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MiR-150 suppressed cell viability, invasion and EMT *via* HMGA2 in oral squamous cell carcinoma

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Abstract. – OBJECTIVE: Oral squamous cell carcinoma (OSCC) accounts for 90% of head and neck cancers, and its 5-year overall survival is very poor. MiR-150 is usually downregulated and acts as tumor suppressor in multiple cancers. The aim of our study is to explore the functions of miR-150 in OSCC.

PATIENTS AND METHODS: Expressions of miR-150 and HMGA2 mRNA in OSCC tissues and cells were analyzed by qRT-PCR. Methyl Thiazolyl Tetrazolium (MTT) and transwell assays were conducted to assess the cell viability and invasive abilities. Western blot was conducted to assess the protein levels of epithelial-mesenchymal transition (EMT) markers. Luciferase reporter assay was carried out to verify miR-150 directly binding to HMGA2 in SCC25 cells.

RESULTS: MiR-150 was low expressed and HMGA2 was highly expressed in OSCC tissues and cells. Downregulation of miR-150 or upregulation of HMGA2 predicted poor prognosis of OSCC patients. MiR-150 overexpression inhibited the abilities of viability, invasive and the EMT by targeting HMGA2 in OSCC cells. HMGA2 was a target gene of miR-150 and its expression was regulated by altering the expression of miR-150 in OSCC cells. HMGA2 reversed partial roles of miR-150 on cell viability and invasion in OSCC.

CONCLUSIONS: MiR-150 impaired cell viability, invasion and EMT *via* binding to HMGA2 of OSCC. Our research demonstrates that miR-150 plays a critical role in the progression of OSCC. miR-150 might be a candidate molecular marker and a novel therapy target for OSCC patients.

Key Words:
MiR-150, HMGA2, OSCC, Prognosis, EMT.

Introduction

Oral squamous cell carcinoma (OSCC) is a frequent oral malignancy, accounting for 90% of head and neck cancers[1,2]. Despite advances in surgical techniques and adjuvant therapies, local recurrences and metastases still occur, with a 5-year overall survival rate that still remains low[3,4]. Due to the lack of suitable markers, a large amount of patients with OSCC were at advanced stage at the first diagnosis[5,6]. Thus, it is necessary to investigate the biomarkers for early diagnosis of OSCC.

MicroRNAs (miRNAs) regulate genes expression through targeting their 3'-untranslated region (UTR) sequences at post-transcriptional level[7]. Recently, increasing evidence elucidated that miRNAs participated in cancer pathogenesis, such as cell viability, differentiation and apoptosis[8]. MiR-150 was low expressed in multiple tumors, including leukemia, ovarian cancer and leiomyoma[9-11]. MiR-150, acted as tumor suppressor, inhibited viability, induced cell cycle arrest and cell apoptosis in colon adenocarcinoma[12]. Moreover, miR-150 impaired cell growth and malignant behavior in papillary thyroid carcinoma[13]. However, miR-150 suppressed cell viability and metastasis in hepatocellular carcinoma[14]. However, miR-150 enhanced cell proliferation and EMT through SRCIN1 in cervical carcinoma[15]. Thus, it is urgent to investigate the function of miR-150 on in OSCC.

The high mobility group AT-hook 2 (HMGA2) acts as a transcriptional regulating factor which contains structural DNA-binding domains[16]. HMGA2, acted as a biomarker for cisplatin resistance in bladder cancer[17]. HMGA2 improved long-term engraftment and myeloerythroid differentiation in human hematopoietic stem and progenitor cells[18]. HMGA2 promoted cancer malignancy by suppressing the proliferation and inducing apoptosis in breast

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cancer[19]. In addition, HMGA2 impaired EMT and lymph node metastasis *via* ATR/Chk1 signaling pathway in cervical cancer[20]. However, the correlation of HMGA2 and miRNAs in OSCC are still unclear.

In this study, the effects of miR-150 on the viability, invasion and EMT of OSCC cells were investigated with the miR-150/HMGA2 axis as the entry point, in order to provide some experimental basis for the clarification of the molecular mechanism of tumor recurrence and metastasis and the selection of therapeutic targets.

