

Magnesium Isoglycyrrhizinate Induces an Inhibitory Effect on Progression and Epithelial–Mesenchymal Transition of Laryngeal Cancer via the NF- κ B/Twist Signaling

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Background: Magnesium isoglycyrrhizinate (MI) was extracted from roots of the plant *Glycyrrhiza glabra*, which displays multiple pharmacological activities such as anti-inflammation, anti-apoptosis, and anti-tumor. Here, we aimed to investigate the effect of MI on the progression and epithelial–mesenchymal transition (EMT) of laryngeal cancer.

Methods: Forty laryngeal cancer clinical samples were used. The role of MI in the proliferation of laryngeal cancer cells was assessed by MTT assay, Edu assay and colony formation assay. The function of MI in the migration and invasion of laryngeal cancer cells was tested by transwell assays. The effect of MI on apoptosis of laryngeal cancer cells was determined by cell apoptosis assay. The impact of MI on tumor growth in vivo was analyzed by tumorigenicity analysis using Balb/c nude mice. qPCR and Western blot analysis were performed to measure the expression levels of gene and protein, respectively.

Results: We identified that EMT-related transcription factor Twist was significantly elevated in the laryngeal cancer tissues. The expression of Twist was also enhanced in the human laryngeal carcinoma HEP-2 cells compared with that in the primary laryngeal epithelial cells. The high expression of Twist was remarkably correlated with poor overall survival of patients with laryngeal cancer. Meanwhile, our data revealed that MI reduced cell proliferation, migration and invasion and enhanced apoptosis of laryngeal cancer cells in vitro. Moreover, MI decreased transcriptional activation and the expression levels of NF- κ B and Twist, and alleviated EMT in vitro and in vivo. MI remarkably inhibited tumor growth and EMT of laryngeal cancer cells in vivo.

Conclusion: MI restrains the progression of laryngeal cancer and induces an inhibitory effect on EMT in laryngeal cancer by modulating the NF- κ B/Twist signaling. Our finding provides new insights into the mechanism by which MI inhibits laryngeal carcinoma development, enriching the understanding of the anti-tumor function of MI.

Keywords: laryngeal cancer, progression, EMT, magnesium isoglycyrrhizinate, Twist, NF- κ B signaling

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Introduction

Laryngeal cancer is one of the most prevalent malignancies of the respiratory system, accounting for 26% to 30% of head and neck cancers.^{1,2} Almost 95% of the histologic pathogeny of laryngeal cancer is laryngeal squamous carcinoma, and the survival incidence of patients is low.³ Despite the development of new strategies

in surgery, chemotherapy, and radiation, the targeted treatment of laryngeal cancer is still a challenge.⁴ Thus, identification of safe and effective treatment candidates for laryngeal cancer is urgently needed. Epithelial–mesenchymal transition (EMT) serves as a cellular program, in which cells drop their epithelial features and gain mesenchymal characteristics.^{5,6} EMT is correlated with multiple tumor progressions, such as resistance to therapy, blood intravasation, tumor initiation, tumor cell migration, tumor stemness, malignant progression, and metastasis.^{7–9} As a critical process of cancer development, EMT contributes to the development of laryngeal cancer. It has been reported that the combination of photodynamic therapy and carboplatin suppresses the expression of MMP-2/MMP-9 and EMT of laryngeal cancer by ROS-inhibited MEK/ERK signaling.¹⁰ EZH2 increases metastasis and aggression of laryngeal squamous cell carcinoma through the EMT program by modulating H3K27me3.¹¹ However, investigation about the inhibitory candidates of EMT in laryngeal cancer remains limited.

Nutraceutical agents display a unique therapeutic activity to treat diseases such as inflammation and cancer.^{12–17} Glycyrrhizic acid (GA) is extracted from the roots of licorice and serves as a major component of licorice, presenting multiple biomedical activities, such as anti-oxidant and anti-inflammatory.¹⁸ Magnesium isoglycyrrhizinate (MI), refined from GA, is α 18- α -GA stereoisomer magnesium salt and demonstrates a better activity than 18- β -GA.¹⁹ As a natural and safe compound, MI shows many biomedical activities such as anti-inflammation capacities,²⁰ anti-apoptosis²¹ and anti-tumor.²² It has been reported that MI inhibits fructose-modulated lipid metabolism disorder and activation of the NF- κ B/NLRP3 inflammasome.²³ MI reduces paclitaxel in patients with epithelial ovarian cancer managed with cisplatin and paclitaxel.²⁴ Moreover, MI attenuates high fructose-induced liver fibrosis and EMT by upregulating miR-375-3p to overcome the TGF- β 1/Smad pathway and JAK2/STAT3 signaling.²⁵ However, the role of MI in cancer development is unknown. The effect of MI on the progression and EMT of laryngeal cancer remains unreported.

Many transcription factors, such as Twist, serve as molecule switches in EMT progression.²⁶ Twist is a crucial transcription factor for the modulating of EMT, which advances cell invasion, migration, and cancer metastasis, conferring tumor cells with stem cell-like properties and providing therapy resistance.^{27,28} The function of Twist in EMT of cancer development has been well reported. Disrupting the diacetylated Twist represses the progression

of Basal-like breast cancer.²⁹ The activation of Twist promotes progression and EMT of breast cancer.³⁰ The inhibition of Twist limits the stem cell features and EMT of prostate cancer.³¹ Twist serves as a critical factor in promoting metastasis of pancreatic cancer.³² Besides, tumor necrosis factor α provokes EMT of hypopharyngeal cancer and induces metastasis through NF- κ B signaling-regulated expression of Twist.³³ NF- κ B/Twist signaling is involved in the mechanism of Chysin inhibiting stem cell characteristics of ovarian cancer.³⁴ Repression of the NF- κ B/Twist axis decreases the stemness features of lung cancer stem cell.³⁵ Down-regulation of TWIST reduces invasion and migration of laryngeal carcinoma cells by controlling the expression of N-cadherin and E-cadherin.³⁶ The expression of Twist displays the clinical significance of laryngeal cancer.³⁷ However, whether NF- κ B and Twist are involved in the biomedical activities of MI is unclear.

In this study, we aimed to explore the function of MI in the development and EMT process of laryngeal cancer. We identified a novel inhibitory effect of MI in the progression and EMT of laryngeal cancer by regulating the NF- κ B/Twist signaling.

