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RESEARCH ARTICLE

Chemosensitizing effect of honokiol in oral carcinoma stem cells via regulation of IL-6/Stat3 signaling

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Abstract

Oral squamous cell carcinoma (OSCC) is one of the most common cancers worldwide with poor prognosis. Numerous studies have attempted to explore alternative regimens aimed at reducing cancer stem cells (CSCs) without compromising the efficacy of conventional chemoradiotherapy. The present study sought to assess the effect of a natural compound honokiol on the reduction of elevated cancer stemness, metastatic capacity, and chemoresistance of oral carcinoma stem cells (OCSCs). Our results demonstrated that honokiol attenuated the cell survival and self-renewal of OCSCs in a dose-dependent manner. Moreover, honokiol downregulated the expression of 2 selective markers of OCSCs, ALDH1, and CD44, as well as the migration and invasion abilities, indicating its potential to suppress cancer stemness. We showed that honokiol reduced the secretion of IL-6 and phosphorylation of STAT3, and the honokiol-inhibited self-renewal, invasion and colony formation were reversed by administration of IL-6. Most importantly, our data demonstrated that honokiol was able to potentiate the effect of Cisplatin, leading to a lower proportion of OCSCs and the decreased cancer stemness features. Taken together, this study demonstrated the benefits of utilizing honokiol as an adjunct therapy for OSCC treatment.

KEYWORDS

cancer stem cells, chemo-sensitivity, honokiol, oral squamous cell carcinoma

1 | INTRODUCTION

Oral squamous cell carcinoma (OSCC) constitutes the most common malignancy in the head and neck region with increasing incidence.[1,2] Apart from drug resistance and local invasion, the great propensity for metastasis in the cervical lymph nodes also results in poor prognosis of OSCC.[3] Moreover, majority of OSCC patients present at a late stage due to delay in seeking professional advice and treatment.[4] Hence, it is necessary to emphasize the public education to reduce the consumption of areca nut, alcohol and tobacco smoking. Additionally, emerging evidence has suggested that failure of cancer therapies may be due to the existence of cancer stem cells (CSCs).[5,6] It has been known that CSCs were capable of remaining vital after treatments

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and retaining their ability to repopulate again. As such, elimination of CSCs became the key issue that needs to be considered when tailoring the treatment for the individual patient.

Among various factors that affect the sensitivity of drug resistance, interleukin (IL)-6 has been shown to be associated with the poor response in drug-resistant head and neck cancer.[7] It has been demonstrated that stromal IL-6 defined the tumorigenic capacity of CSCs from the head and neck cancer via increasing CSCs motility.[8,9] The IL-6 blockade was shown to enhance the chemosensitivity of lung cancer organoids integrated by CSC-like cells[10] and decrease the fraction of CSCs of head and neck cancer.[11] Besides, IL-6 was sufficient to convert non-stem cancer cells into CSCs in genetically different breast cell lines.[12] Consequently, the current study was proposed to examine the effect of a strategy targeting CSCs via modulation of IL-6.

Honokiol is a biphenyl-type neolignan extracted from the bark of traditional Chinese herbal medicine Magnolia species.[13] This bioactive compound possesses multiple pharmacological activities, such as neurotrophic, anti-oxidant, anti-inflammatory, and anti-cancer properties.[13,14] Honokiol has been shown to exhibit the anti-proliferative effect on OSCC through the regulation of specificity protein 1[15] and it has been proven to eliminate CSCs and reduce drug resistence in glioblastoma cells.[16] Also, honokiol has been demonstrated to reduce the IL-6-induced Akt activation and suppressed STAT3 activity in hepatocellular carcinoma cells.[17] One of the recent studies even showed that honokiol was capable of attenuating the sphere formation and xenograft growth of OCSCs.[18] Accordingly, we assessed the cancer stemness, cell motility, and chemoresistance of OCSCs subsequent to the treatment of honokiol in the present study to further elucidate the efficacy of using honokiol as an adjuvant agent in the eradication of OSCC.

2 | MATERIALS AND METHODS

2.1 | Reagent and cell culture

Honokiol was purchased from Sigma Chemical Co. The CSCs derived from OSCC cell lines SAS (tongue cancer cells), OECM-1 (gingival squamous cell carcinoma cells), and GNM (neck metastasis of gingival carcinoma cells) as well as normal human gingival epithelioid cell line (SG) were cultivated as previously described.[19,20]

2.2 | Cell viability assay

Cell survival was determined by MTT assay (Sigma, St. Louis, Missouri) to evaluate the cytotoxicity of honokiol. Cells were seeded in 96-well plates (1×10^4 cells/well) in the presence of various concentration of honokiol or vehicle at 37°C for 24 hours followed by incubation with MTT reagent. The blue formazan crystals were dissolved in DMSO and then evaluated spectrophotometrically at 570 nm. The DMSO-treated group was set as 100%, and data were presented as a percentage of the DMSO control.

