Abstract. Oral squamous cell carcinoma (OSCC) is the most common cancer of the oral cavity. Curcumin (Cur), a naturally derived compound, is reported to have broad-spectrum anticancer activity and is considered as an effective nuclear factor-κB (NF-κB) inhibitor. The present study aimed to clarify the detailed molecular mechanism through which Cur regulates NF-κB pathway activity in OSCC. The viability of HSC3 and CAL33 cells following treatment with Cur was determined using a Cell Counting Kit-8 assay. The protein and mRNA expression of specificity protein 1 (Sp1), p65 and heat shock factor 1 (HSF1) was determined by western blotting and reverse transcription-quantitative PCR analysis, respectively. The NF-κB activity was measured by Dual-Luciferase reporter assay. Short hairpin RNA targeting Sp1 or control RNA was transfected into HSC3 cells using X-treme GENE HP DNA Transfection System. Colony formation assays were performed using crystal violet staining. The results demonstrated that Cur significantly inhibited the viability and colony formation ability of HSC3 and CAL33 cells. In addition, Cur decreased the expression of Sp1, p65 and HSF1 by suppressing their transcription levels. Cur decreased NF-κB activity in OSCC cells, and Sp1 downregulation enhanced the effect of Cur. The findings from the present study suggested that Cur may inhibit the proliferation of OSCC cells via a Sp1/NF-κB-dependent mechanism.

Introduction

Oral squamous cell carcinoma (OSCC) is the most frequent type of head and neck cancer in China (1). The current treatment modalities for OSCC include surgery, which can be functionally debilitating or disfiguring, radiotherapy and chemotherapy. For patients with terminal or advanced cancer, the survival times may be decreased to a few months, which highlights the urgent need for novel therapeutic strategies (2,3).

Curcumin [Cur; 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a natural phenolic compound commonly known as the dietary spice turmeric (Curcuma longa), which is derived from the rhizome of an East Indian plant (4). Cur has been considered as pharmacologically safe for dietary consumption over several centuries (5). Extensive research has revealed that Cur is an effective molecular agent with a broad spectrum of biological activities against aging, inflammation and cancer (6,7). Previous studies have reported that Cur inhibits proliferation, metastasis and initiation of several malignances, including lung, breast, hepatocellular, pancreatic and gastric cancers (8-12). In addition, epidemiological study has suggested that a diet rich in Cur may decrease the incidence of colon cancer (13).

Cur significantly inhibits the activity of the nuclear factor κB (NF-κB) pathway in various human carcinomas, such as liver and thyroid carcinoma (14,15). Cur may therefore, be considered as an inhibitor of NF-κB by regulating the proteins associated with the NF-κB pathway. It was demonstrated that Cur can decrease the expression of p65, which specifically targets cancer stem cell populations and kills liver cancer cells (16). Under hypoxic tumor microenvironment, Cur can effectively inhibit hypoxia-inducible factor 1α (HIF-1α) and nuclear p65 expression in breast and lung cancer cells, which leads to tumor growth inhibition (10). In addition, Cur can inhibit NF-κB activity in an AKT-dependent manner in head and neck squamous cell carcinoma (17).

Specificity protein 1 (Sp1) is a member of the Sp-family, which is involved in multiple biological processes, including cell apoptosis, differentiation, cell cycle progression and proliferation (18-20). The expression and activation of Sp1 have been reported to be associated with the development of human cancers and prognosis (21). Sp1 is overexpressed...
in several cancers, including pancreatic and gastric cancers, and appears to be associated with poor prognosis, suggesting that Sp1 could be a potential therapeutic strategy for cancer treatment (22,23). Sp1 is also associated with proliferation, apoptosis and metastasis in oral cancer. Suppression of the tissue inhibitor of metalloproteinase-3 by promoter methylation of Sp1 contributes to oral cancer metastasis (24). Interfering Sp1 with small interfering (si)RNA or mithramycin A increasingly trigger apoptosis in oral cancer cells (25). A series of Sp1 special target inhibitors were reported to be effective for OSCC treatment, including honokiol, β-lapachone and esculetin (26-29). In addition, Sp1 can regulate NF-κB pathway activity by increasing the transcriptional level of p65 and p50 in pancreatic cancer and OSCC (30,31).

