Hypoxia-induced ROS promotes mitochondrial fission and cisplatin chemosensitivity via HIF-1α/Mff regulation

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Abstract
Chemotherapy treatment based on Cisplatin (CDDP) is established as the drug of choice for head and neck squamous cell carcinoma (HNSCC). Malignant tumors respond to microenvironment alteration through a dynamic balance of mitochondrial fission and fusion. HNSCC is known to have hypoxic conditions, yet the effects and underlying mechanisms of hypoxia on chemosensitivity and mitochondrial dynamics remain unclear. We found that hypoxia promoted mitochondrial fission and CDDP sensitivity in HNSCC cells. Importantly, Mff was shown to be correlated with chemosensitivity in clinical samples of HNSCC that underwent a hypoxic condition. Hypoxia-inducible factor 1 α-subunit (HIF-1α) dramatically increased Mff transcriptional expression and directly bound to Mff. Hypoxia enhanced the release of reactive oxygen species (ROS) and upregulated the expression of Mff via HIF-1α in HNSCC cells. ROS depletion in HNSCC cells attenuated HIF-1α, Mff expression, and mitochondrial fission. Moreover, a knockdown of Mff suppressed hypoxia-induced mitochondrial fission and decreased CDDP chemosensitivity *in vivo* and *in vitro*. Our findings revealed that the hypoxia-induced release of ROS promoted mitochondrial fission and CDDP chemosensitivity via the regulation of HIF-1α/Mff in HNSCC cells, indicating that Mff may serve as a new biomarker to predict neoadjuvant chemosensitivity in HNSCC patients.

Key words: hypoxia; cisplatin sensitivity; mitochondrial fission; ROS; head and neck squamous cell carcinoma

Introduction

Head and neck squamous cell carcinoma (HNSCC), an aggressive and common malignant tumor causing high morbidity and mortality, is a major health challenge worldwide.[1, 2] Although great advancements in surgery, chemotherapy, and radiotherapy treatments have been achieved in the past few decades, the overall 5-years survival rate of HNSCC patients has remained approximately 50%.[3] For HNSCC patients, cisplatin (CDDP) based chemotherapy has been established as the drug of choice.[4] However, chemoresistance continues to be responsible for adverse effects from HNSCC treatments, which has led to poor outcomes. Thus, a better understanding of the molecular mechanisms underlying chemoresistance is critical for establishing the most appropriate treatment strategies.

The role of the tumor microenvironment and hypoxia in cancer progression has garnered much attention in recent years.[5, 6] Normally, the O_2 level of tissues ranges from 1% to 11%, while cells are often cultured in a 20% O_2 environment.[7] Rapid proliferation and unmatched angiogenesis in cancer cells leads to a hypoxic condition in most solid malignant tumors.[8] A recent study has shown that HNSCC experiences hypoxic conditions, which may positively affect anti-EGFR therapies.[9] Previous studies have also shown that excessive accumulation of reactive oxygen species (ROS) could promote cancer progression,[10] and hypoxia induces increased ROS.[8, 11] To alter cellular adaptive responses to hypoxia, the activity of hypoxia-inducible factor 1 α-subunit (HIF-1α) acts as a key mechanism.[12] HIF-1α, a transcription factor, could transactivate various target genes to participate in malignant tumor biological behavior.[13, 14]

Previous studies have shown that abnormal mitochondrial dynamics are correlated with diseases via the regulation of apoptosis.[15, 16] Tumor cells respond to microenvironment alteration through the dynamic balance of mitochondrial fission and fusion.[17] Accumulated evidence has revealed that mitochondrial fission promotes tumor cell CDDP sensitivity in tongue squamous cell carcinoma.[18, 19] However, how mitochondrial fission contributes to CDDP sensitivity in hypoxia-cultured HNSCC cells is still unclear. Furthermore, the molecular mechanism of mitochondrial fission and fusion in modulating CDDP sensitivity in hypoxic-cultured HNSCC cells has not been elucidated.

In the present study, we aimed to investigate the effect of hypoxia on CDDP-cultured HNSCC cells and mitochondrial fission. This study revealed that hypoxia-induced ROS accumulation promotes mitochondrial fission and CDDP chemosensitivity via the regulation of HIF-1α/Mff in HNSCC cells.