Methods

Laryngeal Cancer Clinical Samples

A total of 40 laryngeal cancer clinical samples used in this study were obtained from the Second Affiliated Hospital, Harbin Medical University between June 2016 and August 2018. All the patients were diagnosed by clinical, radiographic, and histopathological analysis. Before surgery, no systemic or local therapy was performed on the subjects. The laryngeal cancer tissues ($n = 40$) and adjacent normal tissues ($n = 40$) obtained from the patients were immediately frozen into liquid nitrogen and stored at -80°C before use. The clinical laryngeal cancer samples were separated into two groups according to the mean expression of Twist. The overall survival was analyzed by Kaplan–Meier survival analysis. The patients and healthy controls provided written informed consent, and that the Ethics Committee of The Second Affiliated Hospital, Harbin Medical University approved this study.

Cell Culture and Treatment

The human laryngeal carcinoma cells HEP-2 and primary laryngeal epithelial cells LEC-P were purchased from American Type Tissue Culture Collection. Cells were cultured in DMEM (Solarbio, China) containing 10% fetal bovine

serum (Gibco, USA), 0.1 mg/mL streptomycin (Solarbio, China) and 100 units/mL penicillin (Solarbio, China) at 37 °C with 5% CO₂. The Magnesium isoglycyrrhizinate (MI) (purity > 98%) was obtained from Zhengda Tianqing Pharmaceutical Co., Ltd (Jiangsu, China). Cells were treated with MI of indicated dose before further analysis.

Quantitative Reverse Transcription-PCR (qRT-PCR)

Total RNAs were extracted using TRIZOL (Invitrogen, USA), followed by reverse transcription into cDNA. The qRT-PCR reactions were prepared using the SYBR Real-time PCR I kit (Takara, Japan). GAPDH was used as the internal control. The qRT-PCR experiment was conducted in triplicate. The primer sequences were as follows:

Twist forward: 5'-CGCTGAACGAGGCATTTC-3'

Twist reverse: 5'-CCAGTTTGAGGGTCTGAATC-3'

Slug forward: 5'-GTGTTTGCAAGATCTGCGGC-3'

Slug reverse: 5'-GCAGATGAGCCCTCAGATTTGA-3'

ZEB1 forward: 5'-CCCCAGGTGTAAGCGCAGAA-3'

ZEB1 reverse: 5'-TGGCAGGTCATCCTCTGGTAC

AC-3'

Snail forward: 5'-CCACACTGGTGAGAAGCCTTT

C-3'

Snail reverse: 5'-GTCTGGAGGTGGGCACGTA-3'

GAPDH forward: 5'-AAGAAGGTGGTGAAGCAG

GC-3'.

GAPDH reverse: 5'-TCCACCACCCAGTTGCTGT

A-3'

MTT Assays

MTT assays were conducted to measure cell viability of HEP-2 cells. Briefly, about 1 × 10⁴ HEP-2 cells were put into 96 wells and cultured for 12 h. Cells were then added with 10 μL MTT solution (5 mg/mL) (Sigma, USA) and cultured for another 4 h. The culture medium was discarded, and 150 μL/well DMSO (Thermo, USA) was added to the wells. An ELISA browser was used to analyze the absorbance at 570 nm (Bio-Tek EL 800, USA).

EdU Assays

The cell proliferation was analyzed by EdU assays using EdU detecting kit (RiboBio, China). Briefly, HEP-2 cells were cultured with EdU for 2 h, followed by fixation with 4% paraformaldehyde at room temperature for 30 min. Then, cells were permeabilized with 0.4% Triton X-100 for 10 min and stained with staining cocktail of EdU at room

temperature for 30 min in the dark. Next, nuclear of the cells was stained with Hoechst at room temperature for 30 min. Images were analyzed using a fluorescence microscope.

Colony Formation Assay

About 1 × 10³ HEP-2 cells were layered in 6 wells and incubated in DMEM at 37 °C. After two weeks, cells were cleaned with PBS Buffer, made in methanol for 30 min, and dyed with 1% crystal violet. The number of colonies was then calculated.

Transwell Assays

Transwell assays were conducted to evaluate the impacts of MI on cell invasion and migration of HEP-2 cells using a Transwell plate (Corning, USA). Briefly, the upper chambers were plated with about 1 × 10⁵ cells. Cells were then solidified using 4% paraformaldehyde and dyed with crystal violet. The invaded and migrated cells were recorded and calculated.

Analysis of Cell Apoptosis

Approximately 2 × 10⁵ HEP-2 cells were plated on 6-well dishes. Cell apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit (CST, USA) following the manufacturer's instructions. Briefly, cells were collected and washed with binding buffer (BD Biosciences, USA), and then dyed at 25 °C, followed by flow cytometry analysis.

Luciferase Reporter Gene Assay

Luciferase reporter gene assay was performed using the Dual-luciferase Reporter Assay System (Promega, USA). Briefly, cells were treated MI as indicated dose, followed by transfection with pGL3-NF-κB and pGL3-Twist using Lipofectamine 3000 (Invitrogen, USA). Luciferase activities were detected and Renilla was used as a normalized control.

Western Blot Analysis

Total proteins were extracted from cells or tumor tissues using RIPA buffer (CST, USA). Protein concentrations were measured using the BCA Protein Quantification Kit (Abbkine, USA). Same amount of protein samples was separated by SDS-PAGE (12% polyacrylamide gels), transferred to PVDF membranes (Millipore, USA) in the subsequent step. The membranes were blocked with 5% milk and incubated with the primary antibodies for Twist (1:1000) (Abcam, UK), E-Cadherin (1:1000) (Abcam, UK), occluding (1:1000) (Abcam, UK), vimentin (1:1000) (Abcam, UK), N-cadherin (1:1000) (Abcam, UK), Ki-67 (1:1000) (Abcam, UK), IKK (1:1000) (Abcam, UK), NF-κB p65

(1:1000) (Abcam, UK), NF- κ B (1:1000) (Abcam, UK), and β -actin (1:1000) (Abcam, UK) at 4 °C overnight, in which β -actin served as the control. Then, the corresponding second antibodies (1:1000) (Abcam, UK) were used to incubate the membranes at room temperature for 1 h, followed by visualization using an Odyssey CLx Infrared Imaging System. ImageJ software was used to quantify the results.