The present study aimed to investigate the effects and underlying mechanisms of Cur in OSCC. The results indicated that Cur inhibited the proliferation and NF-κB activity of OSCC cells. Importantly, Cur decreased the expression of Sp1, p65 and HSF1 in OSCC cells. Finally, Sp1 knockdown contributed to the effect of Cur on p65 and HSF1 in OSCC cells, resulting in a decreased NF-κB activity and cell viability.

Materials and methods

Cell lines and reagents. HSC3 and CAL33 cell lines were used in the present study. The HSC3 cell line was kindly provided by Dr Xin Zhang (University of Wuhan). The CAL33 cell line was a gift from Dr Juhua Zhou (Hubei University of Medicine). All cells were cultured in DMEM (HyClone; Cytiva) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotic (penicillin/streptomycin; Sigma-Aldrich; Merck KGaA) secondary antibodies. An ECL kit (cat. no. 1705060; Bio-Rad Laboratories, Inc.) was used to detect the signal. Western blot gray values were determined using ImageJ software (version 1.8.0; National Institutes of Health).

Reverse transcription-quantitative (RT-q)PCR. HSC3 and CAL33 cells (1x10^4 cells/well) were seeded in 24-well plates overnight. Cells were washed with PBS, treated with DMSO (0.01%) or Cur (10 or 20 µM) and incubated at 37°C for 24 h. Cells were washed with PBS and total RNA was extracted using 0.5 ml TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions. cDNA levels were quantified using the SYBR Premix Ex Taq kit (Takara Bio, Inc.) with the CFX96 Real-Time PCR Detection System. The thermocycling conditions were as follows: Denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec and extension at 72°C for 15 sec. The replication cycles were repeated 30 times. The sequences of the primers were as follows: p65, forward, 5'-GGGGATTGTACTGCTCCAAGC-3' and reverse, 5'-CTCCAGTGCCGCTCTTCT-3'; HSF1, forward, 5'-ACCTCTATCGGAACACCTCAGAG-3' and reverse, 5'-CTGTTAGGCTGGGAAAGATTTAGG-3'; Sp1, forward, 5'-GGGAGCACAACGACAGAC-3' and reverse, 5'-AAGGTGATTTGGGCTTCTG-3'; and GAPDH, forward, 5'-AGCTCGGTGTAACGGATTTC-3' and reverse, 5'-GTGAGACATGATTTGGAGTC-3'. The relative expression levels were normalized to endogenous control and were expressed as 2^ΔΔCt (36).

Cell transfection. HSC3 cells were transfected with Sp1-specific short hairpin RNA (shRNA; 5'-GCATATTGCCCATCACATCCAGG-3', Sp1-Homo-1828; GenePharma, Co., Ltd.) or a non-specific control (NC; 5'-TCTCCGAGTGTGTCAGT-3'; Shanghai GenePharma Co., Ltd.) using X-treme GENE HP DNA Transfection Reagent (Roche Diagnostics; 40 pmol for each shRNA) according to the manufacturer's instructions. Furthermore, PCDNA3.1 control vector and pCDNA3.1-Sp1 plasmid were donated by Dr Zilong Gao (Taihe Hospital, Shiyian). HSC3 cells were transfected with pCDNA3.1 and pCDNA3.1-Sp1 (2 µg) by using X-treme GENE HP DNA Transfection Reagent. At 4 h following transfection, Cur or DMSO control was added. Cells were incubated and harvested for the next experiments as follows: Western blotting (48 h),
Dual-Luciferase reporter assay (12 h) and Cell Counting Kit-8 assay (24 h).

