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Research Article

Microfibrillar-associated protein-2 facilitates aggressive progression of oral squamous cell carcinoma cells through Kelch-like E3 ubiquitin ligase-associated protein 1/nuclear factor erythroid 2-related factor 2 signaling pathway

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ABSTRACT

Objective: This study aims to explore the role of microfibrillar-associated protein-2 (MFAP2) in oral squamous cell carcinoma (OSCC).

Material and Methods: Analysis of MFAP2 expression in diverse cancers and its relationship with head-and-neck squamous cell carcinoma (HNSC) prognosis. MFAP2 abundance was identified in OSCC cells and in human oral epithelial cells using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot assays. Knockdown and overexpression techniques were utilized to examine the mechanism by which MFAP2 and nuclear factor erythroid 2-related factor 2 (NRF2) affect OSCC malignancy. Cell viability, proliferation, and apoptosis were assessed using cell counting kit-8, colony formation, flow cytometry, wound healing, and Transwell tests. Messenger ribonucleic acid expression was detected using qRT-PCR, whereas protein level was analyzed using Western blot.

Results: MFAP2 and Kelch-like E3 ubiquitin ligase (ECH)-associated protein 1 (KEAP1) had high expression levels in numerous tumors, including OSCC, and the high expression level of MFAP2 was associated with unfavorable HNSC outcomes. MFAP2 was abundantly expressed in five OSCC cell lines, with the peak expression observed in squamous cell carcinoma (SCC)-15 and SCC-9 cells, making them suitable for subsequent studies. MFAP2 knockdown hindered the proliferative and mobile capacity of OSCC cells, yet it supported cell apoptosis. MFAP2 silencing led to a notable drop in KEAP1 and NRF2 expression levels in OSCC cells. NRF2 overexpression could counteract the effects of MFAP2 knockout, which included diminished proliferation and movement and heightened apoptosis in OSCC cells.

Conclusion: The results of this study indicated that MFAP2 facilitated the malignant progression of OSCC and provided insights into the downstream regulatory mechanism of the KEAP1/NRF2 axis, highlighting the potential application of MFAP2 in OSCC management.

Keywords: Aggressive driving, Kelch-like ECH-associated protein 1, Microfibrillar-associated protein-2, Nuclear factor erythroid 2-related factor 2, Oral squamous cell carcinoma

INTRODUCTION

Among head-and-neck squamous cell carcinomas (HNSCs), oral squamous cell carcinoma (OSCC) ranks as the foremost type, which is noted for its strong local invasiveness and tendency

to metastasize to cervical lymph nodes.[1] Global statistics from 2022 show about 390,000 new oral cancer cases and over 180,000 deaths.[2] Comprehensive treatments, including surgery, have notably enhanced the control, yet the 5-year survival rate of patients suffering from OSCC continues to hover near 50%[3] primarily due to the spread, local invasion, and distant metastasis of OSCC.[4] Consequently, an in-depth examination of the molecular factors that contribute to the emergence and advancement of OSCC, along with identifying crucial regulatory molecules that influence its growth and metastasis, is clinically important to OSCC prevention, treatment, and prognosis.

Microfibrillar-associated proteins (MFAPs), including MFAP1-MFAP5, are glycoproteins in the extracellular matrix, which are involved in microfibril assembly and tissue equilibrium.[5] MFAPs are found in different human tissues, and they have various functions in normal and disease states.[6] MFAP2, which is referred to as microfibrillar-associated protein 2, is situated at 1p36.13 and is important for impacting cell movement.[7] Recently conducted studies have shown that MFAP2 remarkably influences tumor progression, which could result in the creation of new cancer biomarkers or therapeutic approaches.[8,9] Research has increasingly shown that MFAP2 is expressed more abundantly in various tumor tissues, including breast cancer,[10] osteosarcoma,[11] esophageal squamous cell carcinoma (ESCC),[12] colorectal cancer,[13] gastric cancer (GC),[14] tongue squamous cell carcinoma (TSCC),[15] and OSCC,[16] compared with the normal tissues nearby. In ovarian cancer, MFAP2 boosts cell proliferation and glycolysis.[17] Blocking MFAP2 curtails the malignancy of gastric cancer cells *in vitro* and *in vivo*.[18] The poor survival in patients with hepatocellular carcinoma is linked to increased MFAP2 expression. Experiments conducted in uterine corpus endometrial carcinoma cells reveal that decreasing MFAP2 levels curtails the proliferative and migratory abilities.[19] Jiang and Jiang found a strong link between MFAP2 and the disease-free survival rate in laryngeal squamous cell carcinoma.[20] In TSCC cells, POU domain, class 2, transcription factor 1 upregulates the expression level of MFAP2, which induces them to proliferate, migrate, and invade.[15] By triggering the Wnt/β-catenin pathway, MFAP2 boosts cancerous autophagy, leading to heightened proliferative, migratory, and invasive capacities, while preventing apoptotic ability in OSCC cells.[16] However, the involvement and fundamental mechanism of MFAP2 in OSCC remain unknown.

Considerable research indicates that sustained oxidative stress can cause chronic inflammation, thereby facilitating the progression of numerous long-term illnesses, particularly tumors. Nuclear factor erythroid 2-related factor (NRF2) is essential for oxidative defense in cells, and its malfunction is connected to a range of diseases, including cancers.[21] Normally, NRF2 is associated with Kelch-like E3 ubiquitin ligase (ECH)-associated protein 1 (KEAP1), which possesses ubiquitin ligase E3 activity. When oxidative stress occurs, KEAP1's ubiquitin ligase E3 is deactivated, thereby hindering the ubiquitination that causes NRF2 to degrade. Then, NRF2 is relocated to the nucleus, where it plays a role in the transcription of specific genes.[22] The disruption of KEAP1/NRF2 signaling is recognized as a crucial factor in the advancement of tumors, including OSCC.[23,24] The known effects of NRF2 activation in cancers include supporting cell survival, preventing apoptosis, and inducing resistance to chemotherapy and radiation treatments. OSCC samples show increased NRF2 expression when compared with normal tissues, with its upregulation potentially fostering cancerous traits in OSCC cells.[25] In HNSC, the interaction between tumor necrosis factor-α-induced protein 2 (TNFAIP2) and KEAP1 stabilizes NRF2, resulting in resistance against cisplatin.[26] However, the regulatory mechanism upstream of the KEAP1/NRF2 axis in OSCC remains unclear. MFAP2 has been shown to modulate various signaling pathways associated with cell proliferation, invasion, and autophagy in multiple cancers, including OSCC; thus, MFAP2 might affect cellular oxidative stress responses through the KEAP1/NRF2 pathway. However, the potential regulatory relationship between MFAP2 and KEAP1/NRF2 signaling in OSCC remains largely unexplored and requires further investigation.

This study aimed to examine the influence of MFAP2 on the aggressive traits of OSCC cells and its potential association with the KEAP1/NRF2 signaling pathway in OSCC. Our discoveries might illuminate the complicated regulatory network that propelled OSCC advancement and spread, potentially aiding in the formulation of targeted treatments and better clinical results of patients with OSCC.

MATERIAL AND METHODS

Bioinformatics

The Tumor IMmune Estimation Resource (TIMER) 2.0 database (http://timer.cistrome.org/) was used to compare MFAP2 and KEAP1 messenger ribonucleic acid (mRNA) levels in different tumor tissues, while the Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn/) was used to analyze MFAP2 level in normal and HNSC tissues. The University of ALabama at Birmingham CANcer data analysis Portal (UALCAN) database (https://ualcan.path.uab.edu/) was also used to analyze the link between MFAP2 level and prognosis of patients with HNSC.

