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Original Article

Ubiquitin-like with phd and ring finger domains 1 as a potential therapeutic target in smoking-associated oral squamous cell carcinoma

Jia-Rong Wu [a], Che-Ting Yeh [b], Chu-Yen Chien [c], Ying-Chen Chen [d], Tsai-Wang Huang [e*], Che-Yi Lin [f], Alexander Cheng-Ting Hsieh [g], Yi-Jen Hung [h**], Yi-Shing Shieh [a,i,j,k***]

[a] *Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan*
[b] *School of Dentistry, National Defense Medical Center, Taipei, Taiwan*
[c] *Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan*
[d] *Molecular and Cell Biology, Taiwan International Graduate Program, Academia Sinica and Graduate Institute of Life Science, National Defense Medical Center, Taipei, Taiwan*
[e] *Division of Thoracic Surgery, Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan*
[f] *Department of Oral and Maxillofacial Surgery, Chi Mei Medical Center, Tainan, Taiwan*
[g] *Department of Medical Education, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan*
[h] *Division of Endocrinology and Metabolism, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan*
[i] *Department of Dentistry, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan*
[j] *School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[k] *Department of Oncology, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan*

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* Corresponding author. Division of Thoracic Surgery, Department of Surgery, Tri- Service General Hospital, National Defense Medical Center, No.161, Sec. 6, Min- Chuan East Rd., Nei-Hu, Taipei, 114, Taiwan.
** Corresponding author. Division of Endocrinology and Metabolism, National Defense Medical Center, No.161, Sec. 6, Min-Chuan East Rd., Nei-Hu, Taipei, 114, Taiwan.
*** Corresponding author. Graduate Institute of Life Sciences, National Defense Medical Center, No.161, Sec. 6, Min-Chuan East Rd., Nei-Hu, Taipei, 114, Taiwan.
E-mail addresses: chi-wang@mail.ndmctsgh.edu.tw (T.-W. Huang), metahung@yahoo.com (Y.-J. Hung), ndmcyss@ndmctsgh.edu.tw (Y.-S. Shieh).

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KEYWORDS
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Abstract *Background/purpose:* Oral squamous cell carcinoma (OSCC) is a prevalent and aggressive malignancy with poor clinical outcomes. Epigenetic dysregulation, particularly involving the ubiquitin-like with phd and ring finger domains 1 (UHRF1), is increasingly recognized in cancer progression. However, the role of UHRF1 in OSCC and its regulation by cigarette smoking remains unclear. Therefore, the aim of this study was to investigate whether cigarette smoking influences UHRF1 expression in OSCC.
Materials and methods: UHRF1 expression was analyzed in OSCC tissues using publicly available Gene Expression Omnibus (GEO) datasets. Quantitative PCR and western blotting were employed to evaluate UHRF1 levels in OSCC cell lines. Functional assays, including colony formation, wound healing, and transwell invasion, were conducted following UHRF1 knockdown or overexpression. To investigate the impact of cigarette smoking on UHRF1 expression, cells were treated with cigarette smoke condensate (CSC) in a time-dependent manner. In vivo, a xenograft mouse model was used to assess the effect of CSC treatment on tumor growth and UHRF1 expression.
Results: As compared to normal tissue, UHRF1 was significantly overexpressed in OSCC tissues. Functional assays revealed that UHRF1 promotes OSCC cell proliferation, migration, and invasion. CSC treatment upregulated UHRF1 expression in vitro and enhanced tumor growth in vivo. Immunohistochemical analysis of xenograft tumors confirmed elevated UHRF1 expression following CSC exposure.
Conclusion: This study provides the first evidence that UHRF1 functions as an oncogenic driver in OSCC and may mediate smoking-induced tumorigenesis. These findings highlight UHRF1 as a potential biomarker and therapeutic target, particularly in smoking-associated OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most prevalent malignant tumors of the oral cavity and continues to pose a significant global health challenge due to its high recurrence rate and poor survival outcomes.[1] Although the precise etiology of OSCC remains incompletely understood, epidemiological studies have identified several major risk factors, including betel quid chewing, cigarette smoking, and alcohol consumption.[2] Despite notable advancements in diagnostic techniques and therapeutic strategies, the prognosis for OSCC patients remains dismal,[3] underscoring the urgent need for the identification of novel molecular targets for therapeutic intervention.