Materials and Methods

Ethics

The study was approved by the Ethics Committee of the Second Xiangya Hospital (Approval No. 2020530, 2020/9/14), and written informed consent was obtained from all participants prior to enrollment. All experimental methods abided by the Helsinki Declaration. All animal studies were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the Second Xiangya Hospital Animal Ethical and Welfare Committee (Approval No. 2020489, 2020/9/9).

Patients and Specimens

All HNSCC samples were obtained from the Department of Oral and Maxillofacial Surgery at the Second Xiangya Hospital of Central South University. More specifically,

72 pairs of tumor and adjacent normal tissues were collected from primary HNSCC patients who underwent an initial surgical treatment between July 2017 and June 2019. Patients who received preoperative treatment and had a malignant history were excluded. Clinical parameters were obtained from medical records. Moreover, clinical stage and pathological differentiation were performed according to the World Health Organization Classification of Tumors and the tumor node metastasis staging system from the Union for International Cancer Control.

Cell cultures and reagents

HN6 and HN30 were kindly provided by the University of Maryland Dental School, USA. Cal27 and 293T cells were purchased from the American Type Culture Collection (MD, USA). Cal27, HN6, HN30, and 293T cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, CA, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, CA, USA), streptomycin (100 µg/mL), and penicillin (100 units/mL) at 37°C in a humidified 5% CO_2 atmosphere. For hypoxia, tumor cells were cultured in a tri-gas incubator (ESCO, Singapore), consisting of 5% CO_2, 94% N_2, and 1% O_2. Hydrogen peroxide (H_2O_2), CDDP, and N-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich (USA).

RNA extraction and real-time PCR analysis

The RNA was extracted from the tumor samples and cells using TRIzol reagent (Takara, Japan), and cDNA was synthesized with a PrimeScript RT Reagent Kit (Takara, Japan). A real-time PCR (RT-PCR) was performed using the SYBR Premix Ex Taq Reagent Kit (Takara, Japan) with the StepOne RT-PCR System (Life Technologies, USA) according to the manufacturer's instructions. The mRNA levels were normalized to β-actin levels. The primer sequences used in the present study are listed in Supplementary Table 1.

MTT assays

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay. Tumor cells were seeded in a 96-well plate at a density of 2000 cells per well with 100 µL of medium at 37°C. The cells were incubated with 100 µL of 0.5 mg/mL MTT (Sigma-Aldrich, USA) in Dulbecco's modified Eagle's medium for 4 h. Twelve hours later, the cells were cultured with a gradient concentration of CDDP for 48 h. The formazan was solubilized in 150 µL dimethyl sulfoxide. Absorbance was measured using a multi-well plate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 490 nm. The half maximal inhibitory concentration (IC50) was used to evaluate the degree of tumor cell response to CDDP.

Apoptosis analysis

Apoptosis was assessed by flow cytometric analysis and terminal deoxynucleotidyl trans-ferase-mediated dUTP nick end labeling (TUNEL). TUNEL assays were performed using the Click-iT[TM] Plus TUNEL assay kit (Invitrogen, USA) according to the manufacturer's instructions. Flow cytometric analysis was performed using the Annexin V FITC Apop Dtec Kit (BD Pharmingen, USA) according to the manufacturer's instructions. The cells were trypsinized and centrifuged at 300 × g for 10 min at 4°C. Afterward, the cell pellets were resuspended in binding buffer and stained with Annexin V FITC and propidium iodide for 15 min on ice. Apoptotic cells were analyzed by flow cytometry (BD FACS Calibur, USA).

Western blot analysis

Tumor cells were harvested at the indicated times in SDS lysis buffer (Beyotime, China). Protein samples were separated using 10% polyacrylamide gels and transferred to 0.45-μm poly (vinylidene fluoride) membranes (Merck Millipore, USA). The membranes were soaked in 10% skim milk for 1 h at room temperature and incubated overnight at 4°C with the primary antibody. Subsequently, the membranes were probed with horseradish peroxidase-labeled secondary rabbit antibodies (Beyotime, China) for 1 h. The electrochemiluminescence (New Cell Molecular Biotech, China) developing solution was used to detect signals on a chemiluminometer (Amersham Imager 600). β-Actin was used as a control. The antibodies (1:1000) against Mff, HIF-1α, Mfn2 and β-actin were purchased from Cell Signaling Technology (USA).

