Inhibitory effect of saffron on head and neck squamous cell carcinoma via targeting of ESR1 and CCND1 by its active compound crocetin

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Abstract

Background: Traditional Chinese medicine is promising for managing challenging and complex disorders, including cancer, and in particular, saffron is applied in treating various cancer types. However, its potential therapeutic efficacy and active components in managing squamous cell carcinoma of the head and neck (HNSCC) remain unclear yet.

Methods: Using network pharmacology approaches, active ingredients of saffron, their target genes, and HNSCC-related genes were identified. Enrichment analyses were conducted for determining molecular functions and pathways enriched by genes that overlapped between the saffron target gene set and the HNSCC gene set. Among the four known active ingredients of saffron, crocetin was found to have the strongest inhibitory impact on HNSCC, based on the findings of cell viability and migration assays. Therefore, the potential target genes of crocetin in HNSCC cells were examined using molecular docking experiments and were confirmed by qPCR.

Results: Four active ingredients of saffron and 184 of their target genes were identified. Further, a total of 34 overlapping saffron-/HNSCC-associated targets related to the four active ingredients were screened, and crocetin was chosen for further investigation because it had the strongest inhibitory effect on HNSCC cells. Molecular docking experiments indicated that ESR1 and CCND1 were the target genes of crocetin. These results were confirmed through qPCR analysis, in which crocetin was found to lower the expression of the ESR1 and CCND1 genes in AMC-HN-8 and FaDu cells.

Conclusion: According to our results, crocetin is a primary active anti-cancer component of saffron that may have potential in the development of novel HNSCC-treating medications. However, more thorough molecular research is necessary for confirming these results and elucidating the anti-cancer mechanism underlying saffron.

Keywords: saffron; hub genes; crocetin; network pharmacology analysis; HNSCC; ESR1; CCND1
Head and neck squamous cell carcinoma (HNSCC) represents the term referring to a majority of head and neck malignancies that originate from mucosal epithelium of the larynx, pharynx, and oral cavity [1]. Its typical features include unknown etiology, non-specific early symptoms, rapid tumor growth with a high malignancy rate, and a high risk of recurrence. At the initial visit, 75% of HNSCC cases are already at a locally advanced stage (60%) or have metastasized (15%), with a median survival of only 6 months. At present, surgery combined with radiotherapy is used to treat non-metastatic HNSCC, but this approach has serious side effects. There is a need for alternative and adjunctive treatments that can reduce the side effects and improve the outcomes of HNSCC.

In China, traditional Chinese medicine (TCM) is applied in treating illnesses for centuries, and modern Chinese medicine has been found to have a strong impact on managing various challenging and complex diseases, including cancers [2, 3]. When used in combination with other therapeutic approaches, TCM is important for preventing and treating cancers. For example, for advanced cancers, including HNSCC, the TCM drugs triptolide, quercetin, cryptotanshinone, and Lycii Fructus have been shown to be effective supplemental or alternative agents. Triptolide has been found to induce pyroptosis in HNSCC, and quercetin reduced drug resistance and affected tumor cell proliferation in laryngeal squamous cell carcinoma. Further, cryptotanshinone was found to significantly induce apoptosis of oral squamous cell carcinoma cells and inhibit their growth, and similarly, and L. barbarum extract inhibited oral cancer cell growth, migration and adhesion. A commonly used herb in TCM is the dried stigma of Crocus sativus L., also called saffron, which belongs to the iris family that has been used in culinary, medicinal, and spiced. Saffron extract and its primary component, carotene, have been proven to have anti-cancer chemopreventive capabilities in a growing number of studies [4]. The potential mechanisms include apoptosis induction, cell cycle blocking, inhibition of matrix metalloproteinases, regulation of phase II detoxification enzymes, and decrease in the levels of inflammatory factors. So far, it is not clear whether saffron may have a therapeutic effect on HNSCC.