Analysis of Tumorigenicity in Nude Mice

The effect of MI on tumor growth *in vivo* was analyzed in nude mice of Balb/c. Mice were randomly separated into two groups ($n = 3$). To establish the *in vivo* tumor model, HEP-2 cells were treated with MI (300 mg/kg) or equal volume of saline. And about 2×10^6 cells were subcutaneously injected into mice. After 7 days of injection, tumor growth was measured every 7 days. The mice were sacrificed after 35 days of injection and tumors were scaled. Tumor volume (V) was observed by estimating the length (L) and width (W) with calipers and measured with the formula $(L \times W^2) \times 0.5$. The expression levels of Ki-67 of tumor tissues were measured by immunohistochemical staining with the Ki67 antibody (1:1000) (Abcam, UK). Protein expression levels in tumor tissues were determined by Western blot analysis using Twist (1:1000) (Abcam, UK), E-Cadherin (1:1000) (Abcam, UK), occluding (1:1000) (Abcam, UK), vimentin (1:1000) (Abcam, UK), N-cadherin (1:1000) (Abcam, UK), Ki-67 (1:1000) (Abcam, UK), IKK (1:1000) (Abcam, UK), NF- κ B p65 (1:1000) (Abcam, UK), NF- κ B (1:1000) (Abcam, UK), and β -actin (1:1000) (Abcam, UK). Animal care and experimental procedures in this study were approved by the Animal Ethics Committee of the Second Affiliated Hospital, Harbin Medical University.

Statistical Analysis

Data were presented as mean \pm SD, and statistical analysis was performed using SPSS software (version 18.0). The unpaired Student's *t*-test was applied for comparing two groups, and one-way ANOVA was applied for comparing multiple groups. $P < 0.05$ was considered as statistically significant.

Results

Twist is Potentially Correlated with the Progression and Poor Prognosis of Laryngeal Cancer

To assess the potential correlation of EMT-related transcription factors with laryngeal cancer, the expression of Twist, Slug, ZEB1, and Snail in the clinical laryngeal samples and

laryngeal cells were measured by qPCR assays. The data showed that the expression levels of Twist, Slug, ZEB1, and Snail were significantly elevated in laryngeal cancer tissues ($n = 40$) compared to that in normal tissues ($n = 40$), among which Twist displayed the highest expression levels ($P < 0.01$) (Figure 1A). Meanwhile, the expression levels of Twist, Slug, ZEB1, and Snail were also enhanced in human laryngeal carcinoma HEP-2 cells compared with that in primary laryngeal epithelial cells (LEC-P), and Twist exerted the highest expression levels among these transcription factors ($P < 0.001$) (Figure 1B), implying that Twist is closely associated with the development of laryngeal cancer. To determine whether Twist was able to serve as the potential biomarker for patients with laryngeal cancer, we separated the clinical laryngeal cancer samples into two groups according to the mean expression of Twist. We observed that high expression levels of Twist was remarkably correlated with the poor overall survival ($P < 0.01$) (Figure 1C), suggesting that Twist may play a crucial role in the progression of laryngeal cancer.

Magnesium Isoglycyrrhizinate (MI) Attenuates Cell Proliferation of Laryngeal Cancer *in vitro*

Then, the role of MI in the modulation of laryngeal cancer proliferation was investigated, and the structure formula of MI is shown in Figure 2A. To evaluate the effect of MI on the progression of laryngeal cancer, MTT assay, colony formation assay, and EdU assay were performed in HEP-2 cells treated with MI. MTT assay demonstrated that MI significantly inhibited cell viability in a dose-dependent manner, and the half-maximal inhibitory concentrations (IC₅₀) of MI in HEP-2 cells was 3.22 mg/mL ($P < 0.01$) (Figure 2B). Hence, we selected the MI dose of 3.22 mg/mL in the following experiments. Similarly, colony formation assay showed that the colony numbers of HEP-2 cells were remarkably reduced by MI treatment ($P < 0.01$) (Figure 2C). Besides, EdU assay showed that MI notably decreased number of EdU-positive cells ($P < 0.01$) (Figure 2D). These data suggested that MI was able to inhibit cell proliferation of laryngeal cancer.

MI Inhibits Laryngeal Cancer Cell Migration and Invasion and Enhances Cell Apoptosis *in vitro*

The role of MI in HEP-2 cell migration, invasion, and apoptosis was then evaluated. Transwell assays revealed that MI treatment remarkably reduced cell migration ($P < 0.01$) (Figure 3A). Similarly, cell invasion was significantly

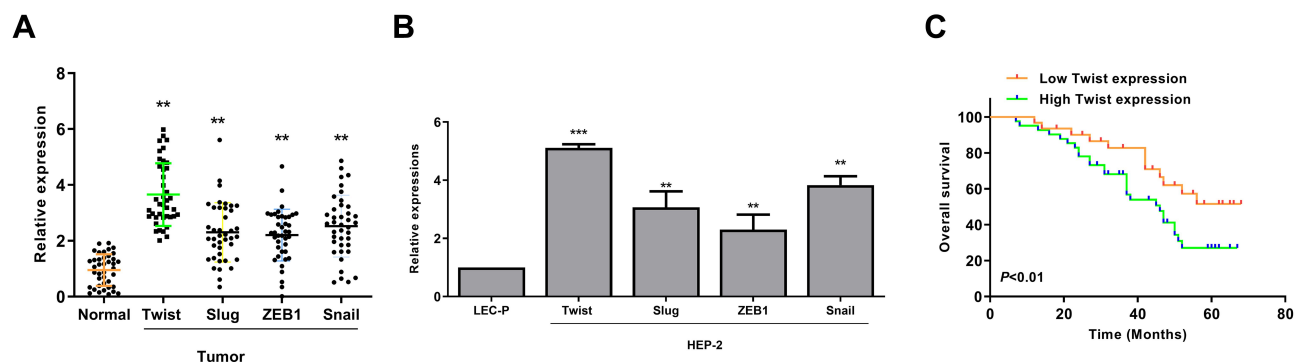


Figure 1 Twist is potentially correlated with the progression and poor prognosis of laryngeal cancer. **(A)** The expression levels of Twist, Slug, ZEB1, and Snail were measured by qPCR in the laryngeal cancer tissues ($n = 40$) and adjacent normal tissues ($n = 40$). **(B)** The expression levels of Twist, Slug, ZEB1, and Snail were assessed by qPCR in HEP-2 cells and primary laryngeal epithelial cells. **(C)** The clinical laryngeal cancer samples were separated into two groups according to the mean expression of Twist. The overall survival was analyzed by Kaplan-Meier survival analysis. Data are presented as mean \pm SD. Statistic significant differences were indicated: ** $P < 0.01$, *** $P < 0.001$.

