HMGB1 promotes the proliferation and invasion of oral squamous cell carcinoma via activating epithelial-mesenchymal transformation

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Abstract: This study aimed to investigate the role of high mobility group box 1 (HMGB1) expression in oral squamous cell carcinoma; HMGB1 promoted the proliferation and invasion of oral squamous cell carcinoma via activating epithelial-mesenchymal transformation (EMT). In this study, RNA transfection was used to silence the expression of HMGB1 in oral squamous cell carcinoma cells. CCK-8, cell clone formation and trans-well assays were used to detect the proliferation and invasion of cells before and after HMGB1 silencing. qRT-PCR and Western blot were used to detect changes in EMT marker protein expression before and after transfection. HMGB1 was significantly higher in OSCC tissues than in adjacent tissues, and of the cell lines examined, HMGB1 was highest in SCC-9 cells. Additionally, HMGB1 silencing decreased SCC-9 cell proliferation and viability. Down-regulation of HMGB1 expression inhibited not only the proliferation but also the invasion of SCC-9 cells. The expression of N-cadherin, Snail, and Slug, but not E-cadherin, were promoted after silencing HMGB1. The expression of HMGB1 in OSCC tissue and cell lines was higher, and HMGB1 silencing decreased SCC-9 cell proliferation and invasion, suggesting that HMGB1 has positive effects on OSCC development. Down-regulation of HMGB1 expression regulates EMT markers, suggesting that HMGB1 promotes OSCC cell proliferation and invasion is likely to be associated with EMT activation.

Introduction

Oral cancer is the general term for a series of oral malignancies, approximately 90% of which are oral squamous cell carcinoma (OSCC) (Andersson and Harris, 2010). According to reports, the number of OSCC cases continues to increase, with an annual increase of approximately 275,000 cases (Anggayasti et al., 2017). Studies have shown that smoking and drinking are the two most common and important causes of the high incidence of oral cancer (Balagurunathan et al., 2018). During clinical diagnosis and treatment, the symptoms of early OSCC manifest as severe inflammatory reactions, such as oral ulcers, damage, lumps, or ulcers (Chen et al., 2013). Although the methods for treating OSCC are constantly improving, the 5-year survival rate of OSCC patients remains at approximately 66% (Cologna et al., 2018).

High mobility group box 1 (HMGB1) is a highly conserved nuclear protein widely distributed in mammalian cells; HMGB1 is abundantly expressed in cells, especially in tumor cells, and promotes inflammation (Doll et al., 2015). As a chromatin binding factor, HMGB1 regulates the transcription of many genes by binding to DNA, and it plays an important role in regulating the expression of multiple genes in the nucleus. HMGB1 binds to specific sites in DNA to regulate the transcription of many tumor-associated genes and promotes the expression of DNA-binding proteins, thus playing a key role in tumor cells (Frank et al., 2016). In addition to regulating nuclear function, HMGB1 can participate in the tumor microenvironment in a variety of ways. Its most important role is to bind to the Toll-like receptor on the surface of tumor cells, thereby playing an important role in tumor cell migration and differentiation and focal metastasis (Gonaus et al., 2017). HMGB1 is up-regulated in various cancers, such as prostate cancer, bladder cancer, liver cancer, stomach cancer, and lung cancer (He et al., 2018). In addition, up-regulation of HMGB1 is associated with all the features of cancer, including unlimited replication potential, apoptosis avoidance, and tissue invasion. HMGB1 may be a new potential target for the treatment of cancer (Huang et al., 2015).

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'Epithelial-mesenchymal transformation (EMT)' refers to the process through which cells that have no ability to invade and migrate under specific physiological and pathological conditions acquire the ability to invade and migrate (Jiang and Dong, 2017). After EMT, the expression level of the marker protein E-cadherin is decreased (Ladiz et al., 2017). E-cadherin acts as a direct adhesion molecule between cells. A lack of E-cadherin causes epithelial cells to separate from primary tumors and gain motility, promoting EMT (Naqvi et al., 2017). The E-cadherin gene CDH1 is thought to inhibit tumor cell invasiveness. In contrast to the decrease in E-cadherin expression, intracellular neuro-cadherin (N-cadherin), Snail, and Slug expression levels are increased with EMT (Nishibori, 2018).