Detection of relative ROS levels

Intracellular ROS levels were detected using 2,7-dichloro-dihydrofluorescein diacetate (Sigma-Aldrich, USA) and dihydroethidium (Sigma-Aldrich, USA) by flow cytometry (BD FACS Calibur, USA). This ROS analysis technique has been described in a previous study.[8]

Mitochondrial staining and analysis of mitochondrial fission

The cells were cultured on coverslips and stained with MitoSpy[TM] Red CMXRos (BioLegend, USA) for 30 min at 37°C. Afterward, the cells were fixed with 4% paraformaldehyde and stained with 4'-6-diamidino-2-phenylindole (DAPI; Sangon Biotech, China). Cells were viewed using a TCS SP2 laser-scanning confocal microscope (Leica Microsystems, Germany). Mitochondrial morphology was then quantified and assessed as described previously.[8]

Analysis of immunofluorescence

The cells grown on coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 10% goat serum, and incubated with the Mff antibody (CST, 1:500) and the HIF-1α antibody (CST, 1:500) at 4°C overnight then incubated with the Alexa Fluor 488-cojugated secondary antibody (Invitrogen, USA) or Alexa Fluor 549-cojugated secondary antibody (Invitrogen, USA) at room temperature in the dark. Cells were then stained with DAPI (Sangon Biotech, China) to detect the nuclei and viewed using a TCS SP2 laser-scanning confocal microscope (Leica Microsystems, Germany).

Co-immunoprecipitation analysis

A co-immunoprecipitation (Co-IP) analysis was performed in our previous study.[20] In brief, the cells were lysed with IP buffer and incubated with the specific antibodies previously mentioned at 4°C overnight, followed by incubation with protein A/G Magbeads (Merck) for 6 h at 4°C. The proteins were separated from the beads for 10 min at 105°C. The supernatants were then used for subsequent immunoblotting analyses.

Cell transfection

SiRNA (Ribobio, China) was transfected into 293T cells using Lipofectamine RNAiMAX (Invitrogen, USA) according to the manufacturer's instructions. Twenty-four hours later, the cells were harvested and the corresponding experiments were performed. The siRNAs used in the present study were designed and synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China).

Luciferase analysis

In brief, the 293T cells (2×10^5), which were cultured on a 6-well plate, were co-transfected with Mff response luciferase plasmid and Renilla luciferase vector using the Lipofectamine 3000 reagent (Invitrogen, USA), according to the manufacturer's instructions. After 48 h, the dual luciferase system (Promega, USA) was used to detect luciferase activity.

Lentiviral transduction and stable strain screening

The Mff interfering lentiviral vector[21] (LV-Mff) and interfering control lentiviral vector (LV-NC), which were constructed by HanYin Biotechnology Co, Ltd (China), conferred puromycin resistance. Lentiviral transduction was performed according to the manufacturer's instructions. After 72 h of transfection, the culture medium with puromycin at a final concentration of 10 μg/mL was used to screen for stably stained cells.

Xenograft mouse experiments

The animal experiments, using BALB/C nude mice (Shanghai Laboratory Animal Center) aged 4 weeks old, were performed in accordance with the ethical standards and national guidelines. Cal27 cells (1×10^6) stably expressing LV-Mff or LV-NC were resuspended in 100 μl of phosphate buffered saline and subcutaneously injected into the dorsal flanks of mice (n = 6 in each group). Tumor sizes were measured using a caliper every three days. CDDP and saline were intraperitoneally injected at 5 mg/kg body weight in every 3 days from day 7 to 33. After day 33, the mice were sacrificed to collect tumor samples and measure tumor weights.

Statistical analysis

To compare the differences in expression between HNSCC and matched adjacent normal tissue, paired t-tests were performed. The Kruskal-Wallis tests and Mann-Whitney U tests were used to analyze the association between mRNA (SLC2A1, PDGFB, VEGFA and HIF-1α) levels and clinical parameters. The comparison between the groups was analyzed using one-way ANOVA or Student's t-test. The correlation between two variables was analyzed using Pearson's correlation. All statistical analyses were performed using SPSS 19.0 (SPSS, Chicago, IL, USA). All values were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Hypoxia facilitates CDDP chemosensitivity in HNSCC cells

