Curcumin inhibits epithelial-mesenchymal transition in oral cancer cells via c-Met blockade

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Abstract. Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer. OSCC cells are highly invasive, a characteristic that involves epithelial-mesenchymal transition (EMT); the conversion of immotile epithelial cells into motile mesenchymal cells. EMT is involved in the progression of various types of cancer by promoting tumour cell scattering and conferring to these cells cancer stem cell (CSC)-like characteristics, such as self-renewal. Hepatocyte growth factor (HGF) signalling plays an important role in EMT induction and, therefore, contributes to cell invasion and metastasis in cancer. Due to its potential chemopreventative and anti-tumour activities, curcumin has attracted much interest and has been shown to act as a potent EMT inhibitor in various types of cancer. However, at present, the potential effects of curcumin on HGF-induced EMT in OSCC have not been investigated. Here, we demonstrated that HGF signalling could induce EMT in the HSC4 and Ca9-22 OSCC cell lines via the HGF receptor c-Met and downstream activation of the pro-survival ERK pathway. Notably, curcumin inhibited HGF-induced EMT and cell motility in HSC-4 and Ca9-22 cells via c-Met blockade. Therefore, these findings establish curcumin as a candidate drug for OSCC treatment. Furthermore, curcumin was able to effectively inhibit the HGF-induced increase in the levels of vimentin by downregulating the expression of phosphorylated c-Met, an ERK. In conclusion, the results of the present study demonstrated that curcumin was able to reverse HGF-induced EMT, possibly by inhibiting c-Met expression in oral cancer cells, providing a strong basis for the development of novel approaches for the treatment of oral cancer.

Introduction

Despite progress in the treatment of cancer through surgery, radiotherapy, and chemotherapy, the incidence of cancer remains high, which is largely due to the aging and growth of the world population. This high incidence of cancer is also associated with an increase in cancer-causing behaviours, particularly smoking, in economically developing countries (1), which is concomitant to moderate improvements in the overall five-year survival rate for oral cancer (2,3). Therefore, new treatments are needed to improve the care and outcome of oral cancer patients. Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer. Notably, OSCC cells are highly invasive, which frequently leads to local recurrence and distant lymphatic metastasis (4,5). Hence, a better understanding of tumour cell invasion mechanisms is essential to develop more potent treatments for OSCC.

Initially identified during embryogenesis, epithelial-mesenchymal transition (EMT) has been described as an essential process related to cell differentiation and morphogenesis (6). Importantly, EMT induction has also been associated with tumour progression, tumour cell invasion, and metastasis (7,8), including in OSCC (9). When EMT occurs, cancer cells lose epithelial characteristics and acquire mesenchymal properties, including fibroblast-like morphology, changes in gene expression patterns, and increased motility. Moreover, the outgrowth of metastases is generally associated with self-renewal, a defining trait of cancer stem cells (CSCs). Notably, EMT induction has also been shown to confer CSC-like characteristics to tumour cells (10), and such changes have been associated with increased cell invasion, metastasis, and resistance to chemotherapy (11-13).

Hepatocyte growth factor (HGF) and its receptor c-Met have been implicated in EMT in numerous types of cancer (14). Notably, activation of the HGF/c-Met signalling pathway has been shown to promote cancer cell scattering and invasion. Curcumin has been extensively studied for its potential chemopreventive and anti-tumour activities in colorectal cancer (15,16). Furthermore, curcumin has been shown to reduce the expression of EMT markers and act as a potent inhibitor of EMT in various

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Abbreviations: OSCC, oral squamous cell carcinoma; EMT, epithelial-mesenchymal transition; CSCs, cancer stem cells; HGF, hepatocyte growth factor; MMPs, matrix metalloproteinases

Key words: curcumin, HGF, EMT, c-Met, ERKs, OSCC

cancers (17-20). However, the potential effects of curcumin on EMT in oral cancer cells remain to be clarified. In this study, we aimed to examine the potential effects of curcumin on HGF-induced EMT in an OSCC cell line. In addition, we analysed invasion by an invasion assay and gelatin zymography, and metastasis by a scratch wound healing cell migration assay. This study may serve as a stepping stone towards the development of novel treatments for oral cancer.

