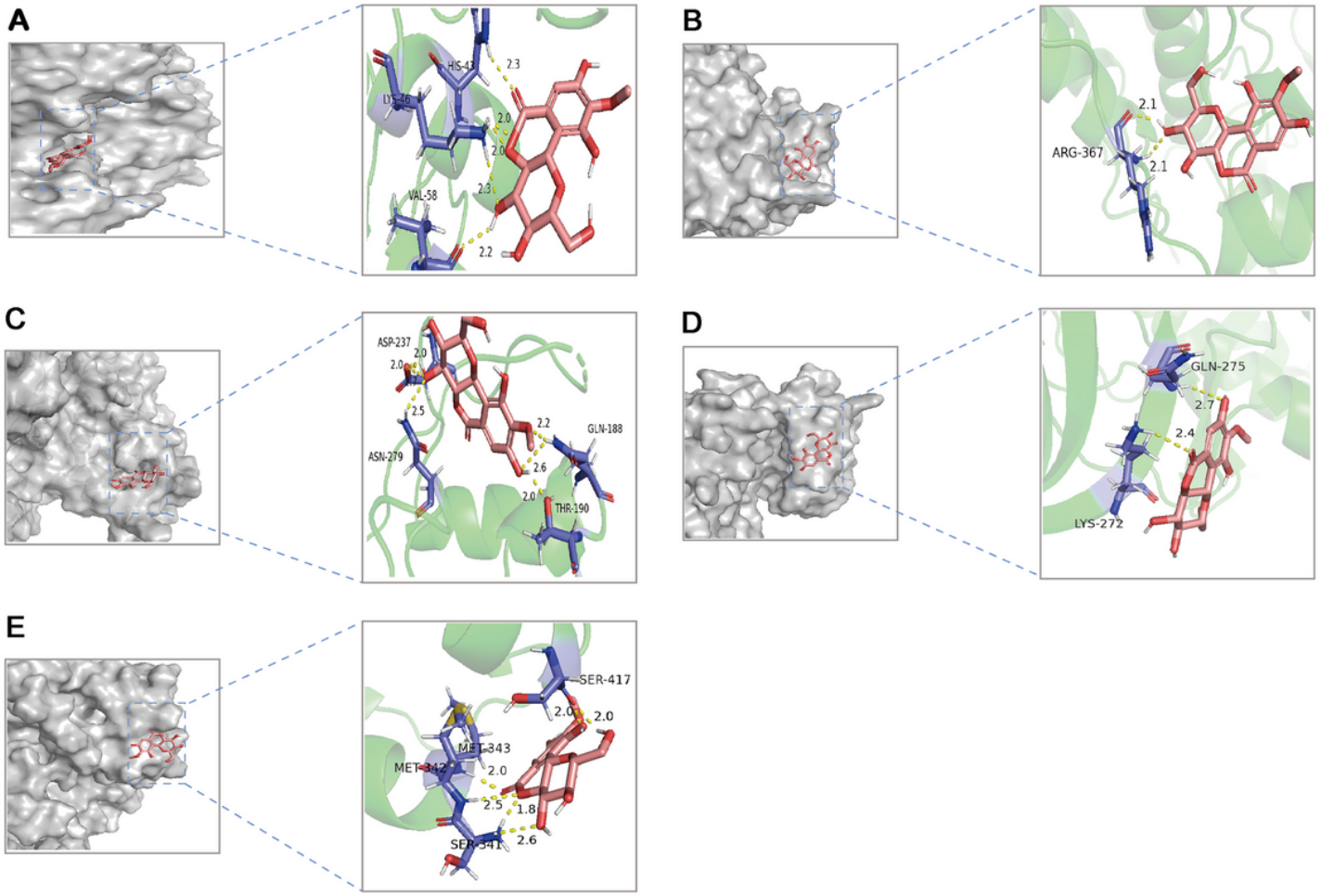


Figure 5

Molecular docking of bergenin and the core targets. (A) Bergenin docking pattern with GAPDH residues HIS-43, LYS-46 and VAL-58; (B) Bergenin docking pattern with GAPDH residues; (C) Bergenin docking pattern with CCND1 residues ASP237, ASN-279, GLN-188 and THR-190; (D) Bergenin and SRC residues GLN275and LYS-272 docking pattern; (E) Docking pattern of bergenin with ESR1 residues MET-342, MET-343, SER-341 and SER-417.

