Oxymatrine suppresses oral squamous cell carcinoma progression by suppressing CXC chemokine receptor 4 in an m^{6}A modification decrease dependent manner

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Abstract. Oxymatrine has been revealed to exert antitumor activity; however, its role in oral squamous cell carcinoma (OSCC) remains unclear. In the present study, the effects and underlying molecular mechanisms of oxymatrine in OSCC were explored. The antineoplastic effects of oxymatrine were measured using Cell Counting Kit-8, apoptosis and Transwell assays. The inhibitory effect of oxymatrine on tumor growth was evaluated *in vivo*. The regulation of oxymatrine on the CXC chemokine receptor 4 (CXCR4) was analyzed using western blotting, reverse transcription-quantitative PCR, RNA stability and methylated RNA immunoprecipitation assays. The present results revealed that oxymatrine inhibited the proliferation and migration of OSCC cells and promoted cell apoptosis. Furthermore, oxymatrine reduced CXCR4 mRNA and protein expression levels by promoting CXCR4 mRNA degradation. Mechanistically, oxymatrine inhibited the methylation at the N6-position of adenosine (m^{6}A modification) of CXCR4 mRNA by decreasing the expression of the methyltransferase-like 3 (METTL3) gene. In addition, oxymatrine inhibited tumor growth *in vivo*. Taken together, our findings demonstrated the antitumor effect of oxymatrine on OSCC. Mechanistically, oxymatrine inhibited the progression of OSCC by downregulating METTL3 and degrading CXCR4 mRNA by decreasing the level of m^{6}A modification.

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Abbreviations: OSCC, oral squamous cell carcinoma; CXCR4, CXC chemokine receptor 4; METTL3, methyltransferase-like protein 3; METTL14, methyltransferase-like protein 14; m^{6}A, N6-methyladenosine; CCK-8, Cell Counting Kit-8; RT-qPCR, reverse transcription-quantitative PCR; MeRIP, methylated RNA immunoprecipitation; BMI1, B-cell-specific moloney murine leukemia virus insertion site 1; Bax, Bcl-2 associated X; Bcl-2, B-cell lymphoma-2; ALKBH5, alkB homolog 5; FTO, fat mass and obesity associated; WTAP, WT1-associated protein

Key words: oxymatrine, OSCC, m^{6}A, CXCR4, METTL3

Introduction

Oral squamous cell carcinoma (OSCC) is a common malignant head and neck tumor with a poor prognosis, accounting for 90% of oral cancers (1,2). Despite the progress in comprehensive treatments, the overall 5-year survival rate of patients with OSCC remains unsatisfactory owing to high recurrence and metastasis rates (3). Therefore, effective treatments for OSCC are urgently needed.

Natural compounds play an important role in cancer therapies. Oxymatrine is an analog of matrine, which is extracted from the roots of the Chinese herb *Sophora flavescens*, also named 'Ku-shen' (4). Oxymatrine is commonly used to treat hepatitis B and C viral infections (5). Its effects on hepatic fibrosis and ischemia-reperfusion injury, as well as its analgesic and cardioprotective effects, are well-defined (6,7). Previously, an increasing number of studies have demonstrated the antitumor activities of oxymatrine, including inhibition of cancer cell proliferation, induction of apoptosis and reversal of multidrug resistance (8-12). Therefore, oxymatrine is used as a novel antitumor agent for the treatment of different types of cancer. However, the antitumor effect of oxymatrine suppressor in OSCC has not been well elucidated.

Dysregulation of the CXC chemokine receptor 4 (CXCR4) is implicated in multiple malignancies. It plays a major role in tumor progression, such as angiogenesis, metastasis and survival of cancer cells (13,14). A previous study reported that CXCR4 downregulation inhibited tumor growth by inducing cell apoptosis and cycle arrest (15). CXCR4 knockdown attenuates tumor metastasis by inhibiting epithelial-mesenchymal

transition (16). Additionally, CXCR4 is remarkably increased in OSCC and is associated with poor prognosis (17,18). Therefore, CXCR4 overexpression is critical for OSCC occurrence and development.

N6-methyladenosine (m^6A), the most abundant modification in eukaryotic mRNA, is involved in RNA splicing, stability, translation and nucleation, and plays a role in tumor progression (19,20). Methyltransferase-like protein 3 (METTL3) is an enzyme that catalyzes m6A and plays a key role in promoting tumor progression in myeloid leukemia (21), breast (22) and colon cancer (23). In addition, METTL3 promotes tumor progression by regulating m^6A methylation of B-cell-specific moloney murine leukemia virus insertion site 1 (BMI1) in OSCC, indicating that METTL3 may be an oncogenic factor in OSCC (24).

The present study aimed to investigate the effect of oxymatrine on OSCC and its underlying mechanisms. It was identified that oxymatrine inhibits CXCR4 expression by reducing its m^6A modification level by downregulating METTL3, thereby inhibiting the proliferation and migration of OSCC.

Materials and methods

Cell culture and transfection. Human normal squamous epithelial hNOK cells (cat. no. CRL-2692) and OSCC cell lines SCC-15 (cat. no. CRL-1623) and CAL-27 (cat. no. CRL-2095) were provided by the American Type Culture Collection (ATCC). Both cell lines were cultured using complete DMEM medium containing 10% fetal bovine serum (FBS; both from Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin solution (Procell Life Science & Technology Co.) at 37˚C with 5% CO_2. METTL3- and CXCR4-expressing plasmids (pcDNA3.1-METTL3 and pcDNA3.1-CXCR4) and negative control were obtained from Shanghai GeneChem Co., Ltd. Plasmids (~4 μg) were used for cell transfections and the transfections were performed using the Lipofectamine® 3000 kit (cat. no. L3000015; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After transfection, cells were cultured at 37˚C with 5% CO_2 and collected for further experiments 1-2 days later.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). TRIzol® reagent (cat. no. 15596018; Thermo Fisher Scientific, Inc.) was used for the extraction of total RNA from OSCC cells. After RNA isolation, NanoDrop1000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) was used to detect the quality and concentration of total RNA at the wavelength of 260/280 nm. Subsequently, Primescript RT Reagent (cat. no. RR047A; TaKaRa Bio, Inc.) was used to reverse-transcribe the total RNA into cDNAs according to the manufacturer's protocol. RT-qPCR reactions were conducted on a LightCycler 480 (Roche Diagnostics) using SYBR qPCR Master Mix (cat. no. Q711-02; Vazyme Biotech Co., Ltd.). For qPCR, the following thermocycling conditions were applied: Initial denaturation at 95˚C for 5 min, subsequently denaturation at 95˚C for 10 sec for 40 cycles, 60˚C for 20 sec of annealing and elongation and final extension at 72˚C for 20 sec. GAPDH was used as an internal reference gene. The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative gene expression (25). The following primers were used in the present study: METTL3 forward, 5'-AACAGAGCAAGAAGGTCGGG-3' and reverse, 5'-TCGGTCTGCACTGGAATCAC-3'; CXCR4 forward, 5'-ACTACACCGAGGAAATGGGCT-3' and reverse, 5'-CCCACAATGCCAGTTAAGAAGA-3'; and GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'.