Materials and methods

Cell culture and reagents. The human tongue-derived OSCC cell line HSC-4 and Ca9-22 was purchased from the RIKEN BioResource Center (Ibaraki, Japan). HSC-4 and Ca9-22 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) in a humidified incubator at 37°C and 5% CO_2. DMEM and FBS were purchased from Gibco (Life Technologies, Tokyo, Japan). HGF and curcumin were purchased from Sigma-Aldrich. All antibodies used in this study were commercially available and included antibodies against c-Met and phosphorylated-cMet (phospho-c-Met, Tyr1234/1235) (Cell Signaling Technology), α-tubulin (Sigma-Aldrich), E-cadherin and vimentin (Merck Millipore), and ERK and phospho-Tyr204-ERK (Tyr204) (both from Santa Cruz Biotechnology).

Scratch wound healing cell migration assay. Cell migration was determined using a scratch wound healing assay, as described previously (21,22), except for the following modifications. Briefly, semi-confluent cells in 12-well plates were treated for 4 h with 10 μg/ml mitomycin C to block cell proliferation, and an artificial wound was made by scraping the bottom of the dish. The cells were subsequently wounded with a sterile 200-μl pipette tip to generate a cell-free gap, ~0.3 mm in width. Cells were then washed with phosphate-buffered saline (PBS) and photographed to record the wound width at 0 h (~0.3 mm). Finally, one group of cells was cultured for 48 h in DMEM supplemented with 10% (v/v) FBS as a control, while the other groups were treated with 20 ng/ml HGF (23). For curcumin pre-treatment, cell monolayers were scratched and incubated for 2 h with DMEM supplemented with 0.5% (v/v) FBS and 15 μM curcumin before HGF treatment. The concentration of curcumin was selected based on the study by Tong *et al*, who reported that the effective concentration of curcumin is 10-20 μM (24). At the end of the incubation, photographs were taken, and the wound width was measured to evaluate cell migration.

Matrigel cell invasion assay. *In vitro* cell invasion assays were performed following the manufacturer's recommendations. HSC-4 cells (3x10⁵ cells/ml) incubated in the presence or absence of HGF were seeded in a 6-well plate fitted with a BioCoat Matrigel Invasion Chamber (Corning; Becton Dickinson). Cells were then cultured for 48 h in DMEM supplemented with 10% (v/v) FBS in the presence or absence of 20 ng/ml HGF and 15 μM curcumin. After 48 h, non-invading cells were removed from the surface of the membrane by scrubbing, and the invading cells were fixed with 100% methanol and then stained using the Diff-Quick staining kit after fixation with methanol. Finally, the number of invading cells in six random fields was counted using a

microscope equipped with a x200 objective and the Image Pro Express software (Meyer Instruments) to evaluate the invasion index.

Gelatin zymography. The gelatinolytic activity of HSC-4 cells was examined by gelatin zymography. Proteins from serum-free conditioned medium were diluted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and separated using a 12.5% (w/v) polyacrylamide gel containing 0.1% (w/v) gelatin. For each sample, the amount of material loaded onto the gel was corrected for the number of cells in culture and corresponded to the proteins secreted by ten cells. Electrophoresis was carried out for 2 h at 60 mA and 4°C, and the gel was incubated overnight in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ and 1X Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). Finally, the gel was stained using 0.25% Coomassie Blue in a methanol:acetic acid:water (50:10:40) solution and destained using the same solution without dye. Negative staining (white bands against a dark background) indicated proteolysis and, therefore, gelatinolytic activity.

