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Original Article

E3 ligase STUB1 attenuates stemness and tumorigenicity of oral carcinoma cells via transglutaminase 2 regulation

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Background/Purpose: Oral cancer is amongst the most prevalent cancers worldwide with rising incidence. Various attempts have been made to elucidate its pathogenesis, and we sought to examine the function of a ubiquitin E3 ligase that was encoded by STUB1.
Methods: The mRNA expression of STUB1 in oral cancer samples and normal counterparts was determined by qRT-PCR. Numerous assays to assess the features of cancer cells, including self-renewal capacity, invasion and migration abilities were conducted following knockdown or overexpression of STUB1.
Results: The expression level of STUB1 was reduced in oral cancer, which was associated with a reduced relapse-free survival. Two oral cancer cell lines with low expression of STUB1 (SAS and HSC3) were chosen for the overexpression of STUB1. We showed that ectopic expression of STUB1 led to the downregulation of TGM2, a multifunctional protein that contributed to cancer progression in several cancers. Our results demonstrated that overexpression of STUB1 suppressed the cancer aggressiveness, while restoration of TGM2 reverted the effects. Last, we showed that STUB1 silencing resulted in enhanced cancer features.

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Conclusion: The abnormal downregulation of STUB1 may lessen its suppressive effect on TGM2, which induced the onset or exacerbated the progression of oral cancer. The therapeutic approach to enhance the expression of STUB1 could be a promising direction for cancer therapy.

Introduction

Oral cancer is one of the most common type of head and neck cancers worldwide, which represents around 4% of all cancer cases and is predicted to rise by 62% by 2035.[1] It has been shown that the incidence of oral cavity cancer was highest in south-central Asia, and consistently greater among men than among women.[1] One of the recent retrospective analyses has shown that the sites of predilection for oral cancer were the lateral border of tongue and floor of the mouth, and around 45% of patients who died of distant metastasis had their primary disease in the tongue.[2] Even if we have known that the major risk factors for oral cancer included tobacco smoking,[3] alcohol consumption,[4] and HR-HPV infection,[5] the prognosis of patients with recurrence was still poor.[2] Hence, more efforts were required to elucidate the pathogenic mechanism and mitigate the high burden of oral cancer.

STUB1 (STIP1 homology and U-Box containing protein 1) is a protein-coding gene that codes for carboxyl-terminus of HSP70-interacting protein (CHIP). This protein was discovered as a co-chaperone protein that interacts with Hsp70 and negatively regulated chaperone functions.[6] Another function was to serve as a ubiquitin E3 ligase to ubiquitinate and target various oral cancer-associated factors, such as p53[7] and HIF-1α[8] for subsequent degradation. It has been reported that CHIP inhibited the sphere formation capacity through degradation of CD166 in several head and neck cancer cell lines (HN13, HN30, Cal27 and UMSCC12).[9] This finding showed that CHIP may play a critical role in the development or progression of oral cancer. Therefore, we sought to examine its significance in clinical samples and functional role in the selected oral cancer cell lines.

In the current study, the expression of STUB1 was evaluated in normal and cancerous samples. We aimed to examine the significance of STUB1 in various cancer stemness features and reveal the downstream factor of STUB1. Also, we tested whether targeting STUB1 could ameliorate the characteristics of oral cancer cells.

Materials and methods

Tissues acquisition and cell culture

Oral cancer tissues (T, n = 30) and normal paired noncancerous tissues (N, n = 30) were collected after obtaining written informed consent. All procedures were conducted in accordance with the Declaration of Helsinki, and approved by The Institutional Review Board in Chung Shan Medical University Hospital (ethical approval code:CSMUH No:CS2-17016). Tumor tissues were snap frozen in liquid nitrogen and stored at −80 °C for quantitative real-time reverse transcription-PCR (qRT-PCR). The OSCC cell lines SAS, GNM, SCC9, HSC3, OECM1, FaDu cells, and normal SG cells were cultivated as previously described.[10]

Quantitative RT-PCR

Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to isolate the total RNA of cells according to the manufacturer's instruction. Superscript III first-strand synthesis system (Invitrogen Life Technologies, Carlsbad, CA, USA) was utilized to reversely transcribe the total RNAs. qRT−PCR was performed using TaqMan miRNA assays with specific primer sets (Applied Biosystems, Carlsbad, Calif). qRT-PCR reactions on resulting cDNAs were performed on an ABI StepOne™ Real-Time PCR Systems (Applied Biosystems).[10]