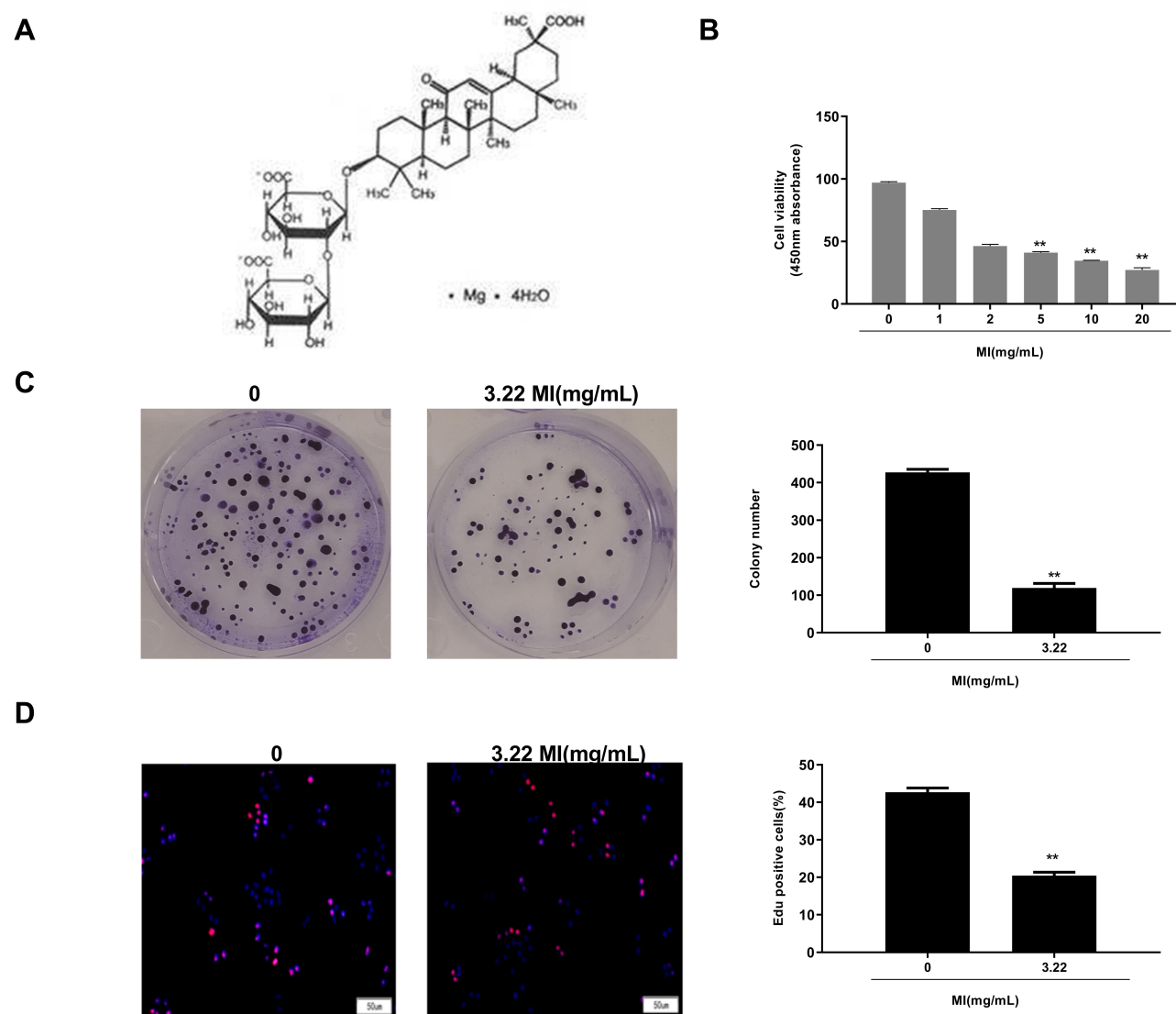


Figure 2 Magnesium isoglycyrrhizinate (MI) attenuates cell proliferation of laryngeal cancer in vitro. **(A)** The structure formula of MI was shown. **(B)** The cell viability was analyzed by MTT assays in the HEP-2 cells treated with MI at indicated dosage. **(C)** The cell proliferation was measured by colony formation assays in the HEP-2 cells treated with MI at indicated dosage. **(D)** The cell proliferation was examined by EdU assays in the HEP-2 cells treated with MI at indicated dosage. Data are presented as mean \pm SD. Statistic significant differences were indicated: ** $P < 0.01$.