Western blotting. Following treatment, HSC-4 and Ca9-22 cells were collected, washed with PBS, and lysed using RIPA buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholic acid, 0.1% (w/v) SDS, 5 mM ethylenediaminetetraacetic acid, 1X Halt Protease Inhibitor Cocktail, and 1X Halt Protein Phosphatase Inhibitor (Thermo Fisher Scientific, Inc.). The protein concentration of the cell lysates was determined using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.), and equal amounts of protein were subjected to SDS-PAGE. The separated proteins were transferred onto a PVDF membrane (GE Healthcare), which was then blocked for 1 h at room temperature with 5% (w/v) bovine serum albumin in Tris-buffered saline (TBS)/Tween-20 (TBS-T) to prevent non-specific binding. The membrane was incubated overnight at 4°C with antibodies diluted in TBS-T, washed, and incubated with HRP-conjugated secondary antibodies diluted in TBS-T. Finally, antibody-antigen complexes were detected using the ECL Plus Western Blotting Detection Reagent (GE Healthcare).

Statistical analysis. All data are presented as the mean ± standard deviation from three independent experiments unless stated otherwise. The statistical significance of a difference between two groups was evaluated using unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Curcumin inhibits HGF-induced invasion and migration of HSC-4 cells. To assess the effect of curcumin on oral cancer cells, we first examined HGF-induced cell motility of HSC-4 cells using an invasion assay (Fig. 1). HGF-induced cells exhibited a 2-fold increase in the number of invasive cells compared to control cells (P<0.05). In stark contrast, curcumin pre-treatment significantly inhibited HGF-induced invasion of HSC-4 cells (P<0.05), with a number of invasive cells that was comparable to that of the control cells.
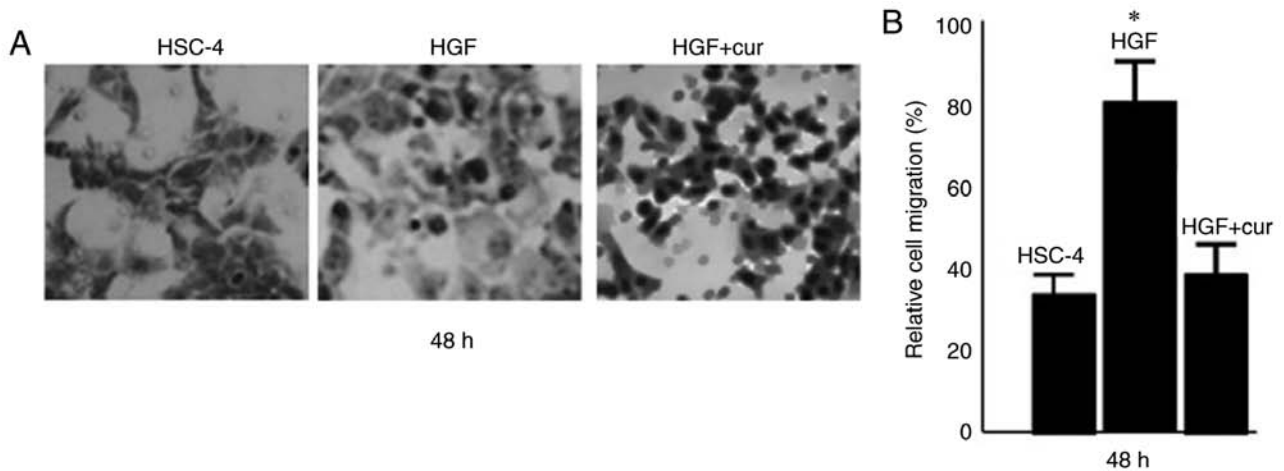
Figure 1. Cur inhibits HGF-induced cell invasion. Control and HGF-induced HSC-4 oral cancer cells with and without cur pre-treatment were cultured in Matrigel chambers, and the number of invading cells was scored after 48 h. (A) Representative bright-field microscopy images showing invading cells in the Matrigel chambers. Magnification, x20. (B) Histogram showing relative cell migration (%) of invading cells after 48 h of culture, compared with HGF-induced cells. Data are presented as the mean ± SD. n=3. *P<0.05 vs. HSC-4. Cur, curcumin; HGF, hepatocyte growth factor.
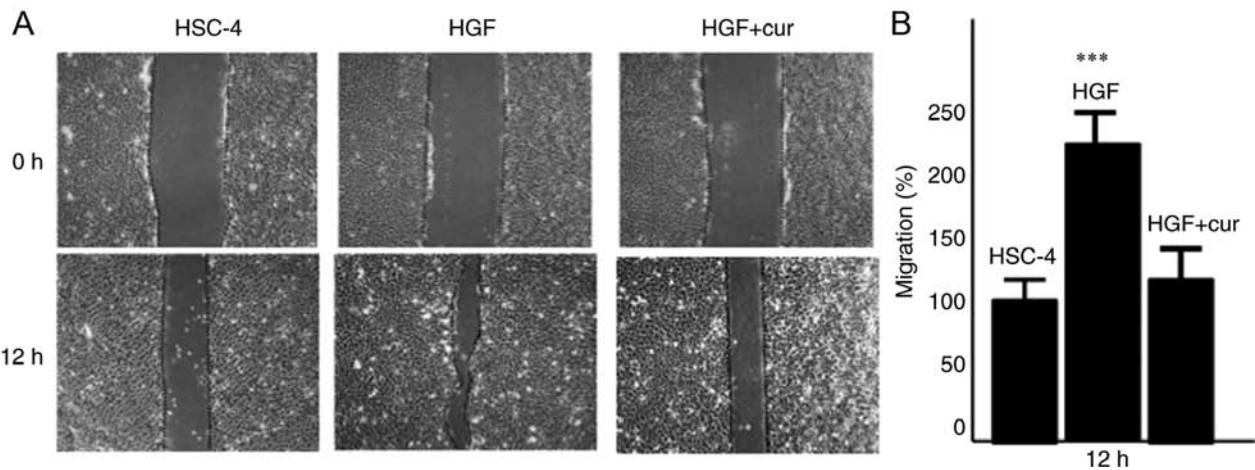