Patients and Methods

Patients and Tissues

51 patients who underwent OSCC was included in this study from at Yantaishan Hospital. We placed all the fresh tissues in liquid nitrogen and stored at -80°C. The inclusion criteria for patients were complete medical records and follow-up data, postoperative pathological diagnosis of CC, no preoperative chemotherapy, radiotherapy, endocrine therapy, and other anti-tumor treatments, and no hormones have been used recently. The exclusion criteria for patients were patients with other malignant tumors, systemic infectious diseases, severe liver and kidney dysfunction, and mental illnesses who cannot cooperate with treatment. Written informed consent was obtained from all patients. This present research was approved by the Ethics Committee of Yantaishan Hospital.

Cell Culture

OSCC cell lines (SCC25 and HSC3) and normal keratinocytes cell line RT7 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). SCC25 and HSC3 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640), while RT7 cells were cultivated in K-SFM (Life 75 Technologies, Carlsbad, CA, USA). All the mediums were contained 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) at 37 °C with 5% CO_2.

Western Blot

Total proteins were separated by RIPA lysis buffer contained PMSF on ice, following by centrifuged at 4°C with 12,000 × g for 20 min. Bicinchoninic acid (BCA) protein assay kit (Bio-Rad, Shanghai, China) was utilized to assess the protein concentration. Proteins were separated sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche Diagnostics, Basel, Switzerland). The membrane was blocked with 5% skim milk powder and then incubated with the primary antibodies overnight at 4°C. The primary antibodies were HMGA2, E-cadherin, N-cadherin and GAPDH. Next, the secondary antibody was employed to incubate the membranes for 1 h. The bands were measured using ECL Kit (KeyGEN BioTECH, China) on a Tanon-5200 Chemiluminescent Imaging System (Millipore, Bedford, MA, USA).

RNA Isolation and RT-qPCR

TRIzol was applied to extract RNAs and followed miScript Reverse Transcription kit and PrimeScript reagent kit with cDNA Eraser was conducted to perform reverse transcription. Next, qRT-PCR assay was carried out using SYBR Premix Ex Taq kit (TaKaRa, Otsu, Shiga, Japan) on an ABI PRISM 7900 System. The primer sequences used for qRT-PCR are shown in Table I. GAPDH or U6 was used as the normalization of miRNA or mRNA using $2^{-\Delta\Delta Cq}$ method[21].

Table I. Primer sequences for RT-qPCR.

Gene		Primer sequences
miR-150	Forward	5'-TCTCCCAACCCTTGTACC-3'
	Reverse	5'-GAATACCTCGGACCCTGC-3'
U6	Forward	5'-AACGCTTCACGAATTTGCGT-3'
	Reverse	5'-CGCTTCACGAATTTGCGTGTCAT-3'
HMGA2	Forward	5'-TGGGAGGAGCGAAATCTAAA-3'
	Reverse	5'-TCCCTGGAGAAGAGCTACG-3'
GAPDH	Forward	5'-AGAAGGCTGGGGCTCATTTG-3'
	Reverse	5'-AGGGGCCATCCACAGTCTTC-3'

Cell Proliferation Assay

Methyl Thiazolyl Tetrazolium (MTT) assay was used to measure cell proliferative ability. SCC25 cells were seeded into 96-well plates and after cultured 24, 48, 72 and 96 h, added 10 μl MTT (Santa Cruz Biotechnology, Santa Cruz, CA, USA) into each well. 150 μl DMSO dimethylsulfoxide (DMSO) (Amresco, Solon, OH, USA) was added into each well, and then absorbance of cell proliferation was measured at 490 nm.

Invasion Assay

The cell invasive ability was detected by transwell (Millipore, Billerica, MA, USA) with Matrigel (BD Biosciences, San Jose, CA, USA) pre-covered. In brief, SCC25 cells suspended in normal medium without FBS were plated in the top well. Meanwhile, we filled the bottom well with DMEM medium contained with 20% FBS, which functioned as chemoattractant. After incubated 24 h, we removed the uninvaded cells using cotton swabs. For the invaded cells, we fixed them using methanol and stained using crystal violet solution. Finally, we counted the invaded cells under microscope (Olympus, Tokyo, Japan).