2.3 | Secondary sphere assay

Cells were dissociated and cultured in the modified medium of DMEM/F-12 supplemented with N2 (R&D Minneapolis, Minnesota), epidermal growth factor (Invitrogen, Carlsbad, California), basic fibroblast growth factor (Invitrogen), and penicillin/streptomycin at 10^3 cells/low-attachment 6-well plate (Corning Inc, Corning, New York). The culture medium was changed every other day. Secondary spheres/10 000 cells were presented as the percentage of control.

2.4 | Flow cytometry

Single-cell suspension of 5×10^6 from trypsinized cells of spheres were stained with aldehyde dehydrogenase 1 (ALDH1) (ALDEFLUOR assay kit; StemCell Technologies, Durham, North Carolina) for ALDH1 activity. For CD44 expression, cells were stained with anti-CD44 antibody conjugated to phycoerythrin (Miltenyi Biotech., Auburn, California) according to the manufacturer's instructions.

2.5 | Migration and invasion assays

The 24-well plate Transwell system with a polycarbonate filter membrane of 8-μm pore size (Corning, United Kingdom) was used to evaluate the migration and invasion abilities of cells. The membrane was coated with Matrigel (BD Pharmingen, Franklin Lakes, New Jersey) for invasion. The cell suspensions were seeded to the upper chamber of the Transwell insert within serum-free medium at the cell density of 1×10^5 for migration and invasion assays. The lower chamber was filled with media supplemented with 10% serum. After 24 hours of incubation, the filter membrane was stained with crystal violet (Sigma-Aldrich). The migrated and invasion cancer cells were then visualized and counted from 5 different visual areas of 100-fold magnification under an inverted microscope.

2.6 | Colony formation assay

Each well of a 6-well culture dish was coated with 1 mL of bottom agar (Sigma-Aldrich) mixture (DMEM/F-12, 15% [vol/vol] FBS, 0.525% [wt/vol] agar). After the bottom layer was solidified, 1 mL of top agar-medium mixture (DMEM/F-12, 15% [vol/vol] FBS, 0.3% [wt/vol] agar) containing 4×10^4 cells were added, and the dishes were incubated at 37°C for 2 weeks. Plates were stained with 0.01% crystal violet, and then, the colonies were counted.

2.7 | Enzyme-linked immunosorbent assay

The secretion of IL-6 was determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) and quantified at 490 nm according to the manufacturer's instructions. Cells were cultured in 6-well plates with various concentration of honokiol for 24 hours. Cell supernatants were collected and centrifuged to remove dead cells. Each individual sample was analyzed in triplicate.

2.8 | Western blot analysis

Cell protein extraction was performed as previously described.[21] The sample was separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Amersham, Arlington Heights, Illinois). The primary antibodies against phosphor-STAT3 and STAT3 were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, California). After blocking, the membranes were incubated with appropriate primary antibodies

followed by corresponding secondary antibodies. The immunoreactive bands were developed using an ECL-plus chemiluminescence substrate (Perkin-Elmer, Waltham, Massachusetts) and captured by ImageQuant LAS 4000 Mini (GE Healthcare, Piscataway, New Jersey).

2.9 | Statistical analysis

Data were expressed as the mean ± SD and analyzed by ANOVA (Tukey's HSD as post hoc) using SPSS Statistics version 13.0. A value of $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Cell survival of normal gingival epithelial cells and oral CSCs in response to honokiol

Sphere-forming cells with cancer stem-like cell traits are often used to study OCSCs.[22] To determine the cytotoxic effect of honokiol on cell viability of normal gingival epithelial SG cells and oral CSCs, we treated the SG cells as well as GNM-sphere, SAS-sphere, and OECM-1-sphere cells with various concentration (0-10 μM) of honokiol for 24 hours followed by MTT assay. As shown in Figure 1A, honokiol demonstrated a dose-dependent decrease in cell survival in OCSCs without damaging normal SG cells. Besides, secondary sphere formation ability of honokiol-treated cells was examined. It was shown that honokiol significantly inhibited the self-renewal property of these OCSCs (Figure 1B). These results indicated that honokiol may be beneficial to suppress proliferation and self-renewal capacities of OCSCs.