Western blot analysis

Western blot was conducted as previously described.[11] The sample was separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL, USA). The primary antibody against CHIP1 and TGM2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The corresponding secondary antibody was added to the membrane subsequent to the primary antibody incubation. GAPDH was used as protein loading control. The immunoreactive bands were developed using an ECL-plus chemiluminescence substrate (PerkinElmer, Waltham, MA, USA) and captured by LAS-1000 plus Luminescent Image Analyzer (GE Healthcare, Piscataway, NJ, USA).[10]

Overexpression of STUB1 and TGM2

STUB1 cDNA will be cloned into pLV-EF1a-MCS-IRES-Puro (BioSettia, Cat. No: cDNA-pLV01; San Diego, CA, USA). Lentivirus production will be performed by co-transfection of plasmid DNA mixture with vector plus helper plasmids (VSVG and Gag-Pol) into 293T cells (American Type Culture Collection, Manassas, VA) using Lipofectamine 2000 (LF2000, Invitrogen, Calsbad, CA, USA).[10]

Lentiviral-mediated STUB1 knockdown

The pLV-RNAi vector was purchased from Biosettia Inc. (Biosettia, San Diego, CA, USA). The method of cloning the

double-stranded shRNA sequence was described in the manufacturer's protocol. Oligonucleotide sequence of lentiviral vectors expressing shRNA that targets STUB1 was synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. The target sequences for are: Sh-STUB1-1: 5'- AAAAGGAGATGGAGAGCTATGATTT GGATCCAAATCATAGCTCTCCATCTCC-3'; The target sequences for are: Sh-STUB1-2: 5'- AAAAGGCCCCTATACA-TAGTTTATTGGATCCAATAAACTATGTATAGGGGCC-3'.[10]

Self-renewal assay

Cells were dissociated and cultured in the modified DMEM/F-12 supplemented with N2 (R&D Minneapolis, MN, USA), epidermal growth factor (Invitrogen, Carlsbad, CA, USA), basic fibroblast growth factor (Invitrogen, Carlsbad, CA, USA), and penicillin/streptomycin at 10^3 live cells/low-attachment six-well plate (Corning Inc., Corning, NY, USA). Medium was changed every other day until the secondary sphere formation was observed in about 2 weeks. Cell density/10,000 cells were presented as the percentage of control.[10]

Migration and invasion assays

24-well Transwell system with an 8.0 μm porous transparent polyethylene terephthalate membrane was used. Cells (1×10^5/well) were added to the upper chambers (filter coated with 1 mg/mL Matrigel for invasion assay). Medium supplemented with higher concentration of serum were added as a chemoattractant in the lower chamber. Cells that had migrated through the membrane to the lower surface were stained with crystal violet and counted from 5 different fields.[10]

Statistical analysis

Three replicates of each experiment were performed. Data were expressed as the mean ± SD and analyzed by Student's *t*-test. The result was considered to be significant if *p*-value was <0.05.

Results

First of all, the relative mRNA expression of STUB1 in the oral cancer tissues (n = 30) was determined and found abnormally lower than the normal counterparts (n = 30) (Fig. 1A). Moreover, oral cancer patients with low expression of STUB1 have reduced relapse-free survival (Fig. 1B). These findings suggested that STUB1 may be implicated in the malignant progression of oral cancer. Hence, we examined the expression of STUB1 in seven commonly used oral cancer cell lines and one normal control, including SAS (tongue squamous cell carcinoma), GNM (gingival carcinoma neck metastasis), SCC9 (tongue squamous cell carcinoma), HSC3 (tongue squamous cell carcinoma), OECM1 (oral cavity squamous cell carcinoma), FaDu (hypopharyngeal squamous cell carcinoma), SCC4 (tongue squamous cell carcinoma), and SG (normal human gingival epithelioid) cells. As shown in Fig. 2A, the gene expression of STUB1 was

downregulated in several cell lines of tongue squamous cell carcinoma (eg. SAS, HSC3, and SCC4) and GNM cells. In accordance with the results of the relative gene expression, protein expression of STUB1 in these cells was also suppressed (Fig. 2B). We then selected SAS and HSC3 for further examination as a high proportion of these two cells was from Asian genetic ancestry, while SCC4 was of European origin.