Cell Transfection

MiR-150 mimic, inhibitor and HMGA2 overexpressing (pcDNA3.1-HMGA2) vectors were purchased from GenePharma. SCC25 cells were placed in 6-well plate, and the transfections of special vectors were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Luciferase Reporter Assay

HMGA2 was predicted as a target gene of miR-150 by TargetScan. To verify miR-150 binding to HMGA2 mRNA 3'-UTR, the putative sequences of miR-150 on HMGA2 mRNA 3'-UTR were mutated from UGGGAG to ACCCUC. The wild type or the mutated sequences of HMGA2 mRNA were cloned into pmiRGLO vector, designated as pmiRGLO-HMGA2-WT (WT) and pmiRGLO-HMGA2-MUT (MUT) respectively. The SCC25 cells were co-transfected miR-150 mimic and WT or MUT using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Dual-Luciferase reporter assay system was applied to calculate the Firefly Luciferase activity, with Renilla Luciferase activity acted as normalization.

Statistical Analysis

All the data were analyzed using SPSS software 19.0 (Armonk, NY, USA) and GraphPad Prism 7.0 (San Diego, CA, USA). The comparison between two or more groups was carried out using Student's *t*-test or one-way ANOVA. The survival data were plotted and analyzed by Kaplan-Meier, log-rank test. Statistically significant was recorded as $p< 0.05$.

Results

MiR-150 Predicts Worse Prognosis of OSCC Patients

RT-qPCR was applied to measure the expression of miR-150 in 51 pairs of OSCC and non-tumor tissues, and we discovered that miR-150 was low expressed in OSCC tissues versus matched non-tumor tissues ($p< 0.05$) (Figure 1A). Moreover, Kaplan-Meier method was used to assess the association between the expression of miR-150 and 5-year overall survival of all the 51 patients. Not unfortunately, low expression of miR-150 predicted poor 5-year overall survival of OSCC patients ($p< 0.05$) (Figure 1B).

Meanwhile, RT-qPCR also elucidated the expression of miR-150 in OSCC and normal cells, and miR-150 was found to be downregulated in OSCC cell lines SCC25 and HSC3 versus normal cell line RT7 ($p< 0.05$) (Figure 1C). To explore the mechanism of miR-150 in OSCC, the expression of miR-150 was enhanced by miR-150 mimic ($p< 0.05$), while it was decreased by miR-150 inhibitor in SCC25 cells ($p< 0.05$) (Figure 1D).

MiR-150 Inhibits Cell Viability, Invasion and EMT of OSCC

MTT assay was used to measure cell viability. Cell viability was calculated to be decreased by transfecting miR-150 mimic, whereas it was elevated by transfecting miR-150 inhibitor in SCC25 cells ($p< 0.05$) (Figure 2A). Meanwhile, transwell assay elucidated that the miR-150 mimic reduced invasive ability ($p< 0.05$), while miR-150 inhibitor could improve the invasive ability of SCC25 cells ($p< 0.05$) (Figure 2B). What's more, the EMT markers, such as E-cadherin, N-cadherin, were evaluated by Western blot assay. We discovered that the miR-150 mimic inhibited the expression of E-cadherin, while enhanced the expression of N-cadherin. In contrary, miR-150 inhibitor facilitated the expression of E-cadherin whereas it reduced the expression of N-cadherin (Figure 2C), which validated that miR-150 suppressed the EMT of OSCC cells.