3.2 | Honokiol diminishes the ALDH1 activity and CD44 expression in OCSCs

ALDH1, a cytosolic isoenzyme, and CD44, a transmembrane glycoprotein, are 2 selective markers that are utilized to identify CSCs from head and neck cancer.[23,24] Hence, we assessed the ALDH1 activity and CD44 expression after honokiol treatment to examine whether honokiol affected the cancer stemness. Results from flow cytometry showed that both ALDH1 activity and CD44 expression were both downregulated in a dose-dependent manner subsequent to honokiol treatment (Figure 2A,B), indicating that the proportion of OCSCs was gradually decreased as the concentration of honokiol increased.

3.3 | Honokiol attenuates the migration and invasion abilities of OCSCs

OCSCs are critical to metastasis, which is one of the primary hurdles in cancer therapy.[25] As a consequence, we evaluated the migration and invasion capacities of these cells after honokiol treatment. Our results showed that the migration (Figure 3) and invasion (Figure 4) abilities were both inhibited in a dose-dependent fashion

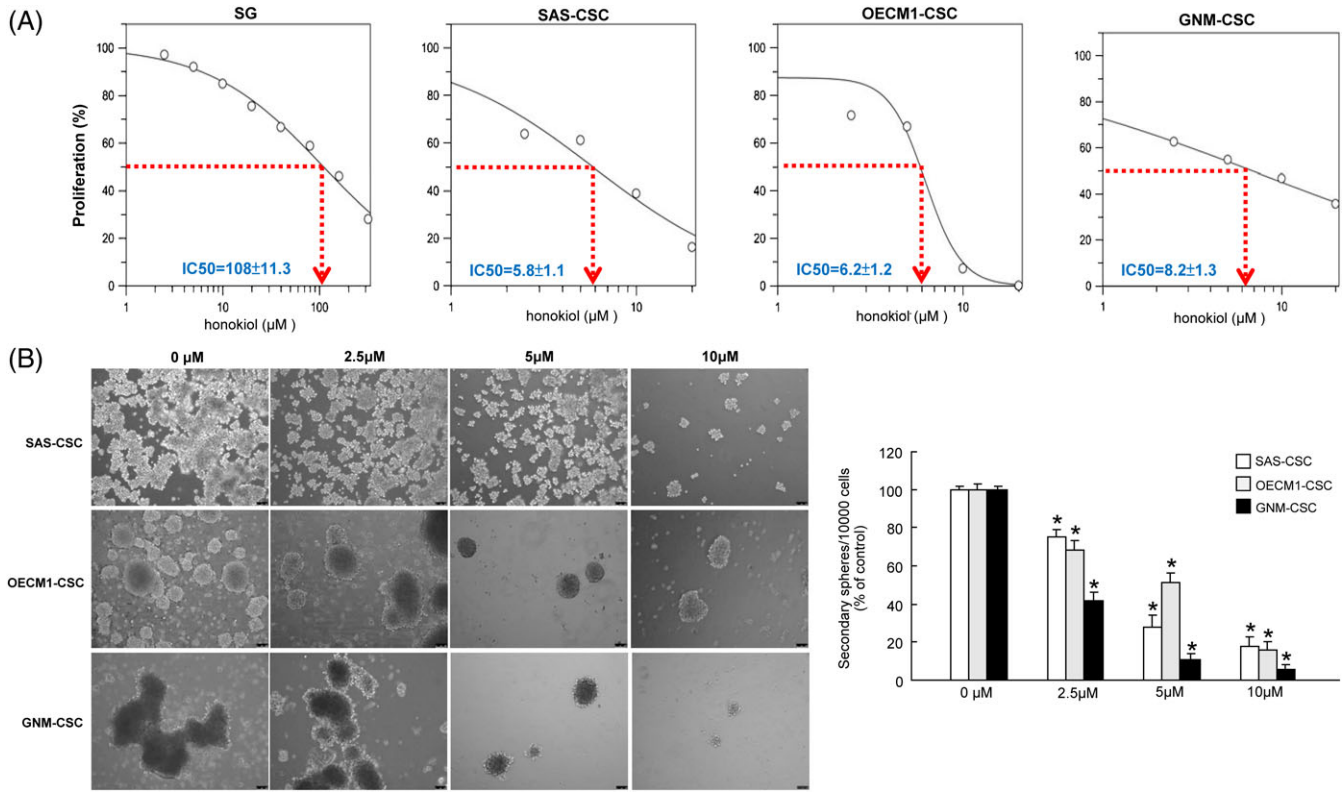

FIGURE 1 The inhibitory effect of honokiol on cell survival and self-renewal of OCSCs. A, The cell survival/proliferation of normal SG cells and various OCSCs following treatment of honokiol was determined by MTT assay. IC50 values were calculated by GraFit software. B, Secondary sphere formation was assessed after treatment of various concentration of honokiol. Results are means ± SD. *$P < .05$ compared to no treatment group [Color figure can be viewed at wileyonlinelibrary.com]