Epigenetic regulation plays a pivotal role in modulating gene expression,[4] and ubiquitin-like containing phd and ring finger domains 1 (UHRF1) has emerged as a key epigenetic regulator, primarily involved in DNA methylation and histone modifications.[5] Dysregulation of UHRF1 has been implicated in tumorigenesis, making it a critical focus of cancer research.[6] Functionally, UHRF1 acts as an E3 ubiquitin ligase, recognizing hemimethylated DNA and facilitating the recruitment of DNA methyltransferase 1 (DNMT1) to maintain DNA methylation.[7] This process occurs predominantly during the G1/S and G2/M phases of the cell cycle. Structurally, UHRF1 comprises multiple functional domains: the UBL (ubiquitin-like) domain,[8] which interacts with the RFTS (replication foci targeting sequence) domain of DNMT1 to activate the enzyme; and the SRA (SET-and-RING-associated) domain, which specifically binds hemimethylated DNA and aids in the separation of the RFTS domain from DNMT1's catalytic domain (CD), thereby ensuring proper DNA methylation.[9] Through these coordinated mechanisms, UHRF1 plays a central role in the epigenetic regulation of gene expression. Its dysregulation can alter DNA methylation landscapes and contribute to cancer progression.[10] An increasing body of evidence has demonstrated that UHRF1 is frequently overexpressed in a variety of cancers and is associated with poor clinical outcomes.[11,12] While UHRF1 has been widely studied in several malignancies, its specific role and mechanistic contributions in OSCC remain largely uncharacterized. Thus, the present study seeks to explore the involvement of UHRF1 in OSCC progression.

Cigarette smoking is a well-documented risk factor for OSCC and has been associated with aberrant DNA methylation patterns, particularly the hypermethylation of tumor suppressor genes (TSGs).[13] However, the molecular mechanisms by which smoking drives these epigenetic changes remain unclear. Given UHRF1's critical role in maintaining DNA methylation, it is hypothesized that UHRF1 may mediate smoking-induced epigenetic alterations in OSCC. Therefore, the aim of this study is to investigate whether cigarette smoking influences UHRF1 expression in OSCC. The findings may provide important insights into the link between smoking and epigenetic dysregulation in OSCC and may identify UHRF1 as a potential molecular target for therapeutic intervention.

Materials and methods

Cell culture and treatment

The human OSCC cell lines YD38, OEC-M1, SCC25, and YD10B were cultured in RPMI-1640 medium (Cat. No. 31800-

022, Thermo Fisher, Pittsburgh, PA, USA) supplemented with 10% fetal bovine serum (FBS, Cat. No. 10437-028, Gibco, Eggenstein, Germany). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. For cigarette smoke exposure experiments, cells were treated with 125 μM of cigarette smoke condensate (CSC, Murty Pharmaceuticals Inc., Lexington, KY, USA).

UHRF1 silencing and overexpression

To investigate the functional role of UHRF1, gene expression was silenced using small interfering RNA (siRNA) targeting UHRF1 (Cat. No. D-006977-03, siGENOME, Dharmacon, Cambridge, UK). The siRNA sequence used was 5′-GGAACAGUCUUGUGAUCAG-3′, and a non-targeting control siRNA (5′-UAGCGACUAAACACAUCAA-3′) was used as a negative control. Knockdown efficiency was confirmed by qPCR and Western blot analysis.

For overexpression studies, cells were transfected with a Human UHRF1 ORF cDNA expression plasmid (Cat. No. HG17896-UT, Sino Biological, Beijing, China) according to the manufacturer's protocol. UHRF1 expression levels were verified via qPCR and Western blot analysis.

Western blot analysis

Total protein was extracted from OSCC cell lines using RIPA buffer supplemented with a protease and phosphatase inhibitor cocktail (Cat. No. PPC2020, Sigma—Aldrich, St. Louis, MO, USA). Protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Cat. No. 23225, Thermo Fisher Scientific, Waltham, MA, USA). A total of 30 μg of protein lysate was resolved on 8—10% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked with 5% nonfat milk in TBST for 1 h at room temperature, followed by overnight incubation at 4 °C with the following primary antibodies: Mouse anti-UHRF1 (Cat. No. MABE308, Sigma—Aldrich); Rabbit anti-GAPDH (Cat. No. 5174, Cell Signaling Technology, Danvers, MA, USA). After washing with TBST, membranes were incubated with appropriate HRP-conjugated secondary antibodies, and protein bands were visualized using an ECL detection kit (Cat. No. JT96-K004M, T-Pro Biotechnology, New Taipei City, Taiwan). Images were captured using the UVP ChemStudio PLUS Touch imaging system (Analytik Jena, Jena, Germany).

RNA extraction and quantitative reverse transcription-PCR

Total RNA was extracted from OSCC cells using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. For reverse transcription, 2000 ng of total RNA was used to synthesize cDNA. qPCR was performed using the SYBR Green Master Mix (Cat. No. 98005, Bioline, London, UK) on a real-time PCR system. The relative mRNA expression levels were quantified using the $2^{-}-\Delta\Delta Ct$ method, with GAPDH serving as the internal control. The primer sequences used were as follows: UHRF1 forward, 5′- AAA TGGCCT CAAGGGGACTC, UHRF1- reverse, 5′- CACTTGCACGTGACTTCGTG; GAPDH-forward, 5′- CCACATCGCTCAGACACCAT, GAPDH-reverse, 5′-TGACCAGGCGCCCAATA.