Hypoxia can regulate malignant tumor vascularization, invasion, chemoresistance, and metastasis in various types of cancer.[8, 22, 23] To understand whether the HNSCC microenvironment was hypoxic, hypoxia markers[8] (solute carrier family 2 member 1 (SLC2A1), platelet-derived growth factor B (PDGFB), vascular endothelial growth factor A (VEGFA), and HIF-1α) were detected in 72 samples of tumor tissues paired with adjacent normal tissues. The expression levels of hypoxia markers were significantly higher in tumor tissues than in the normal tissues (Fig. 1A). The levels of PDGFB were related to patient age (≥ 60 years and < 60 years), sex (male and female), smoking history (yes or no), and alcohol history (yes or no) in our study (Supplementary Table 2). An analysis of The Cancer Genome Atlas (TCGA) database further showed that these hypoxia markers were also significantly upregulated in HNSCC samples (Supplementary Fig. 1A). These data confirm the hypoxic environment in HNSCC tumors.
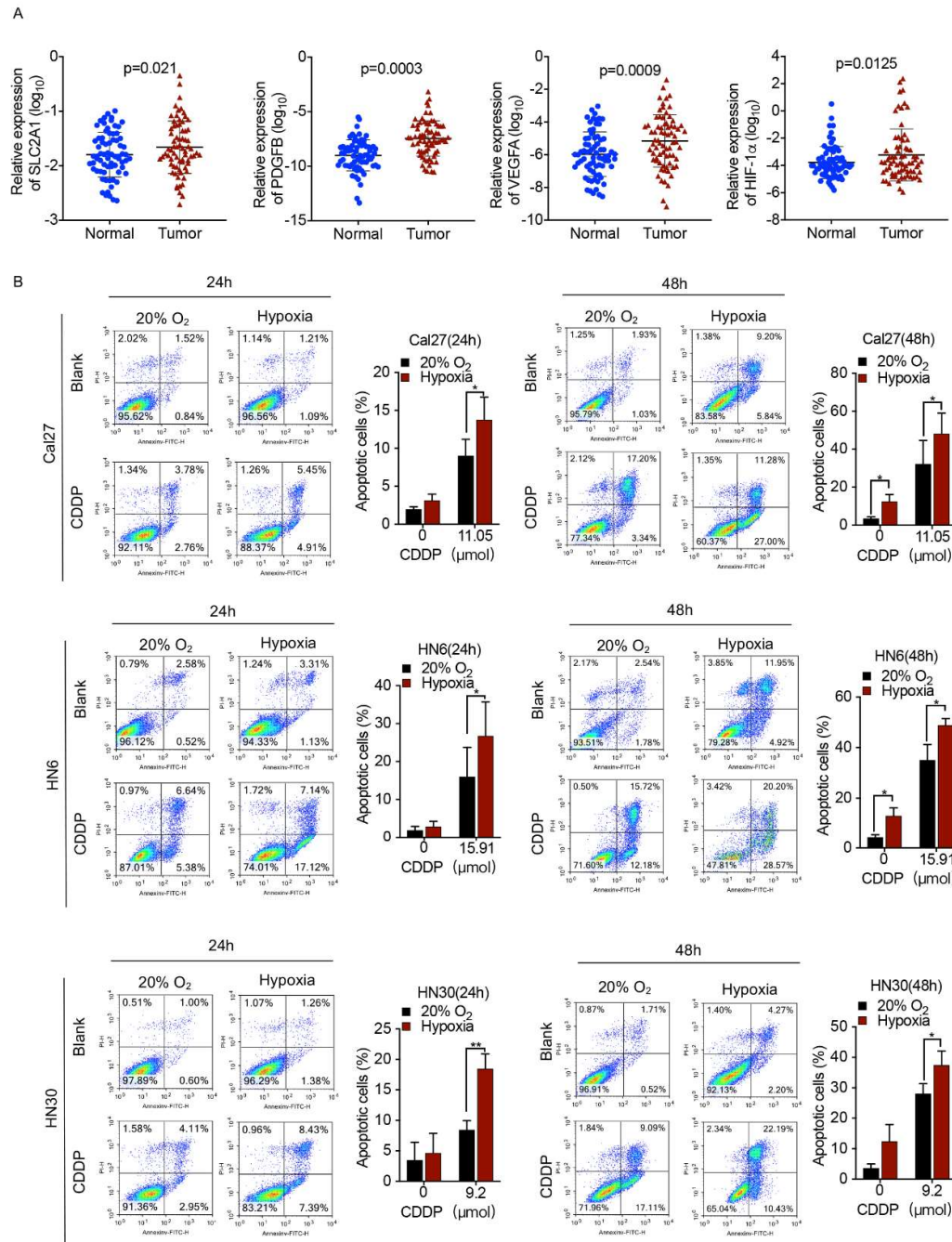
Figure 1. Hypoxia induces CDDP chemosensitivity in HNSCC cells

(A) The expression of hypoxia markers (SLC2A1, PDGFB, VEGFA, and HIF-1α) was upregulated in HNSCC samples (n=72).