This work focused on investigating the potential therapeutic efficacy of saffron in HNSCC, its targets and its active components, with the help of network pharmacological analysis, which accounts for the novel strategy for analyzing molecular regulatory mechanism underlying the active components of TCM [5]. In addition, molecular docking, cell proliferation, and qPCR assays were used for confirming saffron’s inhibition against HNSCC proliferation and its targets. This research offers a conceptual and experimental foundation for applying saffron in treating HNSCC and reports potential treatment targets, too.

**Materials and methods**

**Selection of effective components of saffron**

In this study, we utilized the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) database (https://tcmspw.com/) for predicting major ingredients, effective compounds, and target genes of saffron. We chose the saffron chemical compounds having druggable properties and absorption, distribution, metabolism, and excretion properties to be our effective components to predict targets. Effective components were selected by a kinetic index of oral bioavailability ≥ 30% and drug-likeness ≥ 0.18, which yielded five components [6].

**Screening of target genes of saffron and HNSCC-related target genes and establishment of a component-target network**

The Swiss Target Prediction database was adopted for predicting possible protein targets of saffron’s effective components in line with their 3D structures. The saffron target genes associated with these components were detected and used to build a component target network with Cytoscape 3.3.0 software [7]. In addition, this work used the following three databases to collect HNSCC-related target genes: OMIM database (https://omim.org/), DrugBank database (https://www.drugbank.com/datasets/), and GeneCards database (https://www.genecards.org/). The identified HNSCC-related genes (the keywords “HNSCC” and “head and neck squamous cell carcinoma”) and target genes of saffron selected from the TCMSP database (mentioned earlier) were compared to identify overlapping genes.

**Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis using Metascape**

Through applying ClusterProfiler R package version, GO and KEGG analysis was performed with Metascape on saffron/HNSCC-associated genes to identify three categories, including biological process (BP), cell component (CC), as well as molecular function (MF), and pathways that were enriched for these genes [8]. Bioinformatics analysis (http://www.bioinformatics.com.cn/) was conducted for visualizing the obtained data.

**Protein–protein interaction (PPI) network construction and hub gene identification**

Overlapping genes between saffron target gene set and HNSCC-related gene set were imputed into String database (https://cn.string-db.org/), the protein interaction functional enrichment analysis website that can be used for constructing a PPI network upon the threshold of high confidence at 0.4 [9]. Based on the interaction intensity and average degree of each node, the first four genes were selected as hub genes.

**Cell proliferation assay**

Four components of saffron were used to treat the HNSCC cell lines. The human AMC-HN-8 and FaDu hypopharyngeal carcinoma cells were provided by Chinese Academy of Sciences. Certified fetal bovine serum (10%; VivaCell, Shanghai, China) and 1% antibiotics solution comprising penicillin and streptomycin (SparkJade Co., Ltd., Qingdao, China) were added to RPMI 1640 (VivaCell, Shanghai, China) and Dulbecco’s modified Eagle’s media (VivaCell, Shanghai, China) for culture of AMC-HN-8 and FaDu cells, respectively, followed by incubation under 37 °C and 5% CO2 conditions. The proliferation assay for both cell lines was performed using the CCK8 kit (SparkJade Co., Ltd., Qingdao, China). CCK8 was added to 96-well plates containing AMC-HN-8 or FaDu cells for 48 h. Next, every well was introduced with CCK8 solution (10 μL), followed by 1–2 h incubation in a 37 °C incubator, and then absorbance was measured at 450 nm [10]. The IC50 (half-maximal inhibitory concentration) values were
determined with GraphPad Prism 8 software.

Wound healing assay
Cell scratch (repair) method is a simple and convenient method for determining cell migration and repair capabilities [11]. Briefly, both cell lines (at the 30% density) were evenly plated into 6-well plates prior to culture under 5% CO2 and 37 °C conditions. A pipette tip was used to wound the cell monolayer and images were obtained at 0, 12, 24, and 48 h to observe cell migration. Photographs of the scratch were then processed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA), and the data were processed and a graph was created using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) to determine cell migration rate.