Cell counting kit 8 (CCK-8). Cell proliferation was evaluated using CCK-8 assays. OSCC cells (~5,000) were seeded in a 96-well plate and cultured for 24-48 h. Afterwards, CCK-8 solution (10 μl; cat. no. CK04; Dojindo Laboratories, Inc.) was added to each well. After incubation for ~1 h at 37˚C, the absorbance of each well was measured at 450 nm by a microplate reader (Infinite M200 PRO; Tecan Group, Ltd.).

Cell migration assay. Transwell assays were performed to detect cell migration using chambers (Sigma-Aldrich; Merck KGaA). Cells were first resuspended in DMEM (serum-free) and then ~$2x10^4$ cells were seeded into the upper chamber. Then, 500 μl DMEM (containing 10% FBS) was added to the lower chamber. After 24 h, migratory cells on the lower surface were fixed with 4% paraformaldehyde (20 min) at room temperature. Cells were stained with 0.1% crystal violet (Beyotime Institute of Biotechnology) for 30 min at room temperature. After washing with phosphate-buffered saline (PBS) thrice, images of the cells that migrated through the membrane were captured using a light microscope (magnification, x20; Nikon Corporation).

Flow cytometry. The Annexin V-FITC Apoptosis Detection kit (cat. no. 556547; BD Biosciences) was used for early and late apoptosis detection. Briefly, $5x10^5$ OSCC cells were treated with oxymatrine for ~24 h, harvested by trypsinization and centrifuged (300 x g at room temperature) for 5 min. Cells were then washed with ice-cold PBS, resuspended in 100 μl Annexin V-FITC binding buffer, and mixed with Annexin V-FITC (5 μl) and propidium iodide (5 μl). After incubation at room temperature in the dark (15 min), 300 μl of Annexin V-FITC binding buffer was added. Flow cytometry (BD FACSAria III Flow Cytometer; BD Biosciences) was performed and the results were analyzed using CellQuest™ software (version 3.3; Becton Dickinson and Company).

Western blot analysis. To isolate total cell proteins, OSCC cells were lysed using RIPA buffer (Beyotime Institute of Biotechnology). A BCA protein assay kit (cat. no. P0012S; Beyotime Institute of Biotechnology) was used to assess the concentrations of the isolated proteins. Equal amounts of protein (60 μg) were separated using 10% SDS-PAGE. Subsequently, the separated proteins were transferred onto PVDF membranes. Membranes were blocked with 5% fat-free milk for ~1 h at room temperature and then incubated with primary antibodies overnight at 4˚C. TBST (with 0.1% Tween) was used to wash the membranes thrice. The membranes were then incubated with the corresponding secondary antibodies, HRP-labeled goat anti-mouse (1:2,000; cat. no. A0216) and HRP-labeled goat anti-rabbit (1:2,000; cat. no. A0208) diluted in TBST (cat. no. ST671; Beyotime Institute of Biotechnology), at room temperature for 1 h. Finally, the membranes were exposed to enhanced chemiluminescence (ECL; Thermo Fisher Scientific,

Inc.). Primary antibodies against anti-CXCR4 (1:1,000; cat. no. ab181020), Bax (1:1,000; cat. no. ab182734), anti-Bcl-2 (1:1,000; cat. no. ab32124), anti-METTL3 (1:1,000; cat. no. ab195352), anti-alkB homolog 5 (ALKBH5; 1:1,000; cat. no. ab195377), anti-WT1-associated protein (WTAP; 1:1,000; cat. no. ab195380), anti-METTl14, (1:1,000; cat. no. ab220030), anti-fat mass and obesity associated (FTO; 1:2,000; cat. no. ab126605) and β-actin (1:2,500; cat. no. ab8226) were obtained from Abcam. Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.) was used to semi-quantify relative protein expression levels, and β-actin was used as the loading control.

RNA-binding protein immunoprecipitation (RIP) analysis. A Magna RIP kit (cat. no. 17-704; MilliporeSigma) was used for the RIP assay in accordance with the manufacturer's protocol. Briefly, total cell lysate was mixed with RIP buffer. Then 50 μl Protein A + G magnetic beads were cultured with the mixture at 4˚C for ~12 h. The beads were conjugated with anti-METTL3, anti-Ago2 (cat. no. ab186733) or anti-IgG (cat. no. ab172730; both from Abcam), respectively. Finally, RT-qPCR was performed to quantify the immunoprecipitated RNAs using the RIP assay.

Methylated RNA immunoprecipitation (MeRIP). MeRIP was used to detect m^{6}A levels in CXCR4 mRNA. Total RNA was extracted from OSCC cells by using Dynabeads^{TM} mRNA Purification kit (cat. no. 61006; Thermo Fisher Scientific, Inc.). The isolated RNAs were treated with DNase R (Qiagen GmbH). Anti-m^{6}A antibody (5 μg, cat. no. ab208577; Abcam) was used to immunoprecipitate chemically fragmented RNA. After being washed thrice with IP buffer, elution buffer was used to elute RNA from the beads at 4˚C for 1 h. RT-qPCR was used to detect the RNA expression in input and immunoprecipitation (IP) groups.

m^{6}A enrichment assay. The enrichment of m^{6}A in cells and tissues was measured by using EpiQuik™ m^{6}A RNA Methylation Quantification kit (cat. no. P-9005; EpiGentek) according to the manufacturer's protocol. Briefly, total RNA was isolated from cells and tissues firstly. RNA (~200 ng) was added to interact with strip wells by using RNA high binding solution, followed up with incubation at 37˚C for 90 min. Afterwards, the wells were washed with wash buffer for three times. Each well was then added with 50 μl capture antibody and cultured at room temperature for 1 h. Subsequently, 50 μl detection antibody was added into each well for 30 min incubation at room temperature. After being washed with wash buffer, each well was added with 50 μl enhanced solution and incubated for 30 min at room temperature. Then, 100 μl developer solution was added into each well for another incubation for 10 min in the dark at room temperature and the stop solution was subsequently added. Finally, the detected signal was enhanced and then quantified calorimetrically by measuring the absorbance at optical density (OD)=450 nm in a microplate reader (Infinite M200 PRO, Tecan Group, Ltd.). The amount of m^{6}A is proportional to the OD intensity measured.