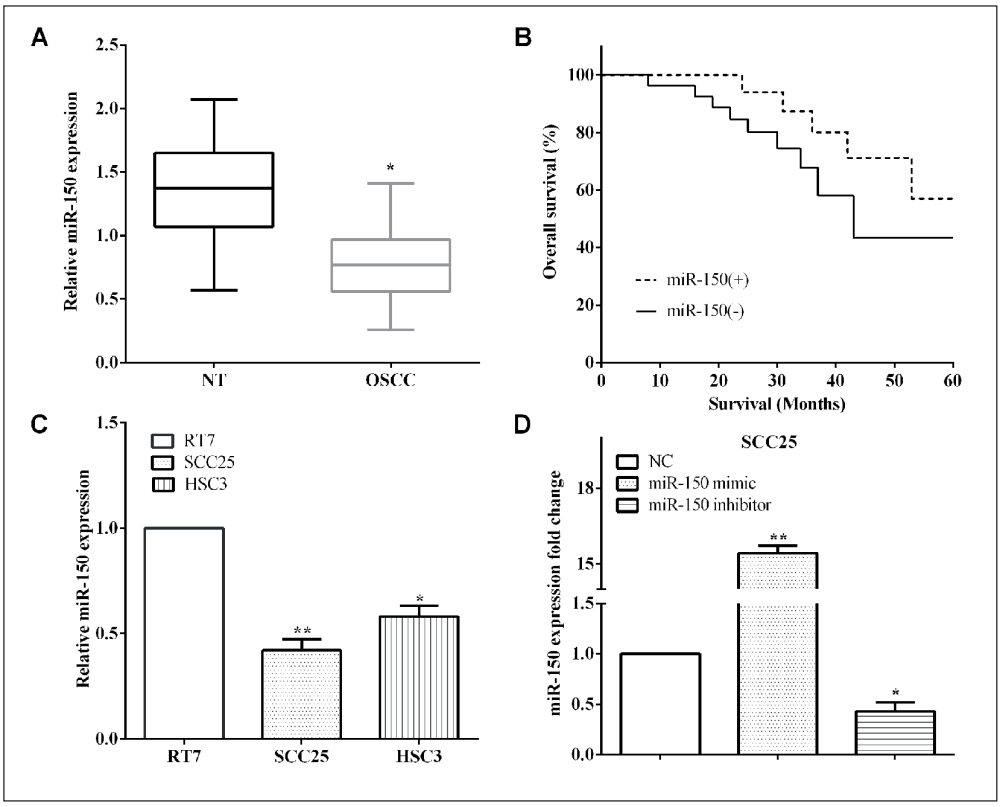

Figure 1. The expression of miR-150 in OSCC. **A**, MiR-150 was low expressed in OSCC tissues than non-cancerous tissues. **B**, Down-regulation of miR-150 predicted poor prognosis of OSCC patients. **C**, MiR-150 was low expressed in OSCC cells versus normal cells. **D**, MiR-150 mimic and miR-150 inhibitor were utilized to up- or down-regulate the expression of miR-150.