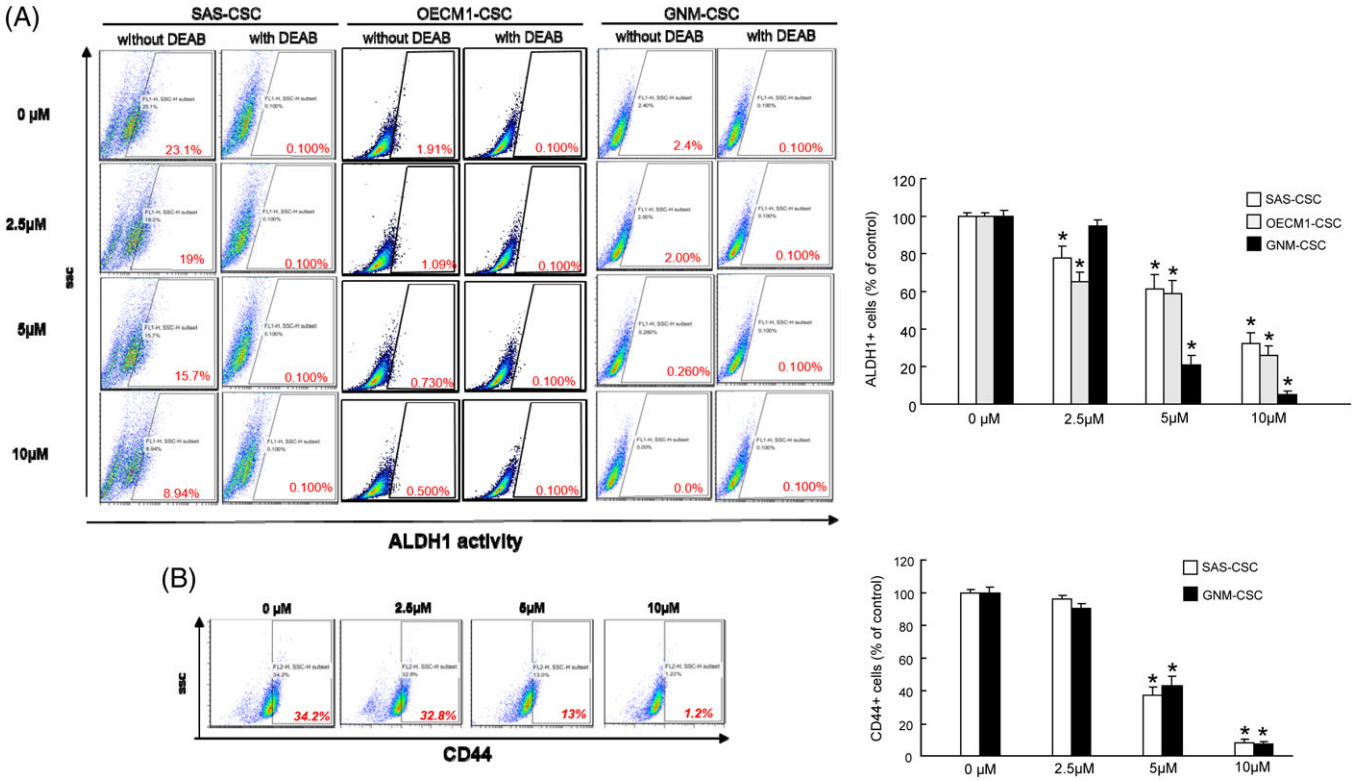

FIGURE 2 Suppressive effects of honokiol on the expression of cancer stemness markers. A, ALDH1 activity and (B) CD44 expression in OCSCs were examined in response to various concentration of honokiol by flow cytometry. Results are means ± SD.*$P < .05$ compared to no treatment group [Color figure can be viewed at wileyonlinelibrary.com]

following honokiol administration. These findings suggested that honokiol possessed the inhibitory effect on the aggressiveness of OSCC.