RNA stability assay. SCC-15 and CAL-27 cells (~5x10^5) were treated with actinomycin D (Act-D, 5 μg/ml; MedChemExpress) for 0, 2, 4, and 6 h. At the indicated times, total RNA was isolated and quantified by qPCR. Linear regression analysis was used to estimate the mRNA degradation rate.

Animal model. A total of 12 BALB/c female nude mice (age, 4-weeks old; weight, ~20 g) were obtained from GemPharmatech. All mice were raised in a standard barrier environment, under specific-pathogen-free conditions at 22˚C with a 12 h light/dark cycle and free access to food and water. Mice were randomly divided into two groups (control and oxymatrine-treated). CAL-27 cells (~1x10^6; PBS solution was used for suspension) were subcutaneously injected into the bilateral hind legs of the nude mice. After a week, one group was injected with PBS as a control, and the other was treated with oxymatrine (30-50 mg/kg) every 3 days. Tumor size was recorded every 3 days according to the following formula: V=(width^2 x length)/2. At total of 4 weeks later, the mice were euthanized by tail vein injection of sodium pentobarbital (200 mg/kg). Tumor tissues were harvested from mice and preserved in -80˚C for further study. All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals. The present study was approved (approval no. 20190612) by the Ethics Committee of the Guangzhou Hospital of Integrated Traditional and Western Medicine (Guangzhou, China).

Immunohistochemistry analysis (IHC). For IHC, the slides were heated at 60˚C for 2 h, followed by the removal of paraffin using xylene. After being rinsed with ethanol, antigen recovery was conducted for 10 min and the samples were immersed in 3% hydrogen peroxide and subsequently blocked with 10% serum (cat. no. SL034; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 15 min. The slides were incubated with primary antibodies anti-CXCR4 (1:1,000; cat. no. ab181020) and anti-METTL3 (1:1,000; cat. no. ab195352; both from Abcam) at 4˚C overnight and then incubated with the corresponding secondary antibody, HRP-labeled goat anti-rabbit (1:2,000) for 1 h at room temperature. The slides were then stained with DAB work solution (cat. no. P0202; Beyotime Institute of Biotechnology) at room temperature, counterstained with hematoxylin work solution (cat. no. C0107; Beyotime Institute of Biotechnology) at room temperature and identified with 1% hydrochloric acid alcohol. Finally, slides were fixed with neutral balsam (cat. no. G8590; Solarbio Science & Technology Co., Ltd.) at room temperature and visualized under a light microscope (magnification, x20; Nikon Corporation).

Statistical analysis. GraphPad Prism version 7 (GraphPad Software, Inc.) was used for statistical analysis. Data are from three independent experiments and were presented as the mean ± SD. The differences between two groups were analyzed using unpaired Student's t-test. One-way ANOVA followed by Turkey's multiple comparison test was applied to analyze differences among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Oxymatrine inhibits proliferation and migration of OSCC cells. A total of 2 OSCC cell lines, SCC-15 and CAL-27,

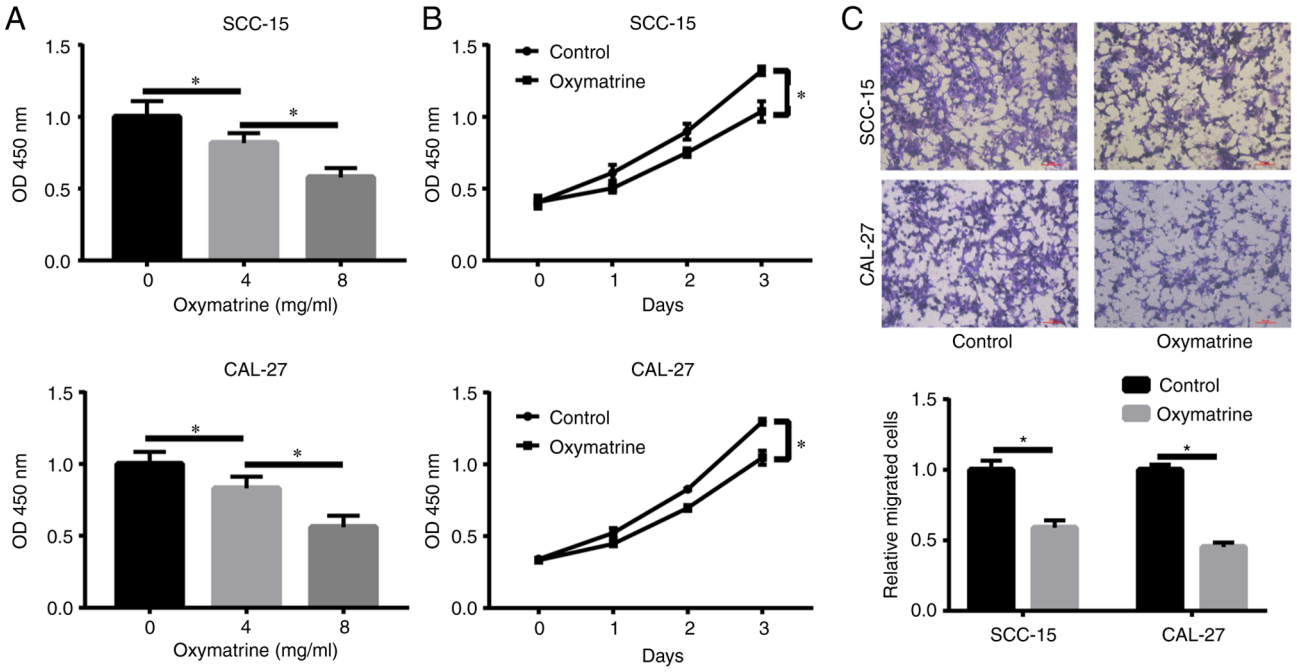

Figure 1. Oxymatrine inhibits proliferation and migration of OSCC cells. (A) Oxymatrine inhibited proliferation of SCC-15 and CAL-27 cells in a dose-dependent manner. (B and C) Viability and migration of OSCC cells was significantly attenuated when treated with 8 mg/ml oxymatrine. The data of each group were normalized compared with the mean of the control group or 0 mg/ml group. *P<0.05. OSCC, oral squamous cell carcinoma.