To investigate the role of STUB1 in oral cancer aggressiveness, we overexpressed STUB1 in SAS and HSC3 cells and assessed the expression of transglutaminase 2 (TGM2) since our previous report has indicated that upregulated the expression of STUB1 led to reduced TGM2 expression in oral myofibroblasts.[12] Result from Western blot showed that the expression level of TGM2 was inhibited in the STUB1-overexpressing SAS and HSC3 cells (Fig. 3A), and there was a negative correlation between STUB1 and TGM2 via analysis of the oral cancer samples in the Cancer Genome Atlas (TCGA) database (Fig. 3B). Next, we sought to evaluate whether the effect of STUB1 on the progression of oral cancer was mediated by TGM2. Our data showed that ectopic expression of STUB1 diminished the self-renewal capacity and invasion ability in both SAS and HSC3 cells (Fig. 4A and B), whereas overexpression of TGM2 reversed these effects. Altogether, these findings suggested that STUB1 promoted the cancer stemness through upregulation of TGM2.

To examine whether the downregulation of STUB1 can alleviate the cancer progression, the knockdown experiment of STUB1 was conducted in FaDu cells and the efficiency was present in Fig. 5A. As expected, the self-renewal capacity, migration, and invasion abilities were all enhanced in two FaDu cells with STUB1-shRNA (Fig. 5 B-D). Collectively, our results demonstrated that suppression of STUB1 may show the potential to delay the progression of oral cancer.