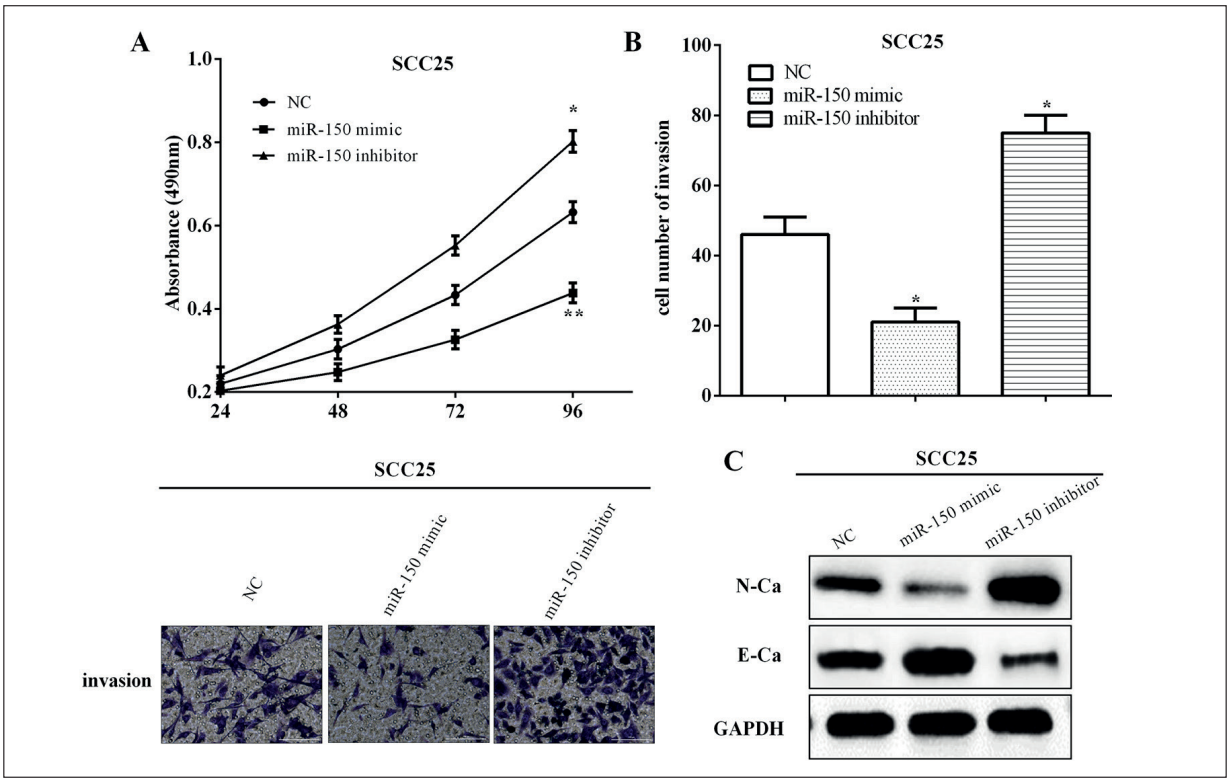

Figure 2. MiR-150 suppressed cell viability, invasion and the EMT. **A**, MiR-150 reduced the proliferative ability in SCC25 cells. **B**, MiR-150 inhibited invasive ability, which was enhanced by miR-150 inhibitor in SCC25 cells. **C**, MiR-150 suppressed the EMT ability in SCC25 cells (magnification 200×).

MiR-150 Targeting HMGA2 in OSCC

The target genes of miR-150 were predicted by TargetScan, and HMGA2 was selected as a candidate. To explore whether miR-150 directly binding to the 3'-UTR of HMGA2 mRNA, the potential binding sequences were mutated from UGGGAG to ACCCUC (Figure 3A). As expected, the luciferase activity of cells that co-transfected with the miR-150 mimic and wild type HMGA2 was reduced ($p< 0.05$). Inversely, the Luciferase activity has no alteration of cells that transfected with mutated HMGA2 mRNA and miR-150 mimic in comparison with co-transfected with control ($p> 0.05$) (Figure 3B). In addition, the expression of HMGA2 was measured in SCC25 cells after transfection of miR-150 mimic or miR-150 inhibitor by RT-qPCR. As expected, miR-150 mimic reduced the expression of HMGA2 ($p< 0.05$), while miR-150 inhibitor facilitated the expression of HMGA2 in SCC25 cells ($p< 0.05$) (Figure 3C).

Overexpression of HMGA2 Predicts Worse overall survival of OSCC patients

The expression of HMGA2 was measured in OSCC tissues and the matched non-tumor tissues by RT-qPCR. Not unfortunately, HMGA2 was found to be high expressed in OSCC tissues versus matched normal tissues ($p< 0.05$) (Figure 4A). Simultaneously, HMGA2 was upregulated in OSCC cells SCC25 ($p< 0.01$) and HSC3 ($p< 0.05$) versus normal cells RT7 (Figure 4B). In addition, Kaplan-Meier method indicated upregulation of HMGA2 was associated with worse 5-year overall survival of OSCC patients ($p< 0.05$) (Figure 4C).

HMGA2 Reverses Partial Roles of miR-150 in SCC25 Cells

To investigate whether HMGA2 participated in miR-150-mediated tumor-suppression in OSCC, we transfected pcDNA3.1-HMGA2 plasmid in miR-150 mimic-transfected SCC25 cells to re-express HMGA2 ($p< 0.05$) (Figure 5A). Moreover, the MTT assay revealed that cell viability was impaired by recovering the expression of HMGA2 in miR-150 mimic-transfected SCC25 cells (Figure 5B). Meanwhile, transwell assay revealed that overexpression of HMGA2 partially reversed the inhibitory effects of miR-150 on cell invasion of SCC25 cells (Figure 5C). In addition, overexpression of HMGA2 enhanced the EMT ability by suppressing E-cadherin expression, while improving N-cadherin expression in SCC25 cells