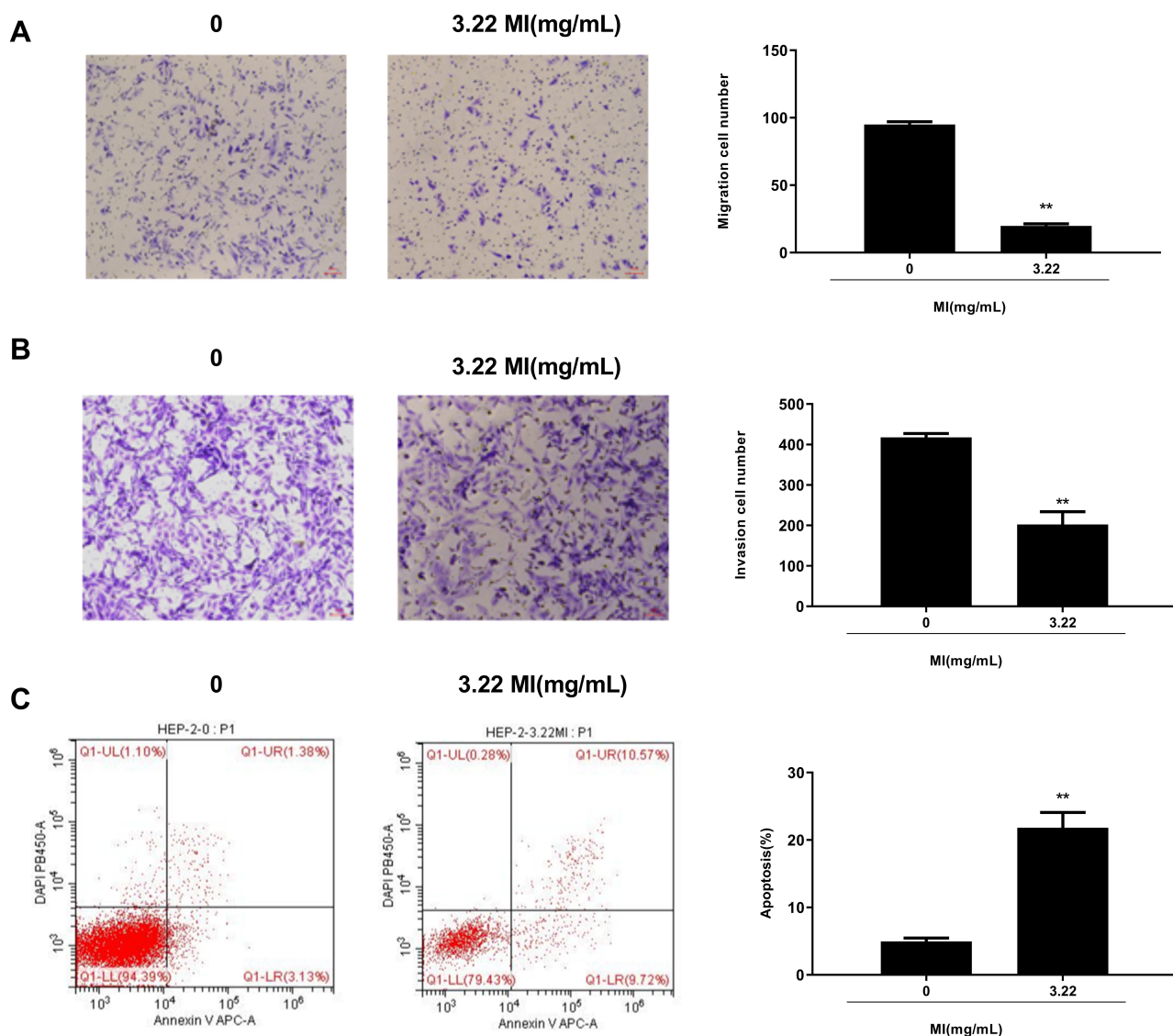


Figure 3 MI inhibits laryngeal cancer cell migration and invasion and enhances cell apoptosis in vitro. **(A)** The cell migration was examined by transwell assays in the HEP-2 cells treated with MI at indicated dosage. **(B)** The cell invasion was examined by transwell assays in the HEP-2 cells treated with MI at indicated dosage. **(C)** The cell apoptosis was measured by flow cytometry analysis in the HEP-2 cells treated with MI at indicated dosage. Data are presented as mean \pm SD. Statistically significant differences were indicated: ** $P < 0.01$.

decreased by MI in HEP-2 cells ($P < 0.01$) (Figure 3B). Moreover, flow cytometry analysis showed that cell apoptosis was notably increased by MI treatment in the system ($P < 0.01$) (Figure 3C). These results indicated that MI inhibited laryngeal cancer cell migration and invasion and enhanced cell apoptosis in vitro.

MI Inhibits Transcriptional Activation and the Expression of NF- κ B and Twist and Alleviates EMT in Laryngeal Cancer Cells

Next, the underlying mechanism of the effect of MI on the development of laryngeal cancer in HEP-2 cells was further

explored. It showed that MI treatment significantly reduced the luciferase activities of NF- κ B in the cells ($P < 0.01$) (Figure 4A), suggesting that MI may inhibit NF- κ B at transcriptional level. Meanwhile, dual-luciferase reporter gene assays further revealed that MI treatment remarkably decreased the transcriptional activities of Twist in the cells ($P < 0.01$) (Figure 4B). Furthermore, Western blot analysis demonstrated that the total expression (Figure 4C) and nucleus expression (Figure 4D) of NF- κ B and Twist were significantly down-regulated by MI in HEP-2 cells ($P < 0.01$), suggesting that MI may inhibit Twist by modulating NF- κ B. Moreover, to assess the effect of MI on the EMT of laryngeal cancer, the expression of EMT markers, including