Discussion

In the present study, we showed that the expression of STUB1 in the oral cancer tissues was abnormally repressed. Additionally, the lower expression of STUB1 was associated with poorer relapse-free survival, which was consistent with a previous study reporting that the expression of CHIP in the specimen of head and neck cancer was associated with the differentiation status.[13] One of the recent studies has been revealed that CHIP suppressed the cancer stemness characteristics through targeting CD166 in head and neck cancers.[9] In fact, the clinical significance of CHIP differed according to the types of cancer. In addition to oral cancer, CHIP served as a tumor suppressor in breast cancer as well since it has been shown that low nuclear expression of CHIP correlated with poorer breast cancer-specific survival.[14] On the other hand, CHIP behaved as an oncogene in colorectal cancer and the expression of CHIP was positively correlated with lymph nodes invasion and TNM stage.[15] This inconsistency may result from the diverse targets of CHIP in various types of cells. For instance, CHIP functioned as an oncogene in colorectal and thyroid cancers via activation of MAPK and AKT pathways.[16] Nevertheless, CHIP inactivated
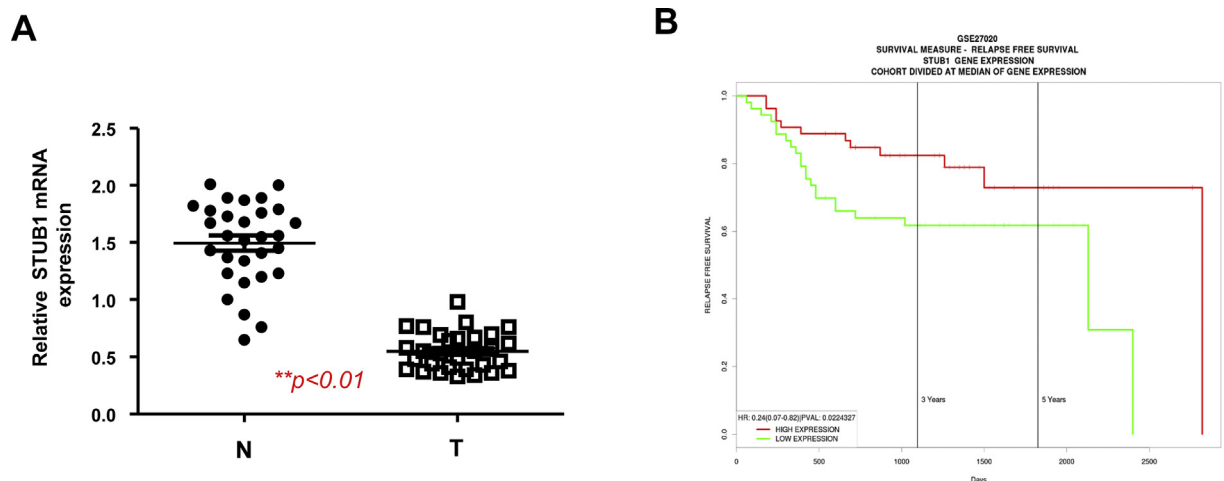
Figure 1 STUB1 is downregulated in oral cancer tissues and associated with relapse-free survival. (A)The relative expression level of STUB1 in oral cancer (T) and normal counterparts (N) (n = 30) was determined by qRT-PCR; (B) Relapse-free survival is estimated by the method of Kaplan–Meier.
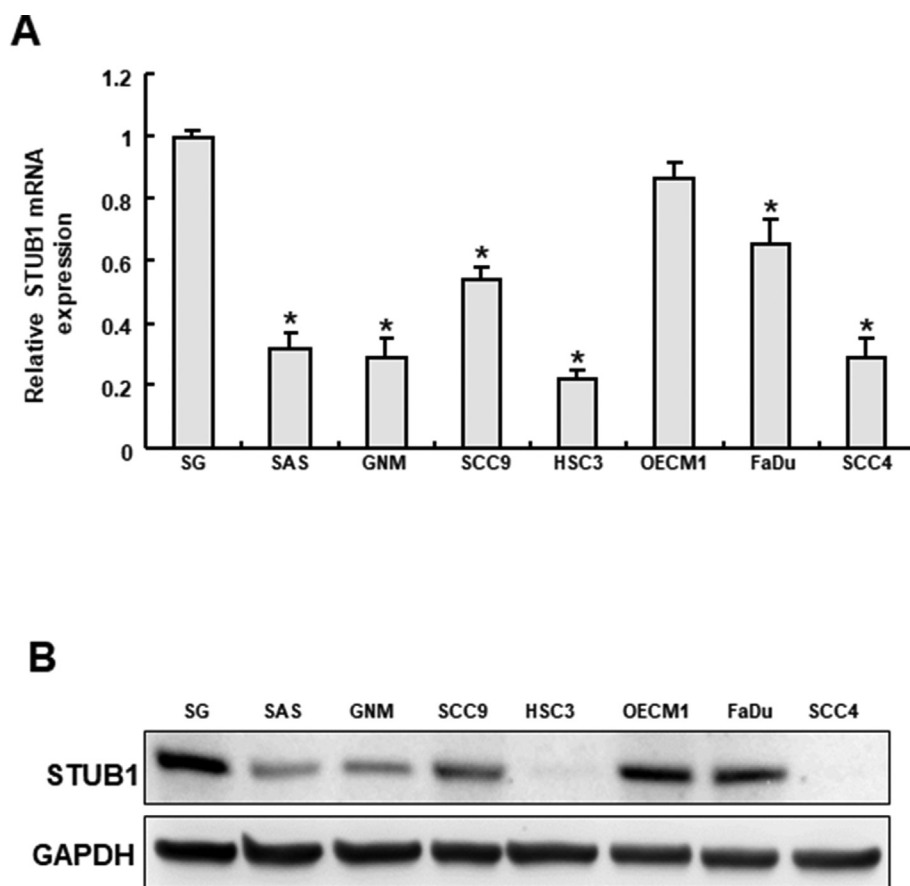

