Antitumor activity of the plant extract morin in tongue squamous cell carcinoma cells

YAWEN JI1,2, LINGLU JIA1,2, YUNPENG ZHANG1,2, YIXIAO XING1,2, XUAN WU1,2, BIN ZHAO1,2, DONDJIAO ZHANG1,2, XIN XU1,2 and XU QIAO3

1Department of Oral Maxillofacial Surgery, School of Stomatology, Shandong University; 2Department of Oral Maxillofacial Surgery, Shandong Provincial Key Laboratory of Oral Tissue Regeneration; 3School of Control Science and Engineering, Shandong University, Jinan, Shandong 250012, P.R. China

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Abstract. Morin is a naturally occurring bioflavonoid originally isolated from members of the Moraceae family of flowering plants and it possesses antitumor activity in various human cancer cells. The present study explored the antitumor effects of morin in tongue squamous cell carcinoma (TSCC) cells in vitro and investigated the underlying molecular events. A TSCC cell line was treated with different doses of morin for up to 48 h. Analyses of cell viability, using Cell Counting Kit-8 (CCK-8), EdU incorporation, colony formation, flow cytometric analysis of cell cycle distribution and apoptosis, wound healing assay, western blot analysis and qRT-PCR assays, were then performed. The data revealed that morin treatment reduced Cal27 cell proliferation and reduced the migration capacity of tumor cells in a dose-dependent manner. Morin treatment also significantly upregulated mammalian sterile 20-like 1 (MST1) and MOB kinase activator 1 (MOB1) phosphorylation in CAL27 cells, but suppressed nuclear translocation of yes-associated protein (YAP) through the induction of YAP phosphorylation in Cal27 cells. Moreover, the expression of YAP-targeting genes, such as CTGF, CYR61 and ANKRD, was downregulated in morin-treated TSCC cells, indicating that morin was able to activate the Hippo signaling pathway to inhibit YAP nuclear translocation and YAP-related transcriptional activity in TSCC cells. In conclusion, the data from the present study demonstrated that morin produces anti-TSCC activity in vitro through activation of the Hippo signaling pathway and the downstream suppression of YAP activity in TSCC cells. Future studies should assess the clinical antitumor effects of morin.

Introduction

Head and neck cancer remains as one of the most debilitating human neoplasms, and is the sixth most commonly diagnosed cancer worldwide. Histologically, 90% of head and neck cancers are head and neck squamous cell carcinoma (HNSCC), and tongue squamous cell carcinoma (TSCC) contributes to a large proportion of HNSCC (1). In recent decades, advancements have been achieved in the conventional treatment of these cancers, including surgery, radiotherapy and chemotherapy. However, the prognosis of TSCC remains poor (2). Thus, a greater understanding of the pathogenesis of TSCC is needed in order to identify effective antitumor drugs that can be used to clinically control TSCC.

To this end, the present study focuses on a natural flavonoid that possesses anticancer effects. The potential chemotherapeutic effects of many flavonoids have been tested in different clinical trials (3). Morin is a naturally occurring bioflavonoid originally isolated from members of the Moraceae family of flowering plants. Morin can be isolated from amygada (P. guajava L.) as a yellow pigment (4,5). Morin exerts anticancer activity through the modulation of various gene-signaling pathways that are involved in cell proliferation and differentiation (6-10). Previous studies have also demonstrated that morin is able to induce apoptosis in prostate cancer, human leukemia HL-60 and multiple myeloma cells. Recently, studies have demonstrated that treatment with morin induces tumor cell apoptosis by activating the mitochondrial and caspase-3 pathway (11,12).