Transwell invasion assay

Cell invasion assays were performed using FluoroBlok™ cell culture inserts with 8-μm pores (Corning Inc., Corning, NY, USA) coated with Matrigel (Cat. No. 354234, Corning Inc). Matrigel was spread evenly in the inserts and incubated at 37 °C for 30 min to allow solidification. Subsequently, 2.5×10^4 cells suspended in serum-free RPMI medium were seeded into the upper chamber. The lower chamber was filled with 700 μL of RPMI medium containing 10% FBS as a chemoattractant. After a 24 h incubation, non-invading cells and Matrigel from the upper surface were gently removed using a cotton swab. The inserts were fixed with methanol for 20 min and stained with propidium iodide overnight at 4 °C. Invaded cells were visualized using an optical microscope and quantified using ImageJ software.

Wound healing assay

The wound healing assay was used to assess cell migration. Cells were seeded at a density of 1×10^6 cells per well in six-well plates and cultured until reaching 90—100% confluence. A linear scratch was created across the cell monolayer using a 1.0 mL pipette tip. Baseline images (0 h) were captured under a microscope. The cells were then incubated in serum-free RPMI medium for 24 h, after which wound closure was imaged again. The extent of migration was evaluated by comparing the scratch area over time, and quantitative analysis was performed using ImageJ software.

Colony formation assay

For the colony formation assay, 3000 cells were seeded per well in six-well plates in triplicate and cultured for 7 days. Colonies were then fixed with 4% formaldehyde for 15 min and stained with 0.01% crystal violet. Colonies were imaged using a microscope, and the number of colonies was quantified using ImageJ software.

Tumor xenograft model

All animal experiments were approved by the Laboratory Animal Center of National Defense Medical Center (NDMC-LAC, Taipei, Taiwan). Four-week-old male nude mice were obtained from the National Center for Biomodels (Taipei, Taiwan). A total of 1×10^6 SCC25 cells were injected subcutaneously into the right dorsal flank of each mouse. Mice were randomly divided into PBS (control) and CSC treatment groups ($n = 3$ per group) using a simple randomization method. Tumor volume was measured weekly and calculated using the formula: $V = (\text{length} \times \text{width}^2)/2$. After 28 days, the mice were sacrificed, and tumors were harvested for further analysis.

Immunohistochemistry (IHC)

Tumor tissues were fixed in 4% formaldehyde, embedded in paraffin, and sectioned. Sections were deparaffinized in xylene and rehydrated through a graded series of ethanol solutions. Antigen retrieval was performed by heating the sections in Tris–EDTA buffer at 100 °C for 30 min. Immunostaining was conducted using the Novolink™ Polymer Detection System (RE7140-K, Leica Biosystems Newcastle, Newcastle upon Tyne, UK) according to the manufacturer's protocol. Sections were incubated overnight at 4 °C with primary anti-UHRF1 antibody (Cat. No. MABE308, Sigma–Aldrich). Signal detection was performed according to standard IHC protocols.

Statistical analysis

Statistical comparisons between groups were performed using the student's t-test. All data analyses and graphical representations were conducted using GraphPad Prism version 9.5.0 (GraphPad Software, La Jolla, CA, USA). A p-value less than 0.05 ($P < 0.05$) was considered statistically significant.

Results

UHRF1 is overexpressed in OSCC

To investigate the role of UHRF1 in oral squamous cell carcinoma (OSCC), we conducted an external validation using publicly available datasets from the Gene Expression Omnibus (GEO) database. Four datasets (GSE30784, GSE23558, GSE74530, and GSE37991) were analyzed, all of which consistently demonstrated upregulated expression of UHRF1 in OSCC tissues compared to normal oral tissues (Fig. 1A–D).

Subsequently, we examined UHRF1 expression across several OSCC cell lines. Quantitative PCR and Western blot analyses revealed relatively low expression of UHRF1 in YD8, YD10B, YD15, SAS, and SCC25 cells, while higher expression levels were observed in YD38, and OEC-M1 cells (Fig. 1E and F). Based on these findings, we established stable UHRF1-overexpressing cell lines using YD10B and SCC25, and generated UHRF1 knockdown models in YD38 and OEC-M1 cells for subsequent functional analyses.

UHRF1 exhibits oncogenic properties in oral squamous cell carcinoma

To explore the functional role of UHRF1 in OSCC, gene silencing was carried out in YD38 and OEC-M1 cells using small interfering RNA (siRNA). Knockdown efficiency was confirmed by qPCR and Western blot analysis (Fig. 2A and B). The colony formation assay revealed a significant reduction in proliferation following UHRF1 knockdown (Fig. 2C and D). Additionally, wound-healing assays demonstrated that UHRF1 silencing impaired the migratory capacity of OSCC cells compared to control cells (Fig. 2E and F). Consistently, Transwell invasion assays showed a marked decrease in invasive potential upon UHRF1 knockdown (Fig. 2G and H)., indicating that UHRF1 contributes to OSCC cell invasiveness.