**Dual-Luciferase reporter assay.** The NF-κB activity plasmid and PRL-TK plasmid were kindly provided by Professor H. Shu (University of Wuhan). HSC3 cells (1x10^5 cells/well) were seeded into 24-well plates and incubated at 37˚C overnight. Plasmids were co-transfected using X-treme GENE HP DNA Transfection Reagent into the cells for 24 h. Cells were treated with Cur (10 or 20 µM) or DMSO (0.01%) for 12 or 24 h, then collected and analyzed using the Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega Corporation). The intensity of NF-κB activity was normalized to the Renilla luciferase activity from the PRL-TK plasmid.

**Statistical analysis.** Experimental data were presented as the mean ± standard deviation of three independent experiments. Student's t-test was used for comparing significance between two groups. One-way ANOVA followed by Newman-Keuls or Bonferroni post hoc tests was used to compare data between >2 groups. Statistical analysis was performed using SPSS version 12.0 (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Cur inhibits OSCC cell proliferation.** To determine the effect of Cur on the viability of OSCC cells, HSC3 and CAL33 cells were treated with various doses of Cur for 24 or 48 h. Cell growth inhibition was compared with DMSO control alone. As presented in Fig. 1A and B, Cur significantly decreased the viability of HSC3 and CAL33 cells in a concentration-dependent manner (20 µM; P<0.001). A colony formation assay was performed to further confirm these results. HSC3 and CAL33 cells were treated with DMSO or Cur for 14 days and subjected to crystal violet staining. As presented in Fig. 1C, Cur significantly inhibited the colony formation of both HSC3 and CAL33 cells. These results suggested that Cur inhibited the proliferation of HSC3 and CAL33 cell lines.

**Cur downregulates Sp1, p65 and HSF1 expression in OSCC cells.** The expression of Sp1, p65 and HSF1 following treatment with DMSO (control) or Cur (5, 10 and 20 µM) for 48 h was evaluated in HSC3 and CAL33 cells. Compared with control, Sp1 expression was significantly decreased after Cur treatment in both HSC3 and CAL33 cells (P<0.001; Fig. 2A and B). It has been reported that Sp1 is a transcription factor of p65 and HSF1 (31). Subsequently, the expression of p65 and HSF1 was also evaluated. As presented in Fig. 2A and B, Cur treatment significantly decreased the expression of p65 (P<0.001) and HSF1 (P<0.001) in OSCC cells compared with the control.

**Cur decreases the expression level of Sp1, p65 and HSF1 in OSCC cells.** In order to determine whether the decreased protein expression in OSCC cells was associated with decreased mRNA levels, RT-qPCR was performed. HSC3 and CAL33 cells were incubated with or without Cur for 12 or 24 h. As presented in Fig. 3A and D, the mRNA expression of Sp1 in HSC3 [12 h (P=0.0036) and 24 h (P<0.001)] and CAL33
[12 h (P<0.001) and 24 h (P<0.001)] cells was decreased following treatment with 20 µM Cur. Furthermore, Cur treatment significantly downregulated the expression levels of p65 [Fig. 3B (12 h, P=0.0052; 24 h, P<0.001) and Fig. 3E (12 h, P=0.0083; 24 h, P=0.0021)] and HSF1 [Fig. 3C (12 h, P<0.001; 24 h, P<0.001) and Fig. 3E (12 h, P<0.001; 24 h, P<0.001)] in both OSCC cell lines. These results suggested that Cur decreased the expression of Sp1, p65 and HSF1 in OSCC cells by downregulating their transcriptional levels.

Cur decreases NF-κB activity in OSCC cells. Downregulation of p65 was reported to decrease the activity of the NF-κB pathway (31). To determine whether Cur could inhibit NF-κB activity in OSCC cells, HSC3 and CAL33 cells were incubated with DMSO (control) or Cur, and the activity of the NF-κB pathway was determined via Dual-Luciferase reporter assay. As presented in Fig. 4A and B, after 24 h treatment, 20 µM Cur significantly decreased the activity of NF-κB by 88.2 and 95.4% in HSC3 and CAL33 cell lines, respectively.