were cultured with different concentrations of oxymatrine (0, 4 and 8 mg/ml) for 24 h and cell proliferation was assessed using CCK-8 assays. The results revealed that oxymatrine significantly reduced cell proliferation in a dose-dependent manner (Fig. 1A). A concentration of 8 mg/ml was selected for further experiments owing to its strong effects. As revealed in Fig. 1B, oxymatrine inhibited proliferation of OSCC cells at the indicated time points. To explore the effect of oxymatrine on tumor metastasis, a Transwell assay was conducted, and the results showed that oxymatrine attenuated SCC-15 and CAL-27 cell migration (Fig. 1C). Notably, the same concentration (8 mg/ml) of oxymatrine did not promote apoptosis or inhibit the proliferation (Fig. S1A and B) of the human normal squamous epithelial (hNOK) cells, indicating that oxymatrine exerts antitumor effects but has no side effects on normal cells.

Oxymatrine induces OSCC cell apoptosis. Apoptosis was assessed using flow cytometric analysis. It was identified that, compared with the control group, oxymatrine induced higher rates of apoptosis (Fig. 2A). Moreover, oxymatrine increased the expression of the proapoptotic protein Bax but significantly reduced the antiapoptotic protein Bcl-2 (Fig. 2B). These results indicated that oxymatrine acts as a tumor suppressor in OSCC by inhibiting cell proliferation and migration and promoting cell apoptosis.

Oxymatrine inhibits CXCR4 expression. CXCR4 is markedly increased in OSCC and promotes the occurrence and development of OSCC (17,18); therefore, it was investigated whether oxymatrine treatment in OSCC affects CXCR4 expression. Oxymatrine treatment reduced CXCR4 expression level in a concentration-dependent manner in SCC-15 and CAL-27 cells (Fig. 3A and B). Considering m^{6}A is the most abundant modification in eukaryotic mRNA, which is closely related to the RNA regulation, as well as the stability of RNA, it was hypothesized that oxymatrine may regulate CXCR4 expression and the mRNA stability by modulating the m^{6}A modification level of CXCR4 mRNA. Notably, oxymatrine treatment (8 mg/ml) shortened the half-life of CXCR4 mRNA in both SCC-15 and CAL-27 cells (Fig. 3C). Furthermore, the MeRIP assay revealed that oxymatrine reduced m^{6}A modification of CXCR4 mRNA in SCC-15 and CAL-27 cells (Fig. 3D). These data suggested that oxymatrine reduces CXCR4 expression by downregulating the m^{6}A level of CXCR4 mRNA.

Oxymatrine exerts antitumor effects in OSCC by inhibiting CXCR4 expression. To validate whether oxymatrine functions as a tumor suppressor in OSCC by inhibiting CXCR4, rescue experiments were conducted. Firstly, the transfection efficiency of CXCR4 plasmid was determined at mRNA level by RT-qPCR, and it was found that the transfection of CXCR4 plasmid significantly upregulated the CXCR4 expression in OSCC cells (Fig. S1D). CCK-8 and Transwell assays revealed that CXCR4 overexpression partly reversed the inhibitory effects of oxymatrine on cell proliferation and migration (Fig. 4A and B). CXCR4 upregulation alleviated apoptosis (Fig. 4C). In addition, in oxymatrine-treated SCC-15 and CAL-27 cells, CXCR4 upregulation reduced Bax protein expression level and increased Bcl-2 protein expression level (Fig. 4D).

Oxymatrine downregulates CXCR4 by decreasing the level of m^{6}A modification via inhibiting METTL3 expression. In order to clarify how oxymatrine regulates the m^{6}A modification level of CXCR4, it was first detected whether the expression of m^{6}A methylase (METTL3, METTL14 and WTAP) and transferase (ALKBH5 and FTO) are influenced by oxymatrine.