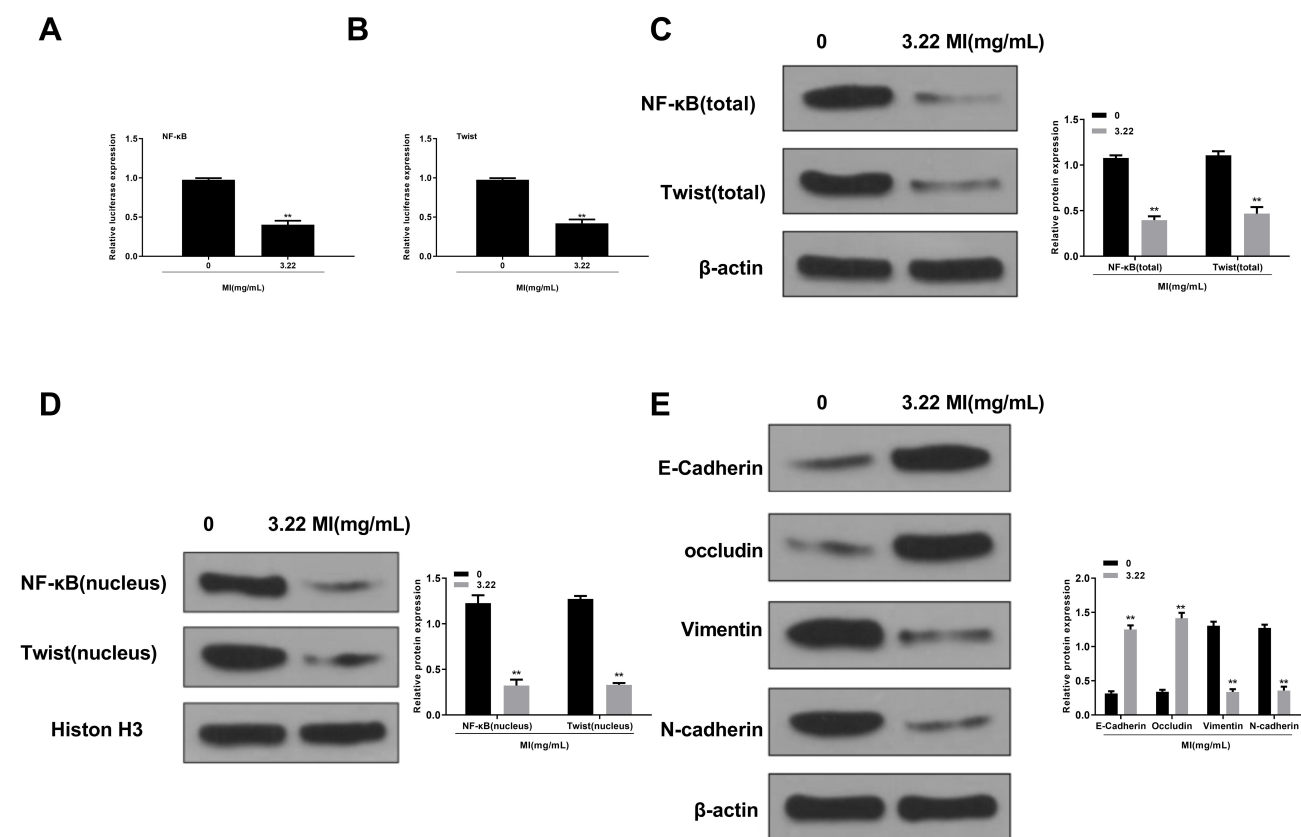


Figure 4 MI inhibits transcriptional activation and expression of NF- κ B and Twist and alleviates EMT in laryngeal cancer cells. **(A)** The luciferase activities of NF- κ B were determined by luciferase reporter gene assays in the HEP-2 cells treated with MI at indicated dosage. **(B)** The luciferase activities of Twist were determined by luciferase reporter gene assays in the HEP-2 cells treated with MI at indicated dosage. **(C)** The total expression of NF- κ B, Twist, and β -actin was measured by Western blot analysis in the HEP-2 cells treated with MI at indicated dosage. The results of Western blot analysis were quantified by ImageJ software. **(D)** The nucleus expression of NF- κ B, Twist, and histone H3 was tested by Western blot analysis in the HEP-2 cells treated with MI at indicated dosage. The results of Western blot analysis were quantified by ImageJ software. **(E)** The expression levels of E-Cadherin, occluding, vimentin, N-cadherin, and β -actin were analyzed by Western blot analysis in the HEP-2 cells treated with MI at indicated dosage. The results of Western blot analysis were quantified by ImageJ software. Data are presented as mean \pm SD. Statistic significant differences were indicated: ** $P < 0.01$.

E-Cadherin, occluding, vimentin, and N-cadherin, in HEP-2 cells treated with MI were measured. Our data showed that the expression levels of E-Cadherin and occluding were enhanced while the expression levels of vimentin and N-cadherin were reduced by MI treatment in HEP-2 cells ($P < 0.01$) (Figure 4E), suggesting that MI can inhibit EMT of laryngeal cancer.

MI Inhibits Tumor Growth and EMT of Laryngeal Cancer via the Twist/NF- κ B Signaling in vivo

The effect of MI on laryngeal cancer development was further investigated in vivo. Tumorigenicity analysis was conducted in nude mice injected with HEP-2 cells, which were treated with MI or corresponding control. The MI treatment significantly reduced tumor size (Figure 5A), tumor weight ($P < 0.01$) (Figure 5B), tumor volume ($P < 0.01$)

(Figure 5C), and the expression levels of Ki-67 in tumor tissues of the mice (Figure 5D), suggesting that MI inhibited tumor growth of laryngeal cancer in vivo.

Moreover, our data showed that MI treatment significantly enhanced the expression levels of E-Cadherin and occluding, whereas reduced the expression levels of vimentin and N-cadherin in tumor tissues of the mice ($P < 0.01$) (Figure 6A and B), indicating that MI attenuated EMT of laryngeal cancer in vivo. Besides, the expression levels of Twist, Ki-67, and NF- κ B signaling proteins containing p65 and IKK were significantly decreased by MI in tumor tissues of the mice ($P < 0.01$) (Figure 6C and D), implying that MI may inhibit EMT of laryngeal cancer via the Twist/NF- κ B signaling.

Discussion

Laryngeal cancer is the second most prevalent head and neck malignancy.³⁸ Laryngeal cancer patients hold poor survival