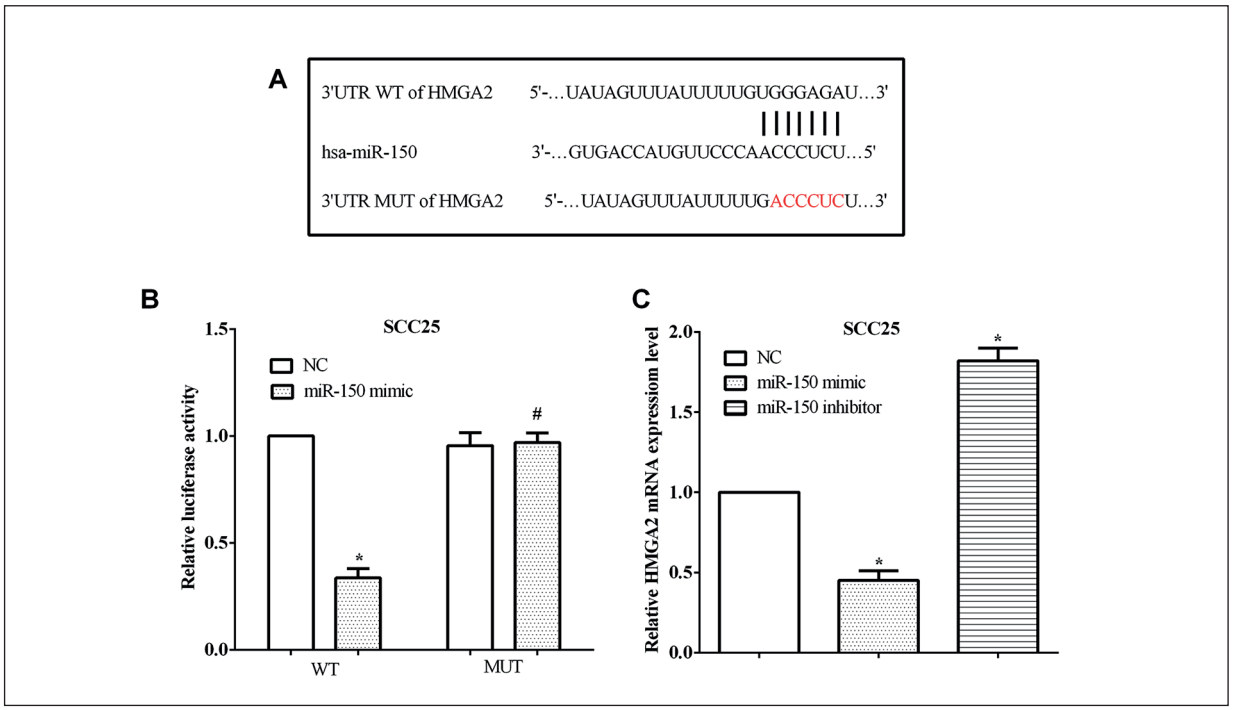

Figure 3. MiR-150 targeted HMGA2 and regulated its expression. **A**, HMGA2 was predicted as a potential target of miR-150. **B**, MiR-150 mimic reduced the Luciferase ability of wild type mRNA 3'-UTR. **C**, MiR-150 mediated the expression of HMGA2 in SCC25 cells.