Cell culture and transfection

The human oral epithelial cells (HOECs; CP-H203) were acquired from Procell (Wuhan, China). All OSCC cell lines, including squamous cell carcinoma (SCC)-4 (CRL-1624),

SCC-9 (CRL-1629), SCC-15 (CRL-1623), SCC-25 (CRL-1628), and Cal27 (CRL-2095), were obtained from ATCC (Rockefeller, MD, USA). All cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide (CO_2) using Dulbecco's modified Eagle medium (DMEM; 10569010, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; 26170043, Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (15140122, Gibco, Carlsbad, CA, USA). All cells were authenticated using short tandem repeat profiling, and mycoplasma testing was performed to avoid contamination.

For MFAP2 silencing, two small interfering ribonucleic acids (siRNAs) targeting MFAP2 (siMFAP2-1 and siMFAP2-2) along with the matching negative control siRNA (siNC) were provided by GeneChem (Shanghai, China). The siRNA sequences are shown in Table 1. For NRF2 overexpression, an NRF2 overexpression plasmid (oeNRF2) was developed by inserting the NRF2 gene sequence into the pcDNA3.1 plasmid, with an empty vector serving as a overexpressing negative control (oeNC). Oligonucleotide fragments, including siMFAP2-1, siMFAP2-2, siNC, oeNRF2, and oeNC, were transfected into SCC-15 and SCC-9 cells using Lipofectamine 3000 (L3000150, Invitrogen, Grand Island, NY, USA). In brief, the cells were seeded in six-well plates at a density of 5×10^5 cells/well with 2.5 mL of DMEM and incubated until reaching 70% confluence. Plasmid DNA was diluted with a serum-free medium, mixed gently, and then combined with Lipofectamine 3000 reagent (pre-diluted with a serum-free medium). The mixture was incubated for 10 min at room temperature to form DNA-lipid complexes. Afterward, 50 nM DNA–lipid complexes were added to the cells, followed by gentle swirling. The cells were incubated at 37°C with 5% CO_2 for 48 h before replacing the medium with a fresh complete medium and collected for further analyses.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The extraction of total RNA from tissues and cells involved the use of TRIzol reagent (15596018CN, Invitrogen, Grand Island, NY, USA). The PrimeScript RT Reagent Kit (Perfect Real Time; RR037B, TaKaRa Bio, Tokyo, Japan) was utilized to convert total RNA into complementary deoxyribonucleic acid, as outlined by the manufacturer. mRNA expression was measured using a LightCycler480 device (Roche, Indianapolis, IN, USA) in accordance with the operational instructions of the FastStart Universal SYBR Green Master (4913850001, Roche, Indianapolis, IN, USA). For internal control, glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was utilized. Data processing was conducted using the $2^{-\Delta\Delta Ct}$ method. The sequences for the amplification primers are shown in Table 2.

Western blot

The extraction of protein from tissues and cells was performed using radioimmunoprecipitation assay lysis buffer (EX6060, G-CLONE, Beijing, China), followed by concentration measurement using a bicinchoninic acid kit (PN0125, G-CLONE, Beijing, China). Approximately 30 μg of proteins was resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel (P0012AC, Beyotime, Shanghai, China) and moved onto polyvinylidene fluoride membranes (ISEQ00010, Merck Millipore, Darmstadt, Germany), with subsequent blocking for 1 h with 5% bovine serum albumin (SW3015, Solarbio, Beijing, China) and incubating overnight at 4°C with primary antibodies targeting MFAP2 (ab231344, Abcam, Cambridge, UK), KEAP1 (#8047, Cell Signaling Technology, Danvers, MA, USA), NRF2 (#12721, Cell Signaling Technology, Danvers, MA, USA), and GAPDH (#12118, Cell Signaling Technology, Danvers, MA, USA). Next, the membranes were exposed to horseradish peroxidase-conjugated immunoglobulin G (#7074, Cell Signaling Technology, MA, Danvers, USA) for 60 min at 37°C. Protein bands were detected using an enhanced chemiluminescence solution (MW2040, G-CLONE, Beijing, China). The protein bands were exposed using a Western Blot

Table 1: Sequences of siRNAs.

siRNA	Sequence (5′-3′)
siMFAP2-1 sense	CCAUACACAGGCCUUGCAATT
siMFAP2-1 antisense	UUGCAAGGCCUGUGUAUGGTT
siMFAP2-2 sense	CCCACUAUAGCGACCAGAUTT
siMFAP2-2 antisense	AUCUGGUCGCUAUAGUGGGTT
siNC sense	UUCUCCGAACGUGUCACGUTT
siNC antisense	ACGUGACACGUUCGGAGAATT

siRNAs: Small interfering ribonucleic acids, siMFAP2-1 Small interfering microfibrillar-associated protein-2, A: Adenine, C: Cytosine, G: Guanine, T: Thymine

Table 2: Sequences of the primers used in qRT-PCR.

Gene	Sequence (5′-3′)
MFAP2 forward	CTGACCACGTCCAGTACACC
MFAP2 reverse	TCCAGCTCTGCATTTCCTGG
KEAP1 forward	AAGAGGATGAGGAGGAGGAAAG
KEAP1 reverse	CTTCTGGGGATCTGAGGCTG
NRF2 forward	CCAGGTTGCCCACATTCCCAAA
NRF2 reverse	TGGAGAGGATGCTGCTGAAGGA
GAPDH forward	GGTCGGAGTCAACGGATTTG
GAPDH reverse	ATGAGCCCCAGCCTTCTCCAT

qRT-PCR: Quantitative real-time polymerase chain reaction, MFAP2: Microfbrillar-associated protein-2, KEAP1: Kelch-like ECH-associated protein 1, NRF2: Nuclear factor erythroid 2-related factor 2, GAPDH: Glyceraldehyde-3-phosphatedehydrogenase, A: Adenine, C: Cytosine, G: Guanine, T: Thymine

chemiluminescence imager (e-BLOT, LIPOSOMA, Beijing, China) and quantified using ImageJ software (version 1.3.0, National Institutes of Health, Bethesda, MD, USA).

Cell count kit-8 (CCK-8) assay

After preparation, the transfected SCC-15 and SCC-9 cells were gathered and resuspended in DMEM. A total of 1×10^3 cells per well were used to seed 96-well plates filled with 100 μL of medium, and they were incubated for 0, 24, 48, and 72 h. Each well received 10 μL of CCK-8 reagent (ab228554, Abcam, Cambridge, UK). The optical density at 450 nm was read using a microplate reader (Synergy H1, BioTeK, Winooski, VT, USA) after a 2-h incubation period.

Colony formation assay

The transfected SCC-15 and SCC-9 cells were collected and then placed into a 3 cm plate at 1000 cells/well with 2 mL of medium. The cells underwent standard culturing for about 14 days until a single colony contained over 50 cells. A solution containing 0.1% crystal violet (C0775, Sigma-Aldrich, St. Louis, MO, USA) was applied to stain the cell colonies for 10 min. The C1si camera (Nikon, Tokyo, Japan) was used to capture the images, and the colony numbers were counted manually.

Cell apoptosis assay

After transfection, SCC-15 and SCC-9 cells were harvested and re-dissolved in a binding solution. Afterward, a 20 min incubation in the dark was performed, followed by fluorescein isothiocyanate-Annexin V (556420, BD Biosciences, Franklin Lakes, NJ, USA) along with 5 μL of propidium iodide (556463, BD Biosciences, Franklin Lakes, NJ, USA) addition. Apoptosis was examined using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and analysis was performed using Flowjo software (Flowjo, Ashland, OR, USA).