3.4 | The reduced clonogenic growth and chemosensitizing effect of honokiol in

3.4.1 | OCSCs

To investigate the capacity of OCSCs to produce progeny after honokiol treatment, the anchorage-independent growth assay was used to show that the colony formation capacity of OCSCs was reduced in response to honokiol (Figure 5A). Drug resistance is another obstacle to the success of cancer therapy. Therefore, we evaluated whether honokiol could decrease the chemoresistance. As expected, SAS-CSCs were barely affected by Cisplatin compared with parental SAS cells (Figure 5C). Nevertheless, the sensitivity to Cisplatin in SAS-CSCs was dramatically improved in combination with honokiol (Figure 5C). Honokiol not only enhanced the chemosensitivity, it also downregulated the self-renewal (Figure 5D) and invasion (Figure 5E) capacities in the Cisplatin-resistant OCSCs. Besides, the overall response appeared to be synergistic as the combination of Cisplatin and honokiol exhibited

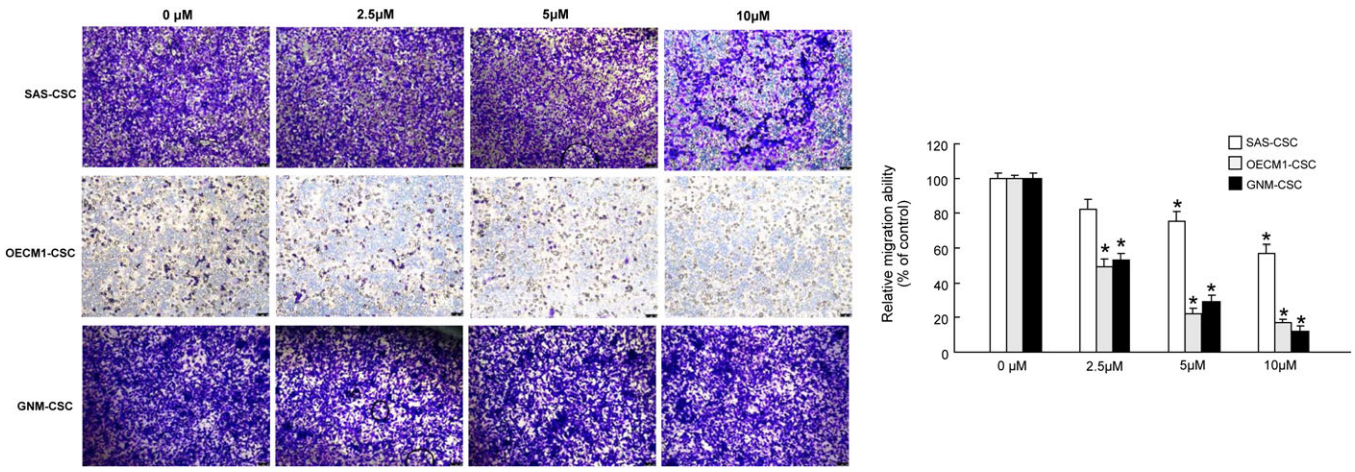

FIGURE 3 Honokiol reduces the migration capacity of OCSCs. Representative images and quantification results showing that administration of honokiol mitigated the increased migration capacity of OCSCs. Data were shown as a percentage of control group. *$P < .05$ compared to control group [Color figure can be viewed at wileyonlinelibrary.com]

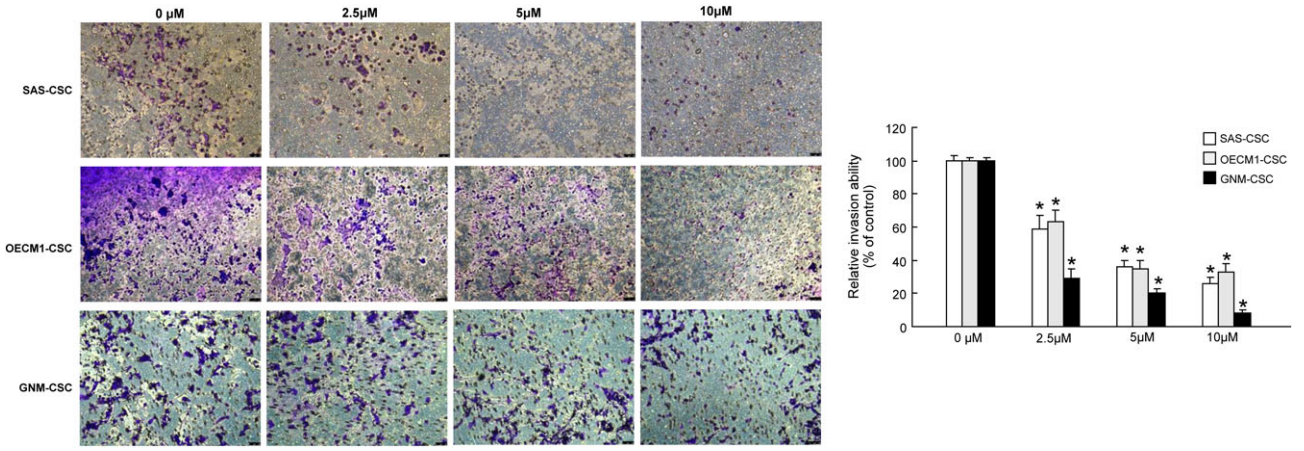

FIGURE 4 Honokiol inhibits the invasive ability of OCSCs. Representative images and quantification results showing that administration of honokiol mitigated the increased invasion capacity of OCSCs. Data were shown as a percentage of control group. *P < .05 compared to control group [Color figure can be viewed at wileyonlinelibrary.com]

lower self-renewal (Figure 5D) and invasion (Figure 5E) abilities compared to Cisplatin only group.