To further validate these findings, we overexpressed UHRF1 by transfecting UHRF1-encoding plasmids into YD10B and SCC25 cells, where elevated expression was confirmed (Fig. 3A and B). Enhanced UHRF1 expression significantly promoted cell proliferation, as evidenced by increased colony formation (Fig. 3C and D). Furthermore, both migration and invasion capabilities were markedly increased in UHRF1-overexpressing cells, as shown by transwell assays (Fig. 3E and F) and wound-healing (Fig. 3G and H), respectively. Collectively, these results demonstrate that UHRF1 promotes OSCC cell proliferation, migration, and invasion, supporting its role as a potential oncogenic driver in oral squamous cell carcinoma.

Cigarette smoke condensate promotes tumor growth and induces UHRF1 expression

Cigarette smoking is a well-established risk factor for oral cancer, particularly in Taiwan,[14] and previous studies have suggested a correlation between UHRF1 expression and cigarette smoking.[15] To investigate this association, we first analyzed public gene expression data from the GEO database, which revealed a significant upregulation of UHRF1 in tissue samples from current smokers compared to never smokers (Fig. 4A). To further validate this observation, we treated OSCC cells with CSC and assessed UHRF1 expression. Both qPCR and Western blot analyses confirmed that CSC treatment markedly increased UHRF1 expression levels in OSCC cells (Fig. 4B and C).

To examine the in vivo effects of CSC, we established a xenograft mouse model using SCC25 cells. Mice were divided into two groups and treated with either PBS or CSC. During the experimental period, no significant differences in body weight were observed between the two groups (Fig. 4D). However, CSC treatment significantly enhanced tumor volume compared to the PBS-treated group (Fig. 4E). Histological analysis of the xenograft tumors further supported these findings. Hematoxylin and eosin (H&E) staining revealed increased nuclear density in CSC-treated tumors. Moreover, immunohistochemical staining demonstrated elevated UHRF1 expression in tumor tissues following CSC administration (Fig. 4F). Collectively, these results suggest that cigarette smoke condensate may promote tumorigenesis in OSCC, potentially through the upregulation of UHRF1 expression.

Discussion

In this study, we provide the first investigation into the role of UHRF1 in oral squamous cell carcinoma. Our findings demonstrate that UHRF1 is significantly overexpressed in OSCC tissues and cell lines and plays a functional role in promoting tumor cell proliferation, migration, and invasion. These findings align with previous reports implicating UHRF1 in the pathogenesis of various cancers, including lung,[15] thyroid,[16] and pancreatic cancers.[17] However, the findings are primarily based on in vitro models and a xenograft mouse system, which may not fully capture the complexity of human disease. Future studies should

