NUPR1 promotes the proliferation and metastasis of oral squamous cell carcinoma cells by activating TFE3-dependent autophagy

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Oral squamous cell carcinoma (OSCC) is the most common type of oral malignancy, and metastasis accounts for the poor prognosis of OSCC. Autophagy is considered to facilitate OSCC development by mitigating various cellular stresses; nevertheless, the mechanisms of autophagy in OSCC cell proliferation and metastasis remain unknown. In our study, high-sensitivity label-free quantitative proteomics analysis revealed nuclear protein 1 (NUPR1) as the most significantly upregulated protein in formalin-fixed paraffin-embedded tumour samples derived from OSCC patients with or without lymphatic metastasis. Moreover, NUPR1 is aberrantly expressed in the OSCC tissues and predicts low overall survival rates for OSCC patients. Notably, based on tandem mass tag-based quantitative proteomic analysis between stable NUPR1 knockdown OSCC cells and scrambled control OSCC cells, we confirmed that NUPR1 maintained autophagic flux and lysosomal functions by directly increasing transcription factor E3 (TFE3) activity, which promoted OSCC cell proliferation and metastasis in vitro and in vivo. Collectively, our data revealed that the NUPR1–TFE3 axis is a critical regulator of the autophagic machinery in OSCC progression, and this study may provide a potential therapeutic target for the treatment of OSCC.

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common form of head and neck neoplasms1,2 and it has high prevalence rate, accounting for estimated 377,713 new global cases in 2020.3,4 Although surgical resection combined with chemotherapy and radiation has been applied in many cases, the overall 5-year survival rate of OSCC patients has not exceeded 50% in recent 20 years, without any obvious developments.5,6 In particular, metastasis is one of the deadliest aspects of oral cancer and can facilitate the dissemination of cancer cells to remote body locations, such as the lung, and the majority of metastatic OSCC patients die within one year.7 Thus, the underlying molecular mechanisms of OSCC proliferation and metastasis could provide vital information for the development of new therapeutic methods to prevent OSCC progression.

Autophagy is a dynamic recycling system that provides internal constituents and energy for cellular homeostasis and renovation under stressful conditions such as the tumour microenvironment.8,9 Some researchers have linked autophagy to the inhibition of OSCC progression; however, other studies have demonstrated that autophagy is positively correlated with OSCC tumorigenesis and development. Tang et al. reported that OSCC had a high degree of autophagy activity, and dual expression of tumour autophagy protein 5 (ATG5) and Beclin-1 (BECN1) expression served as a poor prognostic indicator for OSCC.10 Moreover, in our previous report, we confirmed that OSCC tissues had higher autophagic activity than paracancerous tissues.11 Although upregulated autophagic activity may promote OSCC progression, the underlying mechanism of autophagy in OSCC is still unclear, which limits the targeting of autophagy as a treatment for OSCC.

Nuclear protein 1 (NUPR1) is strongly induced by several types of cellular stress and involves in the chromatin remodelling, cell cycle, and apoptosis.12 Importantly, NUPR1 has been demonstrated to facilitate the progression and promotes the metastasis...
of many malignancies, such as breast cancer, pancreatic adenocarcinoma, hepatocellular cancer and thyroid cancer.\textsuperscript{12–14} Additionally, emerging evidences have demonstrated that NUPR1 could be taken as an autophagic flux master. NUPR1 maintained autophagy process in lung cancer;\textsuperscript{15} moreover, NUPR1 silencing decreased autophagy, leading to the accumulation of sequestosome 1 (SQSTM1) and facilitating multiple myeloma cells death.\textsuperscript{16} Our previous study reported that xenobiotic (e.g., Cd) exposure enhanced NUPR1 expression and initiated autophagy, which led to the OSCC progression.\textsuperscript{17} These reports suggest that NUPR1 may upregulate autophagic flux and drive OSCC metastasis, but the molecular mechanism remains poorly understood.