The Hippo pathway is an important tumor suppressor pathway in various human cancers (13-18). The Hippo kinase cascade involves mammalian sterile 20-like 1/2 (MST1/2, also known as STK4/3), salvador (SAV1), large tumor suppressor 1/2 (LATS1/2), MOB kinase activator 1A/B (MOB1a/b) and Yes-associated protein (YAP) (15,18,19). Activation of the Hippo kinase cascade leads to the phosphorylation of YAP and restriction of YAP translocation into the cytoplasm for the induction of YAP degradation (16,17). However, while the Hippo signaling cascade is inactive, YAP is translocated into
the nucleus where it promotes the expression of various genes involved in cell proliferation and survival (15). In addition, MOB1 is an adapter protein and it can bind to upstream MST1; thus, it can regulate MST1 phosphorylation or bind to the downstream LATS1 protein to enable the trans-phosphorylation of YAP (14). The activity of the Hippo pathway regulates organ size during embryonic development in animals through the regulation of cell proliferation and apoptosis, and alteration of this pathway occurs in various types of human cancer (13,20). Aberrant expression of the Hippo pathway proteins promotes the translocation of YAP into the nucleus and drives transcription of YAP-targeting genes and, therefore, enhances cancer cell proliferation and inhibits apoptosis (20-22). YAP can also stimulate epithelial-to-mesenchymal transition of tumor cells and drive the tumor-initiation capacity of cancer stem cells (23). To date, elevated YAP expression has been observed in many types of human cancers, including lung, liver, ovary, breast and colon cancers (24-28). Previous studies have demonstrated that morin was able to impede YAP nuclear translocation through the activation of Hippo signaling and foster apoptosis in human liver cancer HepG2 cells (29). Additionally, morin has demonstrated antitumor activity in human TSCC cells (6). In the present study, we further assessed whether morin treatment could regulate MST1 and MOB1 expression to constitutively activate the Hippo signaling pathway and inactivate YAP in TSCC cells in vitro. We determined the effect of morin treatment on the modulation of TSCC cell proliferation, apoptosis and migration and on the regulation of the phosphorylation of the protein components of the Hippo pathway and YAP target genes. We aimed to provide insightful information regarding the effect of morin on TSCC cells and to identify morin as a potential treatment strategy or preventative agent for TSCC.

Materials and methods

Cell line culture and drug treatment. The TSCC cell line, CAL27, was obtained from Shanghai Ninth People's Hospital (Shanghai, China) and cultured in a high-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 5% CO2 at 37°C. For morin treatment, tumor cells were cultured in a complete cell culture medium supplemented with different doses of morin (0, 50, 100 and 150 µM; Yuanye Chemicals Co., Ltd., Shanghai, China), while dimethyl sulfoxide (DMSO; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), the solvent, was used as the control treatment for up to 14 days.

Cell viability assay. Tumor cell viability was assayed following morin treatment using a cell viability Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Briefly, Cal27 cells were seeded into 96-well plates at a density of 3,000 cells per well and grown overnight in a complete cell culture medium (see above) and then refreshed with morin-containing medium for 24 to 48 h. At the end of each experiment, cell growth medium was added to 10 µl of CCK-8 working solution and cells were further cultured for 90 min at 37°C. The optical density value (OD) of the plates was then measured using a microplate reader at 450 nm. The experiment was performed in triplicate and repeated at least three times.

 Colony formation assay. Cal27 cells were plated into 6-well plates (1,000 cells/well) and grown overnight. Following this incubation, the cell culture medium was refreshed with morin-containing medium (0, 50, 100 and 150 µM) every 2 days for 14 days. At the end of the experiments, cells were washed with ice-cold phosphate-buffered saline (PBS), fixed using freshly made 4% paraformaldehyde/PBS solution and stained with 0.1% crystal violet. Cell colonies with more than 50 cells were counted under an inverted microscope (Olympus Corp., Tokyo, Japan). The experiment was performed in triplicate and repeated at least three times.

5-Ethynyl-2’-deoxyuridine (EdU) incorporation assay. We performed the EdU incorporation assay using an EdU kit from Guangzhou RiboBio Co., Ltd. (Guangzhou, China) according to the manufacturer's instructions. Specifically, Cal27 cells were plated into 24-well plates at a density of 50,000 cells/well and grown overnight. The cells were then treated with different concentrations of morin (0, 50, 100 and 150 µM) for 24 h. Following this, cell culture was added to 50 µl of the EdU working solution and further cultured for 2 h. At the end of the experiment, cells were washed with ice-cold PBS and fixed in 4% paraformaldehyde for 15 min. The cells were then treated with 2 mg/ml glycine at room temperature for 5 min and stained with Apollo® 567 and Hoechst working solution, in the dark, for 30 min. Finally, the cell images were obtained using fluorescence microscopy (Olympus Corp.).

Wound-healing assay. Cal27 cells were grown and treated with various concentrations of morin (0, 50, 100 and 150 µM) for 24 h. For the tumor cell wound-healing assay, the cells were trypsinized and reseeded into 6-well plates at a density of 1x10^6 cells/well and grown overnight to reach ~90% confluency. The cell monolayer was then scratched with a sterile 200-µl pipette tip across the plates, washed with DMEM twice and cultured for up to 24 h in DMEM/0.1% FBS as well as different the concentrations of morin. At each time-point (0, 6 or 24 h), the cell monolayers were photographed using an inverted microscope (Olympus Corp.). The wound healing areas were measured using ImageJ software 14.8 for Windows (National Institutes of Health, Bethesda, MD, USA). The experiment was performed in triplicate and repeated at least three times.