Wound healing assay

The transfected SCC-15 and SCC-9 cells were placed in 12-well plates at 5×10^4 cells/well with 2 mL of medium. A 200 μL pipette tip was used to make a wound after the cells covered the bottom surface. Once the cells were scratched, they were rinsed with a serum-free medium to remove debris for 24 h incubation at 37°C. The wound was imaged at 0 and 24 h post-scratch using an inverted phase-contrast microscope (BX41, Olympus, Tokyo, Japan). The extent of wound closure served as a measure of cell migration capability.

Transwell assays

The assays for migration and invasion were conducted using Transwell chambers with membranes featuring 8 μm pores. In preparing the invasion assay, the membrane was pre-coated with 40 μL of 1.5 mg/mL Matrigel (356234, BD Biosciences, Franklin Lakes, NJ, USA). The upper chamber received transfected SCC-15 and SCC-9 cells (3×10^4 cells) with 200 μL of medium without FBS, and 800 μL of DMEM with 10% FBS supplement was placed into the lower chamber. The cells were cultured at 37°C with 5% CO_2, allowing them to invade the membrane for 24 h. Afterward, the cells on the membrane's upper surface were wiped away using sterile cotton buds. After penetrating the membrane's underside, the cells were immersed in 4% paraformaldehyde for 30 min, along with 5 min of 0.1% crystal violet (C0775, Sigma-Aldrich, St. Louis, MO, USA) staining. The stained cells in five fields per membrane were counted using an inverted phase-contrast microscope (BX41, Olympus, Tokyo, Japan). The steps for the migration assay mirrored that of the invasion experiment, but the membrane lacked Matrigel pre-coating.

Statistical analysis

Data were displayed as the mean ± standard deviation. The Statistical Package for the Social Sciences (SPSS) version 21.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. In addition, statistical analysis was conducted using one-way analysis of variance, with Tukey's *post hoc* test applied afterward to evaluate the differences among the groups. In comparing two groups, a two-tailed, unpaired Student's *t*-test was used. $P < 0.05$ threshold was applied to establish statistical significance.

RESULTS

MFAP2 was upregulated in OSCC tissues

The expression data from the TIMER2.0 database were examined, covering multiple cancers, to analyze MFAP2 expression in OSCC. MFAP2 was prominently expressed in multiple cancer types, including HNSC [Figure 1a]. Based on the GEPIA database, the expression level of MFAP2 in HNSC tissues was notably elevated compared with that in normal tissues [Figure 1b]. Analysis through the UALCAN database showed that higher MFAP2 expression level was associated with poorer overall survival in individuals with HNSC [Figure 1c]. The data indicated an upregulation of MFAP2 in OSCC, implying its crucial role in the pathogenesis of the condition.

Knocking down MFAP2 curtailed OSCC cell proliferation and facilitated cell apoptosis

The expression of MFAP2 in HOECs and five OSCC cell lines was assessed by qRT-PCR and Western blot analyses, and MFAP2 showed higher expression levels in OSCC cells than

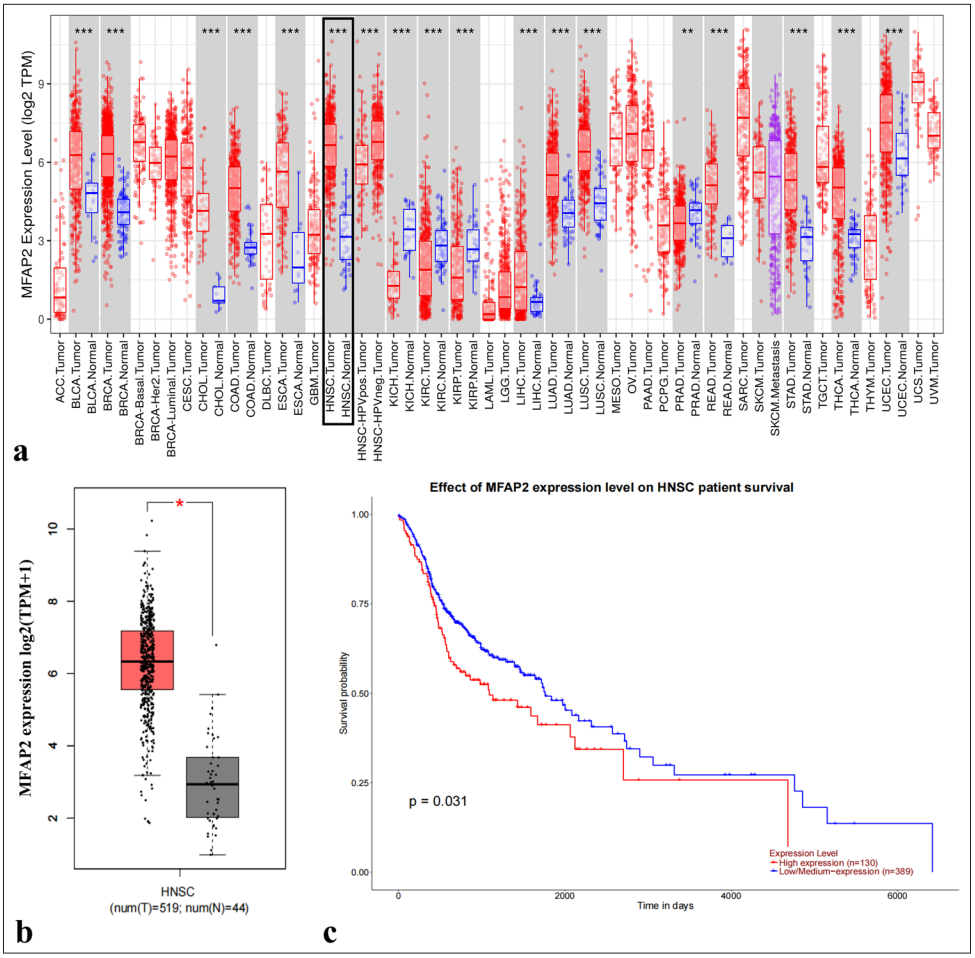

Figure 1: MFAP2 expression level was elevated in OSCC and was associated with a poor prognosis in patients with OSCC. (a) The expression of MFAP2 in different types of cancer was displayed in the TIMER2.0 database, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the normal group. (b) MFAP2 showed high expression level in HNSC, as reported by the GEPIA database, $^{*}P < 0.05$ versus the N group. (c) The UALCAN database provided an overall survival curve plot for patients with HNSC based on low- and high-expression levels of MFAP2 mRNA. MFAP2: Microfibrillar-associated protein 2, OSCC: Oral squamous cell carcinoma, TIMER: Tumor IMmune Estimation Resource, HNSC: Head and neck squamous cell carcinoma, GEPIA: Gene expression profiling interactive analysis, UALCAN: University of ALabama at Birmingham CANcer data analysis Portal, mRNA: Messenger ribonucleic acid. ACC: Adrenocortical carcinoma, BLCA: Bladder urothelial carcinoma, BRCA: Breast invasive carcinoma, CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma, CHOL: Cholangiocarcinoma, COAD: Colon adenocarcinoma, DLBC: Diffuse large B-cell lymphoma, ESCA: Esophageal carcinoma, GBM: Glioblastoma multiforme, HNSC: Head and neck squamous cell carcinoma, KICH: Kidney chromophobe, KIRC: Kidney renal clear cell carcinoma, KIRP: Kidney renal papillary cell carcinoma, LAML: Acute myeloid leukemia, LGG: Lower-grade glioma, LIHC: Liver hepatocellular carcinoma, LUAD: Lung adenocarcinoma, LUSC: Lung squamous cell carcinoma, MESO: Mesothelioma, OV: Ovarian serous cystadenocarcinoma, PAAD: Pancreatic adenocarcinoma, PCPG: Pheochromocytoma and paraganglioma, PRAD: Prostate adenocarcinoma, READ: Rectum adenocarcinoma, SARC: Sarcoma, SKCM: Skin cutaneous melanoma, STAD: Stomach adenocarcinoma, TGCT: Testicular germ cell tumors, THCA: Thyroid carcinoma, THYM: Thymoma, UCEC: Uterine corpus endometrial carcinoma, UCS: Uterine carcinosarcoma, UVM: Uveal melanoma.

