hMOF (human males absent on the first), an oncogenic protein of human oral tongue squamous cell carcinoma, targeting *EZH2* (enhancer of zeste homolog 2)

Qihong Li^{*a}, Haiyan Sun^{*a}, Yao Shu^{*}, Xuan Zou^{*}, Yantao Zhao[†] and Cheng Ge^{*}

^{*}Department of Stomatology, Affiliated Hospital of Academy of Military Medical Sciences, Beijing 100071, China and [†]Department of Orthopaedics, First Affiliated Hospital of the General Hospital of People's Liberation Army, Beijing 100048, China

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Abstract

Objectives: MOF (males absent on the first) is a histone acetyltransferase belonging to the MYST (MOZ, Ybf2/Sas3, Sas2 and TIP60) family. In mammals, MOF plays critical roles in transcription activation by acetylating histone H4 at K16. Human MOF (hMOF) essentially participates in behaviour of several human cancers. However, its role in human oral tongue squamous cell carcinoma (OTSCC) remains elusive, but we propose that hMOF regulates OTSCC cell population growth.

Materials and methods: Real time PCR and western blot analysis were applied, and it was found that *hMOF* level was up-regulated in human OTSCC. High *hMOF* expression predicted poor overall and disease-free survival. *hMOF* knockdown attenuated OTSCC cell growth and transformation.

Results: *EZH2* (enhancer of zeste homolog 2) was up-regulated in human OTSCC tissues and its level positively correlated with level of *hMOF*. *hMOF* knockdown inhibited *EZH2* expression by reducing its promoter activity. Moreover, we have demonstrated that *EZH2* was critically essential for function of hMOF in human OTSCC.

Conclusions: Human males absent on the first regulated OTSCC growth through *EZH2*, thus *EZH2* may serve as a candidate for anti-OTSCC therapy.

Introduction

Oral tongue squamous cell carcinoma (OTSCC) is one of the most common cancers of the oral cavity, with propensity for rapid local invasion and spread, and high-recurrence rate (1–3). It makes up one-third of intraoral cancers whose risk factors include for example, alcohol and tobacco misuse and poor oral hygiene (4–6). According to the American Cancer Society, an estimated 10 990 new cases of tongue cancer are expected each year, accounting for approximately 30% of all oral cavity and pharynx cancers (7). Even with recent advances in its treatment, 5-year survival remains at only around 50% (8). These statistics indicate a major health problem, and point to the immediate need for better understanding of this disease. While attempts have been made to identify genomic alterations that contribute to initiation and progression of OTSCC, most efforts have been focused on protein coding genes.

MOF (males absent on the first) is a member of the MYST family of histone acetyltransferases (9,10). It was first described in *Drosophila melanogaster* as an essential component of X-chromosome dosage compensation male-specific lethal (MSL) complex (10,11). The unique effect of H4K16 acetylation on genome-wide chromatin dynamics shows that it is a key epigenetic modification in regulation of gene transcription (12). hMOF is an orthologue of *Drosophila* MOF and exists in multiple complexes, which include human orthologues of the *Drosophila* dosage compensation proteins MSL1/2/3 (13,14). In mammals, MOF plays critical roles by acetylating histone H4 at K16 and non-histone substrates such as p53 (15). In recent years, various groups have focused on functions of MOF in mammals and recent studies have shown that abnormal expression of *hMOF* gene is involved in certain primary cancers (16).

EZH2 (enhancer of zeste homolog 2) is a critical member of the polycomb group of proteins that regulates a variety of essential biological processes,

Correspondence: C. Ge, Department of Stomatology, Affiliated Hospital of Academy of Military Medical Sciences, Beijing 100071, China. Tel.: +86-010-66947331; Fax: +86-010-66947331; E-mail: cheng_ge76@163.com; and Y. Zhao, Department of Orthopaedics, First Affiliated Hospital of the General Hospital of People's Liberation Army, Beijing 100048, China. Tel.: +86-010-66848865; Fax: +86-010-66848865; E-mail: ytzamms@163.com

^aQihong Li and Haiyan Sun contributed equally to this work.

including embryogenesis and many developmental events (17). It plays an important role in cell proliferation and cell cycle regulation (18). Here, we report that *hMOF* regulated population growth of OTSCC by targeting *EZH2*. Our findings indicate that *EZH2* could serve as a candidate for anti-OTSCC therapy.