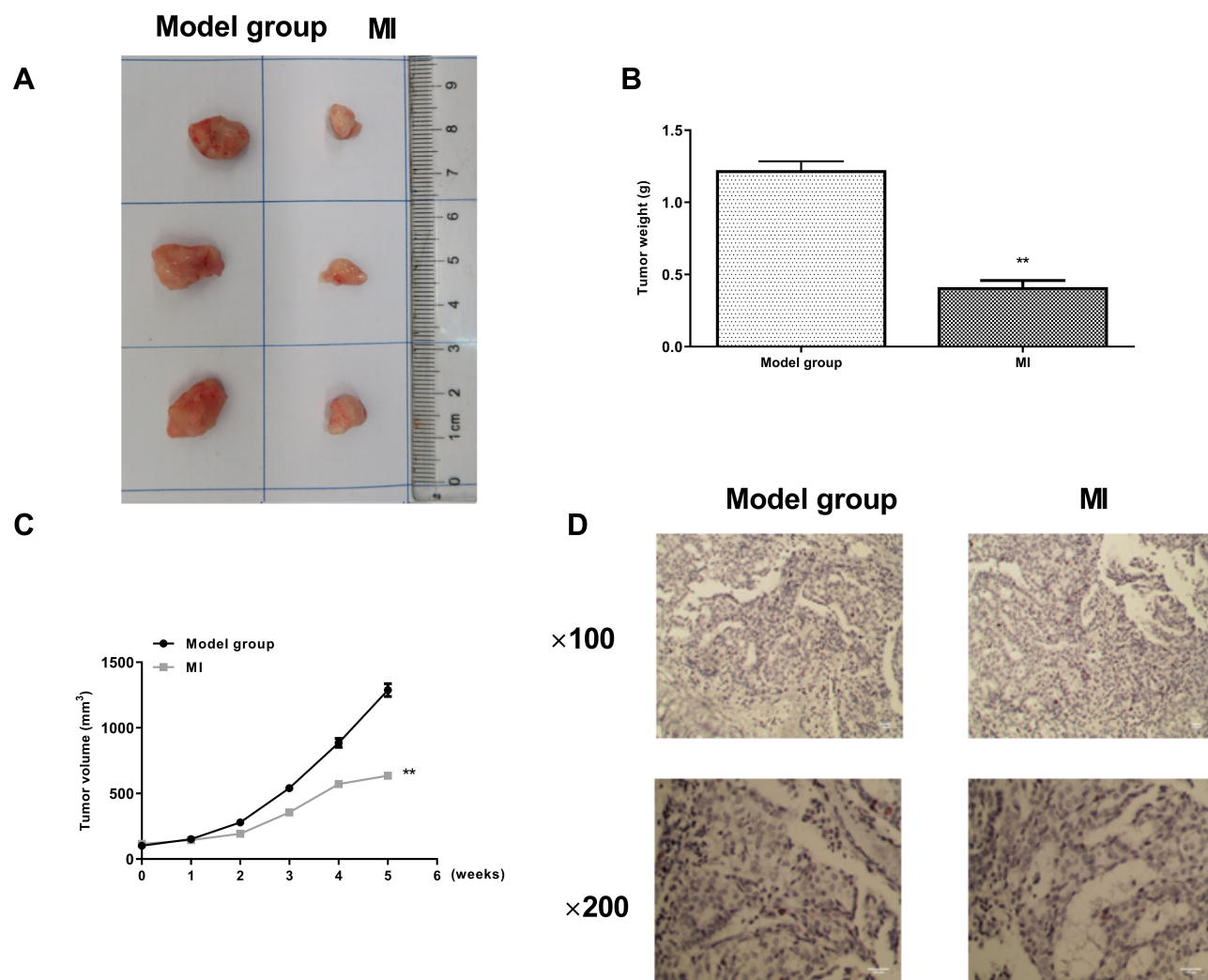


Figure 5 MI inhibits tumor growth of laryngeal cancer in vivo. (A–D) The effect of MI on tumor growth of laryngeal cancer in vivo was analyzed by tumorigenicity assay in nude mice. The HEP-2 cells were treated with MI (300 mg/kg) or equal volume saline and injected into the nude mice ($n = 3$). (A) Representative images of dissected tumors from nude mice were presented. (B) The average tumor weight was calculated and shown. (C) The average tumor volume was calculated and shown. (D) The expression levels of Ki-67 of the tumor tissues were measured by immunohistochemical staining. Data are presented as mean \pm SD. Statistic significant differences were indicated: ** $P < 0.01$.

incidences and low prognosis, and many cases still suffer from recurrence.³⁹ Although improvements have been made in chemotherapy, radiotherapy, and surgery, invasion and metastasis are still the principal reasons for laryngeal cancer-related mortality for patients with advanced grade.⁴⁰ Searching for more safe and practical treatment candidates for the effective therapy of laryngeal cancer is of great importance.⁴¹ As a natural compound, MI has exerted practical potential in the medical application. It has been reported that MI represses cardiac hypertrophy by modulating the TLR4/NF- κ B signaling in mice.⁴² MI preserves against tripolide-produced hepatotoxicity through activating the Nrf2 signaling.⁴³ MI relieves fructose-mediated apoptosis of podocyte by down-regulation of miR-193a to enhance

WT1.⁴⁴ However, the investigation of MI in cancer progression is limited. Previous study identified that the exposure-impact-toxicity was correlated with MI and docetaxel in non-small cell lung cancer mice.²² MI inhibits chemotherapy-caused damage to liver throughout the initial therapy of gastrointestinal tumor patients.⁴⁵ MI decreases paclitaxel in patients with epithelial ovarian cancer treated with cisplatin and paclitaxel.²⁴ The function of MI is associated with its activity to restrain several crucial pathways such as phospholipase A2/arachidonic signaling,⁴⁶ STAT3 signaling,⁴⁷ and NF- κ B signaling.^{23,48} In this study, we firstly identified that MI inhibited cell proliferation, migration, invasion, and enhanced cell apoptosis in laryngeal cancer. MI reduced tumor growth of laryngeal cancer in vivo. Our