in HOECs, with SCC-15 and SCC-9 cells showing the most pronounced expression [Figure 2a-c], which were consistent with previous studies.[15,16] Consequently, SCC-15 and SCC-9 cells were selected for further experiments. In this study,

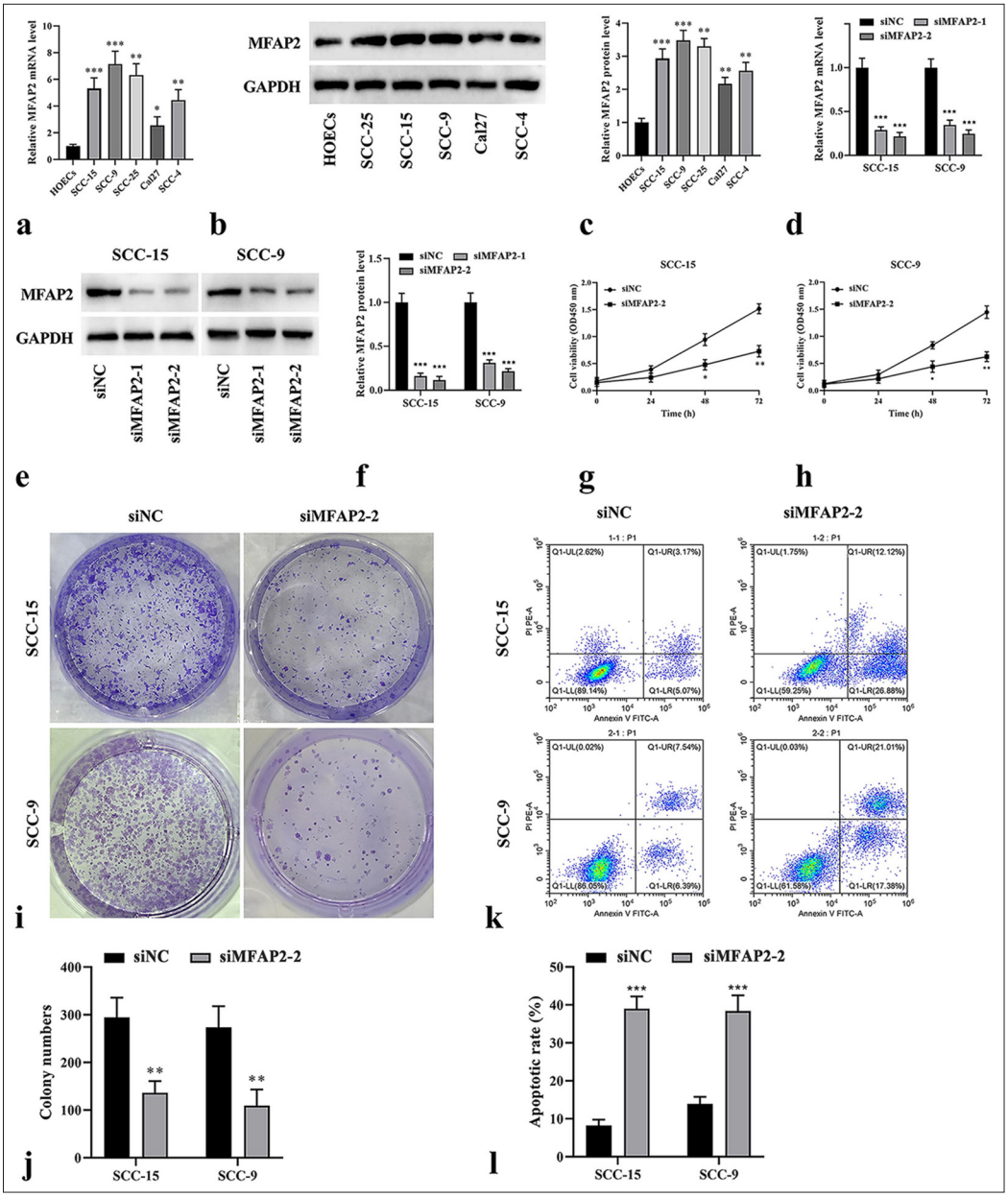

Figure 2: Knocking down MFAP2 curtailed OSCC cell proliferation and facilitated cell apoptosis. qRT-PCR (a) and Western blot (b and c) analyses were conducted to assess the mRNA and protein expression levels of MFAP2 in HOECs and five OSCC cell lines (SCC-15, SCC-9, SCC-25, Cal27, and SCC-4). (d-f) SCC-15 and SCC-9 cells underwent transfection with siNC, siMFAP2-1, and siMFAP2-2 for 48 h. qRT-PCR (d) and Western blot (e and f) were used to evaluate the knockdown effectiveness. (g-l) SCC-15 and SCC-9 cells underwent transfection with siNC and siMFAP2-2 for 48 h. Assessments of cell viability (g and h), proliferation (i and j), and apoptosis (k and l) were conducted using the CCK-8 assay, colony formation assay, and flow cytometry analysis. The results were displayed as means ± SD from at least three repetitions. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus HOECs or the siNC group. One-way ANOVA with Tukey's *post hoc* test was applied for multi-group comparisons (a, c, d, and f). Unpaired two-tailed Student's t-tests were used for comparisons between two groups (g, h, j, and l). MFAP2: Microfibrillar-associated protein 2, OSCC: Oral squamous cell carcinoma, qRT-PCR: Quantitative real-time polymerase chain reaction, HOECs: Human oral epithelial cells, SCC: Squamous cell carcinoma, siMFAP2: Small interfering microfibrillar-associated protein 2, CCK-8: Cell counting kit-8, mRNA: Messenger ribonucleic acid, SD: Standard deviation, ANOVA: Analysis of variance, siNC: Matching negative control siRNA.

HOECs were selected as non-cancerous controls because they share the same tissue origin as the primary tumor samples (oral cavity), minimizing microenvironmental variability, and prior studies have established HOECs as a control for oral cancer research.[27,28] Before elucidating the function of MFAP2 in OSCC, SCC-15 and SCC-9 cells underwent transfection with siMFAP2-1 and siMFAP2-2 to lower the expression level of MFAP2. The siMFAP2-transfected cells exhibited a significant decrease in MFAP2 mRNA and protein levels relative to the siNC group. MFAP2 was clearly lower in the siMFAP2-2 group than in the siMFAP2-1 group, prompting the selection of siMFAP2-2 for the following functional investigations [Figure 2d-f]. To assess the effect of MFAP2 on OSCC cell viability, CCK-8 was conducted. The OD values for siMFAP2-2-transfected SCC-15 and SCC-9 cells were significantly lower at 48 and 72 h than those in the siNC group [Figure 2g and h]. Transfection with siMFAP2-2 resulted in a significantly lower number of colonies relative to siNC transfection [Figure 2i and j]. Based on the flow cytometry results, MFAP2 silencing markedly increased the percentage of cell apoptosis [Figure 2k and l]. The findings indicated that MFAP2 knockdown curtailed the proliferation of OSCC cells by causing apoptosis.

Suppression of MFAP2 hindered the migratory and invasive capacities of OSCC cells

In this study, the functional impact of MFAP2 on OSCC cell movement was investigated. The migrative potential of SCC-15 and SCC-9 cells was notably reduced by MFAP2 silencing [Figure 3a and b]. The Transwell results also revealed a substantial decrease in migrated and invaded cells in the MFAP2-silenced group [Figure 3c-f]. These data indicated that MFAP2 knockdown obstructed OSCC cells from migrating and invading.