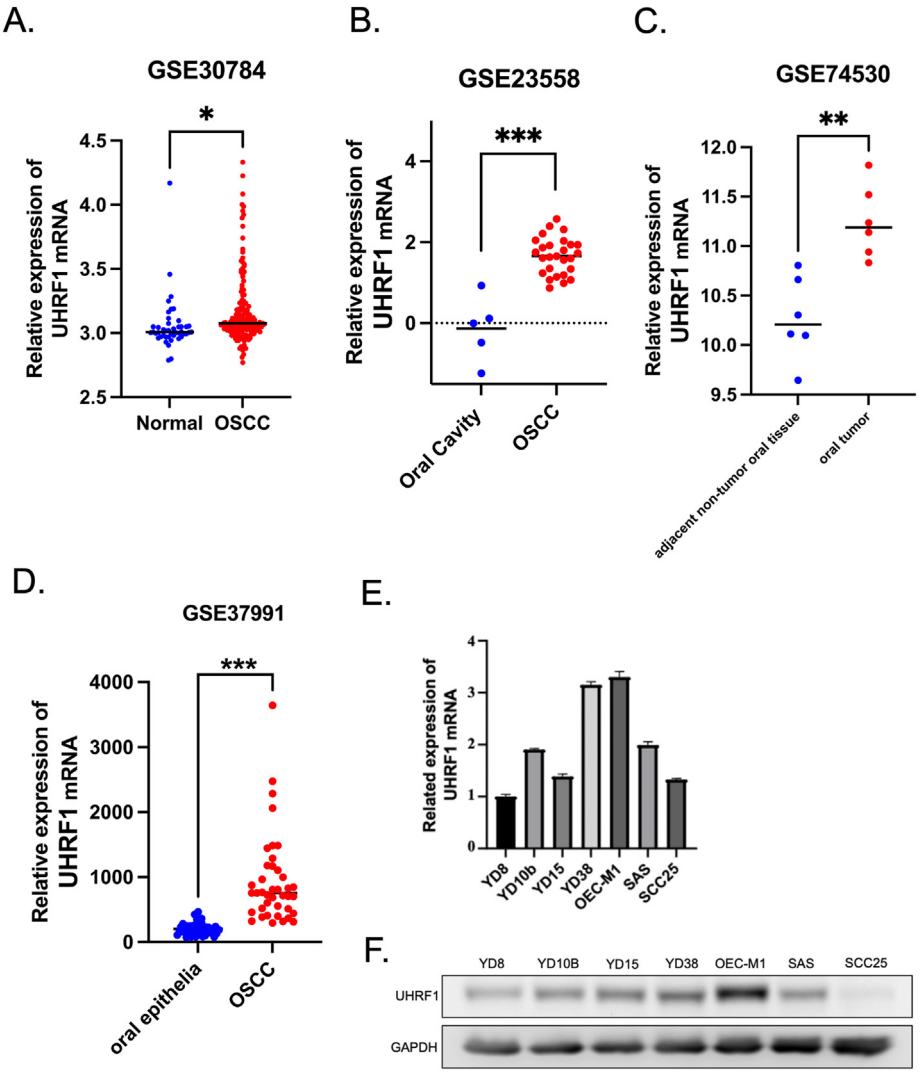

Figure 1 UHRF1 is overexpressed in oral squamous cell carcinoma (OSCC) tissues. (A—D) Gene expression analysis of UHRF1 in OSCC tissues compared to normal tissues using data from the GEO database (datasets: GSE30784, GSE23558, GSE74530, and GSE37991). (E, F) The relative expression levels of UHRF1 in multiple OSCC cell lines were evaluated by qPCR and Western blot analysis. GAPDH was used as an internal control. All data are presented as mean ± standard deviation (SD). Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. OSCC: oral squamous cell carcinoma, UHRF1: ubiquitin-like with phd and ring finger domains 1, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

incorporate analysis of human tissue samples from patients with oral potentially malignant disorders (OPMDs) and OSCC to validate the clinical significance of UHRF1 expression and its association with smoking exposure. Furthermore, correlating UHRF1 expression with clinicopathological features and patient outcomes could establish its potential as a prognostic biomarker and therapeutic target in smoking-associated oral cancer.

Given UHRF1's role in recruiting DNMT1 to maintain DNA methylation, it likely contributes to OSCC progression by facilitating the epigenetic silencing of key tumor suppressor genes (TSGs).[18] Previous studies have shown that UHRF1 mediates the promoter methylation of several well-characterized TSGs, including $p16^{INK4a}$,[19] RASSF1A,[20] and CDH1[21]—all of which are frequently silenced in various malignancies. In the context of OSCC, $p16^{INK4a}$ is of particular interest, as its promoter is often hypermethylated in the oral epithelium of smokers and OSCC patients.[22,23] This gene plays a crucial role in cell proliferation.[24] Similarly, RASSF1A promoter methylation is associated with cigarette smoking in lung cancer.[25] Knockdown of RASSF1A expression has been associated with increased proliferation and migration s in OSCC.[26] In addition, Wu et al. found a significant correlation between CDH1 promoter methylation and smoking in oral cavity cancer.[27,28] Loss of CDH1 enhancing cell motility and invasiveness.[29] These findings suggest that UHRF1 may epigenetically silence multiple tumor suppressor genes in OSCC, especially in response to cigarette smoke, contributing to disease progression.

In the present study, we observed that treatment with CSC upregulated UHRF1 expression in OSCC cells. However, the molecular mechanism underlying this effect remains unclear. Recent studies have highlighted the role of the α7-