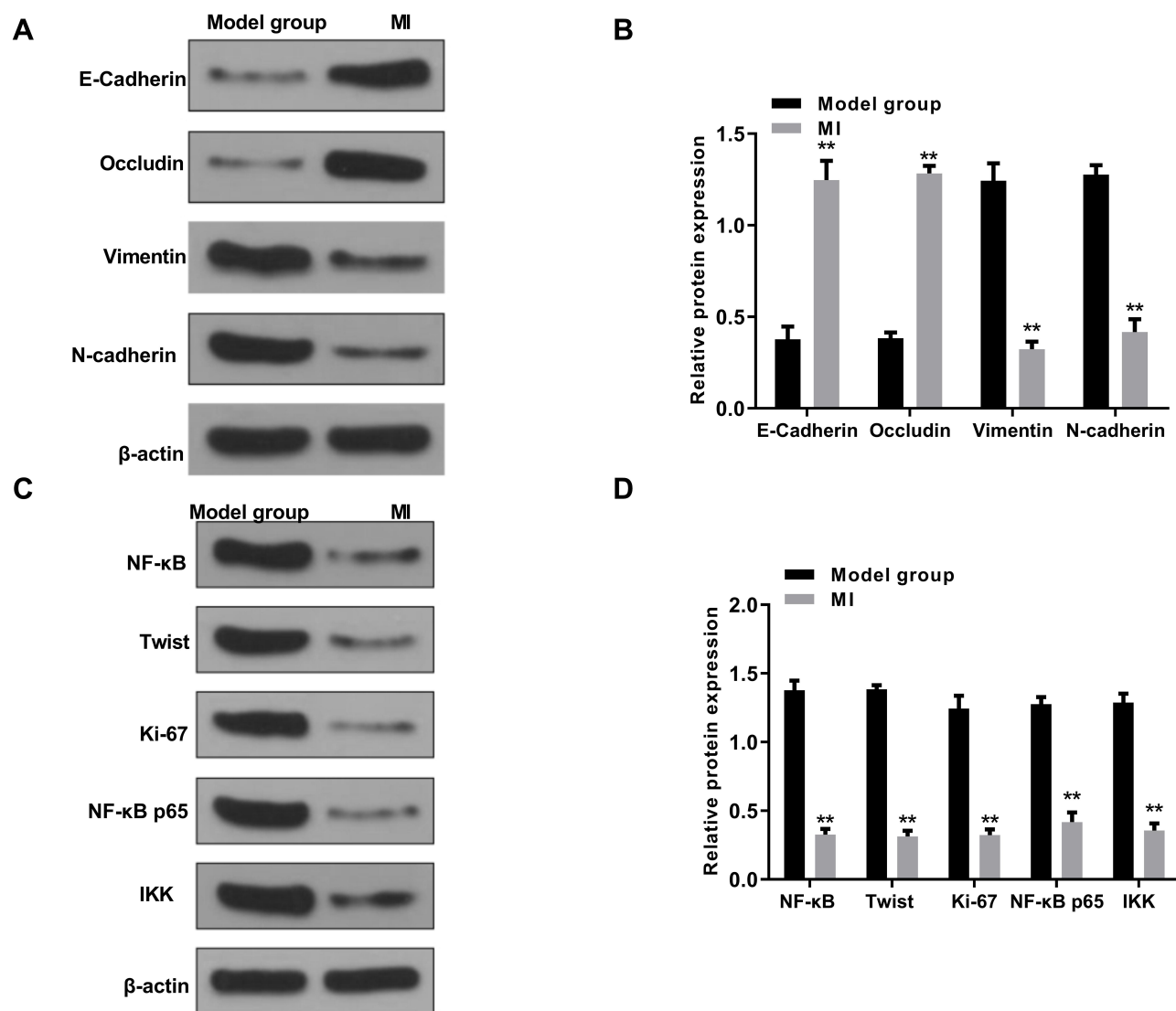


Figure 6 MI inhibits EMT of laryngeal cancer via Twist/NF- κ B signaling in vivo. **(A–D)** The effect of MI on tumor growth of laryngeal cancer in vivo was analyzed by nude mice tumorigenicity assay. The HEP-2 cells were treated with MI (300 mg/kg) or equal volume saline and injected into the nude mice ($n = 3$). **(A)** The expression levels of E-Cadherin, occluding, vimentin, N-cadherin, and β -actin were analyzed by Western blot analysis in the tumor tissues of the mice. **(B)** The results of Western blot analysis in **(A)** were quantified by ImageJ software. **(C)** The expression levels of NF- κ B, NF- κ B p65, Twist, Ki-67, IKK, and β -actin were measured by Western blot analysis in the tumor tissues of the mice. **(D)** The results of Western blot analysis in **(C)** were quantified by ImageJ software. Data are presented as mean \pm SD. Statistic significant differences were indicated: ** $P < 0.01$.

data present a novel inhibitory function of MI in laryngeal cancer and provide valuable insights into the role of MI in cancer development.

EMT plays a critical role in the development of laryngeal cancer, especially in cancer cell metastasis progression by promoting resistance to apoptotic stimulation, invasion, and mobility.⁴⁹ It was reported that TGF- β -induced lncRNA MIR155HG promoted EMT of laryngeal squamous cell carcinoma by regulating the miR-155/SOX10 signaling.⁵⁰ YAP modulates the Wnt/ β -catenin signaling and EMT program of laryngeal cancer.⁵¹ Abnormal methylation and down-regulation of ZNF667

and ZNF667-AS1 enhance EMT of laryngeal carcinoma.⁵² Meanwhile, it was reported that MI alleviated high fructose-caused liver fibrosis and EMT by enhancing miR-375-3p to repress the TGF- β 1/Smad signaling and JAK2/STAT3 signaling in rat.²⁵ Our data demonstrated that MI attenuated EMT of laryngeal cancer via modulating the Twist/NF- κ B signaling. It provides valuable information that MI exerts an inhibitory function of EMT in cancer progression.

As a critical EMT transcription factor, Twist regulates EMT program during cancer development.⁵³ The correlation of Twist with NF- κ B signaling in the modulation of

EMT in cancer progression is well identified. It has been reported that presentation to TGF β combined with TNF α provokes tumorigenesis by modulating NF- κ B/Twist signaling in vitro.⁵⁴ *Antrodia salmonea* represses aggression and metastasis through modifying EMT via the NF- κ B/Twist signaling in triple-negative breast cancer cells.⁵⁵ Twist is involved in the mechanism that ursolic acid restrains EMT of gastric cancer through the Axl/NF- κ B signaling.⁵⁶ NF- κ B activation by the RANKL/RANK pathway enhances the expression of snail and twist and promotes EMT of mammary cancer cells.⁵⁷ Twist plays a crucial role in regulating the NF- κ B and HIF-1 α signaling on hypoxia-induced chemoresistance and EMT in pancreatic cancer.⁵⁸ MiR-153 depletion down-regulates the expression of metastasis-associated family member 3 and Twist family BHLH transcription factor 1 in laryngeal squamous carcinoma cells.⁵⁹ The expression of TWIST is remarkably down-regulated in response to paclitaxel, and TWIST may present a crucial function in paclitaxel-related laryngeal cancer cell apoptosis.⁶⁰ TWIST is involved in the mechanism that TrkB elevates the metastasis of laryngeal cancer by activating the PI3K/AKT signaling.⁶¹ In the present study, we revealed that Twist was significantly elevated in laryngeal cancer tissues and human laryngeal carcinoma HEP-2 cells. It suggests that Twist may play a critical role in the development of laryngeal cancer. NF- κ B/Twist signaling was involved in the mechanism of MI inhibiting EMT of laryngeal cancer. It provides new evidence of the role of Twist in cancer progression.

Conclusion

In conclusion, we discovered that magnesium isoglycyrrhizinate attenuated the progression of laryngeal cancer in vitro and in vivo. Magnesium isoglycyrrhizinate induced an inhibitory effect on epithelial–mesenchymal transition in laryngeal cancer by modulating the NF- κ B/Twist signaling. Our findings provide new insights into the mechanism by which magnesium isoglycyrrhizinate inhibits laryngeal carcinoma development. Magnesium isoglycyrrhizinate may be applied as a potential anti-tumor candidate for laryngeal cancer in clinical treatment strategy.

Abbreviations

EMT, epithelial–mesenchymal transition; MI, Magnesium isoglycyrrhizinate; GA, Glycyrrhizic acid; LEC-P, primary laryngeal epithelial cells; OS, overall survival; IC50, half-maximal inhibitory concentration.

Ethics Approval and Consent to Participate

The patients and healthy controls provided written informed consent, and that the Ethics Committee of The Second Affiliated Hospital, Harbin Medical University approved this study. All animal experiments in this study were approved according to the standards of Care and Use enacted by Laboratory Animals of The Second Affiliated Hospital, Harbin Medical University, and was approved by the Animal Ethics Committee of The Second Affiliated Hospital, Harbin Medical University.

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Disclosure

All the authors have declared no potential conflicts of interest for this research.

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