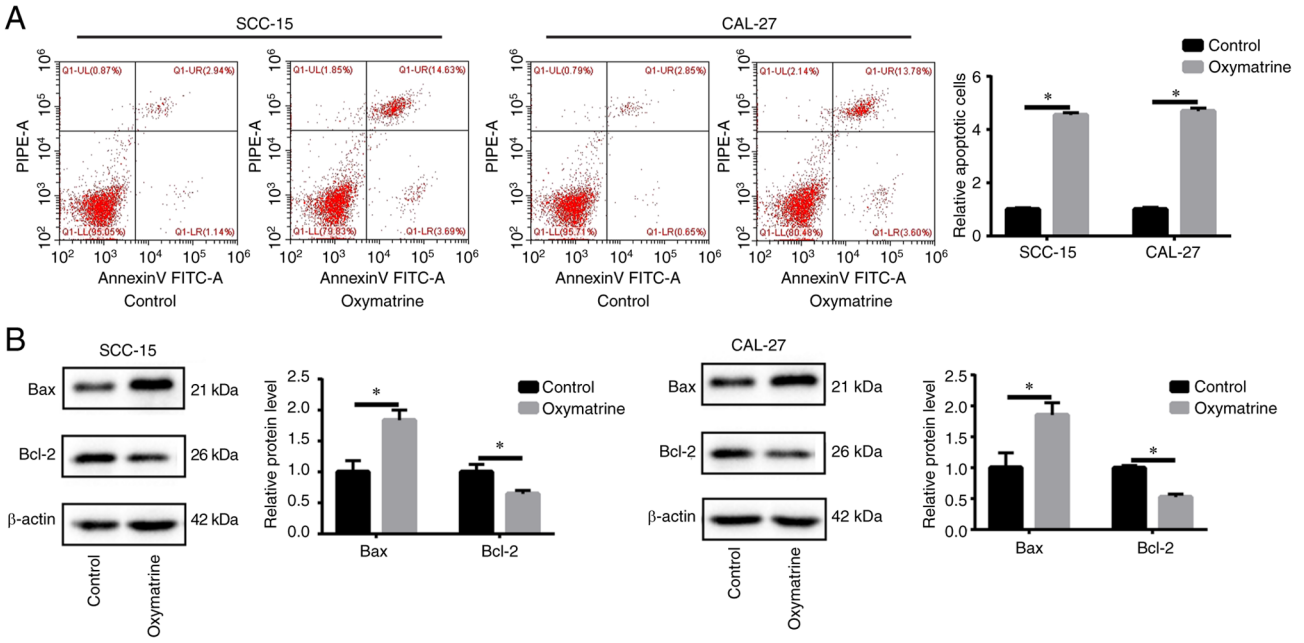

Figure 2. Oxymatrine induces apoptosis of OSCC cells. (A) Flow cytometric analysis revealed that oxymatrine promoted the apoptosis of SCC-15 and CAL-27 cells. (B) Western blotting demonstrated that oxymatrine increased the expression of pro-apoptosis protein Bax but reduced the anti-apoptosis protein Bcl-2 expression in SCC-15 and CAL-27 cells. The data of each group were normalized compared with the mean of the control group. *P<0.05. OSCC, oral squamous cell carcinoma.

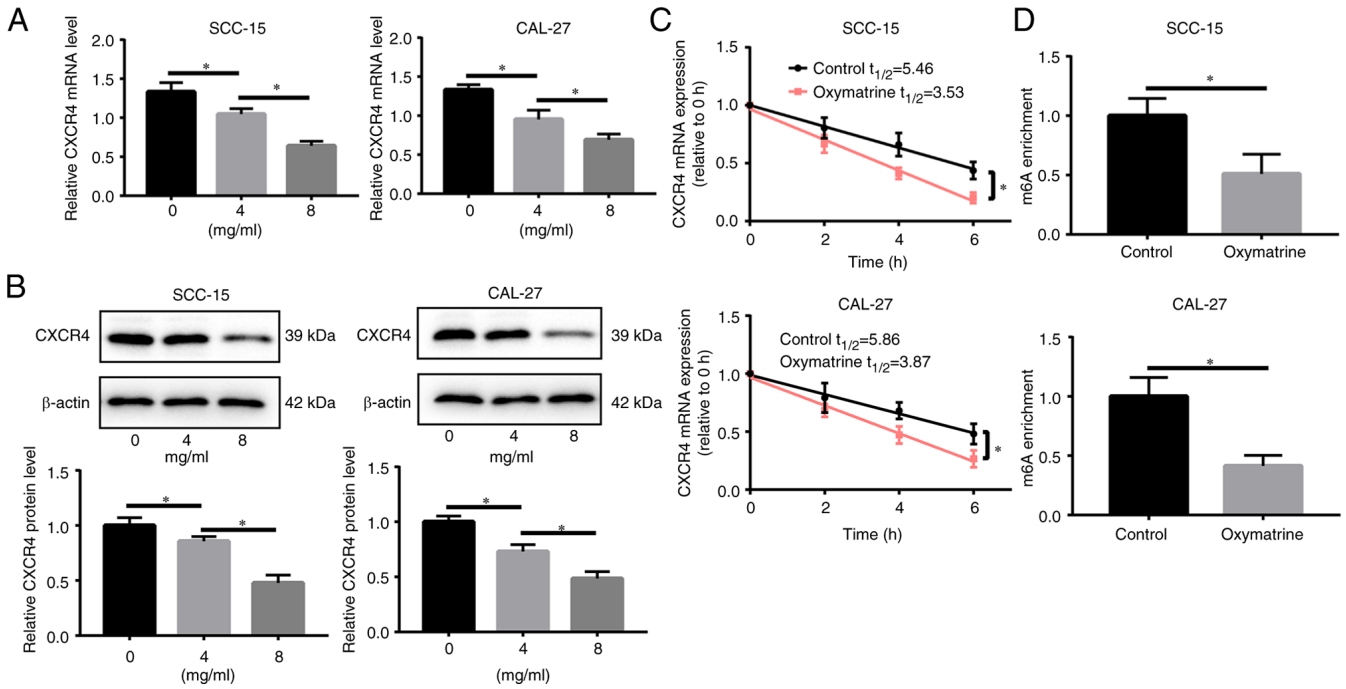

Figure 3. Oxymatrine suppresses CXCR4 expression. (A and B) Oxymatrine decreased CXCR4 (A) mRNA and (B) protein expression level in a dose-dependent manner. (C) Oxymatrine (8 mg/ml) significantly shortened the half-life of CXCR4 mRNAs in SCC-15 and CAL-27 cells. (D) Methylated RNA immunoprecipitation assay showed that oxymatrine (8 mg/ml) decreased the m^{6}A levels of the CXCR4 mRNA in SCC-15 and CAL-27 cells. The data of each group were normalized compared with the mean of the control group or 0 mg/ml group. *P<0.05. CXCR4, CXC chemokine receptor 4; m^{6}A, N6-methyladenosine.

It was identified that the expression of METTL14, WTAP, ALKBH5 and FTO was not significantly changed when treated with oxymatrine (Fig. S1C), whereas METTL3 was down-regulated significantly in a dose-dependent manner (Fig. 5A) indicating that m^{6}A methylase METTL3 may participate in oxymatrine's regulation of CXCR4. Firstly, the transfection efficiency of METTL3 plasmid was determined at mRNA level by RT-qPCR, and it was observed that the transfection of METTL3 plasmid significantly upregulated the METTL3 expression in OSCC cells (Fig. S1D). Furthermore, METTL3 overexpression promoted CXCR4 expression (Fig. 5B). The RIP assay demonstrated that METTL3 binds to CXCR4