Materials and methods

Patients

Oral tongue squamous cell carcinoma samples and adjacent normal tissues were obtained from the Affiliated Hospital of the Academy of Military Medical Sciences. All human tissues were collected according to protocols approved by the Ethics Committee of this institute. Clinical characterization of OTSCC patients is summarized in Table S1. Patients underwent no chemotherapy nor radiotherapy before surgical treatment. The TNM system of tumour staging and histological grade were performed according to World Health Organization guidelines.

Quantitative real-time PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized using a reverse transcriptase reaction kit (Promega, Madison, WI, USA). PCRs were conducted in triplicate for each sample and primers are shown in Table S2. Expression data were normalized to geometric mean of the housekeeping gene glyceraldehydes 3-phosphate dehydrogenase (*GAPDH*).

Immunohistochemistry

Samples were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity, followed by incubation with 5% bovine serum albumin to block non-specific binding (19). After blocking, sections were incubated with *hMOF* antibody (10 µg/ml; R&D Systems, Minneapolis, MN, USA) at 4 °C, overnight, followed by poly-HRP IgG (ZSGB-BIO, Beijing, China) at room temperature for 20 min. All stains were visualised using diaminobenzidine for 2–3 min. Sections were then counterstained with hematoxylin, dehydrated and mounted. Negative control was performed by replacing primary antibody with PBS.

Immunoreactivity was semiquantitatively evaluated on the basis of staining intensity and distribution, using immunoreactive score (IS), where

IS = intensity score × proportion score.

Intensity score was defined as: 0, negative; 1, weak; 2, moderate; or 3, strong, and proportion score was defined as: 0, negative; 1, ≤10% positive cells; 2, 11–50% positive cells; 3, 51–80% positive cells; or 4, >80% positive cells. The immunoreactive score ranged from 0 to 12. Immunoreactivity was divided into two groups on the basis of immunoreactive score: low immunoreactivity defined as total score of 0–4, and high immunoreactivity defined as total score of >4.

OTSCC cells and cell culture

Human OTSCC cell lines, SCC9 and UM1, were maintained in DMEM/F12 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, Gaithersburg, MD, USA). For functional analysis, gene-specific siRNA against *hMOF* or control siRNA were transfected into cells using Dharma FECT Transfection Reagent 1 as described previously (15,16). *hMOF* expression vector or empty vector was transfected into cells using Lipofectamine2000 (Life Technologies, Gaithersburg, MD, USA).

Retrovirus packaging and transduction

Retro-sh-*hMOF* and retro-sh-*EZH2* virus packaging were completed by GenePharma. Cells were selected in the presence of 20 ng/ml colchicine: drug resistant cells were pooled and grown to 80% confluence. Fresh medium lacking colchicine was added to the cells, and virus load was collected after 20–24 h. Virus titre, assayed on SCC9 or UM1 cells, was determined to be 2×10^6 colony-forming units/ml. For transduction of SCC9 or UM1 cells, 5×10^5 cells were plated in 35-mm dishes on day 0. On day 1, 1 ml of virus was added to cells in the presence of 8 µg/ml polybrene. On day 3, 60 ng/ml colchicine was added to select for transduced cells. Colchicine-resistant cells were pooled, and drug resistance was amplified by growing cells in increasing concentrations of colchicine, as described previously (20,21). Amplification was continued until cells were growing in 640 or 1280 ng/ml colchicine.

Western blot analysis

Prior to immunoblotting, cells were washed in ice-cold PBS, resuspended in $2 \times$ lysis buffer, and incubated for 20 min at 4 °C, while rocking. Lysates were cleared by centrifugation (10 min 3913 g, 4 °C) and 50 mg total protein was resolved by SDS-PAGE and transferred to polyvinylidene fluoride membrane (Immobilon;

Millipore, Boston, MA, USA). Membranes were first blocked then incubated with primary antibodies described below for 2 h at room temperature. Secondary antibodies were visualized using LumiGLO Regent and Peroxide (Cell Signaling). Primary antibodies were: anti-hMOF (anti-goat, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-EZH2 (anti-mouse, 1:1000; Cell Signaling Technology, Beverly, MA, USA), anti-vimentin (Abcam, Cambridge, MA, USA) and anti-GAPDH (anti-rabbit, 1:1000; Sigma-Aldrich, St. Louis, MO, USA).