Figure 2. Cur inhibits HGF-induced cell migration. Control and HGF-induced HSC-4 oral cancer cells with and without cur pre-treatment were cultured in monolayers, and cell migration was measured by a scratch wound healing assay. (A) Representative brightfield microscopy images show wound healing at 0 and 12 h. Magnification, x10. (B) Histogram showing relative cell migration (%), compared with control cells. Data are presented as the mean ± SD. n=3. ***P<0.001 vs. HSC-4. Cur, curcumin; HGF, hepatocyte growth factor.

To further assess the effect of curcumin on HGF-induced cell motility, we also examined cell migration using a wound healing assay (Fig. 2). In agreement with our cell migration data, the migration of HGF-induced HSC-4 cells was increased >2-fold compared to control cells (P<0.001). However, as in the case of cell invasion, curcumin pre-treatment dramatically reduced the migration of HGF-induced cells (P<0.05), which was similar to that of control cells. Collectively, these findings strongly suggested that curcumin pre-treatment could inhibit the HGF-induced motility of HSC-4 cells, resulting in reduced cell invasion and migration.

Curcumin suppresses HGF-induced EMT in HSC-4 and Ca9-22 cells. The concomitant down-regulation of the epithelial marker E-cadherin and up-regulation of the mesenchymal marker vimentin is recognised as a hallmark of cells undergoing EMT (25,26) and results in the loss of cell polarity, an important step in EMT induction (27,28). Therefore, we next

performed western blot analyses to assess the expression level of these markers and determine whether the observed inhibitory effects of curcumin on HGF-induced cell motility involved alterations in the EMT process (Fig. 3). As expected, E-cadherin and vimentin expression levels were decreased and increased, respectively, in response to HGF stimulation, which confirmed that HGF-induced HSC-4 and Ca9-22 cells were undergoing EMT. Remarkably, curcumin pre-treatment abrogated HGF-induced changes in E-cadherin and vimentin expression, which strongly suggested that curcumin could inhibit HGF-induced EMT in oral cancer cells.