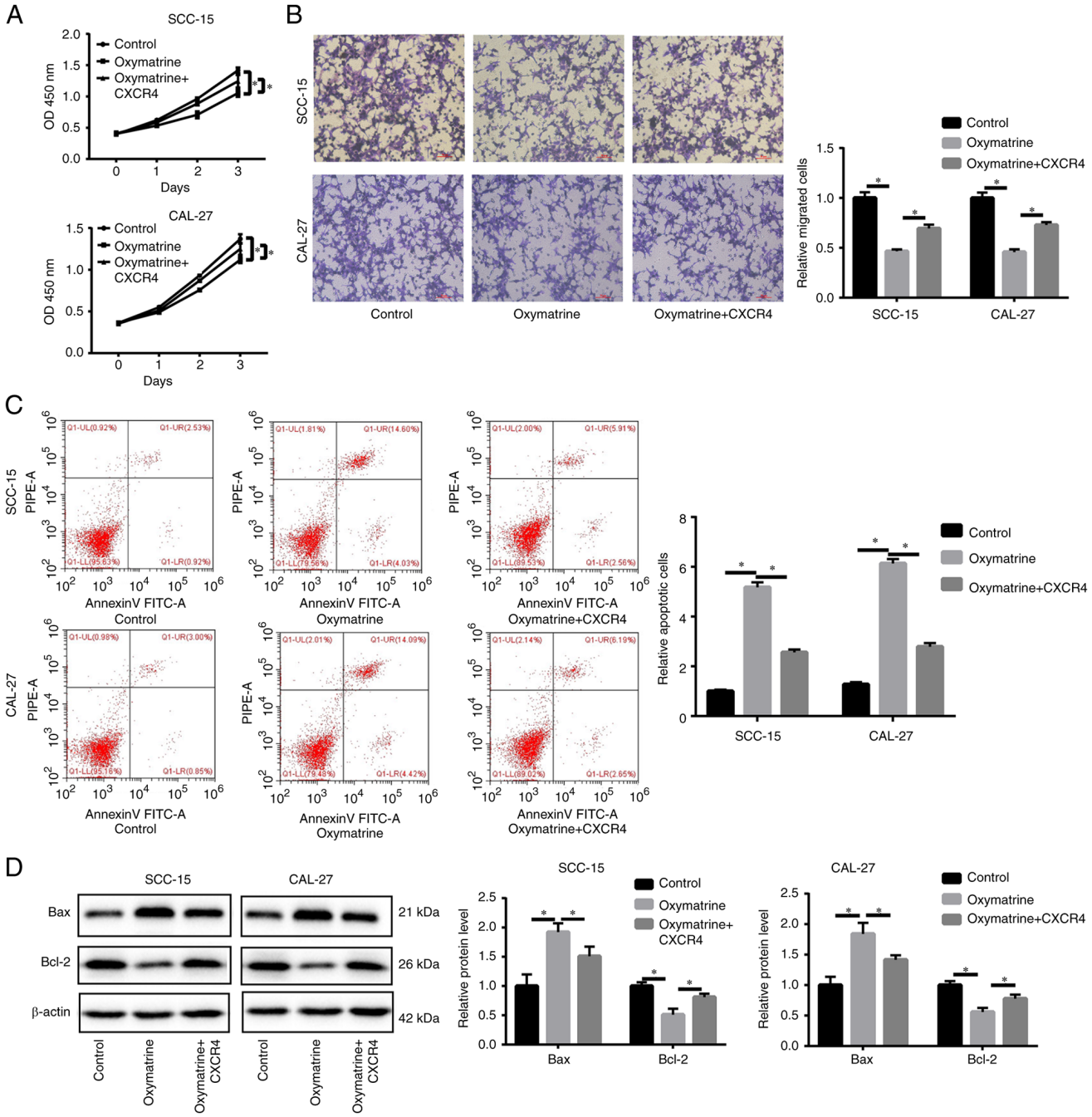

Figure 4. Oxymatrine exerts antitumor functions in OSCC by regulating CXCR4 expression. (A and B) The inhibition of (A) cell proliferation and (B) migration induced by the treatment of oxymatrine (8 mg/ml) were rescued by the overexpression of CXCR4. (C) The upregulation of CXCR4 restored the cell apoptosis induced by oxymatrine (8 mg/ml) treatment in SCC-15 and CAL-27 cells. (D) When the Bax and Bcl-2 protein expression was increased or decreased by oxymatrine, overexpressing CXCR4 could partially rescue it. The data of each group were normalized compared with the mean of the control group. *P<0.05. OSCC, oral squamous cell carcinoma; CXCR4, CXC chemokine receptor 4.

mRNA (Fig. 5C). Although oxymatrine treatment inhibited CXCR4 expression, METTL3 upregulation reversed this effect (Fig. 5D). In addition, METTL3 prolonged the half-life of CXCR4 mRNA, which was reduced by oxymatrine treatment in SCC-15 and CAL-27 cells (Fig. 5E). The MeRIP assay showed that METTL3 overexpression reversed the reduction in the level of CXCR4 mRNA m^{6}A methylation, which was induced by oxymatrine in SCC-15 and CAL-27 cells (Fig. 5F).

To elucidate whether other m^{6}A enzymes participate in regulating the level of CXCR4 mRNA m^{6}A methylation, the expression of other m^{6}A enzymes was detected, including METTL14, WTAP, ALKBH5 and FTO. It was found that the protein expression levels of these enzymes were not significantly changed when treated with oxymatrine (Fig. S1C), indicating that they may not participate in regulating the level of CXCR4 mRNA m^{6}A methylation. Therefore, oxymatrine inhibits CXCR4 expression by reducing the m^{6}A methylation level via downregulating METTL3.

Oxymatrine inhibits tumor growth in vivo. To further evaluate the efficacy of oxymatrine in the treatment of OSCC, xenograft tumors were established in nude mice.