Transcription factor E3 (TFE3) has been identified as a powerful regulator that controls the autophagic flux-related genes expression in various cancers. Perera \textit{et al.} reported that TFE3 enhanced lysosome biogenesis and function and augmented autophagy, which promoted the pathological process of pancreatic ductal adenocarcinoma.\textsuperscript{18} Moreover, our previous report and other study also confirmed that TFE3, which led to autophagic flux enhancement, was positively correlated with the development and poor prognosis of OSCC as well as breast cancer.\textsuperscript{12,19}

In this study, high-sensitivity quantitative label-free quantitative proteomics analysis of formalin-fixed, paraffin-embedded (FFPE) tumour samples was combined with tandem mass tag (TMT) proteomic analysis of the NUPR1 knockdown (KD) OSCC cell line to determine the possible mechanisms of autophagy involved in OSCC proliferation and metastasis. We revealed and confirmed that NUPR1 maintained autophagic flux by activating TFE3 transcription during OSCC progression. Therefore, understanding the NUPR1-TFE3-mediated autophagy process reveals potential new avenues for pharmacological therapies targeting OSCC progression.

\textbf{RESULTS}

\textbf{NUPR1 may be a vital protein involved in OSCC progression}

We first performed high-sensitivity label-free quantitative proteomics analysis of FFPE tumour samples derived from OSCC patients with or without lymphatic metastasis (Fig. S1a). According to the quantification results with MaxQuant software, we identified and quantified 3021 proteins. The differentially expressed proteins (DEPs, \(P\ value <0.05\) and a ratio-fold change \(>1.5\) or \(\leq 0.67\) were clustered through unsupervised hierarchical clustering analysis, which showed the gathering characteristics and proteomic diversity between the two groups (Supplementary Fig. S1b). Compared with samples from OSCC patients without lymph node metastasis (LNM), those from patients with LNM exhibited 208 upregulated proteins and 165 downregulated proteins (Supplementary Fig. S1c). Furthermore, NUPR1 expression was notably upregulated in the LNM group compared with the non-LNM group (top five upregulated proteins) (Supplementary Table S1).

NUPR1 was positively correlated with OSCC progression and adverse prognosis in patients

To further validate the proteomic findings in a larger patient cohort, the protein expression levels of NUPR1 were detected using our OSCC TMA with IHC. The results showed that NUPR1 expression was remarkably increased in OSCC tissues (\(n=88\)) versus normal mucosa tissues (\(n=20\)) (Fig. 1a, c). We further explored the relationship between the expression level of NUPR1 (high or low) and several clinicopathological characteristics of OSCC patients (Supplementary Table S2). NUPR1 expression was categorised based on the median value according to immunohistochemistry (IHC) scores for 88 OSCC tissues. High expression of NUPR1 was statistically associated with pathological differentiation grade (low, \(P=0.000\)), TNM stage (III/IV, \(P=0.008\)) and lymphatic metastasis (N\(^+\), \(P=0.000\)). The expression of NUPR1 positively correlated with TNM stage and lymphatic metastasis (\(n=47, P<0.05\). Figs. 1b, d, e), suggesting that NUPR1 is positively related to OSCC metastasis. Moreover, according to Kaplan–Meier survival analysis and the log-rank test, the overall survival rate was lower for OSCC patients with high NUPR1 expression levels (\(P=0.014\), Fig. 1f).

\textbf{NUPR1 knockdown (KD) inhibited the OSCC progression}

To clarify the role of NUPR1 in OSCC progression, we firstly determined NUPR1 expression in six OSCC cell lines, and we confirmed that the expressions of NUPR1 in Cal27 and HN6 were higher than other cell lines (Supplementary Fig. S2a). Then Cal27 and HN6 cells were stably transfected with lentivirus-containing negative control or shRNA-NUPR1 (Supplementary Fig. S2b). A colony formation assay revealed that NUPR1 KD significantly decreased the colony formation efficiency of Cal27 and HN6 cells (Fig. 1g). In addition, wound healing and Matrigel-coated Transwell assays demonstrated that the migration and invasion abilities of NUPR1-depleted OSCC cells were markedly suppressed (Fig. 2h–j). Overall, NUPR1 KD effectively suppressed the OSCC progression.