Flow cytometric Annexin V/PI apoptosis assay. Cal27 cells were first plated into 6-well plates (1x10^5 cells/well) and incubated overnight at 37°C. Cells were then treated with different doses of morin (0, 50, 100 and 150 µM) for up to 48 h. At the end of each experiment, both detached and attached cells were collected and incubated with the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (eBioscience, Vienna, Austria) according to the manufacturer's protocol. Specifically, ~20,000 cells were suspended in the binding buffer and 5 µl of Annexin V-FITC was added. Cells were incubated for 10 min, followed by a further 5-min incubation after the addition of 5 µl of the PI staining solution at room temperature in the dark.
Finally, the apoptosis level was measured using flow cytometry (FACScalibur; BD Biosciences, San Jose, CA, USA).

**Flow cytometric cell cycle assay.** Cal27 cells were plated into 6-well plates (1x10⁶ cells/well) and incubated overnight. Cells were then treated with different doses of morin (0, 50, 100 and 150 µM) for 24 h. The cells were then harvested and washed with ice-cold PBS once and fixed in 70% ethanol at 4°C for 12 h. On the next day, the cells were washed again with PBS and then stained with PI working solution at room temperature for 30 min in the dark. The cell cycle distribution of the cells was analyzed using flow cytometry (FACScalibur; BD Biosciences).

**Protein extraction and western blot analysis.** Morin-treated Cal27 cells were lysed in radioimmunoprecipitation assay buffer (RIPA buffer; Beyotime Institute of Biotechnology, Shanghai, China) containing 1% phenylmethylsulfonyl fluoride (PMSF; Beyotime Institute of Biotechnology) for 30 min on ice. Both cytoplasmic and nuclear extracts were then extracted using a nuclear fractionation protocol, following the manufacturer instructions (Beijing Solarbio Science & Technology Co., Ltd.). The concentration of each protein sample was assayed using the bicinchoninic acid assay kit (Beijing Solarbio Science & Technology) and equal amounts (20 µg of each) of protein samples were separated using 10% sodium dodecyl sulfate-polyacrylamide gel (Beyotime Institute of Biotechnology) electrophoresis and electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Invitrogen; Thermo Fisher Scientific). For western blotting, the membranes were first incubated in 5% non-fat milk at room temperature for 1 h and then incubated with a rabbit monoclonal anti-human YAP antibody (cat. no. 14074), a rabbit monoclonal anti-human phospho-YAP antibody (cat. no. 13619), a rabbit polyclonal anti-human MST1 antibody (cat. no. 3682), a rabbit polyclonal anti-human phospho-MST1 antibody (cat. no. 3681), a rabbit monoclonal anti-human MOB1 antibody (cat. no. 13730), and a rabbit monoclonal anti-human phospho-MOBI antibody (cat. no. 8699) (all from Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight. The next day, the membranes were washed with Tris-based saline-Tween-20 (TBS-T; 20 mmol/l Tris-HCl, 150 mmol/l NaCl and 0.05% Tween-20) three times for 10 min each and then incubated in 1:5,000 HRP-conjugated goat anti-rabbit IgG (PMSF; Beyotime Institute of Biotechnology) for 30 min on ice and then washed with ice-cold PBS (20 mmol/l Tris·HCl, 150 mmol/l NaCl and 0.05% Tween-20) three times for 10 min each time and then incubated in 1:5,000 HRP-conjugated goat anti-rabbit IgG (PMSF; Beyotime Institute of Biotechnology) for 30 min on ice and then washed with ice-cold PBS (20 mmol/l Tris·HCl, 150 mmol/l NaCl and 0.05% Tween-20) three times for 10 min each time.

**Quantitative RT-PCR (qRT-PCR).** The total cellular RNA was isolated from Cal27 cells that had been treated with morin for 24 h using a TRIzol® reagent (Takara Bio, Inc., Otsu, Japan). The RNA was then reversely transcribed into cDNA using a Reverse Transcriptase kit (Takara Bio) according to the manufacturer’s protocols. These cDNA samples were then amplified with qPCR in 20 µl of the reaction system, which contained the SYBR® Premix Ex Taq™ (Takara Bio), DNA, each primer and RNase-free H₂O, following the manufacturer’s protocol. Amplification conditions were set to an initial step of 95°C for 30 sec and then 45 cycles of 95°C for 5 sec, 60°C for 35 sec, and 72°C for 60 sec and a final step at 40°C for 30 sec. The level of GAPDH mRNA was used as a control and the relative expression level of each RNA sample was calculated using the 2-ΔΔCq method (30). All experiments were performed in triplicate and repeated at least once. The primer sequences were GAPDH, 5'-GCACCGTCAAGGCTGAGA633G-3' and 5'-TGG TGAAGACGCCAGTGGA3-3'; CTGF, 5'-TCTCCAACCTCT CCTACTAC-3' and 5'-GCACGTAGTTCTGACATCCT3'; CYR61, 5'-CCCTTGGGAGCCAGCTGTA3-3' and 5'-ACT TGGGCGGTATTTCTCT-3'; and ANKR D, 5'-AGTAGA GAACCTGTACTGG-3' and 5'-TGGGCTGAGAGTGC TTCAGAT-3'.