3.5 | Honokiol displayed an inhibitory effect on OCSCs via suppression of IL-6/STAT3 axis

Previously, it has been proven that IL-6 and signal transducers and activators of transcription 3 (STAT3) were involved in drug resistance and maintenance of CSC properties in oral cancer.[26,27] To understand the mechanism underlying the regulation of honokiol in the OCSCs characteristics, we examined the IL-6 secretion and STAT3/phospho-STAT3 expression of honokiol-treated OCSCs. We found that the IL-6 production was gradually down-regulated in the presence of increased honokiol concentration (Figure 6A). It has been shown that IL-6 is a major autocrine/paracrine factor to activate STAT3 in OSCC, and our result demonstrated the

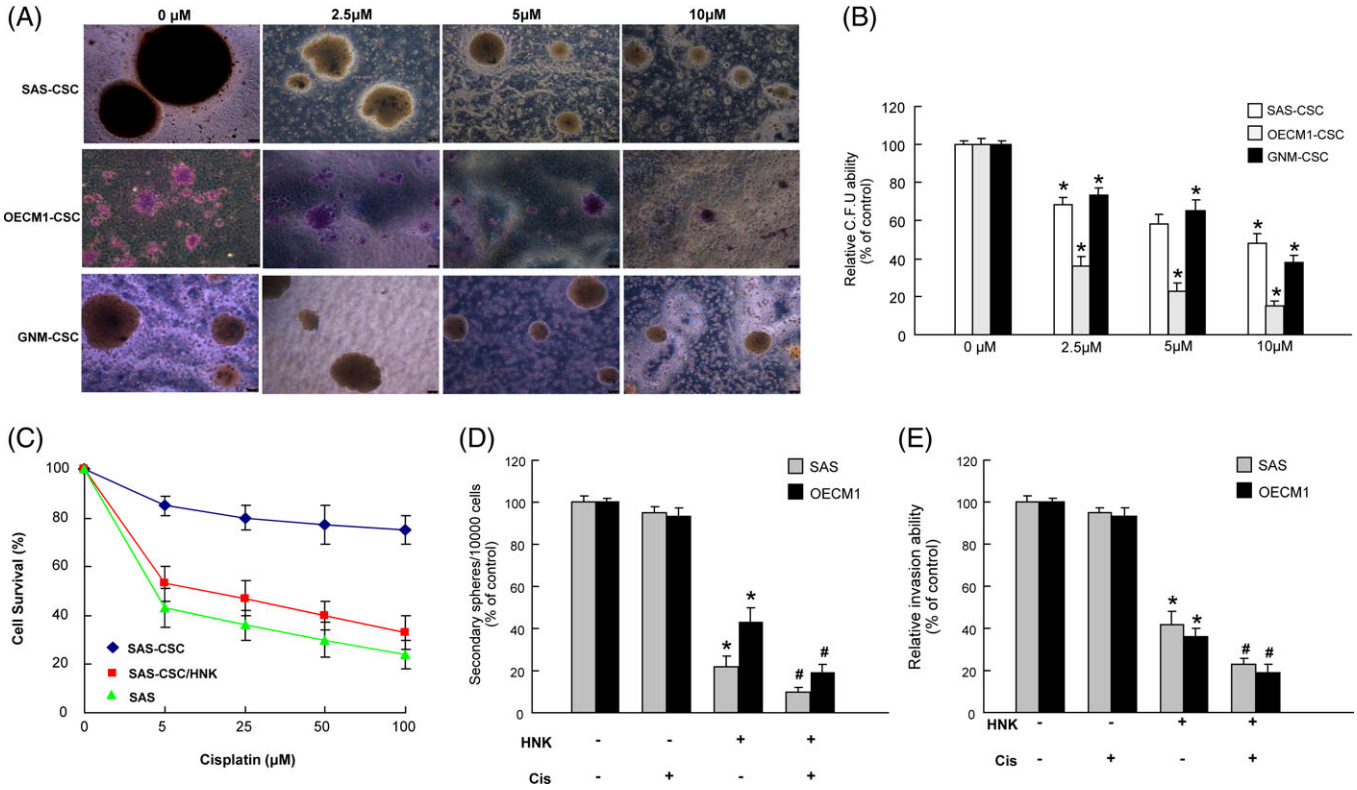

FIGURE 5 Honokiol suppresses the colony formation potential and enhances the effects of chemotherapy. A, Representative images and (B) quantification result of colony formation assay of honokiol-treated OCSCs. Data were shown as a percentage of the control group. *P < .05 compared to control group. C, Cell survival, (D) secondary sphere, and (E) invasive capacities were evaluated