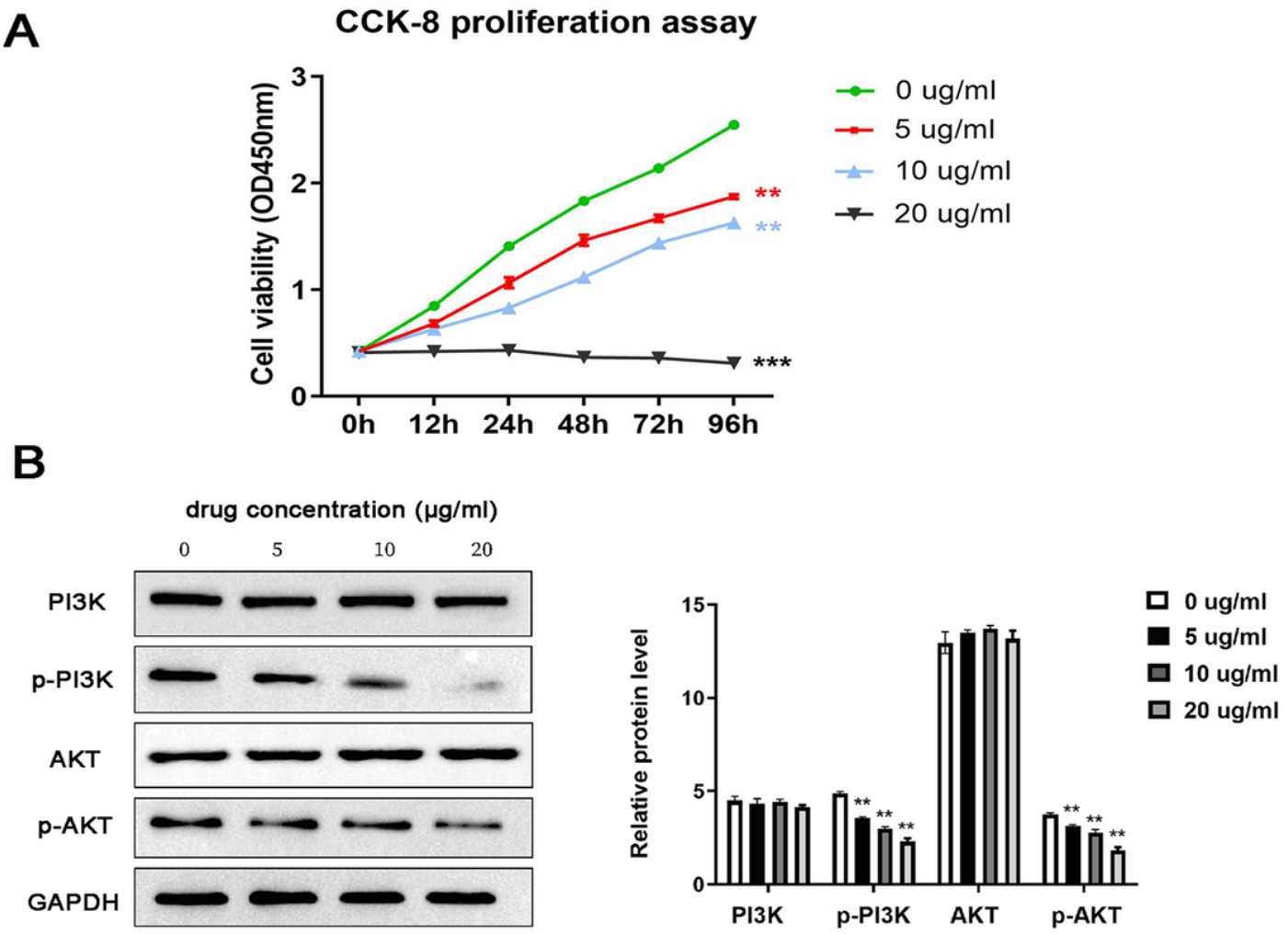


Figure 6

The effect of *A. crenata* on FaDu cells and PI3K/AKT pathway. (A) CCK8 showed the effect of *A. crenata* on the proliferation of FaDu cells (Compared with control group, **P* 0.05; ***P* 0.01); (B) The effect of *A. crenata* on protein expression of PI3K/AKT pathway (Compared with control group * *P* 0.05; ***P* 0.01)