Cell proliferation assay

Cells were seeded into 96-well plates at 1000 cells per well, 24 h after transfection. Effects of hMOF on cell proliferation were detected after seeding, using the MTT assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's instructions. Each assay was performed in five replicates of three independent experiments.

Soft sugar colony formation assay

In vitro tumorigenicity was determined on the basis of cell population expansion in soft agar colony assay,

which was performed in triplicate. Cells (~100 cells/well) were seeded in 24-well plates in DMEM medium containing 0.35% low melting point agar, overlying a 0.7% low melting point agar layer. Cells were cultured at 37 °C in 5% CO₂. Every 7 days, 500 µl fresh medium was added to each well and visible colonies were photographed.

Xenograft mouse experimentation

Four to five week-old female BALB/c nude mice were provided by the Chinese Academy of Medical Sciences (Beijing, China) and animals were housed in micro-isolator cages in a pathogen-free animal biosafety level-2 facility, at 22 ± 2 °C. Tumours were initiated by intraperitoneal injection of SCC9 cells prepared as above. All procedures involving the use and care of mice were approved ethically and scientifically by the university, in compliance with the Practice Guidelines for Laboratory Animals of China.

Luciferase assay

Enhancer of zeste homolog 2 plasmid was transfected into UM1 cells using Cellfectin (Invitrogen, Carlsbad, CA, USA) in serum-free medium, along with plasmid

Figure 1. human males absent on the first (*hMOF*) expression up-regulated in human oral tongue squamous cell carcinoma (OTSCC). (a) *hMOF* mRNA level was higher in human OTSCC, $n = 22$ normal group and $n = 64$ OTSCC group. (b) *hMOF* protein level was up-regulated in human OTSCC. Immunohistopathological analysis was performed to examine *hMOF* of adjacent normal epithelium (Normal), well differentiated primary (well), moderate or poor differentiated OTSCC (poor-mode). Representative images of $\times 200$ magnification. Scale bar = 100 µm.

Figure 2. human males absent on the first (*hMOF*) predicted poor prognosis. Kaplan-Meier curve comparing time to survival between non-small cell lung cancers with low (<25th percentile) versus high (>25th percentile) *hMOF*-positive areas, determined using immunohistochemistry. (a) Overall survival. (b) Disease-free survival.

containing *firefly luciferase* reporter gene, driven by insect cell promoter OpIE2. Luciferase reporter assay was performed on cell lysates using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Experiments were conducted at least 3 times, each in triplicate. Controls were set arbitrarily at 1, and fold increase over control was plotted as mean $\pm$ SD.

Statistical analysis

Data are presented as (mean $\pm$ SD). Statistical analysis was performed using SPSS 13.0 statistical software (SPSS, Inc. Chicago, IL, USA). $P < 0.05$ was considered significant. Expression of *hMOF* in a subset of human OTSCC samples was analyzed using the cumulative hypergeometric distribution and χ^2 testing.

Results

hMOF expression elevated in human OTSCC

To investigate *hMOF* function in human OTSCC, we examined changes in *hMOF* mRNA and protein expression levels in 64 OTSCC tissues and 22 samples of adjacent normal tissue. Data indicated that

hMOF mRNA and protein levels were significantly up-regulated (Fig. 1a; Table S1). *hMOF* was detectable only in basal layers in normal tissue, while there was predominant staining of *hMOF* in nuclei of cancer tissue cells (Fig. 1b). Semiquantitative analysis revealed that *hMOF* was significantly overexpressed in OTSCC when compared to normal tissues. Of the 64 OTSCC samples, 24 (37.50%) had low *hMOF* nuclear staining but in 40 cases (62.50%) it was high (Table S1).

hMOF predicted overall and disease-free survival

Striking difference in prognosis was observed between the high *hMOF* expression group (5-year survival rate $<25\%$) and the low *hMOF* expression one (5-year survival rate $>60\%$). Differences in disease-free survival were also observed (Fig. 2b). These data indicate that high *hMOF* expression correlated with poor overall and disease-free survival than low *hMOF* ($P < 0.05$).