Autophagy plays a crucial role in NUPR1-mediated OSCC progression

To clarify the underlying mechanism of NUPR1 in OSCC progression, we detected the protein profile changes between NUPR1-depleted Cal27 cells and scrambled control cells through TMT quantitative proteomic analysis. The levels of 27 proteins were significantly upregulated and the levels of 28 proteins were remarkably downregulated in the NUPR1-depleted OSCC group compared to the scrambled group (Supplementary Fig. S3a). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated that the autophagy pathway was markedly enriched in the NUPR1 KD group compared to the scrambled group (Supplementary Fig. S3b). These results demonstrated that autophagy may play an essential role in NUPR1-mediated OSCC progression.

\textbf{NUPR1 KD blocked autophagic flux in OSCC cells}

Next, we further investigated whether NUPR1 KD affected autophagic flux in OSCC cells. The MAP1LC3B-I protein plays an essential role in autophagy and is a well-admitted autophagy marker that normally resides in the cytosol, but upon induction of autophagy, it becomes lipidated and embedded in autophagosome membranes (forming the MAP1LC3B-II isoform).\textsuperscript{20} Immunoblotting analysis showed that NUPR1 KD increased the protein expression levels of MAP1LC3B-II (Fig. 2a, b). Additionally, SQSTM1 binds MAP1LC3B-II for cargo recruitment and degradation when it accumulates,\textsuperscript{21} was also apparently elevated in Cal27 and HN6 cells (Fig. 2a, b). Autophagy involves multiple steps, and autophagosome accumulation might occur on account of upregulated autophagy activity or decreased autophagosome turnover. NUPR1 KD led to increased levels of MAP1LC3B-II and expression of SQSTM1, which seems to imply that NUPR1 KD impaired OSCC cell autophagic flux. Chloroquine (CQ) inhibits lysosome activity and disturbs the fusion between autophagosomes and lysosomes to inhibit autophagy,\textsuperscript{22} however, the NUPR1 KD-induced increase in MAP1LC3B-II expression was not affected by cotreatment with CQ (Fig. 2c, d). These results indicated that NUPR1 KD inhibits autophagic flux in OSCC cells.

\textbf{NUPR1 KD did not restrain autophagosome formation or maturation in OSCC cells}

Since autophagy serves as a dynamic recycling system and has a multistep process, we investigated autophagy inhibition induced by NUPR1 KD in the early or late stage. ATG5 conjugated with ATG12 and PIK3C3/VPS34 kinase activity is the requirement of phagophore formation in cells.\textsuperscript{23} We transfected Cal27 and HN6 cells with an ATG5 shRNA plasmid in the presence or absence of
NUPR1 KD and observed a change in GFP-LC3 puncta, which represent phagophores. As shown in Supplementary Fig. S4a, b, ATG5 shRNA attenuated the increase in GFP-LC3 puncta induced by NUPR1 KD in Cal27 and HN6 cells. Similarly, ATG5 KD reduced MAP1LC3B-II accumulation under NUPR1 depletion conditions, indicating that NUPR1 KD does not affect phagophore formation (Supplementary Fig. S4c, d). Polyubiquitinated protein aggregates, whose formation is mediated by SQSTM1, a cargo incorporation of autophagosomes, are crucial components of mature autophagosomes.24 We evaluated autophagosome maturation by assessing
NUPR1 promotes the proliferation and metastasis of oral squamous cell...

Fan et al.