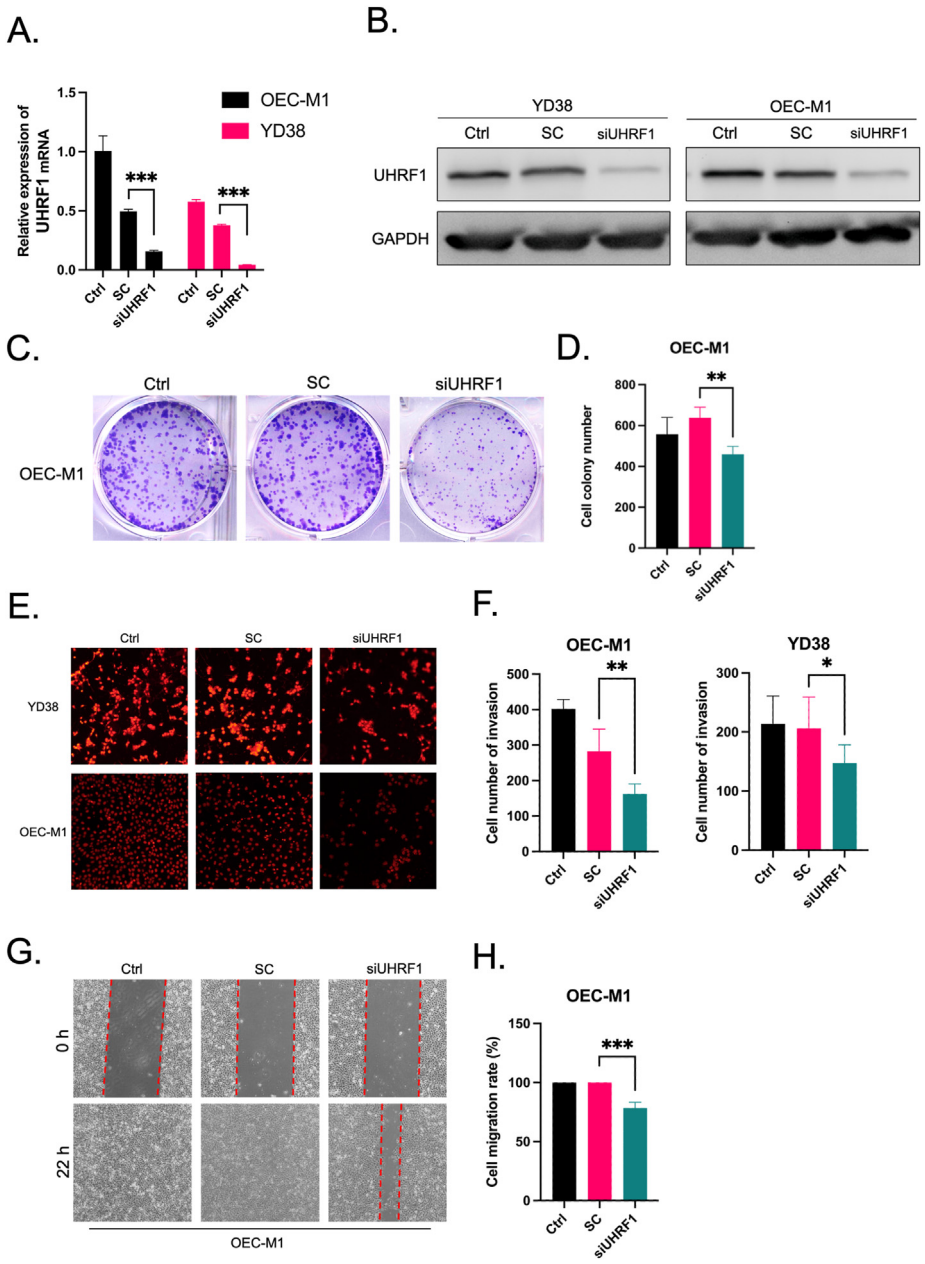

Figure 2 Effects of UHRF1 knockdown on OSCC cells. (A, B) The efficiency of UHRF1 knockdown in YD38 and OEC-M1 cells was assessed by qPCR and Western blot analysis. GAPDH was used as an internal control. (C, D) The impact of UHRF1 knockdown on cell proliferation in OEC-M1 cells was evaluated using the colony formation assay. Colony numbers per well were averaged from three independent experiments. (E, F) The effects of UHRF1 knockdown on cell invasion in YD38 and OEC-M1 cells were assessed using the Transwell invasion assay. (G, H) The influence of UHRF1 knockdown on cell migration in OEC-M1 cells was analyzed using the wound healing assay. All data are presented as mean ± standard deviation (SD). Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. UHRF1: ubiquitin-like with phd and ring finger domains 1, Ctrl: untreated control; sc: scramble control; siUHRF1: UHRF1 knockdown. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, 0 h: 0 h, 22 h: 22 h.

nicotinic acetylcholine receptor (α7-nAChR) as a critical mediator of nicotine-induced oncogenic signaling in various malignancies, including OSCC.[30] Activation of α7-nAChR by nicotine and other tobacco-related carcinogens initiates downstream signaling cascades such as the PI3K/AKT,[31] MAPK/ERK,[32] and JAK/STAT pathways,[33] which are known to regulate genes involved in cell proliferation, migration, invasion, and resistance to apoptosis. In OSCC specifically, α7-nAChR expression has been shown to be elevated, and its activation contributes to enhanced tumor cell aggressiveness, whereas pharmacological inhibition attenuates nicotine-induced malignant behaviors.[34] Given that UHRF1 transcription is influenced by oncogenic transcription factors such as E2F1[6] and c-Myc,[35] both of which are downstream effectors of α7-nAChR-mediated signaling,[36] it is plausible that CSC promotes UHRF1 expression via this