MFAP2 modulated the KEAP1/NRF2 signaling pathway in OSCC

Figure 4a illustrates that KEAP1 had high expression levels in various cancer types, such as HNSC. In SCC-15 and SCC-9 cells with silenced MFAP2, KEAP1 and NRF2 protein expression levels [Figure 4b-d] and mRNA levels [Figure 4e and f] dropped significantly. These outcomes indicated that MFAP2 influenced the KEAP1/NRF2 axis in OSCC.

MFAP2 influenced the proliferative capacity and apoptosis of OSCC cells through the KEAP1/NRF2 axis

A rescue experiment was conducted to see if the KEAP1/NRF2 axis mediated the influence of MFAP2 on OSCC cell multiplication and apoptosis. SCC-15 and SCC-9 cells with oeNRF2 transfection resulted in a notable increase in NRF2 mRNA and protein levels [Figure 5a-d], without altering MFAP2 and KEAP1 protein expression [Figure 5b-d], indicating that NRF2 serves as the downstream of MFAP2 and KEAP1. The CCK-8 findings indicated that NRF2 overexpression lessened the impact of MFAP2 knockdown on the cell viability [Figure 5e and f]. NRF2 overexpression also alleviated the decline in cell proliferation resulting from MFAP2 silencing [Figure 5g and h]. Knocking down MFAP2 can increase apoptosis, which was mitigated by NRF2 being overexpressed in SCC-15 and SCC-9 cells [Figure 5i and j]. This finding indicated that MFAP2 affects the proliferative and apoptotic capacities of OSCC cells by interacting with the KEAP1/NRF2 axis.

MFAP2 affected OSCC cell mobility through the KEAP1/NRF2 pathway

Finally, whether MFAP2 affected OSCC cell movement was investigated by modulating the KEAP1/NRF2 pathway. As shown in Figure 6a and b, NRF2 overexpression boosted the reduced migratory potential caused by MFAP2 depletion in SCC-15 and SCC-9 cells. In addition, the introduction of oeNRF2 resulted in a notable increase in the migrated and invaded cells, which were diminished due to MFAP2 knockdown [Figure 6c-f]. The data indicated that MFAP2 boosted OSCC cell migration and invasion by managing the KEAP1/NRF2 axis.

DISCUSSION

HNSC ranks among the most prevalent cancers globally, with OSCC being the most prevalent type. Despite the remarkable advancements in diagnosing and treating OSCC, certain individuals may still experience local and distant metastases due to variations in their genetic makeup, leading to unfavorable outcomes. Consequently, discovering innovative therapeutic targets might improve the treatment efficacy for patients with OSCC.

A growing literature has indicated that MFAP2 supports the aggressive advancement of cancers. Increased MFAP2 expression level is associated with unfavorable outcomes of individuals with cancer. Consequently, MFAP2 is regarded as a potential indicator for predicting and treating certain tumors, including OSCC.[9,16] The study conducted by Dong *et al*. indicates that MFAP2 might be a useful biomarker for diagnosing and forecasting the development of papillary thyroid cancer.[29] The thorough study conducted by Yu *et al*. highlights that triple-negative breast cancer can be treated by targeting MFAP2, which also serves as a useful biomarker for diagnosis, prognosis, and immunotherapy.[30] In addition, MFAP2 can independently predict stomach adenocarcinoma, and it is related to its prediction.[31] MFAP2 may serve as a diagnostic and prognostic indicator of gastric cancer due to its relation to malignant characteristics.[32] MFAP2 is a

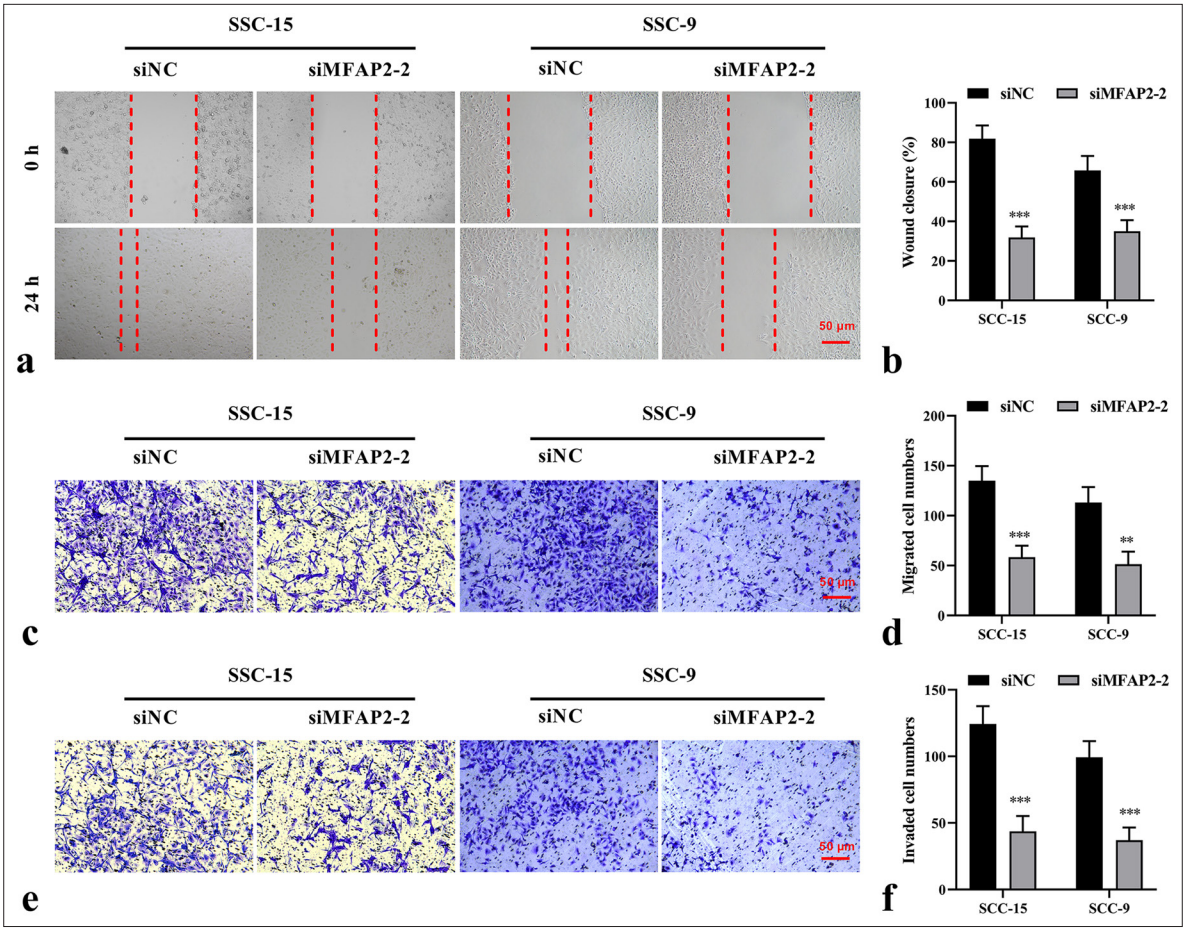

Figure 3: Suppression of MFAP2 hindered the migration and invasion of OSCC cells. SCC-15 and SCC-9 cells underwent transfection with siNC and siMFAP2-2 for 48 h. (a and b) Migratory ability was assessed by wound healing assay. Scale bar: 50 μm. Transwell assays were conducted to measure cell migration (c and d) and invasion (e and f). Scale bar: 50 μm. The results were displayed as means ± SD from at least three repetitions. $^{**}P < 0.01$, $^{***}P < 0.001$ versus the siNC group. Unpaired two-tailed Student's *t*-tests were used for comparisons between two groups (b, d, and f). MFAP2: Microfibrillar-associated protein 2, OSCC: Oral squamous cell carcinoma, SCC: Squamous cell carcinoma, siMFAP2: Small interfering microfibrillar-associated protein 2, SD: Standard deviation, siNC: Matching negative control siRNA.