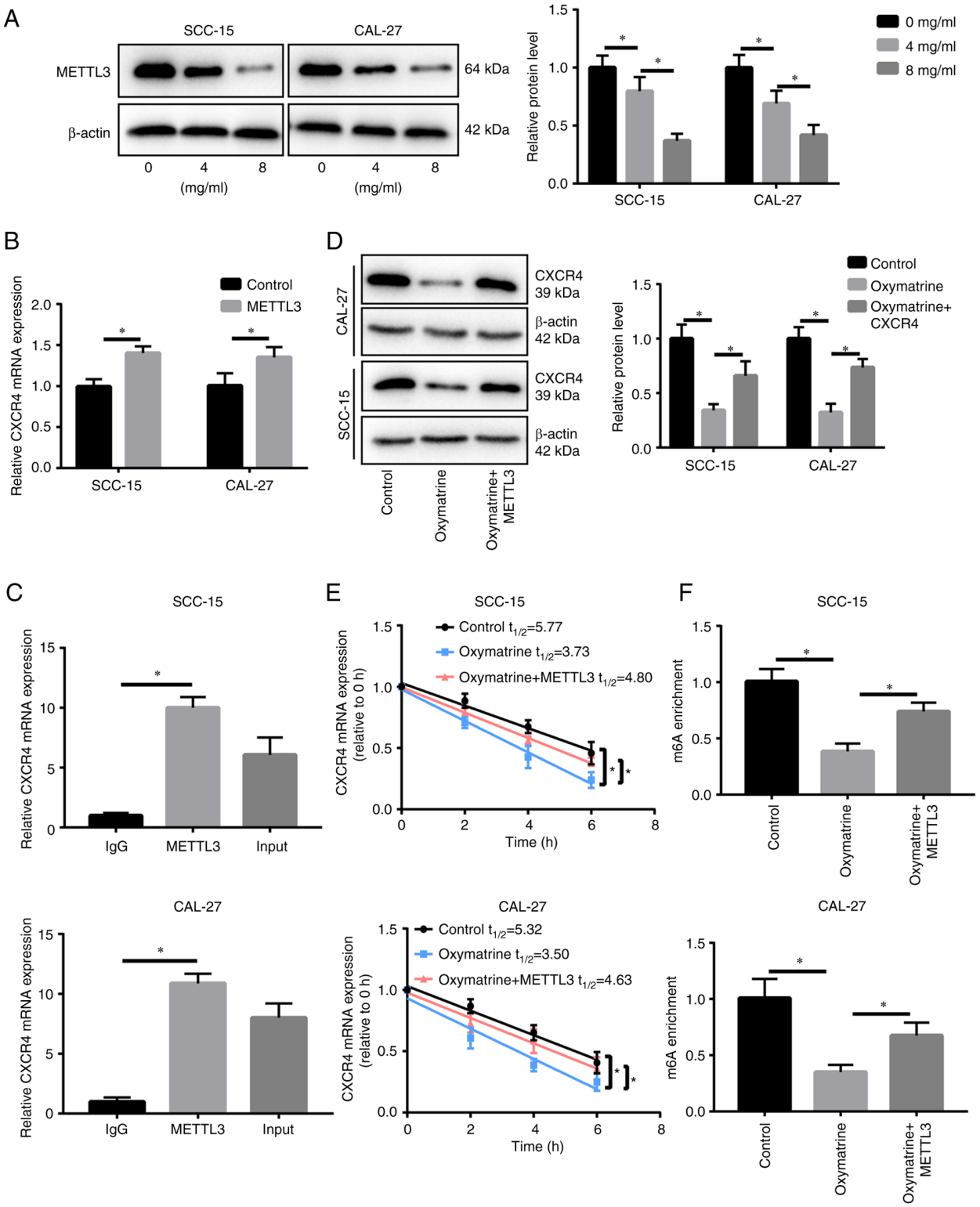

Figure 5. Oxymatrine downregulates CXCR4 through decreasing the m6A modification level by inhibiting METTL3. (A) Oxymatrine inhibited METTL3 protein expression at a dose-dependent manner. (B) Overexpression of METTL3 promoted CXCR4 expression. (C) RNA-binding protein immunoprecipitation assays revealed that METTL3 could bind with CXCR4 mRNA. Overexpression of METTL3 rescued the (D) CXCR4 protein expression, (E) CXCR4 mRNA half-life ($t_{1/2}$) and (F) m^6A enrichment of CXCR4 mRNA which was decreased by oxymatrine (8 mg/ml) in SCC-15 and CAL-27 cells. The data of each group were normalized compared with the mean of the control group or 0 mg/ml group. *P<0.05. CXCR4, CXC chemokine receptor 4; METTL, methyltransferase-like protein; m^6A, N6-methyladenosine.

Compared with the control group, oxymatrine treatment inhibited tumor growth and reduced tumor volume and weight (Fig. 6A-C). Western blotting and IHC assays revealed that CXCR4 and METTL3 were downregulated in the oxymatrine-treated group compared with the control group (Fig. 6D and E). Additionally, m^6A levels in the oxymatrine-treated group were much lower than those in the control group (Fig. 6F).