Molecular docking analysis
Molecular docking analysis is used for identifying novel interested compounds and predicting ligand–target interactions at a molecular level. We obtained relevant data from the RCSB PDB database 10, and downloaded three-dimensional structures of protein (CCND1, ESR1 and MTOR) [12]. Receptor proteins were dehydrated using the PyMOLWin software, and then hydrogenated and charged using the Autodock software [13]. Next, we obtained the 2D structures of ligand compounds in PubChem database11 (https://pubchem.ncbi.nlm.nih.gov). Energy-minimized 3D structures were calculated and exported using the ChemBio 3D software. The structures were later transformed into the mol2 format using OpenBabel-3.1.1, and the docking site parameters were set, as required, for the binding of the receptor proteins with the ligand compounds. We used the Autodock Vina-1.1.2 software for inducing docking of effective components with candidate targets and determining their free binding energies [14].

Reverse-transcriptase quantitative PCR (polymerase chain reaction)
Total RNA was extracted from AMC-HN-8 and FaDu cells cultured in a medium containing crocetin (GlpBio Co., Ltd., Shanghai, China) for 48 h using the Trizol reagent (SparkJade Co., Ltd., Qingdao, China) and transcribed into cDNA using the Accurate Bio reverse transcription kit (SparkJade Co., Ltd., Qingdao, China). The qPCR steps, that is, preparing the PCR reaction solution and setting up the PCR reaction program, were performed using the SYBR Green qPCR Mix kit (SparkJade Co., Ltd., Qingdao, China) in the StepOne Plus fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA). The data were analyzed to determine relative gene expression with the 2^−ΔΔCT method, with β-actin used as a housekeeping gene. The primers used for amplification of human ESR1 and CCND1 are listed in Supplementary Table S1.

Western blotting
The proteins were isolated in AMC-HN-8 and FaDu cells cultured in a medium containing crocetin (GlpBio Co., Ltd., Shanghai, China) for 48 h. 20 μg protein aliquots were subjected to 12% SDS-PAGE Gel Fast Preparation kits (Servicebio Co., Ltd., Wuhan, China) for separation prior to transfer onto polyvinylidene difluoride membranes.

After blocking using 5% Bovine Serum Albumin (SparkJade Co., Ltd., Jinan, China), membranes were further probed using indicated concentration of primary antibodies (GAPDH, ESR1, and CCND1; 1:1,000, Proteintech Co., Ltd., Wuhan, China) under 4 °C overnight. Using an enhanced chemiluminescence reaction kit (Millipore Corporation, Bill Ricka, MA, USA), target proteins were identified after secondary antibodies, goat anti-mouse and anti-rabbit IgG (1:5,000, Affinity Biosciences Pty., Ltd., Melbourne, Australia), were incubated.

Statistical analysis
We used GraphPad Prism 8 to analyze experimental data with unpaired t-tests. P < 0.05 stood for significant difference. Data were represented by mean and standard deviation.

Results
Screening of saffron/HNSCC-related genes
Figure 1 shows the flowchart of network pharmacology and experimental validation. We adopted TCMSP for searching targets of the following five active compounds of saffron [Supplementary Table S2]: crocetin, isorhamnetin, kaempferol, quercetin, and n-heptanial (PubChem CID: 5281132, 52811654, 5280663, 5280543 for the former four). Since n-heptanial does not have a PubChem CID, it could not be used for further experiments. Therefore, the other four ingredients were chosen as the main ingredients, and their 184 valid targets were obtained (Supplementary Table S3).

With the keywords “HNSCC” and “head and neck squamous cell carcinoma” 94, 66, and 317 HNSCC-related genes were identified from the OMIM (https://omim.org/), DrugBank (https://www.drugbank.com/datasets/), and GeneCards (https://www.genecards.org/) databases, respectively. After duplicate elimination, a total of 461 disease targets remained (Supplementary Table S4).

Comparison of the 461 HNSCC-related genes and 184 target genes of saffron revealed that 34 overlapped, as shown in Figure 2. Then we utilized valid targets of the four ingredients for building the component-target network by the Cytoscape 3.9.0 software (Figure 3A).