Cur inhibits cell proliferation and NF-κB activity in a Sp1-dependent manner. To determine whether the effects of Cur on cell viability and NF-κB pathway are dependent of Sp1, HSC3 cells were transfected with control shRNA or shRNA-Sp1, and treated with Cur or DMSO (control). As presented in Fig. 5A, shRNA-Sp1 significantly downregulated the expression of Sp1, p65 and HSF1, and enhanced the inhibitory effect of Cur on the expression of these proteins. In addition, Sp1 knockdown significantly enhanced the effect of Cur on NF-κB activity (Fig. 5B) and proliferation of HSC3 cells (Fig. 5C). To further confirm these results, pcDNA3.1 or pcDNA3.1-Sp1 was transfected into HSC3 cells, which were subsequently treated with Cur or DMSO (control). As presented in Fig. 5D, pcDNA3.1 significantly upregulated Sp1, p65 and HSF1 expression, and attenuated the effect of Cur on the expression of these proteins. In addition, overexpression of Sp1 significantly reversed the effect of Cur on NF-κB activity (Fig. 5E) and proliferation of HSC3 cells (Fig. 5F). These results suggested that Cur may inhibit OSCC cell proliferation and NF-κB activity via Sp1 regulation.

Discussion

Chemotherapy is a treatment of choice for OSCC. Most anticancer drugs (~80%) are derived from natural products or analogues based on natural products, such as triptolide and baicalein (37). Numerous studies have reported the potent antitumor activities of Cur in various types of cancer in vivo and in vitro, including pancreatic, lung, breast, colorectal, ovarian, gastric and head and neck cancers (17,38-42). In phase I clinical trials, Cur is not associated with significant side effects in animals or patients with breast, bladder or pancreatic cancers (43-45). In the present study, Cur significantly inhibited the proliferation of OSCC cells, and significantly decreased the protein and mRNA levels of Sp1, p65 and HSF1. In addition, Cur inhibited NF-κB activity in OSCC cells. Furthermore, the downregulation of Sp1 significantly decreased the expression of p65 and HSF1 and enhanced the inhibitory effect of Cur on cell proliferation and NF-κB activity.

NF-κB highly contributes to tumor cell survival, proliferation and metastasis and exerts anti-apoptotic effects on various types of cancer, including OSCC (46). Tumor necrosis factor-α is known to enhance the invasive and metastatic abilities of OSCC cells by increasing the

Figure 2. Cur inhibits the expression of Sp1, p65 and HSF1 in oral squamous cell carcinoma cell lines. (A) HSC3 and (B) CAL33 cells were treated with either 0.01% DMSO or Cur (5, 10 and 20 µM) for 48 h and then subjected to western blotting. Expression of Sp1, p65 and HSF-1 was measured. Data are presented as the mean ± standard deviation of three independent experiments performed in triplicate. *** P<0.001 vs. control group. Cur, curcumin; Sp1, specificity protein 1; HSF1, heat shock factor 1.
expression of p65 and IKKβ (47). NF-κB has been reported to increase the expression of matrix metalloprotease-9, which is associated with distant lymph node metastasis and poor survival of patients with OSCC (48). It has been demonstrated that upregulated expression of activator protein 1 (AP-1) and NF-κB in OSCC tissues is involved in Bcl-2 gene regulation, which promotes cancer progression and resistance to chemoradiotherapy (49). In previous clinical studies, NF-κB overexpression has been associated with negative prognosis of patients with OSCC, pancreatic cancer and laryngeal squamous cell carcinoma (50-52). p65 may therefore serve a critical role in the development and progression of OSCC and may be considered as a potent therapeutic target.