following treatment of Cisplatin only, honokiol only, or the combination of Cisplatin and honokiol. *P < .05 compared to control group; # P < .05 compared to Cisplatin only group [Color figure can be viewed at wileyonlinelibrary.com]

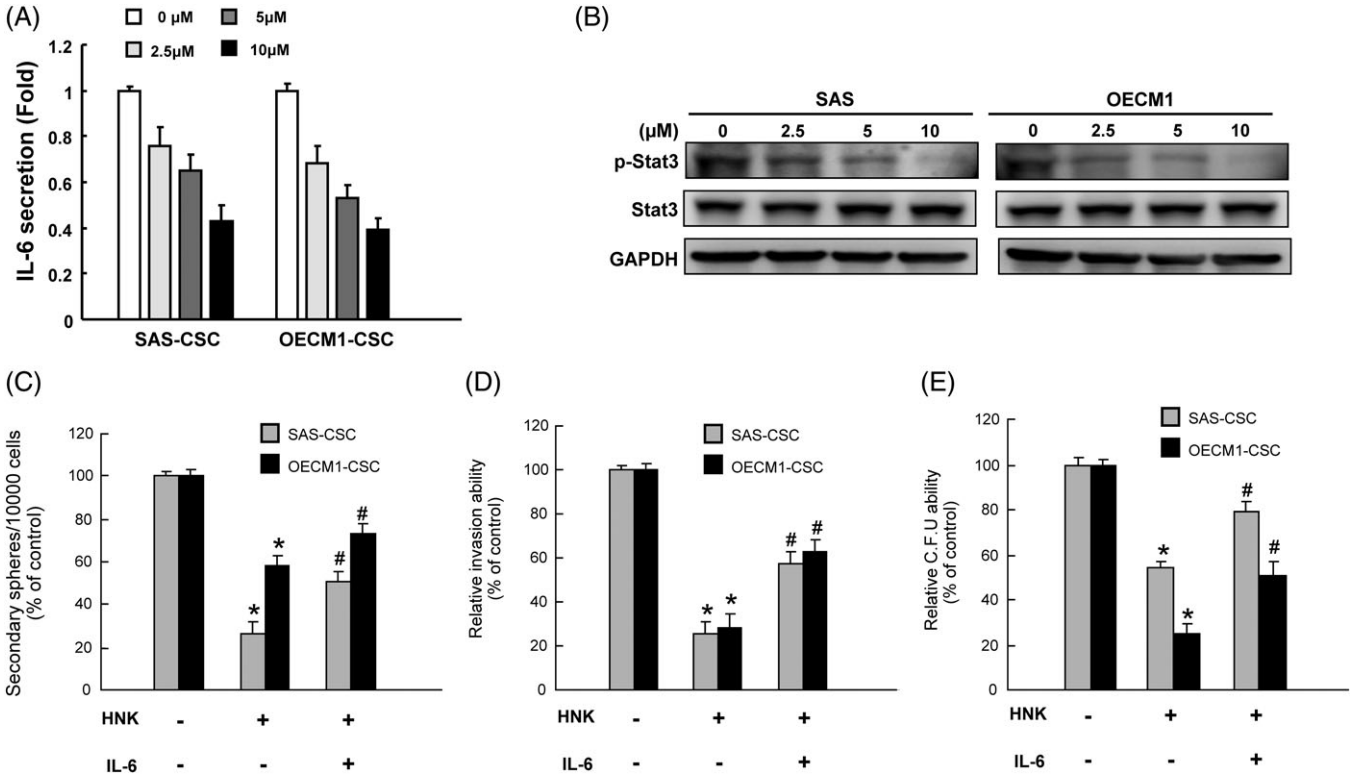

FIGURE 6 The effect of honokiol on cancer stemness was via reduction of IL-6 secretion and phosphorylation of STAT3. A, IL-6 secretion was evaluated by ELISA, and B, the expression of phosphorylated STAT3 was assessed by Western blot in response to various concentration of honokiol. The inhibitory effects of honokiol on (C) secondary sphere formation, (D) invasive capacity, and (E) colony formation ability were reversed by IL-6 administration. *P < .05 compared to control group; # P < .05 compared to honokiol group

corresponding reduction in phospho-STAT3, whereas the level of total STAT3 was not altered (Figure 6B). Furthermore, the inhibitory effects of honokiol on self-renewal (Figure 6C), invasive (Figure 6D), and colony formation (Figure 6E) were reverted by addition of IL-6, indicating the anti-OCSCs potential of honokiol was via modulation of the IL-6/Stat3 axis.