**Statistical analysis.** All data are summarized as the mean ± standard error of the mean (SEM) of at least three replicates of each experiment and statistically analyzed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) with the two-tailed Student’s t-test for two groups of data or the one-way analysis of variance (ANOVA) plus Tukey’s post hoc test for multiple groups of data. P<0.05 indicates statistical significance.

**Results**

**Morin reduces TSCC cell proliferation.** To evaluate the effects of morin on the regulation of TSCC cell proliferation, we performed cell viability (CCK-8), EdU incorporation and colony formation assays. Our CCK-8 assay data revealed that morin inhibited TSCC cell proliferation.

**Morin induces tumor cell cycle arrest and apoptosis.** We further investigated how morin treatment inhibits tumor cell proliferation using flow cytometric apoptosis and cell cycle...
assays. Our data showed that morin treatment, for 24 h, arrested tumor cells at the G1 phase of the cell cycle, and showed a significantly smaller percentage of S phase cells than this percentage in the control group (Fig. 4A and B). Moreover, morin-treated tumor cells underwent apoptosis. Both the percentages of early and late apoptotic cells were significantly higher at 48 h of morin treatment at 100 and 150 µM than in the control cells (Fig. 4C and D).
Morin reduces TSCC cell migration. We then performed the tumor cell wound-healing assay to investigate the effect of morin on the migratory capacity of Cal27 cells. We found that, following morin treatment, cells showed a lower tumor cell migration compared with the tumor cell migration in the control group at 6 h. Treatment of tumor cells with the different morin concentrations showed a significant inhibitory effect on migration at 24 h; *P<0.05, vs. the control (DMSO) group.
Morin activates Hippo pathway proteins and inhibits YAP nuclear translocation. Thus far, we demonstrated the antitumor activity of morin in TSCC cells in vitro. To further this study, we aimed to assess the underlying molecular pathways responsible for the action of morin on TSCC cells. Our western blot analysis revealed that the total levels of MST1 and MOB1 proteins were not significantly altered after morin treatment of TSCC cells (there was no significant difference between the results of the treated cells and the control cells; Fig. 6A and B).
In contrast, the expression levels of phospho-MST1 and phospho-MOB1 proteins were significantly higher in morin-treated TSCC cells than these levels in the control cells (Fig. 6C and D). Furthermore, the cytoplasmic level of YAP protein was significantly upregulated in morin-treated TSCC cells and the ratio of phosphorylated YAP was also greater in the morin-treated tumor cells (Fig. 6E and F). In contrast, TSCC cells treated with high concentrations of morin exhibited a lower level of nuclear YAP protein than that of the control cells (Fig. 6G and H). We further investigated whether the changes in YAP protein localization that were observed following morin treatment correlated with an alteration in YAP-targeting proteins, such as CTGF, CYR61 and ANKR. Our data showed that the expression of CTGF, CYR61 and ANKR genes was significantly lower in the morin-treated Cal27 cells than that in the control cells (Fig. 6I). Taken together, our current data demonstrated that morin treatment upregulated the activity of the Hippo pathway but suppressed YAP nuclear translocation and YAP-related transcriptional activity in Cal27 cells.