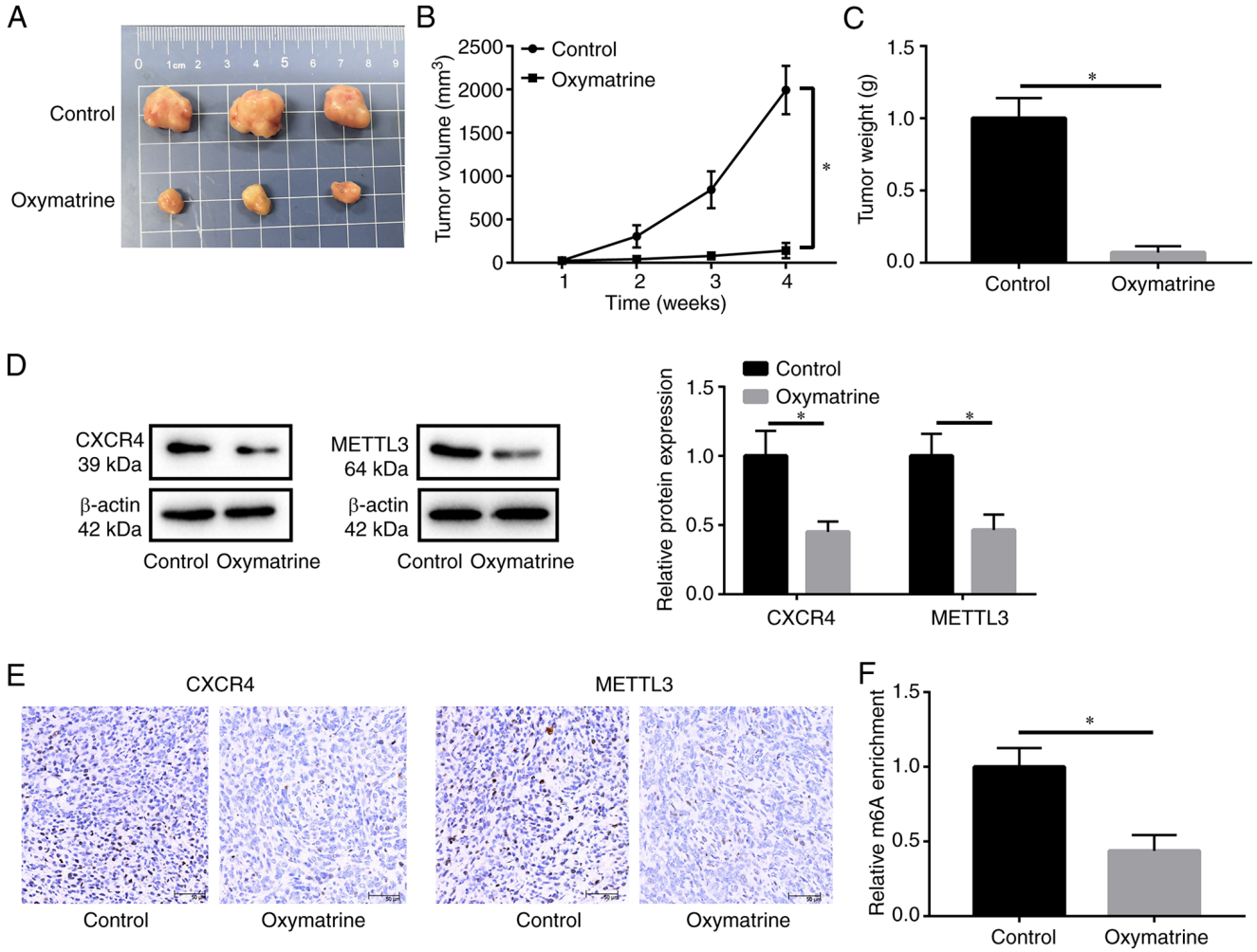

Figure 6. Oxymatrine inhibits tumor growth *in vivo*. (A) The tumor harvested from the mice treated with oxymatrine was markedly smaller than that in control group. (B and C) Oxymatrine significantly reduced tumor (B) volume and (C) weight. (D) The western blotting and (E) immunohistochemical assays revealed that CXCR4 and METTL3 was significantly downregulated in the oxymatrine-treated group compared with the control group. (F) The m^{6}A modification level was significantly reduced in the oxymatrine-treated group compared with the control group. The data of each group were normalized compared with the mean of the control group. *P<0.05. CXCR4, CXC chemokine receptor 4; METTL, methyltransferase-like protein; m^{6}A, N6-methyladenosine.

Discussion

Oxymatrine has been revealed to inhibit the growth of several types of cancer and has gained increasing attention in recent decades. Oxymatrine induces apoptosis in pancreatic cancer cells by influencing the ratio of Bax/Bcl2 and the release of mitochondrial cytochrome c (26). In bladder cancer, oxymatrine suppresses tumor progression by inducing apoptosis and cell cycle arrest (27). However, little is known about the role of oxymatrine in OSCC. In the present study, it was found that oxymatrine attenuated the progression of OSCC by inhibiting tumor cell proliferation and migration and promoting cell apoptosis. The antitumor effect of oxymatrine in OSCC *in vivo* was further confirmed.

CXCR4, a highly conserved seven-span transmembrane G-protein-coupled receptor that binds to the ligand CXCL12 (28), plays an important role in the survival, proliferation, angiogenesis and metastasis of various tumors (14). Dysregulation of CXCR4 has been observed in OSCC. Additionally, OSCC progression is regulated by the miR-139-5p/CXCR4 axis (29). Zerumbone, a bioactive monocyclic sesquiterpene isolated from the rhizomes of tropical ginger, reduces OSCC cell motility and proliferation by targeting CXCR4 (30). Therefore, it was hypothesized that oxymatrine may function as a tumor suppressor by downregulating CXCR4. The present findings showed that oxymatrine treatment reduced the mRNA and protein expression levels of CXCR4 in a dose-dependent manner in OSCC cells. Furthermore, the rescue experiments indicated that ectopic CXCR4 expression partly restored the suppressive effect of oxymatrine on OSCC cell viability and migration, but alleviated oxymatrine-induced cell apoptosis. Therefore, oxymatrine inhibits OSCC tumor progression by inhibiting CXCR4 expression.

m^{6}A is the most common mRNA modification (31,32). Accumulating evidence has revealed that m^{6}A regulators, such as METTL3, are dysregulated in numerous tumors, including OSCC and METTL3 plays a role in tumor cell proliferation, migration and apoptosis by stabilizing its downstream genes (33-37). In the present study, it was demonstrated that oxymatrine reduced the stability of CXCR4 mRNA, which was confirmed by the Act-D assay, suggesting that oxymatrine may influence CXCR4 m^{6}A

methylation level in OSCC cells. MeRIP results revealed that oxymatrine treatment reduced the m^{6}A methylation level of CXCR4 mRNA. Moreover, it was found that oxymatrine also decreased METTL3 expression, indicating that oxymatrine may decrease the m^{6}A methylation level of CXCR4 mRNA by regulating METTL3 expression. The RIP assay showed that METTL3 binds to CXCR4 mRNA and the MeRIP assay identified that METTL3 knockdown reduces CXCR4 m^{6}A mRNA methylation level. Furthermore, oxymatrine treatment in OSCC cells overexpressing METTL3 increased the stability and m^{6}A methylation level of CXCR4 mRNA. Collectively, the results of the present study confirmed that oxymatrine induced a reduction in m^{6}A methylation levels in CXCR4 mRNA by inhibiting METTL3 expression.

In summary, the present study demonstrated for the first time, to the best of our knowledge, that oxymatrine attenuates OSCC progression by inhibiting cell proliferation and migration and inducing cell apoptosis. Mechanistically, it was revealed that oxymatrine directly destabilizes CXCR4 mRNA via inhibiting METTL3-mediated m^{6}A methylation. Therefore, the present findings revealed the therapeutic value of oxymatrine in treating OSCC. However, further studies are needed to determine the validity, safety and pharmacokinetics of oxymatrine.

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Availability of data and materials

The datasets in the present study are available from the corresponding author on reasonable request.

Authors' contributions

JS and ZL designed the present study. RL, YL and LX performed the experiments. RL and YL analyzed the data. JS, RL and YL prepared the draft of the manuscript. All authors read and approved the final version of the manuscript. ZL and RL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The protocols of animal studies were approved (approval no. 20190612) by The Animal Care and Use Committee of the Guangzhou hospital of integrated traditional and West medicine (Guangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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