4 | DISCUSSION

In this study, we demonstrated the anti-OSCC effect of honokiol on cancer stemness (self-renewal capacity and the expression of stemness markers), cancer aggressiveness (migration and invasion abilities), and chemosensitivity. Our results showed that administration of honokiol significantly resulted in a concentration-dependent decrease in sphere formation as well as ALDH1 activity and CD44 expression. This was consistent with the previous studies showing that honokiol abrogated the stem-like phenotype of various cancer cells by reducing the sphere formation, ALDH1 activity, and CD44 expression.[28–30] Furthermore, it has been shown that CD44 could modulate drug resistance via regulation of the anti-apoptotic protein Bcl-xL[31] and inhibition of ALDH1 sensitized the ALDHhiCD44$^+$ breast cancer cells to chemoradiotherapy.[32] In head and neck cancer, ALDH1 expression has been shown to be highly correlated with tumor differentiation and decreased overall survival.[33] Our data demonstrated that honokiol was suitable to serve as an adjunctive agent to reduce ALDH1 activity and CD44 expression in CSCs, thereby suppressing the malignant and metastatic ability.

The attenuated CSCs migration and invasion capacities by honokiol treatment were in accordance with previous findings in bladder and breast cancers.[29,34,35] In OSCC, it has been shown that honokiol downregulate the in vitro and in vivo tumor growth via induction of apoptosis, cell cycle arrest and autophagy.[36] Another study reported that honokiol induced cell cycle arrest in prostate cancer cells by targeting c-Myc.[37] Honokiol has been proven to repress epithelial-mesenchymal transition (EMT),[34,35] thereby decreasing the possibility of metastasis. In addition to EMT, various molecular targets that resulted in anti-tumor effects of honokiol have been identified, such as EGFR, mTOR, MAPK, NFκB, and STAT3.[14,18] In this study, we showed that honokiol inhibited STAT3 phosphorylation, which may be via reduction of IL-6, one of the growth factors that activated STAT3. IL-6/STAT3 signaling has been indicated to be crucial for the maintenance of CSC in oral cancer.[26,27] Cancer-associated fibroblasts also used IL-6/STAT3 pathway to promote CSCs properties of hepatocellular carcinoma cells.[38] It has been shown that honokiol abrogated CSC phenotype in breast cancer through activation of tumor suppressor LKB1 and inhibition of STAT3.[28] Additionally, honokiol inhibited sphere formation of oral cancer by suppression of JAK/STAT signaling pathway.[18] In associated with this findings, we showed that honokiol downregulated the expression of IL-6 and phospho-STAT3. Furthermore, the repressed CSCs features, including self-renewal, metastatic and colony formation capacities after honokiol treatment were reversed by IL-6.

Aside from ameliorating the behavioral and phenotypical characteristics of CSCs, honokiol was suggested to exert a synergistic effect along with chemo/radiotherapy. It has been shown that 5-Fluorouracil in conjunction with honokiol possessed an additive effect on OSCC by inducing apoptosis.[39] In human multiple myeloma, honokiol was found to improve drug resistance by induction of caspase-dependent and -independent apoptosis.[40] It also has been shown to potentiate the cytotoxic effect of gemcitabine in human pancreatic cancer cells.[41] Honokiol in combination with radiation was proven to inhibit colon CSCs.[42] In preclinical multi-drug resistant cancer models, honokiol was able to induce mitochondria-dependent and death receptor-mediated apoptosis, which was associated with inhibition of EGFR-STAT3 signaling.[43] In line with the existing evidence, our data demonstrated the benefits of using honokiol to potentiate the Cisplatin sensitivity. This effect may be related to the reduction of the expression of ALDH1 and CD44, and inhibition of IL-6/STAT3 signaling after honokiol treatment.

Overall, this study showed that honokiol could function as an adjunctive therapy to ameliorate the ALDH1 activity and CD44 expression in OCSCs, which may lead to less drug resistance and tumor recurrence. Our data suggested that honokiol reduced the malignant potential of OCSCs by inhibiting IL-6 secretion and phosphorylation of STAT3. Moreover, honokiol could potentiate the effects of chemotherapy and enhance the efficacy of Cisplatin.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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