**Fig. 1** NUPR1 is correlated with OSCC progression. a Representative images of NUPR1 expression in normal oral mucosa (left) and OSCC (right) by IHC staining; scale bars = 100 μm. b Representative images of NUPR1 expression in non-LNM (left) and LNM (right) OSCC samples by IHC staining; scale bars = 100 μm. c NUPR1 expression was substantially upregulated in OSCC tissues \( (n = 88) \) compared with normal mucosa tissues \( (n = 20) \). d Scale bar indicates the expression level of TFE3, as shown by the qPCR analysis. NUPR1 was reported as an important transcription factor that regulates gene transcription. As shown in Fig. 1e, f, TFE3 promoter activity was distinctly increased in OSCC cells treated with the autophagy agonist rapamycin (0.1 μM) for 24 h but was substantially inhibited in NUPR1-depleted OSCC cells compared to control cells. Together, these results demonstrated that NUPR1 maintains autophagic flux by increasing TFE3 promoter activity and activating TFE3 transcription during OSCC progression. Moreover, correlation and simple linear regression of NUPR1 expressions with TFE3 expressions in 88 samples were analysed and calculated, and a positive correlation between NUPR1 and TFE3 was identified (Supplementary Fig. S6).

We further overexpressed TFE3 in OSCC cells with or without NUPR1 KD. DQ™ Red BSA and LysoSensor Green DND-189 dye detection assays showed that overexpression of TFE3 could significantly rescue the damage to lysosome function caused by NUPR1 KD in Cal27 and HN6 cells (Fig. 4a-d). Moreover, MAP1LC3B-II and SQSTM1 accumulation was also markedly decreased with TFE3 overexpression in NUPR1-depleted OSCC cells (Fig. 4e, f).

NUPR1 promoted OSCC progression by activating TFE3-dependent autophagy in vitro and in vivo

We then performed TFE3 overexpression to observe the change in the progression abilities of NUPR1-deficient OSCC cells in vitro. Plate colony formation assays and wound healing and Matrigel invasion assays were performed to detect the change of NUPR1 KD in Cal27 and HN6 cells (Fig. 5a–d). The invasion results for NUPR1 KD or scrambled Cal27 and HN6 cells at 48 h. Scale bar = 80 μm; magnification, 40×; \( n = 4 \); ** \( P < 0.01 \) vs. the scrambled group.

To elucidate the underlying mechanism of NUPR1-mediated autophagy-lysosomal processes, we next analysed the DEPs through proteomic analysis. Interestingly, NUPR1 KD markedly decreased the expression level of TFE3, as shown by the differentially expressed proteins (Supplementary Table S3). Indeed, TFE3 was markedly downregulated in NUPR1-depleted OSCC cells (Fig. 3a, b). More importantly, we confirmed the expression changes in "TFE3-responsive genes" involved in autophagic flux by real-time PCR (Fig. 3c, d), suggesting that NUPR1 plays a crucial role in autolysosomal events and may be related to TFE3. We performed luciferase reporter assays to detect the relationship between NUPR1 and TFE3. Recently, NUPR1 was reported as a pivotal protein that was remarkably involved in autophagic-lysosomal processing, we next analysed the DEPs through proteomic analysis. Interestingly, NUPR1 was reported as an important transcription factor that regulates gene transcription. As shown in Fig. 3e, f, TFE3 promoter activity was distinctly increased in OSCC cells treated with the autophagy agonist rapamycin (0.1 μM) for 24 h but was substantially inhibited in NUPR1-depleted OSCC cells compared to control cells. Together, these results demonstrated that NUPR1 maintains autophagic flux by increasing TFE3 promoter activity and activating TFE3 transcription during OSCC progression. Moreover, correlation and simple linear regression of NUPR1 expressions with TFE3 expressions in 88 samples were analysed and calculated, and a positive correlation between NUPR1 and TFE3 was identified (Supplementary Fig. S6).