Curcumin represses HGF-induced signalling in HSC4 cells through inhibition of the c-Met/ERK pathway. As mentioned previously, HGF signalling plays an important role in EMT induction. A crucial step is the homodimerisation and autophosphorylation of the c-Met receptor tyrosine kinase upon HGF binding (29,30). Therefore, we performed western blot
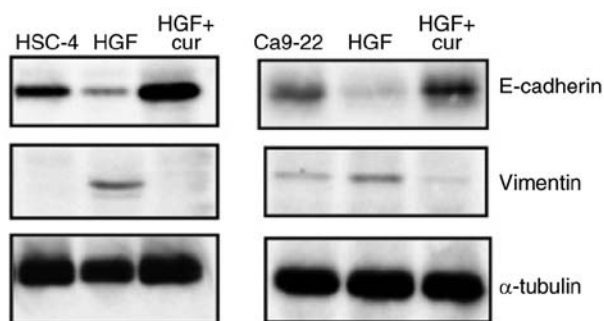
Figure 3. Cur inhibits epithelial-mesenchymal transition in HGF-induced cells. Representative western blot showing the levels of E-cadherin and vimentin in whole cell lysates from control and HGF-induced HSC4 (left) and Ca9-22 oral cancer cells (right) with and without cur pre-treatment. α-tubulin was used as a loading control. Cur, curcumin; HGF, hepatocyte growth factor.
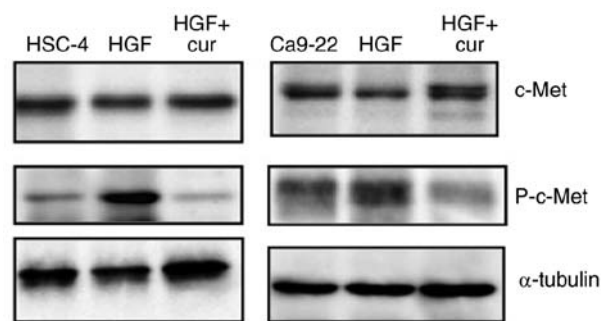

Figure 4. Cur inhibits c-Met phosphorylation in HGF-induced cells. Representative western blot showing the levels of c-Met and p-c-Met in whole cell lysates from control and HGF-induced HSC4 (left) and Ca9-22 oral cancer cells (right) with and without cur pre-treatment. α-tubulin was used as a loading control. Cur, curcumin; HGF, hepatocyte growth factor; p, phosphorylated.

analyses to examine the effect of curcumin pre-treatment on the level of phospho-c-Met. HGF-induced HSC-4 and Ca9-22 cells exhibited an increased level of phospho-c-Met compared to control cells. Remarkably, curcumin pre-treatment abolished the increase in phospho-c-Met level resulting from HGF stimulation (Fig. 4), which strongly suggested that the inhibitory effects of curcumin on HGF-induced EMT involve the down-regulation of HGF signalling.

The role of HGF signalling in the induction of EMT is mediated via the downstream activation of the AKT and ERK effector pathways (31). Accordingly, we assessed the activation status of the ERK pathway to further dissect the mechanisms of EMT inhibition by curcumin. Western blot analyses showed that ERK phosphorylation, a marker of ERK activation, was increased in HGF-induced HSC-4 cells compared to control cells (Fig. 5). In stark contrast, curcumin pre-treatment completely blocked HGF-induced ERK phosphorylation. Collectively, these findings indicated that curcumin could inhibit HGF-induced EMT by repressing c-Met and ERK activation.