HMGB1 acts as a cytokine or inflammatory mediator, and an increasing amount of data indicate that HMGB1 is associated with inflammatory diseases, cancer and autoimmune diseases. However, little is known about the nucleic acid or cytoplasmic functions of HMGB1, especially in cancer cells (Parveen et al., 2017). In this study, we aimed to investigate whether HMGB1 is involved in EMT and to reveal the intrinsic mechanisms that influence the progression of OSCC. Therefore, this study focused on the biological characteristics of cancer cells after HMGB1 silencing and the effect on EMT marker protein expression. The results showed that HMGB1 silencing inhibited OSCC invasion and cell proliferation, suggesting that HMGB1 may promote the malignant biological behavior of OSCC by activating EMT.

Materials and Methods

Tissue samples
From June 2016 to December 2018, cancer tissues and adjacent tissues from OSCC patients (N = 23) treated at the Third Affiliated Hospital of Soochow University were collected. The samples were frozen in liquid nitrogen immediately after collection. All patients and their families were informed of the research methods and purposes of this experiment and signed informed consent. This study was reviewed and approved by the hospital ethics committee.

Cell culture
Human OSCC cell lines, including CAL-27, TCA-8113, SCC-4, SCC-9, and SCC-15 (ATCC, MA), were grown in Dulbecco's modified Eagle's Minimal Essential Medium (DMEM-high glucose; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin solution, in a humidified atmosphere of 5% CO2 in air.

Optimization of transfection
The study was performed in 3 different 6-well plates for SCC-9 cells. Each plate was devoted to 3 study groups (in triplicate): LncRNA HMGB1 si-RNA (si-RNA), negative control (si-NC), and miR-HMGB1-mimics (miR-mimics). Cells were plated in 6-well plates at 2 x 10^4 cells/well and cultured overnight to 50-60% confluence. The cells were subsequently transfected with si-NC, si-RNA, and miR-mimics by using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The effects were examined using PCR analysis after transfection for 48 h. Cells were harvested after 48 h of transfection for the subsequent experiment.

Cell apoptosis assay
Cell apoptosis analysis was performed using Annexin V-FITC/PI Apoptosis Detection Kit (CWBio, Beijing, China). Briefly, transfected HMGB1 siRNA, miR-mimic, and si-NC SCC-9 cells were suspended by binding buffer at the density of 1 x 10^6 cells/mL. 5 μL Annexin V/FITC and 10 μL 20 μg/mL PI were added into 100-μL cell suspension and incubated at room temperature for 15 min in the dark. Stained cells were analyzed with a BD FACSCanto™ II flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Quantitative real-time PCR (qRT-PCR)
A total of 1 mL of Trizol reagent was added to the cells to extract total RNA. RNA was reverse transcribed into cDNA in strict accordance with the instructions of the PrimeScript™ RT Kit (TaKaRa, Dalian, China). qRT-PCR assays were carried out using a CFX96-Real-Time System. The PCR reaction procedure was as follows: 40 cycles at 95°C for 5 s, denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 15 s. Three duplicate wells were used for each sample. The relative mRNA expression levels were calculated using the 2^-ΔΔCt method and GAPDH as a reference standard.

Cell proliferation assay (CCK-8)
Logarithmic growth phase cells were seeded into a 12-well plate at a cell density of 5 x 10^4 cells/mL. After successful transfection for 48 h, the cells were incubated for 1-5 days, followed by the addition of 20 μL of CCK-8 reagent per well. A Synergy H4 Hybrid microplate reader (Bio Tek, Winooski, VT) was used to detect readings at 450 nm.

Colony formation assay
After cells were successfully transfected, they were plated at 200 cells/well in a 6-well plate and cultured for 15 days. The cells were fixed in 10% paraformaldehyde for 10 min and stained with a 0.1% crystal violet solution for 15 min. Finally, the number of clones was observed under a microscope.