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**DISCUSSION**

The high metastasis and recurrence rates, which are associated with a high mortality rate, are some of the main features of OSCC noted recently; due to a lack of powerful treatment strategies, this cancer presents a significant burden to human health.\(^{1,2}\) Seeking the key molecules that regulate the recurrence and metastasis of OSCC is an important direction and prerequisite for developing effective therapeutic drugs. In this study, we first demonstrated (i) NUPR1 was identified as a pivotal protein that was remarkably augmented in LNM patients and positively related to OSCC metastasis and poor prognosis with the high-sensitivity quantita-

GFP-LC3 colocalization with SQSTM1. Compared with the scrambled group, the NUPR1 KD group showed an augmented colocalization coefficient of GFP-LC3 and SQSTM1 in Cal27 and HN6 cells (Supplementary Fig. S4a–c). Together, these results indicated that NUPR1 KD does not facilitate the cargo incorporation or maturation of autophagosomes in OSCC cells.

**NUPR1 KD had no effect on autophagosome-lysosome fusion in OSCC cells**

The fusion of autophagosomes with lysosomes is a fundamental process of autophagic degradation. As shown in Supplementary Fig. S5a–d, the ratio of colocalization between the autophagosomal marker GFP-LC3 and the lysosomal marker LAMP2 was not affected by NUPR1 KD in Cal27 and HN6 cells. The RFP-GFP-LC3B kit can be used as an autophagy sensor to monitor fusion. GFP fluorescence is pH sensitive and is quenched in an acidic environment, but RFP fluorescence is not sensitive to changes in pH. Thus, yellow puncta indicate autophagosomes before fusion, and red puncta indicate complete autophagosome fusion.\(^{25}\) As shown in Supplementary Fig. S5e–h, NUPR1 KD remarkably augmented the ratio of yellow to red puncta; if fusion indeed occurred, lysosomes may exhibit an abnormal pH environment or activities.

**NUPR1 KD inhibited the functions of lysosomes in OSCC cells**

To investigate whether NUPR1 KD inhibited lysosomal functions, the expression levels of lysosome markers located on the surface of the lysosomal membrane, LAMP1 and LAMP2, were examined. The results indicated that NUPR1 KD inhibited the expression of LAMP1 but not that of LAMP2 in Cal27 and HN6 cells (Fig. 2e, f). The fluorescence intensity of DG™ Red BSA is positively associated with proteolytic activity based on the proteolysis of BSA conjugates, which aggregates in acidic organelles, which contain lysosomes, and serves as a pH indicator with an increase in fluorescence intensity dependent on acidification.\(^{25}\) NUPR1 KD decreased the fluorescence intensity of LysoSensor Green DND-189 in Cal27 and HN6 cells (Fig. 2h). Together, these results demonstrated that impairment of autophagic flux by NUPR1 KD may be mediated by the suppression of lysosomal functions in OSCC cells.

TFE3 is responsible for NUPR1-mediated autolysosomal processes in OSCC cells

To elucidate the underlying mechanism of NUPR1-mediated autophagy-lysosomal processing, we next analysed the DEPs between Cal27 cells with NUPR1 KD and scrambled Cal27 cells through proteomic analysis. Interestingly, NUPR1 KD markedly decreased the expression level of TFE3, as shown by the differentially expressed proteins (Supplementary Table S3). Indeed, TFE3 was markedly downregulated in NUPR1-depleted OSCC cells (Fig. 3a, b). More importantly, we confirmed the expression changes in “TFE3-responsive genes” involved in autophagic flux by real-time PCR (Fig. 3c, d), suggesting that NUPR1 plays a crucial role in autolysosomal events and may be...
Fig. 2  
NUPR1 KD restrained autophagic flux and lysosomal function in OSCC cells.  
a, b Immunoblotting analysis of MAP1LC3B-II and SQSTM1 levels in NUPR1 KD or scrambled Cal27 and HN6 cells; n = 3.  
c, d Immunoblotting analysis of MAP1LC3B-II in NUPR1 KD or scrambled Cal27 and HN6 cells in the absence or presence of CQ (10 μM) for 24 h; n = 3.  
e, f Immunoblotting analysis of LAMP1 and LAMP2 in NUPR1 KD or scrambled Cal27 and HN6 cells; n = 4.  
g, h LysoSensor DND-189 fluorescence intensity in NUPR1 KD or scrambled Cal27 and HN6 cells. n = 4; *P < 0.05, **P < 0.01 vs. the scrambled group.
formation or maturation, and autophagosome-lysosome fusion) is a crucial cause of NUPR1-KD-induced autophagic flux impairment and inhibition of OSCC cell proliferation and metastasis. (iii) NUPR1 maintained autophagic flux by increasing TFE3 promoter activities and activating TFE3 transcription in OSCC progression. These findings, therefore, broaden new insights regarding a potential therapeutic target of NUPR1-TFE3-dependent autophagy in OSCC progression.