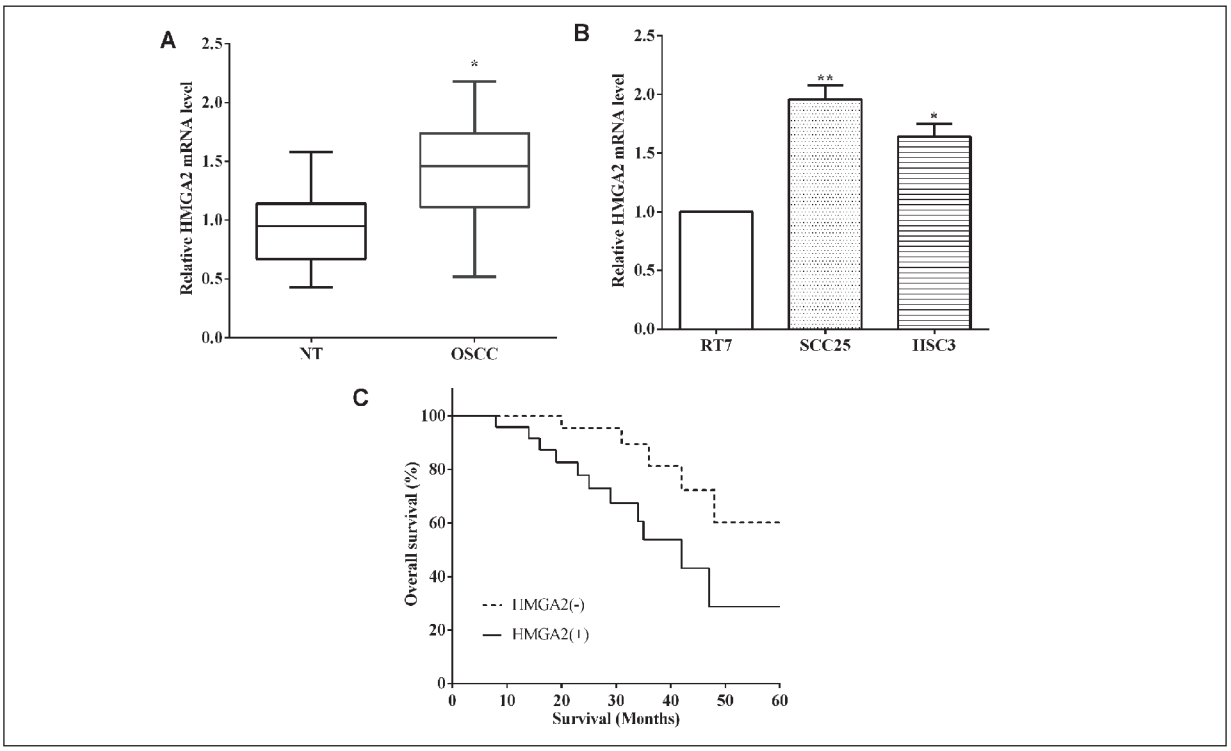

Figure 4. Overexpression of HMGA2 predicted worse outcome in OSCC. **A**, HMGA2 was upregulated in OSCC tissues versus matched non-tumor tissues. **B**, HMGA2 was overexpressed in OSCC cells SCC25 and HSC3 versus normal cell RT7. **C**, Kaplan-Meier curve method elucidated that upregulation of HMGA2 predicted worse prognosis of OSCC patients.