Figure 3. Cur inhibits the transcription levels of Sp1, p65 and HSF1 in oral squamous cell carcinoma cell lines. HSC3 and CAL33 cells were exposed to either 0.01% DMSO or Cur (10 and 20 µM) for 12 or 24 h and then subjected to reverse transcription-quantitative PCR analysis. mRNA levels of (A and D) Sp1, (B and E) p65 and (C and F) HSF1 were examined. Data are presented as the mean ± standard deviation of three independent experiments performed in triplicate. **P<0.01 and ***P<0.001 vs. control group. Cur, curcumin; Sp1, specificity protein 1; HSF1, heat shock factor 1.

Figure 4. Cur inhibits the NF-κB pathway. (A) HSC3 and (B) CAL33 cells were treated with 0.01% DMSO or Cur (10 and 20 µM) for 12 or 24 h. Activity of NF-κB was measured by the Dual-Luciferase reporter assay system. Data are presented as the mean ± standard deviation of three independent experiments performed in triplicate. ***P<0.001 vs. control group. Cur, curcumin; NF-κB, nuclear factor-κB.
Cur is commonly known as a special inhibitor targeting p65 and suppressing NF-κB activity in multiple human cancer cells, including lung, breast and liver cancer cells (10,14). In addition to targeting p65, Cur has been reported to sensitize cancer cells to radiation by decreasing the expression of inhibitor of NF-κB α (IκBα) and inhibiting NF-κB activity (53). Furthermore, Cur can promote paclitaxel-induced apoptosis of human papillomavirus-positive cervical cancer cells via the NF-κB-p53-caspase-3 pathway (54). Previous studies have demonstrated that Cur inhibits OSCC growth by inhibiting NF-κB pathway through various mechanisms (32,33,55). By inhibiting NF-κB activity, Cur has been indicated to enhance OSCC radiosensitivity in vivo and in vitro (32). Cur treatment has been revealed to inhibit the release of epithelial-to-mesenchymal transition (EMT) mediators in carcinoma-associated fibroblasts and induce the reversal of EMT in tumor cells, which was indicated to decrease the invasion efficiency of OSCC cells (33). To the best of our knowledge, the present study was the first to demonstrate that Cur could downregulate p65, which may account for the decreased NF-κB activity and viability of OSCC cells.

It has been reported that Sp1 is overexpressed in OSCC tissues compared with adjacent normal oral mucosal tissues, suggesting that Sp1 may be considered as a potential target for treating OSCC (56). Numerous drugs targeting Sp1, including mithramycin A, have exhibited strong inhibitory effects against OSCC proliferation (56-58). It was previously reported that baicalein decreases the expression of the NF-κB subunits p50 and p65, in a Sp1-dependent manner in OSCC cells, indicating that baicalein may inhibit NF-κB pathway and OSCC cell proliferation (30). Since Cur has been reported to decrease Sp1 expression in various cancer cells, including osteosarcoma and non-small cell lung cancer cells (59,60), the expression of Sp1 was detected in OSCC cells following Cur treatment. The results demonstrated that Sp1 expression was significantly decreased in OSCC cells following Cur treatment. In addition, Sp1 silencing significantly downregulated the expression levels of p65 and HSF1, which were detected to evaluate the effect of Cur on Sp1 expression. Furthermore, shRNA-Sp1 significantly contributed to the effect of Cur on cell viability and NF-κB pathway activity, which suggested that Cur may inhibit OSCC cell proliferation in a Sp1-dependent manner. One limitation of the present study was that transfection experiments were only performed on HSC3 cell line.

In conclusion, the present study demonstrated that Cur inhibited the proliferation and NF-κB activity of OSCC cells.
In addition, Curcumin effectively inhibits p65 and HSF1 in OSCC cells. Sp1 knockdown contributed to the effect of Cur on p65 and HSF1 in OSCC cells, resulting in the decreased activity of NF-kB and cell viability. These findings suggested that Cur may inhibit OSCC cell proliferation through a Sp1/NF-kB-dependent pathway.

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Availability of data and materials

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

Authors' contributions

HL designed the study. TLo performed the experiments. TLi prepared the new reagents/analytic tools. TLo analyzed the data. TLo and TLi wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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