Most tumour tissue specimens archived in hospitals for pathologic diagnosis are FFPE specimens, which represent the pinpoints of tumour tissue and generally contain rich clinical data. Proteomics is an efficient tool to investigate discrepancies in

**Fig. 3** TFE3 was required for NUPR1-mediated autolysosomal processes in OSCC cells. 

- **a, b** Immunoblotting analysis of TFE3 in NUPR1 KD or scrambled Cal27 and HN6 cells; *n* = 3. 
- **c, d** The results of TFE3-responsive genes involved in autophagic flux were detected by real-time PCR in NUPR1 KD or scrambled Cal27 and HN6 cells; *n* = 3. 
- **e, f** TFE3 transcription activities involved in autophagic flux were detected by Secrete-Pair luminescence assay in NUPR1 KD or scrambled Cal27 or HN6 cells; *n* = 3; **P < 0.01 vs. the scrambled group.
Fig. 4  TFE3 overexpression rescued the NUPR1 KD-inhibited autophagic flux in OSCC cells. **a**, **b** DQ-BSA or **c**, **d** LysoSensor DND-189 fluorescence intensity was detected in NUPR1 KD or scrambled Cal27 and HN6 cells transfected with TFE3 plasmid or a control plasmid for 24 h; *n* = 4. **e**, **f** Immunoblotting analysis of MAP1LC3B and SQSTM1 in NUPR1 KD or scrambled Cal27 and HN6 cells transfected with TFE3 plasmid or a control plasmid for 24 h; *n* = 3; *P* < 0.05, **P* < 0.01 vs. the scrambled group. *P* < 0.05, **P* < 0.01 vs. NUPR1 KD group.
protein expression in different tumour tissues, especially in FFPE tissues, and to further analyse the pathogenesis of diseases. In this study, FFPE tumour samples of OSCC patients with or without LNM were comparatively profiled with high-sensitivity quantitative proteomics analysis. NUPR1 was identified and verified to have potential prognostic value; it was highly expressed in OSCC patients with LNM and was significantly associated with low pathological differentiation grades, regional LNM, clinical stage, and decreased overall survival time.

NUPR1 is a transcription factor that is dependent on a basic helix-loop-helix structure present at its C-terminus, and its function may be mediated by various kinases, factors or cellular stressors. NUPR1 was reported to result in cancer development and progression over twenty years ago and has been found to be involved in multiple aspects of cancer, especially transcription regulation of cancer cells. NUPR1 was found to promote metastasis and chemotherapeutic resistance in some cancers, such as breast, lung, and colorectal cancer. Recently, Jiang et al. reported that upregulating the activation of NUPR1 can significantly promote epithelial-mesenchymal transition (EMT) of SCC-9 and Tca8113 OSCC cells. Huang et al. also found that enhancing the expression level of NUPR1 promoted the growth and invasiveness of SCC-9 and HSC-2 OSCC cells. These findings indicated that NUPR1 serves as a key factor mediating OSCC development and progression, which is in accordance with our results and further supports the possibility of targeting NUPR1 for OSCC treatment.