valuable prognostic marker and an effective anticancer target in hepatocellular carcinoma.[33] MFAP2 plays a crucial role in promoting ESCC metastasis, partially through the activation of FAK-AKT signaling, highlighting the potential of MFAP2 as a promising therapeutic target for ESCC.[12] MFAP2 promotes the malignant progression of GC through activating the PI3K/AKT signaling pathway, providing new insights into the early diagnosis and precision treatment of GC.[34] In addition, in OSCC, higher expression levels of MFAP2 are linked to poorer outcomes of patients. Moreover, Zhang *et al.* discovered that in OSCC cases, MFAP2 is heightened and is related to a worse outcome for patients with OSCC.[16] Consistent with these results, by employing bioinformatics and experimental confirmation, MFAP2 was detected to be highly expressed in OSCC, which was associated with its poor outcomes, highlighting the supportive role of MFAP2 in OSCC.

Development requires stringent regulation of cell proliferation, while uncontrolled growth is a primary feature of cancer. Research on hepatocellular carcinoma reveals that MFAP2 is markedly overexpressed and is linked to its stage; knocking down MFAP2 curtails uterine corpus endometrial carcinoma cell proliferation.[19] Downregulating MFAP2 impedes the proliferation of papillary thyroid carcinoma cells.[29] Apoptosis, as a multifaceted regulator, is crucial for preserving development and homeostasis. An imbalance in its process is linked to numerous diseases, with the onset and progression of cancers being the most worrisome. In thyroid papillary carcinoma and OSCC cells, MFAP2 silencing triggers abundant apoptotic cells.[16,29] In addition, the primary traits of cancer include activating invasion and metastasis. Knocking down MFAP2 impedes melanoma cells from migrating and invading.[35] In TSCC and OSCC cells, MFAP2 overexpression demonstrates greater invasive and

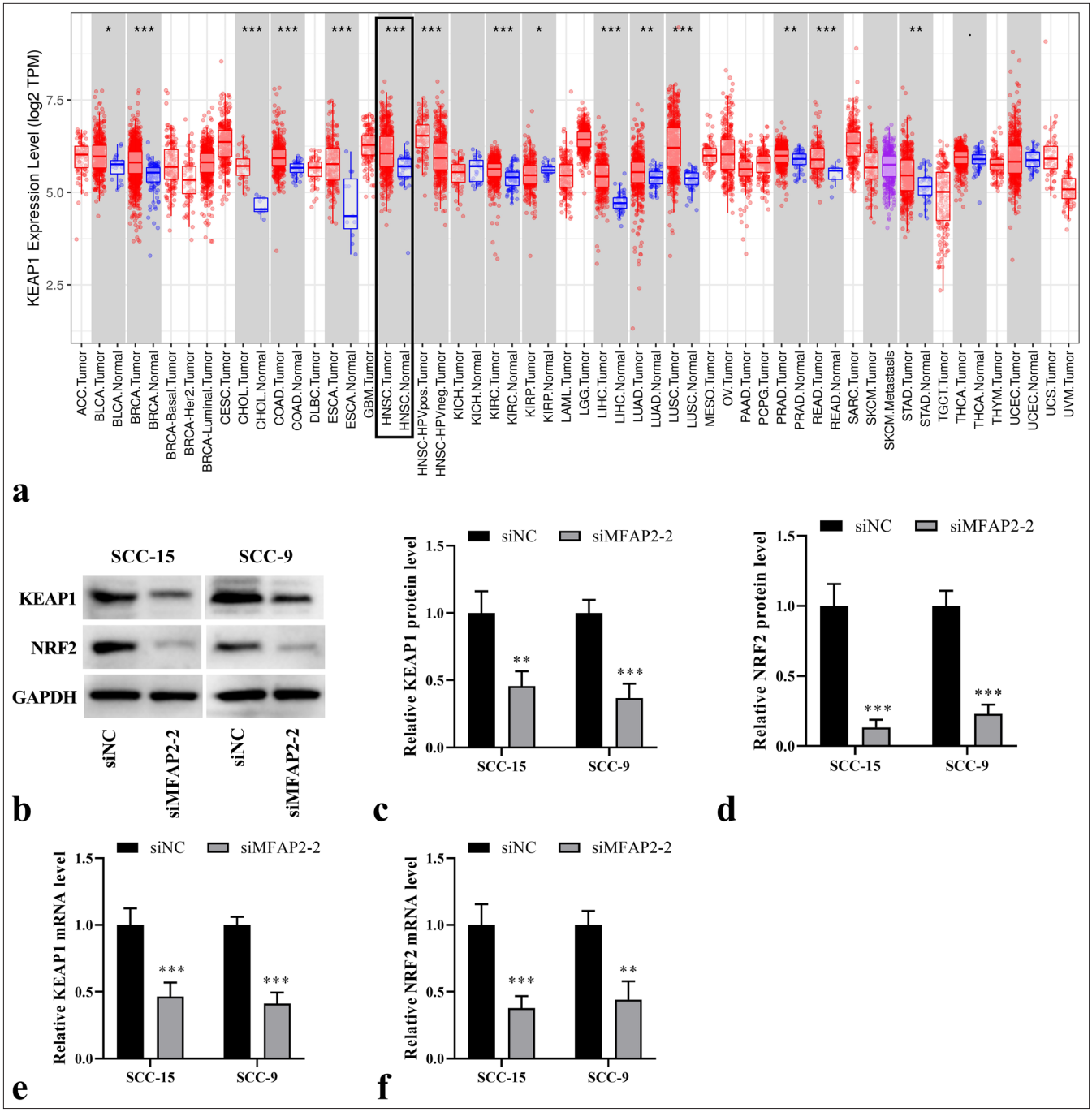

Figure 4: MFAP2 modulated the KEAP1/NRF2 signaling pathway in OSCC. (a) KEAP1 expression in various cancer types was presented in the TIMER2.0 database. (b-d) SCC-15 and SCC-9 cells underwent transfection with siNC and siMFAP2-2 for 48 h. Western blot was conducted to analyze the protein expression level of KEAP1 and NRF2. (e and f) qRT-PCR was conducted to assess the mRNA expression level of KEAP1 and NRF2. The results were displayed as means ± SD from at least three repetitions. $^{**}P < 0.01$, $^{***}P < 0.001$ versus the siNC group. Unpaired two-tailed Student's *t*-tests were used for comparisons between two groups (c-f). MFAP2: Microfibrillar-associated protein 2, OSCC: Oral squamous cell carcinoma, KEAP1: Kelch-like ECH-associated protein 1, NRF2: Nuclear factor erythroid 2-related factor 2, SCC: Squamous cell carcinoma, qRT-PCR: Quantitative real-time polymerase chain reaction, mRNA: Messenger ribonucleic acid, siMFAP2: Small interfering microfibrillar-associated protein 2, SD: Standard deviation, siNC: Matching negative control siRNA.

migratory potential, whereas knocking down MFAP2 plays a role in suppressing proliferation, promoting apoptosis, and inhibiting migration and invasion.[15,16] Our results are consistent with the aforementioned data, showing that