Functional enrichment analyses of saffron/HNSCC-related genes
In addition, GO annotation of 34 intersecting genes was carried out for identifying BP, CC and MF terms that the genes were associated with [15, 16]. The first eight terms of P < 0.01, a minimal overlap of 3, and an enrichment factor value > 1.5 were selected. The BP-related terms that were enriched included protein phosphorylation, response to inorganic substances, response to UV, response to peptides, tube morphogenesis, and regulation of cellular response to stress, among others. The CC enrichment terms included transferring phosphorus-containing groups, extrinsic component of the membrane, nuclear envelope, transcription regulator complex, and membrane raft, among others. Moreover, the MF enrichment terms included protein kinase activity, nitric oxide synthase regulator activity, protein tyrosine kinase activity, transcription coactivator binding, and insulin receptor substrate binding, among others (Figure 3B). These 34 overlapping genes were also used for KEGG enrichment analysis [17]. The top 12 terms were selected, and the main pathways included prostate cancer, pathways in cancer, miRNAs in cancer, bladder cancer, viral carcinogenesis, progesterone-mediated oocyte maturation, EGFR tyrosine kinase inhibitor resistance, transcriptional misregulation in cancer, oxytocin signaling pathway, tuberculosis, nuclear factor-kappa B pathway, and apelin pathway (Figure 3C).

PPI network analysis and hub gene acquisition
A PPI network is a set of systems established based on the physical combination of multiple proteins [18]. An analysis of the interactions that occur among different proteins can greatly contribute to understanding the functional principle of proteins. The 34 saffron/HNSCC-associated genes were imputed in String database 11.5, the website for protein interaction functional enrichment, for constructing a PPI network (Figure 4A) with a cut-off of high confidence of 0.4 [19].

We used the PPI network generated by Cytoscape to sort the network’s hub genes based on the betweenness values [20]. ESR1, AKT1, MTOR, and CCND1 were identified as hub genes since they had the top four betweenness scores (Figure 4B) [21].

Inhibition of crocetin against HNSCC cell migration
Inhibition of four active components in saffron, including crocetin, isorhamnetin, kaempferol, and quercetin, against HNSCC cells were tested using CCK-8 experiments. The action curve was drawn in accordance with the results, and crocetin was found to have the highest inhibitory effect on the HNSCC cell lines. To confirm the
Figure 1 A flowchart illustrating how saffron inhibits HNSCC based on network pharmacological analysis and experimental validation. As shown in the diagram, screening for saffron/HNSCC related genes, using experiments to verify that the most effective component in saffron is crocetin, and screening for its action genes. Intersect the above genes to obtain the co acting genes (ESR1, MTOR, CCND1), and then perform molecular docking on the target protein to obtain the relevant genes (ESR1, CCND1). Finally, use qPCR to verify the expression of the two genes. HNSCC, squamous cell carcinoma of the head and neck; TCMSP, Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform.

Figure 2 Venn diagram of overlapping saffron and HNSCC target genes. From among 461 HNSCC target genes and 184 saffron target genes, 34 common target genes were present. HNSCC, squamous cell carcinoma of the head and neck.
Figure 3 Network map and functional enrichment analyses of the target genes of saffron. (A) Cytoscape was used to create a network map of the 184 target genes that were associated with the identified active components. The four red hexagons represent the four active components of saffron, while the green circle symbolizes saffron. The margin of the blue diamond denotes the interaction between the component and the target gene, and the blue diamond itself denotes the genes connected to saffron. (B) GO and (C) KEGG analyses. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Figure 4 PPI network construction and hub gene identification. (A) PPI network including 34 saffron/HNSCC-related genes. The identified hub genes are ESR1, AKT1, MTOR, and CCND1, represented by the four yellow circles. (B) The hub genes were ESR1, AKT1, MTOR, and CCND1.
Inhibitory effect of crocetin on the expression of ESR1 and CCND1