Cell invasion assay
After transfection, the lower trans-well chamber was filled with 600 μL of DMEM with 10% FBS, and 300 μL of the serum-free cell mixture was added to the upper trans-well chamber. The cells were incubated for 48 h at 37°C under 5% CO2, and then fixed in 75% ethanol and counted after crystal violet staining. We used a Varioscan Flash Microplate Reader (Thermo Scientific, Waltham, MA, USA) to collect the data at a wavelength of 450 nm after 48 h of culture.

Western blotting
Cells were washed three times with cold PBS. Then, the cells were lysed on ice for 30 min and centrifuged at 3500 rpm for 3 h at 4°C; the supernatant was used as the total protein extract. The protein concentration was measured using a bicinchoninic acid (BCA) kit (Sigma). The sample was then mixed with loading buffer, boiled at 100°C for 5 min, and...
run on a prepared SDS gel for separation. After separation, the proteins were transferred to a PVDF membrane, which was incubated with primary antibody at 4°C overnight. The antibody-skimmed milk solution was washed away for 1 h the next day, and labeled secondary antibody was then incubated for 1 h. The secondary antibody was then washed away, and detection was performed with electrochemiluminescence (ECL) reagents. Finally, the blots were quantified by densitometry using a computerized image analysis program (Amercontrol Biosciences, USA).

Statistical analysis
The data were analyzed using GraphPad Prism 6 software. The data from two groups were statistically compared using an unpaired Student’s t-test. One-way ANOVA with Bonferroni’s correction was used for multiple comparisons. The in vitro experiments were repeated at least three times, and the data are presented as the mean ± standard deviation (SD) from at least three independent experiments. p values ≤ 0.05 were considered significant for all analyses.

Results

Expression of HMBG1 mRNA in OSCC tissues and cells
To investigate the role of HMBG1 in OSCC, we first measured the expression of HMBG1 in tumor tissues and matched adjacent non-tumor tissues from patients with OSCC who underwent surgery in The Sixth Affiliated Hospital of Sun Yat-Sen University. We used qRT-PCR to detect differences in the mRNA expression. The qRT-PCR results showed that the expression of HMG1 was significantly higher in OSCC tissues than in adjacent tissues (p < 0.01, Fig. 1(A)). The expression level of HMG1 was higher in the five OSCC cell lines than in HIOEC cells, and the expression level was highest in SCC-9 cells (p < 0.01, Fig. 1(B)). Thus, SCC-9 cells were selected for further experiments.

FIGURE 1. The expression level of HMBG1 was increased in OSCC tissues and cells. (A) The expression level of HMBG1 was significantly higher in the 23 pairs of cancerous tissue than in adjacent tongue epithelial tissue as detected by real-time PCR. (B) HMBG1 mRNA expression levels in CAL-27, TCA-811, SCC-4, SCC-9, and SCC-15 osteosarcoma cells. The highest expression level of HMBG1 was found in SCC-9. The data are expressed as the mean ± SEM. N = 3. * p < 0.05, ** p < 0.01, *** p < 0.001.
FIGURE 2. HMGB1 knockout suppresses the proliferation and invasion of SCC-9 cells. (A) SCC-9 cells were transfected with HMGB1 siRNA, miR-mimic and negative siRNA control. The expression of HMGB1 in SCC-9 cells was confirmed by qRT-PCR. (B, C) Cell proliferation was determined by the CCK-8 and colony formation assays (10 × 40 μm). The results indicated that upregulation of HMGB1 promoted significantly, while downregulation of HMGB1 suppressed cell proliferation. (D) Cell apoptosis was measured by flow cytometry. Overexpression of HMGB1 suppressed the apoptosis, while knockdown of HMGB1 promoted the apoptosis in SCC-9 cells, compared with NC group. (E) Typical representative images and quantitative data showing the invasion of SCC-9 cells after HMGB1 siRNA, miR-mimic and transfection. The data are expressed as the mean ± SEM. N = 3. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).
FIGURE 3. HMGB1 directly targeted EMT marker proteins in SCC-9 cells. (A) Expression of HMGB1 in the nucleus and cytoplasm of SCC-9 cells. (B) The expression levels of EMT marker proteins were examined by qRT-PCR after siRNA transfection. (C) The expression levels of EMT marker proteins were examined by Western blot after siRNA transfection. The data are expressed as the mean ± SEM. N = 3. * p < 0.05, ** p < 0.01, *** p < 0.001.
EMT marker protein expression decreases with HMGB1 downregulation