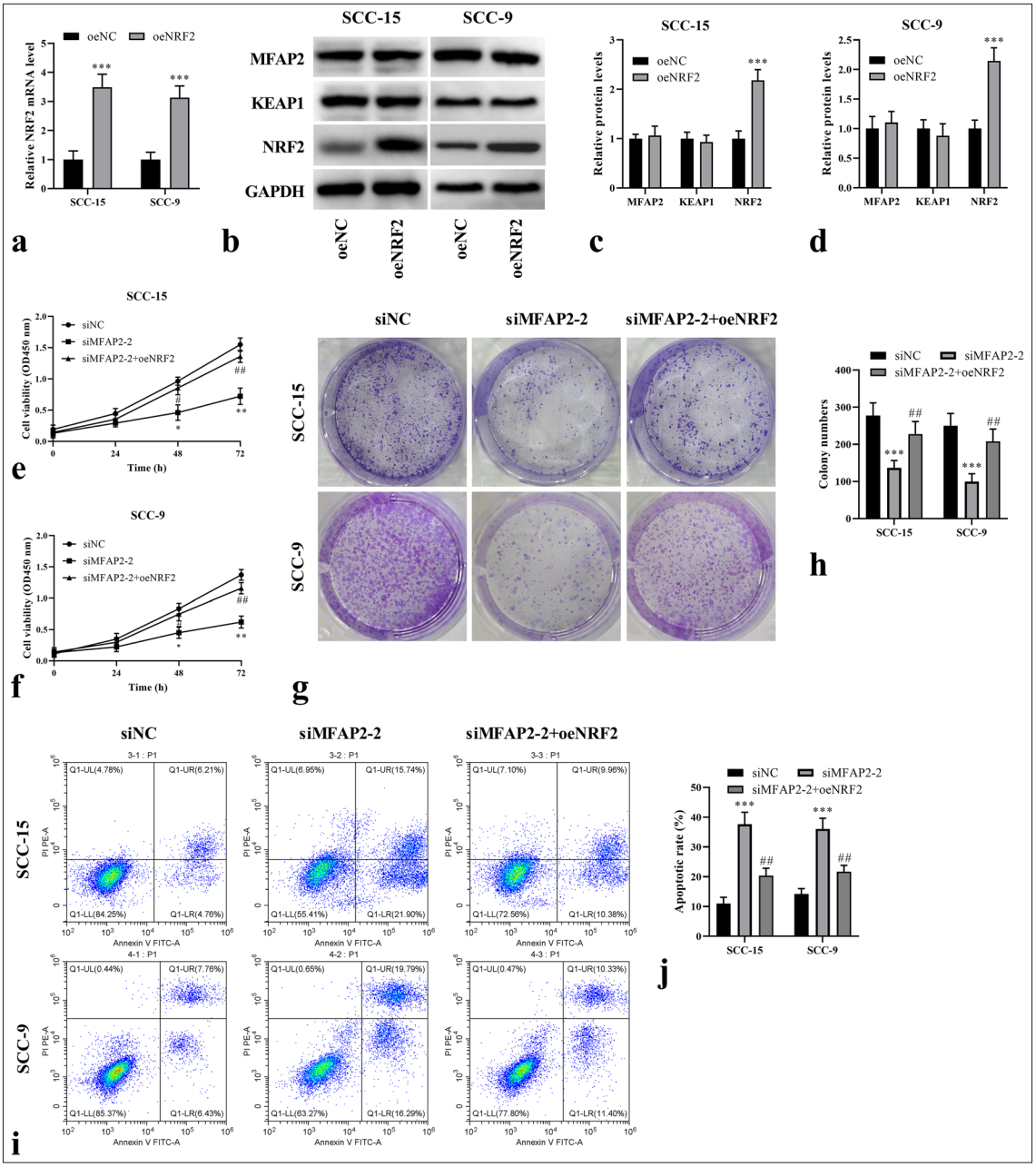

Figure 5: MFAP2 affected the proliferation and apoptosis of OSCC cells through the KEAP1/NRF2 pathway. (a) SCC-15 and SCC-9 cells underwent transfection with oeNC and oeNRF2 for 24 h. qRT-PCR was performed to measure NRF2 mRNA expression. (b-d) SCC-15 and SCC-9 cells underwent transfection with oeNC and oeNRF2 for 48 h, and the protein levels of MFAP2, KEAP1, and NRF2 were examined using Western blot. (e-j) SCC-15 and SCC-9 cells underwent transfection with siNC, siMFAP2-2, and a combination of siMFAP2-2 and oeNRF2. The viability (e and f), proliferation (g and h), and apoptosis (i and j) of the cells were evaluated using CCK-8, colony formation, and flow cytometry assays. The results were displayed as means ± SD from at least three repetitions. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus the siNC group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs the siMFAP2-2 group. Unpaired two-tailed Student's *t*-tests were used for comparisons between two groups (a, c, and d). One-way ANOVA with Tukey's *post hoc* test was applied for multi-group comparisons (e, f, h, and j). MFAP2: Microfibrillar-associated protein 2, OSCC: Oral squamous cell carcinoma, KEAP1: Kelch-like ECH-associated protein 1, NRF2: Nuclear factor erythroid 2-related factor 2, SCC: Squamous cell carcinoma, siMFAP2: Small interfering microfibrillar-associated protein 2, CCK-8: Cell counting kit-8, SD: Standard deviation, ANOVA: Analysis of variance, siNC: Matching negative control siRNA, oeNRF2: NRF2 overexpression.