hMOF regulated OTSCC cell population expansion in vitro and in vivo

To further evaluate the role of *hMOF* in OTSCC cell population expansion, we performed cell proliferation

Figure 3. human males absent on the first (*hMOF*) regulated oral tongue squamous cell carcinoma (OTSCC) cell population growth *in vitro* and *in vivo*. (a, b) *hMOF* knockdown repressed SCC9 cell proliferation. SCC9 cancer cells were infected with retrovirus expressing ctrl shRNA (sh-Ctrl) or shRNA targeting *hMOF* (sh-*hMOF*). Relative cell numbers were evaluated using MTT analysis at the indicated time points. * $P < 0.05$; ** $P < 0.01$ versus sh-Ctrl. (c, d) *hMOF* knockdown blunted SCC9 cell colony formation. SCC9 cells infected with retro-sh-Ctrl or retro-sh-*hMOF* were subjected to soft sugar colony formation assay and colony numbers were evaluated 2 weeks later. (e, f) *hMOF* knockdown attenuated SCC9 cell population growth *in vivo*. SCC9 cells with *hMOF* knockdown were used in xenograft mouse experiments. Tumour weight was evaluated at the end of the treatment period. Representative tumours are shown on the left and quantitative results of 10 mice per group on the right. Bar = 1 cm.

and colony formation experiments *in vitro* and xenograft mouse experiments *in vivo*. SCC9 is an OTSCC cell line with high *hMOF* expression. We chose this cell line for studying how *hMOF* regulated OTSCC cell growth *in vitro*. SCC9 cells were infected with retrovirus expressing ctrl shRNA (sh-Ctrl) or two different sequences shRNA targeting *hMOF* (sh-*hMOF*-1#; sh-*hMOF*-2#) (Table S3). Results showed that low expression of *hMOF* repressed SCC9 proliferation (Figs S1,3a,b). Next, we evaluated colony formation capacity. Results showed that *hMOF* knockdown down-regulated colony formation of SCC9 cells (Fig. 3c,d). We performed the xenograft mouse experiments simultaneously and our results indicated that *hMOF* knockdown reduced average tumour weight (Fig. 3e,f). Taken together, our findings demonstrate that *hMOF* promoted OTSCC growth *in vitro* and *in vivo*.

hMOF targeting *EZH2* in OTSCC

Recently, some studies have found that *EZH2* plays critical roles in progression of cancers. Our results show high *EZH2* mRNA expression in OTSCC compared to normal tissue (Fig. 4a). Due to correlation of *EZH2* and

hMOF with OTSCC progression, we examined relationships of their expression levels, using Pearson's rank correlation coefficient analysis. Expression level of *EZH2* positively correlated with *hMOF* in OTSCC (Fig. 4b; $r^2 = 0.7860$, $P < 0.0001$). UM1 is an OTSCC cell line that expresses high levels of *EZH2* and *hMOF*. Both *EZH2* protein and mRNA expression were reduced ($P < 0.01$) when the cells were treated with retro-sh-*hMOF* (Fig. 4c). *hMOF* knockdown induced poor *EZH2* promoter activity ($P < 0.01$) (Fig. 4d). Moreover, *hMOF* knockdown inhibited UM-1 cell proliferation and colony formation (Fig. S2). When UM1 cells were treated with *EZH2*-specific shRNA or *hMOF*-specific shRNA, cell proliferation was significantly reduced (Fig. 4e). Simultaneously, through knockdown of *EZH2* and *hMOF* in UM1 cells, we found that *EZH2* knockdown blocked effects of *hMOF* on cell proliferation (Fig. 4f). *EZH2* or *hMOF* knockdown in UM1 cells led to reduced UM-1 cell colony formation. However, compared to knockdown of *EZH2*, no apparent change was observed with co-treated of *hMOF* shRNA. These results indicate that *EZH2* knockdown blocked effect of *hMOF* on cell proliferation and colony formation.