Figure 2 Expression of STUB1 in various oral cancer cell lines. The transcripts and protein expression levels of STUB1 in the selected oral cancer cell lines and normal gingival epithelioid S-G cell line were determined by qRT-PCR (A) and Western blot analysis (B). *$p < 0.05$.
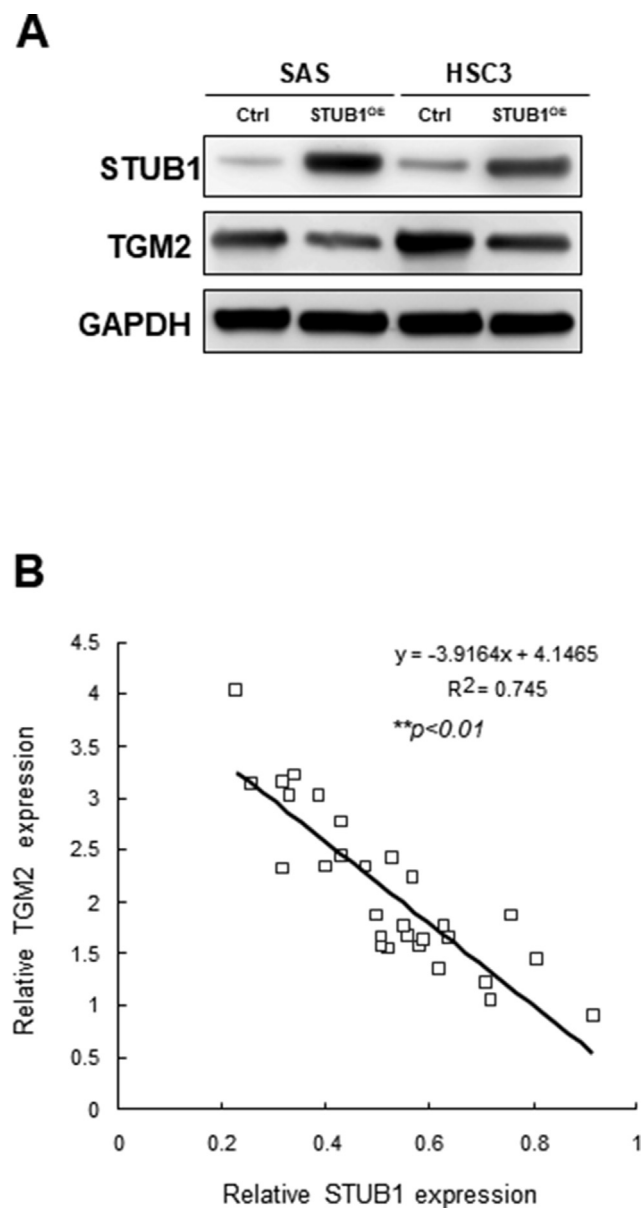
A

B

Figure 3 Overexpression of STUB1 downregulated the expression of TGM2. (A) The expression level of TGM2 was reduced in the STUB1-overexpressing SAS and HSC3 cells; (B) The expression of STUB1 is reversely correlated with TGM2 using the Pearson and Spearman's Correlation analyses from the oral cavity cancer samples retrieved from The Cancer Genome Atlas (TCGA) database.

A

B

Figure 4 STUB1 affects the phenotypes of oral cancer through modulation of TGM2. The self-renewal (A) and invasion (B) capacities were both downregulated in cells (both SAS and HSC3) with STUB1 overexpression. Nevertheless, the ectopic expression of TGM2 reversed these effects. Results were presented as the percentage of control. *$p < 0.05$ compared with control. #$p < 0.05$ compared with STUB1 overexpressing (STUB1^{OE}) cells.

NF-κB and induced TNF receptor-associated factor 2 (TRAF2) degradation in breast cancer to inhibit the cancer invasion.[17] In this work, we demonstrated that CHIP exerted its anti-cancer property through the upregulation of TGM2.