Autophagy plays a crucial role in the maintenance of intracellular homoeostasis in response to cellular stress. The transcription factor NUPR1 serves as a master regulator of cellular clearance through promoting autophagy process. Recently, Wu et al. found that NUPR1 knockdown blocked autophagic flux and increased apoptosis of liver cancer cell, and Wang et al. reported that NUPR1 depletion induced premature senescence in breast cancer cells by impairing the autolysosomal process. Moreover, NUPR1 maintains autophagic flux and is required for the development of lung cancer. In accordance with these results, in this study, we revealed that several proteins were participated in autophagic flux in NUPR1 KD cells via proteomic and bioinformatic analysis. Autophagic flux is considered to be a multistage cascade that generally includes the phagophore formation phase, the expansion of phagophores into an autophagosome stage, the fusion of autophagosomes with lysosomes and finally degradation in lysosomes. Importantly, we further found that NUPR1 KD substantially disrupted lysosomal function but did not disturb other key steps of autophagic flux. Thus, lysosomal dysfunction is a crucial cause of NUPR1 KD-induced autophagic flux blockage and inhibition of OSCC cell proliferation and metastasis.

TFE3 has been recognised as a key regulator of the genes expression that are correlated with autophagosome and autolysosome formation and degradation. Emerging evidence

![Fig. 5](Image)
indicates that TFE3-dependent autophagy–lysosome activation is required for lysosomal function and maintains pH, which significantly promotes the progression of cancers such as renal cancer, pancreatic cancer, and epithelioid haemangioendothelioma.\textsuperscript{18,32} Interestingly, our data indicated that TFE3 expression was markedly decreased following NUPR1 KD and that TFE3 overexpression antagonised the inhibition of lysosome function caused by NUPR1 KD in OSCC cells. NUPR1 can directly regulate genes transcription by combining with platelet-derived growth factor subunit A (PDGFA), synaptosomal-associated protein 25 (SNAP25), or other transcription factors to promote autophagy and lysosome function in OSCC cells.\textsuperscript{33} NUPR1/TFE3 axis is involved in the regulation of autophagic flux and lysosomal function in tumour progression. NUPR1 promotes the proliferation and metastasis of oral squamous cell carcinoma (OSCC),\textsuperscript{19,20} indicating that NUPR1 is a novel therapeutic target for OSCC. NUPR1 promotes proliferation and metastasis of OSCC in vivo as well. NUPR1 KD suppressed the tumour volume and weight by approximately 50\% compared to the scrambled group. NUPR1 KD inhibited tumour growth in xenograft models, as indicated by the calculated volume and weight of xenograft tumours. This result is consistent with previous studies indicating that NUPR1 promotes tumour growth.\textsuperscript{21} NUPR1 KD also attenuated the expression of TFE3-responsive genes involved in autophagic flux, such as autophagy-related genes (ATG7, ATG16L1, CLCN5, CTSB, LAMP1, TRPML1) and lysosomal membrane proteins (LAMP1, LAMP2, LAMP3). These findings suggest that NUPR1 is a key regulator of autophagic flux and lysosome function in OSCC progression.
NUPR1 promotes the proliferation and metastasis of oral squamous cell... Fan et al.

(SNAP25), and other gene promoters.27 In our study, we found that NUPR1 could substantially enhance TFE3 promoter activity, and we also found a positive correlation between NUPR1 and TFE3 in OSCC tissues. This study is the first to examine these aspects of OSCC progression, and the findings indicate the existence of a NUPR1-TFE3 link.

In summary, by combining high-sensitivity label-free quantitative proteomics and TMT-based quantitative proteomics, we speculate that the NUPR1-TFE3 axis-mediated autolysosomal clearance pathway may contribute to OSCC development and that this pathway could be exploited for the prevention and therapy of OSCC (Fig. 6g).

**MATERIALS AND METHODS**

**Ethics statement**

OSCC and oral mucosa tissue samples were obtained from the Department of Oral and Maxillofacial Surgery, the Second Xiangya Hospital of Central South University. All participants provided written informed consent before the study. The protocols were approved by the Clinical Research Ethics Committee of the Second Xiangya Hospital of Central South University (NO. JBWKQA001), and the animal experiments were approved by the ethics statement of the Shanghai Jiao Tong University Institute Animal Care and Use Committee.