Whether HMGB1 regulates the production of EMT by entering the nucleus in SCC-9 cells, we first extracted the cytoplasm and nuclear protein from SCC9 cells for Western blot. The results showed that the expression of HMGB1 in the nucleus of SCC-9 was significantly higher than that in the cytoplasm \( (p < 0.01, \text{Fig. 3(A)}) \). This shows that HMGB1 mainly regulates the occurrence of OSCC EMT by means of nuclear access. As EMT markers, E-cadherin is suppressed, but N-cadherin, Snail, and Slug are increased. To further explore the direct effect of HMGB1 on EMT marker expression, qRT-PCR and Western blotting were conducted. The results showed that silencing HMGB1 significantly promoted the expression of E-cadherin, but not N-cadherin, Snail, and Slug \( (p < 0.01, \text{Figs. 3(B)-3(C)}) \). Therefore, we speculate that HMGB1 likely promotes OSCC cell proliferation and invasion through EMT activation.

Discussion

OSCC is considered to have a high degree of malignancy, and the incidence in developing countries is increasing each year. Although the treatment of OSCC has improved, new targeted therapies for OSCC are still needed due to its low survival rate \( (\text{Anggayasti et al., 2017}) \). In this study, we determined the effect of HMGB1 expression on EMT in OSCC. We concluded that HMGB1 promotes cell proliferation and OSCC invasion by targeting and reducing EMT and reduces its marker expression.

HMGB1 is a highly conserved nuclear protein in cells and is generally considered to be a chromatin binding factor that specifically binds to DNA to promote the transcription of tumor-associated proteins \( (\text{Su et al., 2014}) \). HMGB1 plays a positive role in a variety of malignant tumor biological behaviors \( (\text{Cologna et al., 2018}) \). OSCC has a RAGE receptor, and HMGB1 binds to RAGE and TLR4, thereby promoting autophagy and invasion of OSCC cells and inhibiting apoptosis \( (\text{Doll et al., 2015}) \). It has been suggested that HMGB1 is abnormally highly expressed in various tumor tissues \( (\text{Gonaus et al., 2017}) \).

In addition, HMGB1 is associated with a variety of malignant biological behaviors, such as cell proliferation, tumor tissue angiogenesis, cell invasion and metastasis, and tumor inflammatory microenvironment formation \( (\text{Naqvi et al., 2017}) \). There is little research on the function of HMGB1 in the nucleus and the cytoplasm, especially in OSCC \( (\text{Wang et al., 2019}) \). Therefore, this study focused on changes in biological behaviors, such as cell proliferation and invasion, in the OSCC cell line SCC-9 after silencing HMGB1. First, we successfully constructed specific HMGB1 si-RNA and si-NC and transfected them into SCC-9 cells. The PCR results showed that the expression of HMGB1 was effectively silenced \( (\text{Fig. 2}) \). After HMGB1 was effectively silenced, we evaluated the biological behavior of SCC-9 cells in a variety of ways. The results showed that HMGB1 silencing significantly inhibited the proliferation and invasion of SCC-9 cells. In addition, we found that as an EMT marker protein, E-cadherin decreased with the silencing of HMGB1, but the expression of N-cadherin, Snail, and Slug increased.

In conclusion, our study indicates that HMGB1 plays a very important positive role in OSCC cell proliferation, invasion, and metastasis. Silencing HMGB1 by si-RNA interference technology can effectively inhibit malignant biological behaviors such as OSCC cell proliferation and invasion, suggesting that HMGB1 may be a potential therapeutic target for OSCC.

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Conflicts of Interest

There are no conflicts of interest for the authors.

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