Figure 4. human males absent on the first (*hMOF*) targets enhancer of zeste homolog 2 (*EZH2*) in oral tongue squamous cell carcinoma (OTSCC). (a) *EZH2* expression was up-regulated in human OTSCC. *EZH2* mRNA level was determined using q-PCR in normal versus OTSCC tissues. Boxes represent interquartile range; whiskers represent 10–90th percentile range; bars represent the median. $n = 16$ normal group, $n = 34$ OTSCC group. (b) *EZH2* mRNA level significantly and positively correlated with *hMOF* mRNA level. Linear regression analysis was performed to analyze correlation between *EZH2* expression and *hMOF*. (c) *hMOF* knockdown inhibited expression of *EZH2* at protein and mRNA level. (d) *hMOF* knockdown down-regulated promoter activity of *EZH2*. $**P < 0.01$ versus Ctrl; $##P < 0.01$ versus *EZH2*-Luc+sh-Ctrl. (e) *EZH2* knockdown blocked effects of *hMOF* on cell proliferation. (f) *EZH2* knockdown blocked effects of *hMOF* on colony formation. $*P < 0.05$; $**P < 0.01$ indicate sh-Ctrl versus sh-*EZH2*. $#P < 0.05$; $##P < 0.01$ indicate sh-Ctrl versus sh-*hMOF*.

Discussion

As one of the most common cancers of the oral cavity, various molecular markers and factors have been proposed for risk assessment of OTSCC survival or recurrence; however, none are currently in clinical use (3,23–25). Survival rates have improved only slightly over the past 50 years despite advancements in diagnostic tools and treatment regimes (22). Today there are still no clinically well-established treatments for OTSCC.

Human MOF, as a histone acetyltransferase, is responsible for histone H4K16 acetylation in human cells and in recent years, various groups have focused on functions of MOF in mammals. It has been reported that hMOF enhances transcription of *Hox* genes that coordinate with H3K4 methyltransferase MLL (26). hMOF and H4K16 acetylation are pivotal to DNA damage response and double-strand break repair, after exposure to ionizing radiation (27,28). Moreover, hMOF acetylates p53 at K120, which rapidly occurs after DNA damage. Acetylation at this site can activate proapoptotic target genes, but has only minimal effects on cell cycle progression, which suggests that it is a key switch for apoptosis and cell cycle control (29).

Human MOF has been reported to form a stable complex with histone methyltransferases MLL, WDR5 and MSL1v1 (13). Although hMOF has been shown to be involved in DNA damage repair, cell differentiation and the cell cycle, its functional roles in cancer have remained obscure. Recent studies have demonstrated that *hMOF* is down-regulated in primary breast cancers and medullablastomas (30). In addition, loss of hMOF function in human cells leads to genomic instability, defects in cell cycle progression, chromosomal aberrations and impaired DNA damage response (13,14,31). Studies have also found that comparing 43 paired non-small cell lung cancers (NSCLC) and corresponding tissues, hMOF and H4K16ac were shown to be more frequently over-expressed in NSCLC than normal tissue. We found that hMOF regulated cell proliferation and migration in OTSCC cells. Additionally, hMOF has been reported to mediate S phase entry by regulating H4K16ac in the *Skp2* promoter region of NSCLC cells (32). How hMOF specifically regulates such a variety of distinct cellular outcomes remains an outstanding biological question.

The oncogenic role of *EZH2* has recently been discussed in several cancers (33–36). In this study, we have demonstrated high expression of *EZH2* in human OTSCC. Over-expression of *EZH2* in OTSCC was a strong and an independent predictor of poor overall survival. Thus, *EZH2* expression appears to have the potential to predict prognosis of OTSCC patients. Con-

sequently, *EZH2* protein could possibly served as a novel biomarker for predicting OTSCC cancer patient survival.

We found that *EZH2* was up-regulated in human OTSCC tissues and its level positively correlated with that of *hMOF*. *hMOF* knockdown in UM1 cells inhibited *EZH2* expression by reducing its promoter activity, and *EZH2* knockdown blocked effects of hMOF on UM1cell proliferation and colony formation.

In summary, our results reveal the critical role of hMOF in OTSCC, regulating OTSCC cell population growth through *EZH2*. These findings are very interesting and may provide a new target for OTSCC therapy.

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Conflict of interest

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 hMOF knockdown in SCC-9 cells.

Fig. S2 hMOF regulates UM-1 cell growth and transformation.

Fig. S3 hMOF and EZH2 stably knockdown in SCC-9 cells.

Table S1. Characteristics of patients with OTSCC

Table S2. Primers used for q-PCR

Table S3. Sequences of sh-RNAs