We used crocetin for molecular docking experiments with the four identified hub genes (ESR1, AKT1, MTOR, and CCND1) because the cell proliferation assay showed that crocetin has the strongest inhibitory effect on the tumor cells. By screening the targets of crocetin through a database, ESR1, MTOR, and CCND1 were selected as its targets from among the four hub genes. Molecular docking of crocetin with these three targets revealed that while crocetin had no effective docking site with MTOR, it bound to ESR1 at GLN-441 and ASN-439 (Figure 6A) and to CCND1 at SER-150, SER-28, GLN-98, and ASP-97 (Figure 6B). The affinity values were calculated and evaluated as follows: $> -4$ kcal/mol, extremely weak binding force or no binding; $-7$ to $-4$ kcal/mol, moderate binding strength; $\leq -7$ kcal/mol, strong binding force. ESR1 and CCND1 exhibited crocetin-binding affinity values of $-5.6$ and $-5.5$ kcal/mol, respectively, which are indicative of moderate binding strength. Based on the molecular docking results, expression of the ESR1 and CCND1 genes was examined by qPCR in AMC-HN-8 and FaDu cells after crocetin treatment, confirming that the inhibitory effect of crocetin (when applied at its IC$_{50}$ value of 46.94 µM and 49.32 µM for AMC-HN-8 and FaDu cells, respectively) may be brought about via regulation of ESR1 and CCND1 expression. Consequently, crocetin suppressed ESR1 and CCND1 gene levels in HNSCC cell lines at these two concentrations (Figure 7A–7E).

Discussion

In this study, based on the results of cell viability and wound healing assays, saffron was found to have an inhibitory effect on HNSCC cells. Further, through network pharmacology, molecular docking, and PCR analyses, effective components and target genes of saffron in treating HNSCC were identified.

Crocetin was identified as the main active component of saffron involved in HNSCC suppression. In accordance with the findings,
Figure 6 Target proteins of crocetin identification. (A) Docking sites of ESR1 and (B) CCND1 with crocetin.

Figure 7 Impact of crocetin on the expression of ESR1 and CCND1. (A, B) Impact of crocetin on ESR1 and CCND1 levels within AMC-HN-8 cells determined through qPCR. (C, D) Impact of crocetin on the expression of ESR1 and CCND1 in FaDu cells determined through qPCR. (E) Impact of crocetin on ESR1 and CCND1 protein levels within AMC-HN-8 cells and FaDu cells as determined by western blot.

Protection of the saffron carotenoids crocin and crocetin are widely investigated with regard to the antioxidant effects, as well as tumor growth inhibition and cell death induction [22]. Especially, many articles suggest the effect of crocetin on cancer cell proliferation through suppressing nucleic acid synthesis, promoting antioxidative effects, accelerating apoptosis, while blocking growth factor pathways [23]. However, we were unable to clarify the specific mechanism by which crocetin acts on HNSCC. Nonetheless, the identification of crocetin as the main component is a good starting point for future mechanistic studies on the anti-tumor effect of saffron on HNSCC.
Screening of the target genes of saffron and HNSCC target genes revealed that 34 genes overlapped, and these were subjected to GO and KEGG analyses. Those top terms enriched in GO analysis were protein phosphorylation, transfer of phosphorus-containing groups, and protein kinase activity, and the main pathway that was enriched according to KEGG analysis was pathways in cancer. Thus, the targets of saffron in HNSCC may include phosphorylation-related proteins, protein kinases, and phosphorus-containing groups. Accordingly, protein kinase inhibitors are reported to have a potential to modulate disorders like diabetes, cardiovascular disease, rheumatoid arthritis, cancer and diabetes-related complications. The pathways in cancer includes EGF-EGFR-RAS-ERK pathway, EGF-EGFR-PI3K pathway, cell cycle (cancer), etc. Cell cycle represents the various events occurring within one cell, which promote cell division and production of two novel daughter cells [24]. Inducing cell cycle arrest and apoptosis can inhibit tumor cell proliferation [25]. The present findings highlight that protein kinase and cancer pathways may be promising subjects to explore in relation to anti-tumor mechanism of saffron in HNSCC.