TGM2 is a multifunctional protein that participated in various cellular events. This calcium-dependent cross-linking enzyme post-translationally catalyzed protein m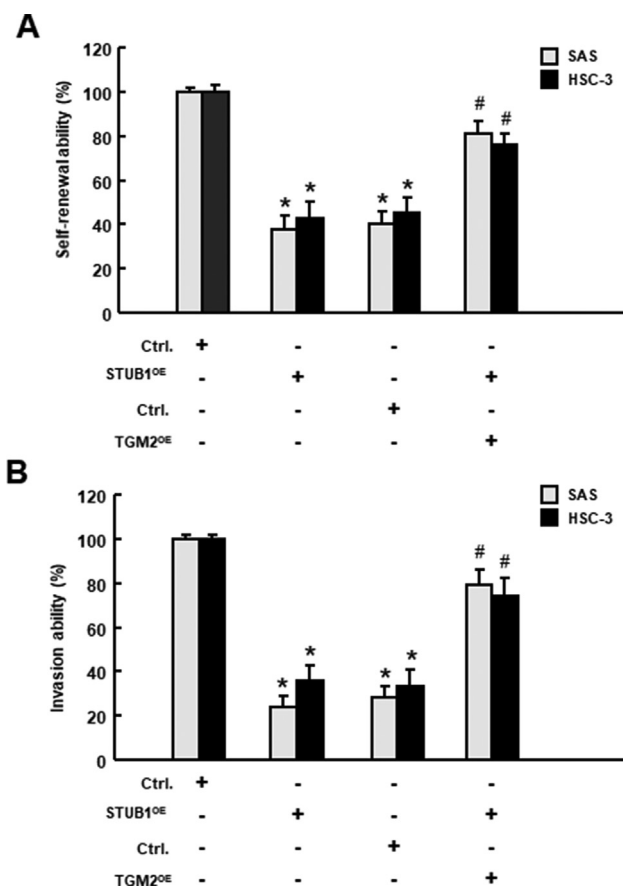odifications and was the most commonly expressed member of the transglutaminase family.[18] It has been reported that the expression of TGM2 was overexpressed in oral cancer tissues, and was higher in lymph node metastases than in the corresponding primary tumors.[19] In breast cancer, the TGM2/integrinβ/fibronectin signaling has been considered to be implicated in the increased survival, invasion and motility.[20] Another study showed that TGM2 activated the PI3K/Akt pathway, upregulated NF-κB activity, and inactivated GSK3β in A431 tumor cells (an epidermoid carcinoma in the skin), which led to an increase in Snail and MMP-9 as well as higher cell motility.[21] TGM2 was also required for cell migration, invasion and wound closure in epidermal squamous cell carcinoma and the GTP binding function of TGM2 was found essential for spheroid formation.[22] Besides, TGM2 has been shown to form a complex with fibronectin, which was implicated in the regulation of spheroids proliferation through Wnt signaling and tumor initiating capacity of ovarian cancer stem cells.[23] It has
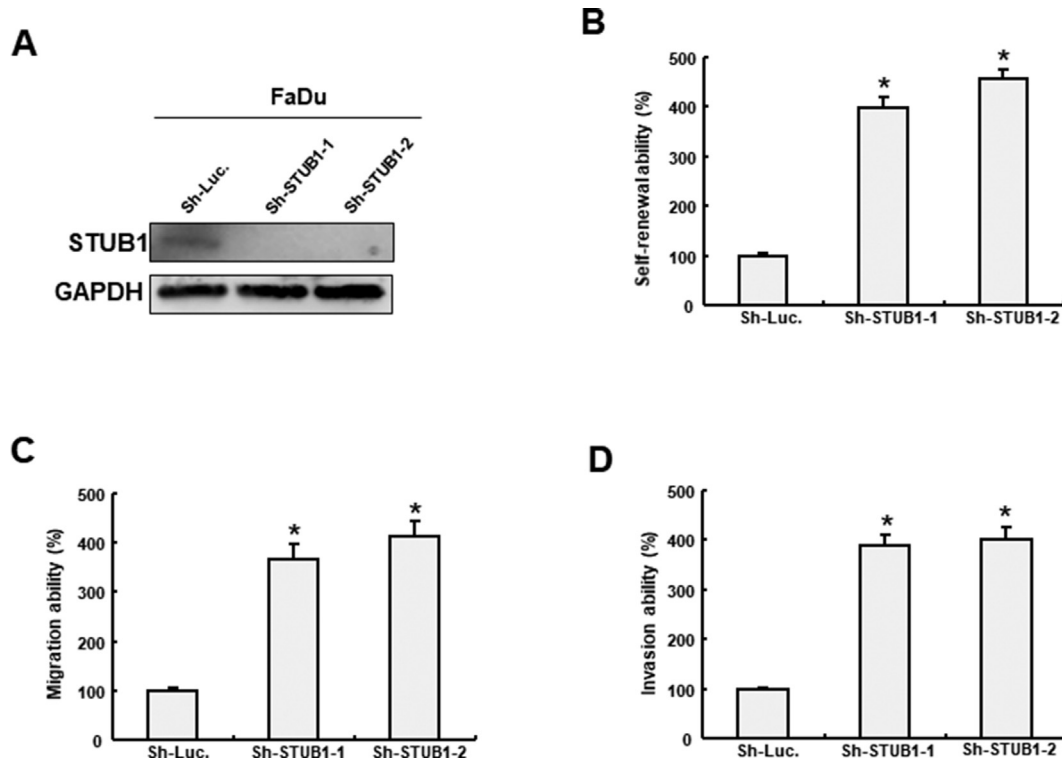
Figure 5 The silence of STUB1 induces the characteristics of oral cancer. (A) Knockdown efficiency in cells transfected with control Sh-Luc, ShSTUB-1, and ShSTUB1-2 was examined using Western blot; (B) Self-renewal, (C) migration and (C) invasion capacities were evaluated. Results were presented as the percentage of control. *$p < 0.05$ compared with Sh-Luc.