Discussion

Morin displays wide-ranging pharmacological activities and low cytotoxicity against different types of human cancer cells (31). For example, previous studies have shown that morin possesses anti-liver cancer activity in the promotion stage of an in vivo liver carcinogenesis model (10). Additionally, morin has been shown to suppress breast cancer malignant behaviors through the inhibition of tumor cell epithelial-mesenchymal transition and Akt activation (32). Morin has also been suggested to induce apoptosis of human histiocytic lymphoma U937 cells through the upregulation of the Bcl-2-associated death promoter (BAD) protein levels (33). In tongue squamous cell carcinoma (TSCC), a recent study demonstrated that human TSCC cells were sensitive to morin-induced tumor cell growth inhibition (6). However, the underlying mechanism of morin in TSCC cells remains to be defined. Thus, the present study assessed the antitumor effect of morin in TSCC Cal27 cells and explored the underlying mechanisms. We found that morin treatment effectively decreased cell proliferation, colony formation, and migration of Cal27 cells in a dose-dependent manner. In the wound healing assay, cells were cultured for up to 24 h. During this period of time cells do not proliferate to a great extent, thus this enabled us to avoid the effect of morin on cell proliferation in the wound healing assay. Our data showed that the wound healing capacity was inhibited to a greater extent in the morin-treated tumor cells than that observed in the control group, over a period of 24 h. Additionally, the cell viability assay showed a significant reduction in tumor cell survival in the morin-treated group after a 24-h incubation period. Taken together, we have reason to believe that the wound assay will show a more significant difference between the drug treatment groups and the control group after 24 h. Furthermore, our cell cycle analysis showed that morin treatment led to cell cycle arrest at the G1 phase, with a significantly lower percentage of cells in the S phase than that noted in the controls. Moreover, the data concerning the apoptosis analysis showed that morin treatment significantly increased the percentage of Cal27 cells that were undergoing early and late apoptosis. The morin-induced inhibition of TSCC cell growth and migration was demonstrated in our in vitro results.

Indeed, previous studies have demonstrated that different phytochemicals, including morin, possess antitumor activity in various types of human cancers (reviewed in ref. 4); morin has shown different pharmacological activities with very low cytotoxicity in humans (29); thus, in the present study, we treated TSCC Cal27 cells with up to 150 µM morin, while a previous study of nude mouse melanoma cell xenographs treated the mice with 50 mg/kg of morin intraperitoneally (34). These doses could clinically be achievable (35). Previous studies have demonstrated that a low dose of morin reduced the cisplatin-induced toxicity of 293 cells and mouse kidney cells (36) and showed neuroprotective effect of morin in lead acetate-induced apoptosis in the rat brain (37). The induction of tumor cell apoptosis in response to morin at higher doses has been further confirmed in our current data.

Upon further exploration of the underlying mechanism of the morin antitumor activity in TSCC, we found that, at the gene level, morin upregulated the phosphorylation of the Hippo pathway proteins and inhibited YAP nuclear translocation. It is known that the Hippo pathway regulates cell growth, proliferation, apoptosis and organ size during embryonic development through alterations of the subcellular localization of YAP (38). For example, Song et al demonstrated that Hippo pathway-inactivated mice exhibited induced YAP phosphorylation with nuclear translocation, which led to the development of hepatocellular carcinoma (HCC) in mice (39). In contrast, re-activation of the Hippo pathway, in the HCC-derived cell line, promoted YAP phosphorylation and the suppression of HCC (39). Immunohistochemical studies of non-small cell lung cancer revealed that an elevated ratio of nuclear localization of YAP protein is associated with advanced tumor features and poor patient outcome (40-42). Furthermore, the level of YAP protein has been found to be upregulated in precancerous lesions in a rat model of liver cancer and upregulation of the nuclear level of YAP protein is frequently found in fully developed HCC (43). Elevated YAP expression and nuclear localization also occurs in mouse models of pancreatic cancer, and in tumors derived from human pancreatic adenocarcinoma cell lines (44,45). The pancreas-specific YAP knockout inhibits tumor progression in a mouse pancreatic cancer model (46). In the present study, we also demonstrated that morin treatment was able to activate Hippo signaling through the upregulation of phospho-MST1 and phospho-MOB1 proteins. Additionally, YAP phosphorylation was induced by morin, and the level of YAP nuclear translocation and the expression of YAP-targeting CTGF, CYR61 and ANKR genes were inhibited.

This study demonstrated that morin possesses antitumor activity in Cal27 cells through the activation of the Hippo pathway and suppression of YAP nuclear translocation in vitro. However, the use of only one cell line is a limitation of this study. Future study with more cell lines is needed to confirm our current data. In addition, we do know that morin, as a photochemical, should be able to target multiple gene or gene pathways, as is seen with other phytochemicals (33,34). Thus, future studies will further explore the gene targets of morin.

In conclusion, the present proof-of-principle study demonstrated that morin treatment inhibited TSCC Cal27 cell
proliferation and migration, but induced TSCC cell apoptosis and arrested tumor cells in the G1 phase of the cell cycle. At the gene level, morin treatment activated the Hippo pathway and inhibited YAP activity, indicating that morin possesses antitumor activity in TSCC in vitro.

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Availability of data and materials
The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions
XX and XQ designed the study and the experiments. YJ and LJ conducted the experiments. YZ, YX, DZ, XW and BZ analyzed the data and YJ wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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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