The hub genes ESR1 and CCND1 were found to be the main targets of crocetin, as their expression was decreased in HNSCC cells treated with crocetin. Located on chromosome 6q24–27, the ESR1-encoding gene contains 7 introns and 8 exons approximately 140 kb long and comprise 2 promoter regions together with 5 functional domains. It is critical for DNA binding, hormone binding, as well as transcription activation due to its effect on encoding the estrogen receptor responsible for mediating hormonal response within estrogen-sensitive tissues [26]. Additionally, abnormal hormone receptor expression or mutations within cancer cells are related to the aggressiveness of cancer [27]. Cyclin D1 is the CCND1 gene-encoded protein (45 kDa) on chromosome 11q13 [28]. Cyclins are important for controlling cell cycle, and they are under the regulation of cyclin-dependent kinases. Cyclins activation can activate cell cycle activation due to its effect on encoding the estrogen receptor responsible for mediating hormonal response within transition from G1 to S phase, thus causing cell proliferation [29]. CCND1 serves as the efficient biomarker for predicting HNSCC prognosis and clinical response, with CCND1 down-regulation being tightly related to chemotherapeutic response and good prognostic outcome, while CCND1 up-regulation predicts chemotherapeutic failure [30].

The main function of CCND1 is to promote cell proliferation, and overexpression of CCND1 can lead to uncontrolled cell proliferation and malignancy. Studies have shown that CCND1 gene overexpression and gene amplification are found in a variety of tumors, including HNSCC. Interferon-induced transmembrane 3 proteins knockdown down-regulated CDK4 and CCND1, which in turn causes apoptosis, senescence, and cell cycle arrest. Ultimately, this reduces the growth of OSCC cells [31]. Long non-coding RNA CASC15 upregulates cyclin D1 within laryngeal squamous cell carcinoma for promoting cell growth [32]. At the same time, other studies have shown that cyclin D1 expression within HNSCC is directly related to tumor recurrence [33].

ESR1-positive breast cancer shows the highest prevalence among breast cancer cases, either early or advanced ones [34]. The mechanism related to ESR1 in breast cancer is studied extensively. It has been discovered that the estrogen-binding ESR1 promotes cell cycle progression by upregulating the expression of MYC and CCND1, and it intensifies mitotic signaling by upregulating the transcription of multiple growth factors crucial for the development of the mammary gland, such as TGFα, IGF-1, and EGF. As a result, blocking ESR1 can greatly reduce the rate at which breast cancer cells proliferate [35]. Several recent studies have shown that 20% of metastatic ESR1(+) disease patients receiving endocrine therapy develop gap-relapsing ESR1 mutations that result in possible enhanced metastatic ability, tumor development, ligand-independent ER activity, and partial drug resistance [36]. However, there are few studies on the importance of ESR1 in HNSCC.

Through molecular docking experiments, we have identified that crocetin possesses the ability to directly bind to ESR1 and CCND1 proteins, consequently influencing the downstream functions of these genes. Subsequent qPCR and western blot experiments have revealed a notable down-regulation in the expression of both ESR1 and CCND1 genes and proteins following crocetin treatment of AMC-HN-8 and FaDu cells, thereby exerting a significant impact on their functionalities. Upon KEGG analysis, CCND1 directly affects cell proliferation, while ESR1 can also contribute to the regulation of cell proliferation through its interaction with CCND1. Furthermore, the wound healing assay has provided evidence that the proliferation of tumor cells is markedly inhibited after exposure to crocetin, suggesting that crocetin may regulate ESR1 and CCND1 either directly or through indirect pathways, thereby impeding the progression of HNSCC (Figure 8).

Based on these findings, future studies on HNSCC therapy could explore further the potential of ESR1 and CCND1 as potential markers and therapeutic targets.

Conclusions

In this study, we identified crocetin as the primary ingredient of saffron that plays an anti-tumor role in the treatment of HNSCC. Further, ESR1 and CCND1 were identified as the main target genes of crocetin in HNSCC cells. The findings imply that the primary mechanism by which saffron targets HNSCC cells is through the regulatory effect of the active component crocetin on ESR1 and CCND1 expression. The above results help to develop novel therapeutic agents for treating HNSCC, but they need to be verified in the future through more detailed research into the underlying molecular mechanisms. In addition, the findings point to the importance of exploring the potential of TCM agents in cancer treatment.

![Figure 8 Cancer pathways associated with ESR1 and CCND1. P, indicating phosphorylation; E2, indicating estradiol.](https://www.tmrjournals.com/tmr)