been revealed that the GTP binding activity of TGM2 rather than its transamidase activity was required for TGM2-dependent cancer stem cell invasion, migration and tumour formation.[22] In line with these findings, we demonstrated that overexpression of TGM2 elicited the increased self-renewal and invasion abilities. Moreover, we showed that the overexpression of TGM2 in oral cancer may be due to the repressed expression of STUB1, which was supported by a previous study showing that CHIP promoted the ubiquitination and proteasomal degradation of TGM2.[24] It has been revealed that CHIP was an YAP1-interacting ubiquitinase that destabilized YAP1,[25] and the dysregulation of YAP1 has been known to contribute to the onset of oral cancer via driving cell proliferation, survival, and migration.[26] Aside from YAP1, we demonstrated that loss of STUB1 promoted oral tumorigenesis through the upregulation of TGM2 as well.

In conclusion, the current data suggested that lower expression of STUB1 was associated with a poorer prognosis. A lack of tumor-suppressive effect from STUB1 may result in overexpression of TGM2, which led to the increased self-renewal and invasion abilities. Approaches to restoring STUB1 may be potential therapeutic interventions for oral cancer.

Declaration of Competing Interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

References

1. Shield KD, Ferlay J, Jemal A, Sankaranarayanan R, Chaturvedi AK, Bray F, et al. The global incidence of lip, oral cavity, and pharyngeal cancers by subsite in 2012. *Ca - Cancer J Clin* 2017;**67**:51−64.
2. Jerjes W, Upile T, Petrie A, Riskalla A, Hamdoon Z, Vourvachis M, et al. Clinicopathological parameters, recurrence, locoregional and distant metastasis in 115 T1-T2 oral squamous cell carcinoma patients. *Head Neck Oncol* 2010;**2**:9.
3. Wyss A, Hashibe M, Chuang SC, Lee YCA, Zhang ZF, Yu GP, et al. Cigarette, cigar, and pipe smoking and the risk of head and neck cancers: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Am J Epidemiol* 2013;**178**:679−90.
4. Marziliano A, Teckie S, Diefenbach MA. Alcohol-related head and neck cancer: summary of the literature. *Head Neck* 2020;**42**:732−8.
5. Combes JD, Franceschi S. Role of human papillomavirus in non-oropharyngeal head and neck cancers. *Oral Oncol* 2014;**50**:370−9.
6. Ballinger CA, Connell P, Wu Y, Hu Z, Thompson LJ, Yin LY, et al. Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins

and negatively regulates chaperone functions. *Mol Cell Biol* 1999;**19**:4535—45.

7. Muller P, Hrstka R, Coomber D, Lane DP, Vojtesek B. Chaperone-dependent stabilization and degradation of p53 mutants. *Oncogene* 2008;**27**:3371—83.

8. Bento CF, Fernandes R, Ramalho J, Marques C, Shang F, Taylor A, et al. The chaperone-dependent ubiquitin ligase CHIP targets HIF-1alpha for degradation in the presence of methylglyoxal. *PloS One* 2010;**5**:e15062.

9. Xiao M, Yan M, Zhang J, Xu Q, Qi S, Wang X, et al. Cancer stem-like cell related protein CD166 degrades through E3 ubiquitin ligase CHIP in head and neck cancer. *Exp Cell Res* 2017;**353**: 46—53.

10. Chou MY, Hu FW, Yu CH, Yu CC. Sox2 expression involvement in the oncogenicity and radiochemoresistance of oral cancer stem cells. *Oral Oncol* 2015;**51**:31—9.