Curcumin represses the production of gelatinolytic activity. Matrix metalloproteinases (MMPs) are major regulators of the extracellular matrix and are known to play important roles in tumour invasion and metastasis (32). Furthermore, activation of the MMP2 and MMP9 gelatinases has been associated with an induction of the ERK pathway (33). Therefore, we used gelatin zymography to evaluate gelatinolytic activity in the conditioned medium of HSC-4 cells (Fig. 6). While control cells exhibited two major types of gelatinolytic activity, which were consistent with pro-MMP9 and pro-MMP2, HGF-induced cells exhibited a marked increase in pro-MMP9 production. Notably, curcumin pre-treatment inhibited the production of pro-MMP9 by HGF-induced cells, whereas the production of pro-MMP2 remained unaffected. This observation suggested that inhibition of HGF signalling by curcumin might decrease cell motility by repressing the production of gelatinolytic activity.

Discussion

EMT is a highly conserved cellular program that allows polarised, immotile epithelial cells to convert into motile mesenchymal cells. Importantly, EMT plays physiological
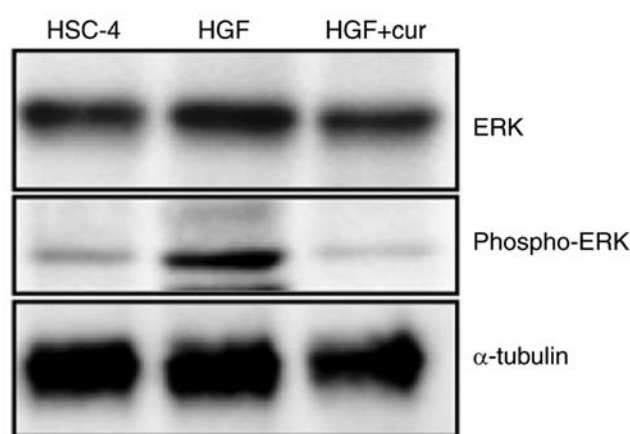

Figure 5. Cur-mediated c-Met blockade results in the downregulation of ERK phosphorylation. Representative western blot showing the levels of ERK and phospho-ERK in whole cell lysates from control and HGF-induced HSC4 oral cancer cells with and without cur pre-treatment. α-tubulin was used as a loading control. Cur, curcumin; HGF, hepatocyte growth factor.

roles during embryonic development, but also pathological roles in cancer. HGF signalling plays an important role in EMT induction and involves homodimerisation and autophosphorylation of its receptor c-Met, which induces the transcription of downstream target genes. Moreover, the activation of c-Met has been shown to promote invasion and metastasis, as well as angiogenesis and tumorigenesis. The functional diversity of HGF signalling has attracted much interest in the clinical setting due to its potential prognostic and therapeutic value (34).

In the present study, we demonstrated that HGF signalling could induce EMT in the HSC-4 OSCC cell line and promote both cell migration and invasion. Importantly, EMT in HSC-4 and Ca9-22 cells involved down- and up-regulation of E-cadherin and vimentin expression, respectively. Grotegut *et al* have previously reported that HGF induces scattering of epithelial cells via up-regulation of Snail, a transcriptional repressor involved in EMT that represses the expression of E-cadherin and other epithelial-related genes (35). Importantly, HGF-induced up-regulation of Snail expression requires the activation of the ERK pathway and its downstream effector early growth response factor-1 (Egr-1).
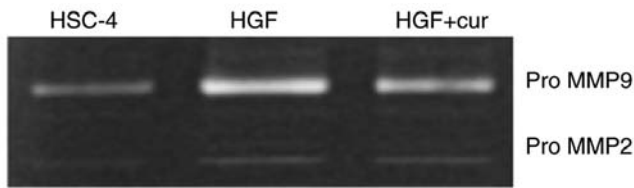
Figure 6. Cur represses the production of gelatinolytic activity. Representative gelatin zymography showing the levels of the two principal gelatinolytic activities (pro-MMP2 and pro-MMP9) in conditioned culture medium from control and HGF-induced HSC4 oral cancer cells with and without cur pre-treatment. Cur, curcumin; HGF, hepatocyte growth factor; MMP, matrix metalloproteinase.