Cell culture and NUPR1 KD OSCC cell line construction

The OSCC cell lines HN4, HN6, and HN30 were kindly gifted by Professor Mao Li from the University of Maryland. SCC9, SCC25, and Cal27 cell lines were purchased from the American Type Culture Collection (ATCC, USA). All these cells except SCC9 and SCC25 were cultured in DMEM (Thermo Fisher, 11995040) supplemented with 10% FBS (Thermo Fisher, 10091155) and 1% (v/v) penicillin/streptomycin (Sigma, P4333) at 37 °C in a humidified 5% CO2 atmosphere. While SCC9 and SCC25 cells were kept in DMEM/F12 medium. To investigate the role of NUPR1 in OSCC development, stable NUPR1 KD HN6 and Cal27 cell lines were constructed at Shanghai GeneChem Co., Ltd.

FFPE tissue preparation and high-sensitivity label-free quantitative proteomics analysis, TMT quantitative proteomic analysis

Detailed information of quantitative proteomics analysis is shown in the Supplementary Method.

Tissue microarray (TMA), Cell migration, invasion assay and colony formation assay

For the methods to determine the OSCC progression in vitro are shown in the Supplementary Method.

Western blot analysis, Plasmid or RFP-GFP-LC3B lentivirus transfection, Immunofluorescence analysis, DQ-BSA proteolytic activity assay, LysoSensor Green DND-189 staining, Real-Time PCR analysis, and Secrete-Pair luminescence assay

The protocols are shown in the Supplementary Method.

Xenograft mouse experiments and in vivo metastasis assay

The tumour xenograft model was established with $1 \times 10^6$ Cal27 cells according to our recent report. The BALB/c nude mice were divided into 2 groups (n = 10 per group): (a) scrambled Cal27 cells and (b) stable NUPR1 KD Cal27 cells. Tumour volumes and tumour weight measurements were performed as previously reported. For the experimental metastasis assay, $1 \times 10^6$ cells (scrambled Cal27 cells or NUPR1 KD Cal27 cells) in 100 μl normal saline were injected into the caudal vein of nude mice (n = 10 per group). The injected mice were euthanized after 7 weeks as previously reported. The lungs were removed, and subsequent experiments were performed. The mRNA levels of NUPR1 and TFE3-responsive genes in tumours and metastatic nodules were quantified.

Statistical analysis

Data were analysed using the student’s t test or non-parametric Mann–Whitney U Test and one-way analysis of variance (ANOVA) in GraphPad Prism version 8. Mann–Whitney U tests were used to analyse the associations between TMA scores and clinical parameters as well as the puncta immunofluorescence study. The log-rank test was used to assess the survival differences and Kaplan–Meier survival analyses were used to estimate the prognostic and diagnostic value. The correlation between NUPR1 and TFE3 was determined by Pearson analysis. P < 0.05 was considered statistically significant. All values are expressed as the mean ± SE.

**DATA AVAILABILITY**

All data are available from the corresponding author upon reasonable request.

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**AUTHOR CONTRIBUTIONS**

H.-F.P., Z.-Z., S.Z., H.-J.W., Z.-P.Y., and Z.-Y.Z. were involved in the study design. F.-T.F., X.-N.W., Y.-J., P.D., Y.-D.L., S.J., Q.W., C.-W.L., G.-C.T., Z.Z., Z.-R.H., B.L., Y.-R.C., Z.-J.H. and M.-L.C. conducted the experiments and data analyses. F.-T.F., X.-N.W., P.D., H.-F.P., and Z.Z. wrote the manuscript. All authors have read and approved the article.

**ADDITIONAL INFORMATION**

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**Competing interests:** The authors declare no competing interests.

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Signal Transduction and Targeted Therapy (2022) 7:130
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