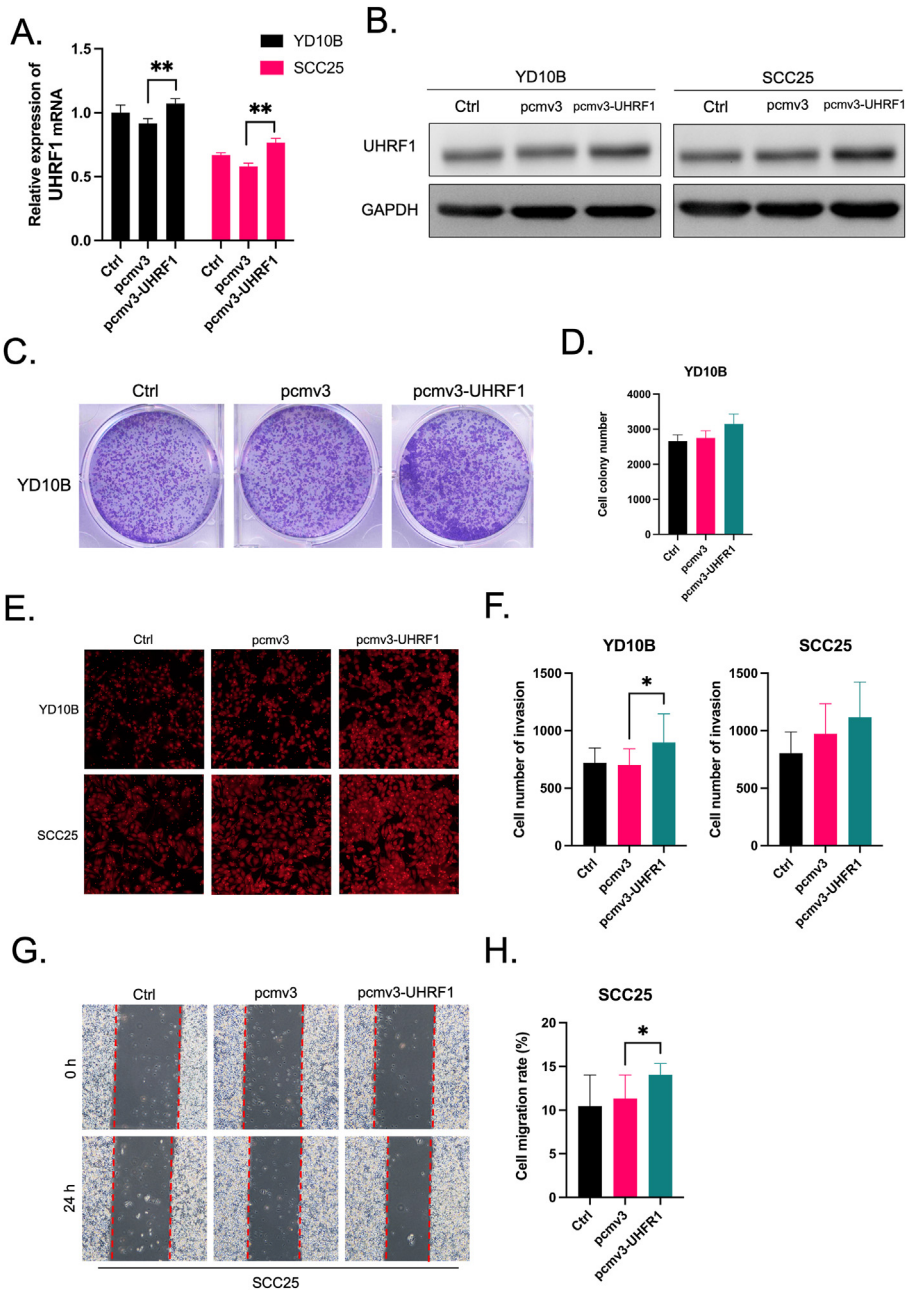

Figure 3 Effects of UHRF1 overexpression on OSCC cells. (A, B) The efficiency of UHRF1 overexpression in YD10B and SCC25 cells was assessed by qPCR and Western blot analysis. GAPDH served as the internal control. (C, D) The impact of UHRF1 overexpression on cell proliferation in YD10B cells was evaluated using the colony formation assay. Colony numbers per well were averaged from three independent experiments. (E, F) The effect of UHRF1 overexpression on cell invasion in YD10B and SCC25 cells was determined by the transwell invasion assay. (G, H) Wound healing assays were performed to assess the influence of UHRF1 overexpression on cell migration. All data are presented as mean ± standard deviation (SD). Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. UHRF1: ubiquitin-like with phd and ring finger domains 1, Ctrl: control; pcmv3: empty pcmv3 vector; pcmv3-UHRF1: UHRF1 overexpression plasmid. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, 0 h: 0 h, 24 h: 24 h.

receptor—driven axis. Moreover, α7-nAChR is implicated in promoting epigenetic remodeling and inflammatory responses,[37] which may further contribute to the upregulation and oncogenic function of UHRF1 in OSCC. These findings underscore a potential role for α7-nAChR as an upstream regulator of UHRF1 and further mechanistic studies are necessary to evaluate whether inhibition of α7-nAChR can attenuate CSC-induced UHRF1 expression and tumor progression in OSCC.