Furthermore, previous studies have shown that the various oncogenic effects related to HGF signalling are mediated by a complex downstream signalling network, which prominently involves the AKT and ERK effector pathways (31,36). In agreement with these findings, our results indicated that the c-Met/ERK pathway mediated HGF-induced EMT in HSC-4 cells. Therefore, we propose that in OSCC, HGF signalling can activate the c-Met/ERK pathway, increasing Snail expression, which induces EMT and increases the invasion and migration of tumour cells.

MMPs are known to play important roles in tumour invasion (31), and the activation of the MMP2 and MMP9 gelatinases has been associated with induction of the ERK pathway (33). We previously reported that EGF increases the promoter activities of MMP9 in oral cancer cells (37). Moreover, it was previously reported that curcumin inhibits colon cancer cell invasion via MMP9 (24). The results of the current study also suggested that curcumin inhibits OSCC cell invasion via MMP9.

In a previous study, Davies et al have shown that inhibition of c-Met expression using a hammerhead ribozyme transgene decreases invasion and metastasis of prostate cancer cells (38). Hence, repression of cell invasion and metastasis by c-Met blockade appears to be an attractive therapeutic approach for oral cancer. In this study, we investigated the potential beneficial effects of curcumin in OSCC. The chemopreventive and anti-tumour activities of curcumin have been extensively documented, establishing it as a promising drug for the prevention and treatment of cancer (39). Indeed, curcumin can modulate multiple molecular pathways involved in carcinogenesis and exert its chemopreventive and anti-tumour activities through several mechanisms, which include induction of apoptosis, inhibition of survival signals, scavenging of reactive oxidative species, and reduction of the inflammatory cancer microenvironment (39). Previous studies have also demonstrated that curcumin can inhibit tumour cell invasion and metastasis. For example, Chen et al have reported that curcumin can inhibit the invasion and metastasis of lung cancer cells through the up-regulation of E-cadherin expression (40). In agreement with these observations, we showed that curcumin pre-treatment could block HGF-induced invasion and migration of HSC-4 cells by preventing EMT induction. Furthermore, we demonstrated that in HSC-4 cells, curcumin could inhibit c-Met and ERK phosphorylation in response to HGF stimulation, which are essential steps in the signalling cascade promoting EMT. Although further work is required to elucidate the underlying mechanisms, we propose that the inhibitory effects of curcumin on HGF-induced EMT and cell motility are mediated via down-regulation of the c-Met/ERK pathway.

The HSC-4 and Ca9-22 human OSCC cell line was used as an experimental model in this study. HSC-4 and Ca9-22 cells are negative for cancer stemness (41) and responsive to HGF and c-Met inhibitor SU11274 (23). Thus, the effects of curcumin on HGF-induced EMT and the invasive and migratory potential of HSC-4 and Ca9-22 cells were investigated.

Moreover, Siddappa et al have recently assessed a curcumin/metformin combination for the treatment of a mouse model of induced oral carcinogenesis (42). They found that this drug combination was an efficient chemopreventive treatment, as demonstrated by the positive clinical response, that specifically inhibited CSCs associated with cancer progression. In vitro studies also showed that administration at an earlier disease stage resulted in improved efficiency of these drugs. Our in vitro data on HGF-induced HSC-4 cells further demonstrated that curcumin is a promising drug that could target cells exhibiting CSC-like characteristics. Nevertheless, additional studies will be necessary to fully understand the molecular mechanisms involved in the chemopreventive activity of curcumin in oral cancer.

In conclusion, this study demonstrated that curcumin could inhibit HGF-induced EMT and cell motility in an OSCC cell line via c-Met blockade and inhibition of the ERK effector pathway. Importantly, these findings suggested that curcumin was a potent drug targeting invasive oral cancer cells. Therefore, we believe that our data provide a strong theoretical and experimental basis for the development of novel approaches and drugs for the treatment of oral cancer.

Acknowledgements

Funding

Availability of data and materials

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

Authors' contributions

YO and MN contributed to the conception and design of the experiments. YO, TS and HY performed the experiments. LZ, HH and HK contributed to the study conception and design. All authors read and approved the final version of the 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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