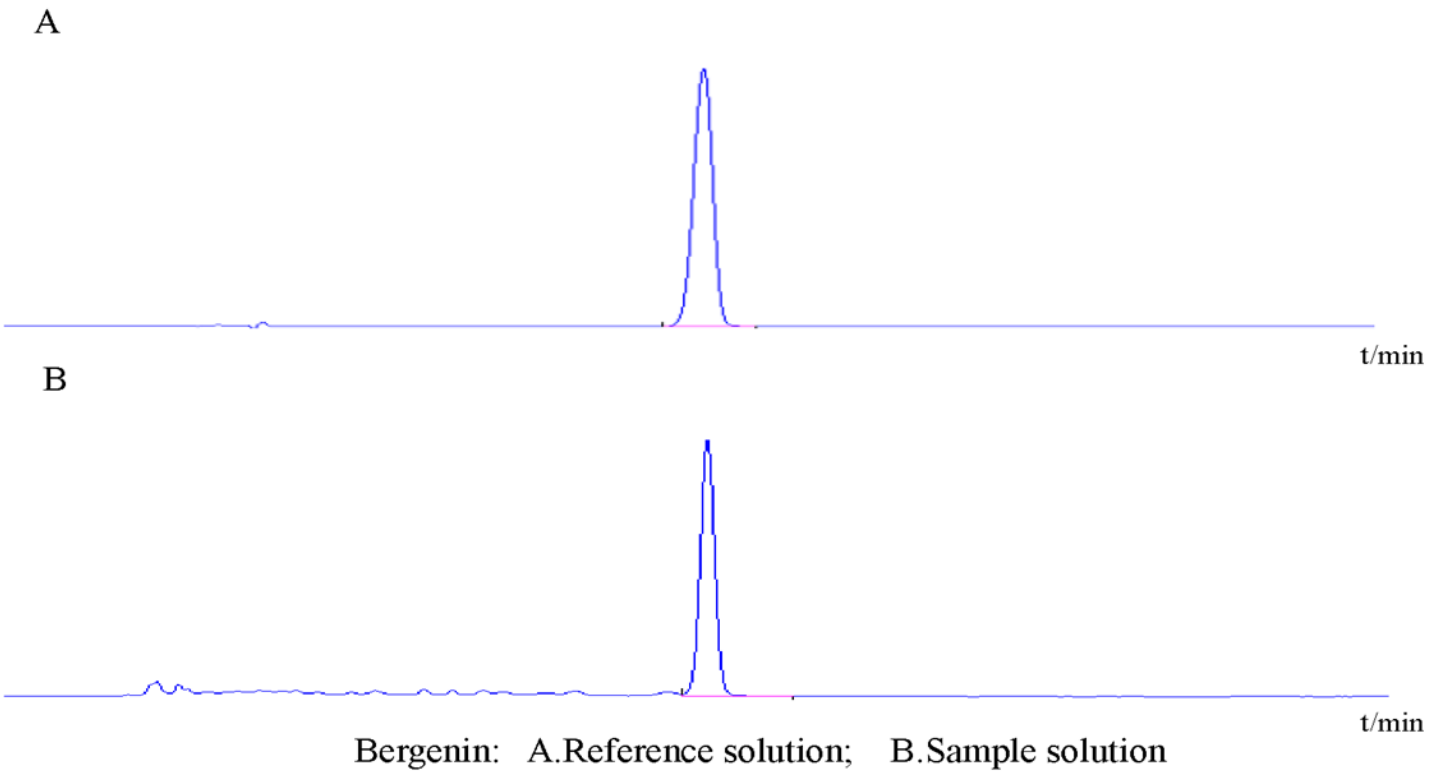


Figure 7

HPLC detection map of bergenin in *A. crenata*

Supplementary Files

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- Westernblot.zip