11. Lin CY, Liao YW, Hsieh PL, Lu MY, Peng CY, Chu PM, et al. LncRNA GAS5-AS1 inhibits myofibroblasts activities in oral submucous fibrosis. *J Formos Med Assoc* 2018;**117**:727—33.

12. Chen SC, Liu CM, Hsieh PL, Liao YW, Lin YJ, Yu CC, et al. E3 ligase carboxyl-terminus of Hsp70-interacting protein (CHIP) suppresses fibrotic properties in oral mucosa. *J Formos Med Assoc* 2020;**119**:595—600.

13. Xiao M, Yan M, Zhang J, Xu Q, Chen W. Carboxy-terminus Hsc70 interacting protein exerts a tumor inhibition function in head and neck cancer. *Oncol Rep* 2017;**38**:1629—36.

14. Luan H, Mohapatra B, Bielecki TA, Mushtaq I, Mirza S, Jennings TA, et al. Loss of the nuclear pool of ubiquitin ligase CHIP/STUB1 in breast cancer unleashes the MZF1-cathepsin pro-oncogenic program. *Canc Res* 2018;**78**:2524—35.

15. Xu J, Zhou J, Dai H, Liu F, Li W, Wang W, et al. CHIP functions as an oncogene by promoting colorectal cancer metastasis via activation of MAPK and AKT signaling and suppression of E-cadherin. *J Transl Med* 2018;**16**:169.

16. Zhang L, Liu L, He X, Shen Y, Liu X, Wei J, et al. CHIP promotes thyroid cancer proliferation via activation of the MAPK and AKT pathways. *Biochem Biophys Res Commun* 2016;**477**:356—62.

17. Jang KW, Lee KH, Kim SH, Jin T, Choi EY, Jeon HJ, et al. Ubiquitin ligase CHIP induces TRAF2 proteasomal degradation and NF-kappaB inactivation to regulate breast cancer cell invasion. *J Cell Biochem* 2011;**112**:3612—20.

18. Fesus L, Piacentini M. Transglutaminase 2: an enigmatic enzyme with diverse functions. *Trends Biochem Sci* 2002;**27**: 534—9.

19. Caffarel MM, Chattopadhyay A, Araujo AM, Bauer J, Scarpini CG, Coleman N. Tissue transglutaminase mediates the pro-malignant effects of oncostatin M receptor over-expression in cervical squamous cell carcinoma. *J Pathol* 2013;**231**: 168—79.

20. Mangala LS, Fok JY, Zorrilla-Calancha IR, Verma A, Mehta K. Tissue transglutaminase expression promotes cell attachment, invasion and survival in breast cancer cells. *Oncogene* 2007;**26**: 2459—70.

21. Lin CY, Tsai PH, Kandaswami CC, Chang GD, Cheng CH, Huang CJ, et al. Role of tissue transglutaminase 2 in the acquisition of a mesenchymal-like phenotype in highly invasive A431 tumor cells. *Mol Canc* 2011;**10**:87.

22. Fisher ML, Keillor JW, Xu W, Eckert RL, Kerr C. Transglutaminase is required for epidermal squamous cell carcinoma stem cell survival. *Mol Canc Res* 2015;**13**:1083—94.

23. Condello S, Sima L, Ivan C, Cardenas H, Schiltz G, Mishra RK, et al. Tissue tranglutaminase regulates interactions between ovarian cancer stem cells and the tumor niche. *Canc Res* 2018; **78**:2990—3001.

24. Min B, Park H, Lee S, Li Y, Choi JM, Lee JY, et al. CHIP-mediated degradation of transglutaminase 2 negatively regulates tumor growth and angiogenesis in renal cancer. *Oncogene* 2016;**35**:3718—28.

25. Tang DE, Dai Y, Lin LW, Xu Y, Liu DZ, Hong XP, et al. STUB1 suppresseses tumorigenesis and chemoresistance through antagonizing YAP1 signaling. *Canc Sci* 2019;**110**:3145—56.

26. Hiemer SE, Zhang L, Kartha VK, Packer TS, Almershed M, Noonan V, et al. A YAP/TAZ-Regulated molecular signature is associated with oral squamous cell carcinoma. *Mol Canc Res* 2015;**13**:957—68.