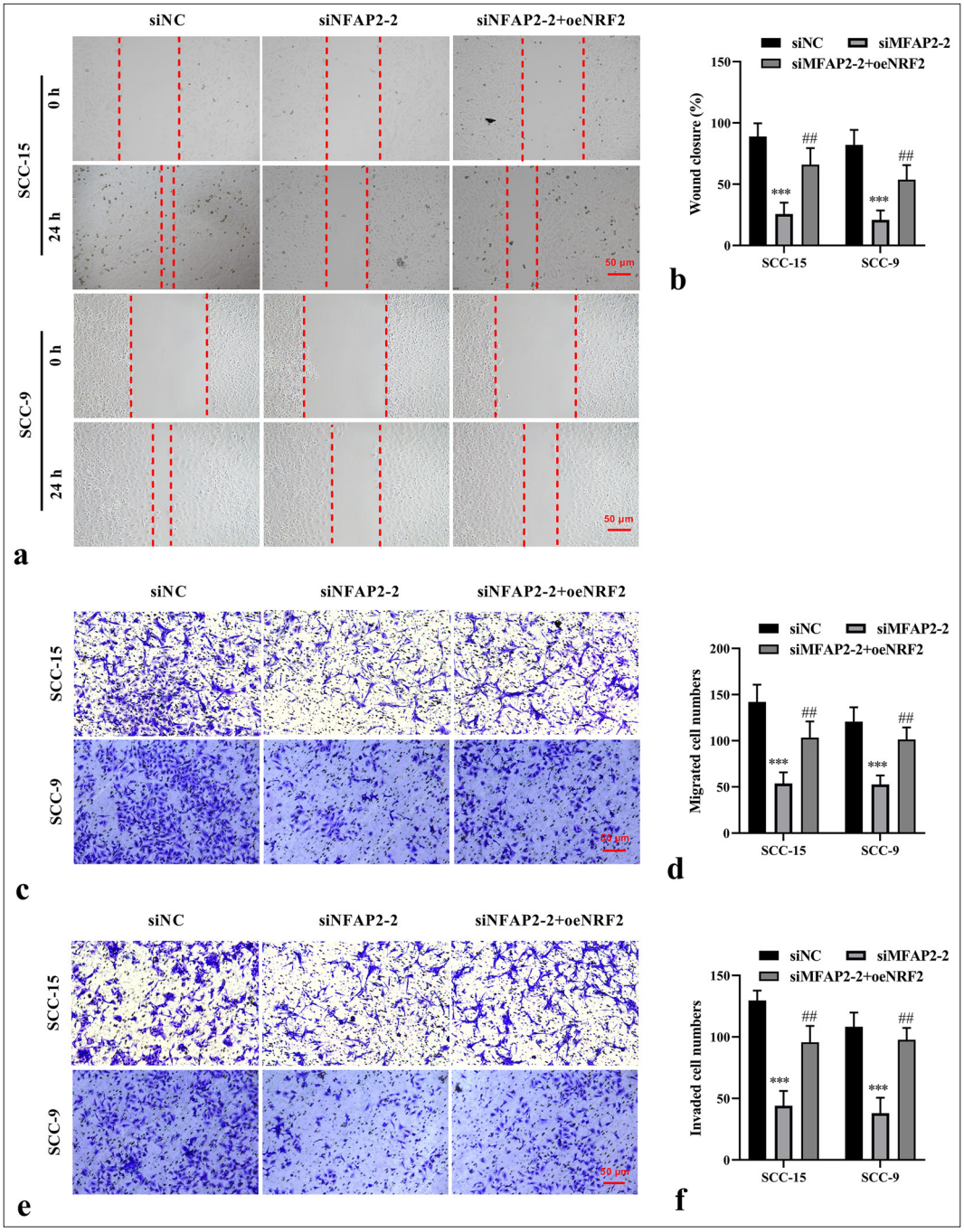

Figure 6: MFAP2 influenced the migration and invasion of OSCC cells through the KEAP1/NRF2 pathway. SCC-15 and SCC-9 cells underwent transfection with siNC, siMFAP2-2, and a combination of siMFAP2-2 and oeNRF2 for 48 h. (a and b) The migratory ability was assessed through a wound healing assay. Scale bar: 50 μm. Cell migration (c and d) and invasion (e and f) were evaluated using Transwell assays. Scale bar: 50 μm. The results were displayed as means ± SD from at least three repetitions. ***$P < 0.001$ versus the siNC group. $^{\#\#}P < 0.01$ versus the siMFAP2-2 group. One-way ANOVA with Tukey's *post hoc* test was applied for multi-group comparisons (b, d, and f). MFAP2: Microfibrillar-associated protein 2, OSCC: Oral squamous cell carcinoma, KEAP1: Kelch-like ECH-associated protein 1, NRF2: Nuclear factor erythroid 2-related factor 2, SCC: Squamous cell carcinoma, siMFAP2: Small interfering microfibrillar-associated protein 2, SD: Standard deviation, ANOVA: Analysis of variance, siNC: Matching negative control siRNA, oeNRF2: NRF2 overexpression.

MFAP2 silencing hindered the proliferative, migratory, and invasive capacities of OSCC cells while promoting apoptotic capability, implying that targeting MFAP2 could be crucial for OSCC treatment.

KEAP1 functions as a sensor for oxidative stress, with a specific focus on targeting NRF2.[36] NRF2 is primarily responsible for governing cell reactions to environmental stress and supervising a variety of genes that provide cytoprotection. Normally, KEAP1 and NRF2 form trimers to facilitate NRF2 degradation by ubiquitination via the proteasome. Evidence indicates that interference with NRF2 activation can cause or intensify numerous diseases. Conversely, atypical NRF2 activation can also lead to intriguing pathological features, particularly in cancer.[37] A comprehensive cancer study reveals immune evasion traits in squamous cancers with high NRF2 activity.[24] When KEAP1 loses its function, it markedly activates NRF2, leading to increased proliferation of cancer cells and enhanced cancer stemness and oxidative stress resistance in HNSC cells.[38] In OSCC cells, overexpressing NRF2 enhanced cancer characteristics, whereas silencing NRF2 suppressed them. Furthermore, activating NRF2 in KEAP1$^{-/-}$ mice causes squamous epithelial cells in the tongue to proliferate excessively.[25] Huang *et al*. found that human OSCC tissues show increased levels of Keap1 and Nrf2.[39] The KEAP1/NRF2 signaling pathway is affected by upstream molecules, and it contributes to the advancement of cancer. For example, through KEAP1/NRF2 signaling, TNFAIP2 lowers cisplatin sensitivity in HNSC.[26] SELENBP1 binds to the KEAP1 promoter and promotes its transcription, leading to a reduction in NRF2 at the protein level through enhancing polyubiquitination and subsequent breakdown, thereby decreasing chemoresistance in OSCC.[40] In this study, we found that KEAP1 was overexpressed in HNSC in accordance with bioinformatic analysis. Knocking out MFAP2 in OSCC cells resulted in lower protein expression levels of KEAP1 and NRF2. The overexpression of NRF2 negated the negative influence of MFAP2 depletion on the aggressive traits of OSCC cells, implying that MFAP2 could drive the malignant advancement through the KEAP1/NRF2 axis.

This study also has some limitations. Given the limited funding and experimental resources, the gain-of-function effects of MFAP2 on the malignant behavior of OSCC were not investigated, such as cell proliferation and migration. We did not incorporate animal experiments or utilize patient-derived OSCC tissues for a more in-depth exploration and analysis. In addition, we did not perform rescue experiments with siRNA-resistant MFAP2 to address the potential off-target effects of siRNA. Hence, in the subsequent investigation, we will explore the effects of MFAP2 overexpression on the malignant phenotypes of OSCC, such as proliferation and migration. We will undertake animal experiments and add OSCC patient samples to further explore the importance of MFAP2 in OSCC. Furthermore, we will conduct rescue experiments with siRNA-resistant MFAP2 to explore the off-target effects of siRNA.

SUMMARY

The expression level of MFAP2 increased in OSCC tissues and cells. Such an increase was associated with the aggressive activities of OSCC cells, such as proliferation, apoptosis, and mobility. By facilitating KEAP1/NRF2 signaling, MFAP2 might enhance OSCC growth and spread, indicating its potential as a biomarker and treatment candidate in patients with OSCC. Our future research will focus on the interaction between MFAP2 and KEAP1/NRF2 signaling to provide theoretical and experimental support for new therapeutic strategies in OSCC treatment. In addition, we will incorporate more comprehensive genomic characterization to address potential mutation-specific effects.

AVAILABILITY OF DATA AND MATERIALS

The data and materials that support the findings of this study are available from the corresponding author on reasonable request.

ABBREVIATIONS

BSA: Bovine serum albumin
CCK-8: Cell counting kit-8
DMEM: Dulbecco's modified eagle medium
ESCC: Esophageal squamous cell carcinoma
FBS: Fetal bovine serum
GC: Gastric cancer
HNSC: Head-and-neck squamous cell carcinoma
KEAP1: Kelch-like ECH-associated protein 1
MFAP2: Microfibrillar-associated protein 2
MFAPs: Microfibrillar-associated proteins
mRNA: Messenger ribonucleic acid
NRF2: Nuclear factor erythroid 2-related factor
OSCC: Oral squamous cell carcinoma
qRT-PCR: Quantitative real-time polymerase chain reaction
SELENBP1: Selenium binding protein 1.
TNFAIP2: TNF-alpha-induced protein 2
TNFAIP2: Tumor necrosis factor-α-induced protein 2
TSCC: Tongue squamous cell carcinoma

AUTHOR CONTRIBUTIONS

WZY: Literature search, experimental studies, statistical analysis, and manuscript preparation; JYT: Design, data analysis, manuscript preparation, manuscript editing, and review; KXD: Design, manuscript editing, and review; KMJ: Design, manuscript editing, and review. All authors read and approved of the final manuscript. All authors meet ICMJE authorship requirements.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval and consent to participate were not required as this study did not involve animal or human experimentation, and the patient data used were obtained from the database.

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Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

EDITORIAL/PEER REVIEW

To ensure the integrity and highest quality of CytoJournal publications, the review process of this manuscript was conducted under a **double-blind model** (authors are blinded for reviewers and vice versa) through an automatic online system.

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