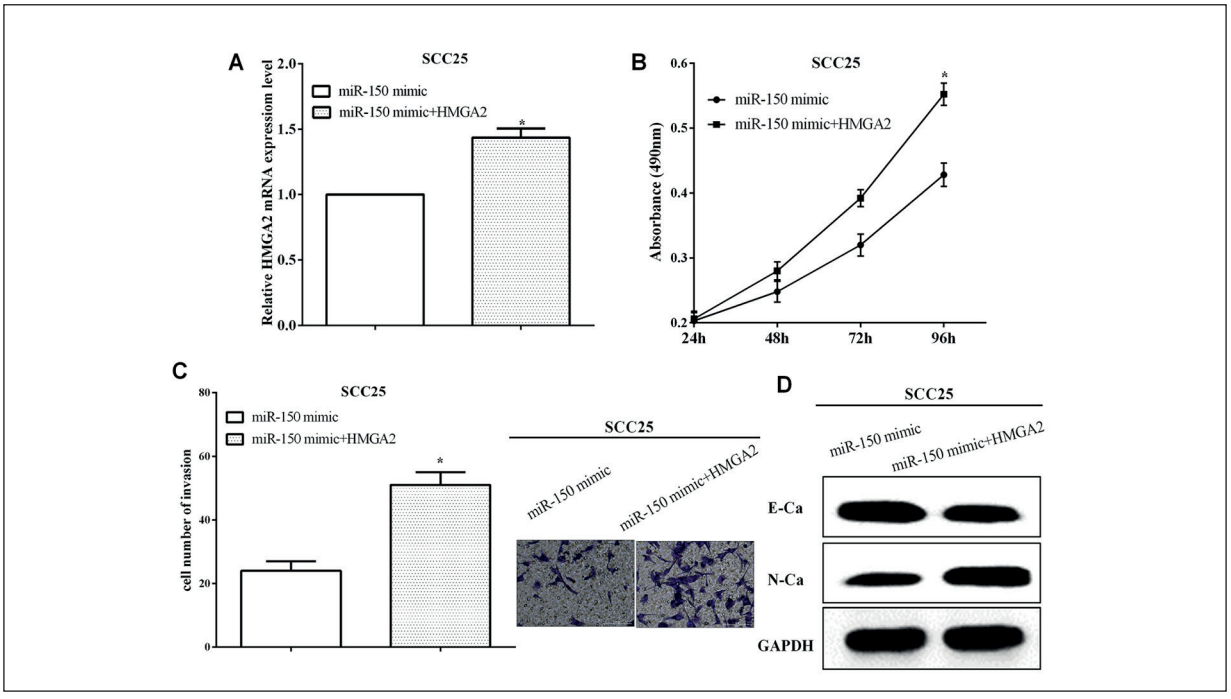

Figure 5. HMGA2 reversed partial tumor-suppressive role of miR-150 in OSCC. **A**, Overexpression of HMGA2 in miR-150 mimic-transfected cells. **B**, HMGA2 reversed partial roles of miR-150 on cell viability. **C**, Cell invasive ability was enhanced by HMGA2 in miR-150 mimic-transfected cells (magnification 200×). **D**, HMGA2 improved the EMT ability in miR-150 mimic-transfected cells.

(Figure 5D). All results elucidated that HMGA2 partially reversed the tumor-suppressive functions of miR-150 in SCC25 cells.

Discussion

A large number of studies have confirmed that miRNA can regulate the occurrence and development of tumors in a variety of cancers, including OSCC. Our findings indicated that miR-150 was low expressed in OSCC tissues versus non-tumor tissues. Also, miR-150 was downregulated in OSCC cells compared to normal cells. The novelty of this study is that we first proposed that miR-150 acted as a survival factor of OSCC patients, which was similar to the findings in prostate cancer[22]. More importantly, Zhang et al. suggested that miR-150 inhibited cell viability and promoted cell apoptosis in Burkitt lymphoma[23]. Similarly, miR-150 impaired cell viability and metastasis in melanoma[24]. It has reported that miR-150 acted as a tumor suppressor to inhibit OSCC progression by suppressing cell migration and aggression [25]. Consistent with this findings, miR-150 overexpression suppressed the viability and invasion in OSCC cells SCC25. Moreover, we first discovered that miR-150 inhibited the EMT ability in OSCC, as similar with the findings in papillary thyroid cancer and prostate cancer[26,27].

HMGA2 was overexpressed in invasive adenomas than in noninvasive adenomas of pituitary neuroendocrine tumors[28]. Upregulation of HMGA2 was connected with worse prognosis of acute myeloid leukemia[29]. HMGA2 enhanced cell metastasis and therapy resistance in pancreatic cancer[30]. Sakata et al[31] have been reported that upregulation of HMGA2 facilitated distant metastasis and poor prognosis by promoting angiogenesis in OSCC. In our findings, the expression of HMGA2 was also higher in OSCC tissues and cells than that of the matched non-tumor tissues and normal cells. At the same time, overexpression of HMGA2 was related to poor overall survival of OSCC patients. This results further confirm the previous research conclusion. What's more, HMGA2 was a target gene of miR-150 by binding to its 3'-UTR of mRNA in OSCC cells, which was consistent with the findings in non-small-cell lung cancer[32]. HMGA2 also promoted cell metastasis and the EMT ability in prostate cancer[33]. In the results of the complement experiment, HMGA2 was involved in the inhibitory effect of miR-150 on OSCC cells. We first explored that HMGA2 partially reversed the functions of miR-150 on cell viability and metastasis in OSCC cells.

Conclusions

In conclusion, downregulation of miR-150 or upregulation of HMGA2 predicted poor prognosis of OSCC patients. MiR-150 can target and regulate the expression of HMGA2, thereby inhibiting the viability, invasive and EMT abilities of OSCC cells. Results first put forward that miR-150 acted as a survival factor of OSCC patients and inhibited the EMT ability in OSCC. We first explored that HMGA2 partially reversed the functions of miR-150 on cell viability and metastasis in OSCC cells. This newly identified miR-150/HMGA2 axis may further confirm the important role of miR-150 in the occurrence and development of OSCC and provide a new idea for the treatment of OSCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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