In conclusion, this study demonstrates for the first time that UHRF1 expression in OSCC tissues. In future, the study of human oral potentially disorders would determine whether UHRF1 expression also relates to smoking-induced oral squamous cell carcinogenesis. These findings highlight

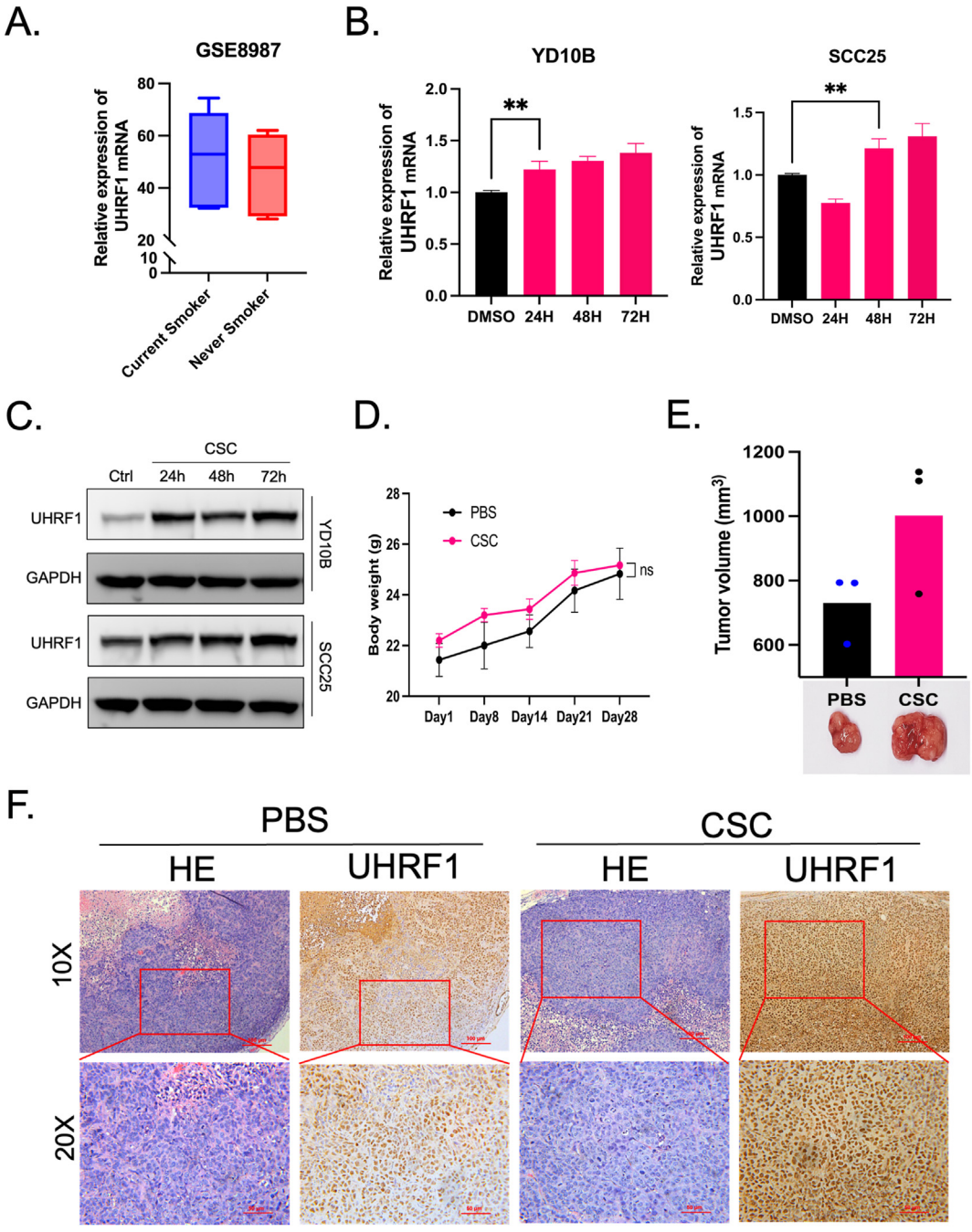

Figure 4 Cigarette smoke condensate increased the expression level of UHRF1 in vitro and in vivo. (A) Relative expression levels of UHRF1 in current smokers and never smokers were analyzed using the GEO database (GSE8987). (B, C) qPCR and Western blot analyses showing UHRF1 expression in YD10B and SCC25 cells following treatment with 125 μM of cigarette smoke condensate for 24, 48, and 72 h. GAPDH was used as an internal control. (D) Effects of CSC treatment on body weight in nude mice. (E) Representative image of xenograft tumors illustrating tumor volume in mice treated with PBS or CSC. (F) Representative H&E staining of SCC25 xenograft tumors from both groups. Immunohistochemical (IHC) detection of UHRF1 expression in tumor tissues from the two treatment groups. Scale bars: 10 × , 100 μm; 20 × , 50 μm. All data are presented as mean ± standard deviation (SD). Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. UHRF1: ubiquitin-like with phd and ring finger domains 1, DMSO: Dimethyl sulfoxide, 24 h: 24 h, 48 h: 48 h, 72 h: 72 h, Ctrl: control, CSC: cigarette smoke condensate. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, PBS: Phosphate buffered saline, H&E: hematoxylin and eosin.

UHRF1 as a potential biomarker and therapeutic target, especially in smoking-associated OSCC. Further research is needed to clarify the α7-nAChR—UHRF1 signaling axis and its therapeutic potential in oral cancer.

Declaration of competing interest

The authors declare that they have no competing interests.

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