(B) Hypoxia promotes cell apoptosis in Cal27, HN6, and HN30 cells, while the apoptotic effect of hypoxia was augmented in HNSCC cells after incubation with CDDP. Cal27, HN6, and HN30 cells were treated with the indicated concentrations of CDDP. Data are presented as the mean ± standard deviation (SD) from 3 independent experiments (*P < 0.05, ** P < 0.01).

To determine the effect of hypoxia on CDDP sensitivity in HNSCC, Cal27, HN6, and HN30 cells were cultured in 20% O$_2$ or a hypoxic environment (1% O$_2$). The IC50 value of CDDP, identified by MTT assay in Cal27, HN6, and HN30 cells, was 11.05,

15.91, and 9.2 μM, respectively (Supplementary Fig. 1B). Cal27, HN6, and HN30 cells were treated with the IC50 of CDDP for apoptosis assays. The proportion of apoptotic cells (positive for Annexin V FITC and propidium iodide staining) in hypoxia-cultured HNSCC cells was higher than that in 20% O_2-cultured HNSCC cells. Moreover, the apoptotic effect of hypoxia was augmented in HNSCC cells after incubation with CDDP. The proportion of apoptotic cells in hypoxia-CDDP-cultured HNSCC cells was dramatically higher than that in the 20% O_2-CDDP-cultured HNSCC cells after 24 h and 48 h (Fig. 1B). These findings showed that hypoxia enhances CDDP sensitivity in HNSCC cells.

Hypoxia increases mitochondrial fission via Mff/Mfn2 and ROS levels in HNSCC cells

In the tumor microenvironment, ROS increases under hypoxic conditions[8, 11] and is produced as a byproduct by mitochondria.[10] To explore the effect of hypoxia on ROS and mitochondrial fission, ROS levels and mitochondrial fission were measured in Cal27, HN6, and HN30 cells. After 24 h or 48 h cultured in hypoxic conditions, Cal27, HN6, and HN30 cells showed a dramatic increase in both the intracellular H_2O_2 and superoxide (O_2^-) levels compared to the 20% O_2-cultured cells (Fig. 2A). Mitochondrial fission in hypoxia-cultured HNSCC cells increased more dramatically than that in 20% O_2-cultured HNSCC cells for 24 h or 48 h, indicated by decreased mitochondrial length (Fig. 2B).

To clarify the molecular mechanism by which hypoxia-induced mitochondrial fission occurs, the major mitochondrial fission and fusion genes (Drp1, Fis1, Mff, Mid49, Mid51, GDAP1, Mfn1, Mfn2 and OPA1) [24] expression levels were detected using RT-PCR in Cal27, HN6, and HN30 cells (Supplementary Fig 1C). Our results showed that the transcriptional expression level of Mff increased more significantly in hypoxia-cultured HNSCC cells than that in 20% O_2-cultured HNSCC cells, and also showed a time-dependent increasing pattern (Fig. 2C). In addition, the expression level of Mfn2 decreased more dramatically in hypoxia-cultured HNSCC cells compared to that in 20% O_2-cultured HNSCC cells (Fig. 2C). To further determine if Mff and Mfn2 proteins were responsible for mitochondrial fission induced by hypoxia, Mff and Mfn2 were detected by western blotting. The post-translational level of Mff increased under hypoxia in a time-dependent manner (Fig. 2D). The post-translational level of Mfn2, however, decreased under hypoxic conditions (Fig. 2D). To confirm hypoxic conditions in HNSCC cells, the protein level of HIF-1α, detected by western blotting, was significantly increased in HNSCC cells under hypoxic conditions (Fig. 2D). These results indicate that hypoxic HNSCC cells undergo mitochondrial fission via Mff upregulation and Mfn2 downregulation.
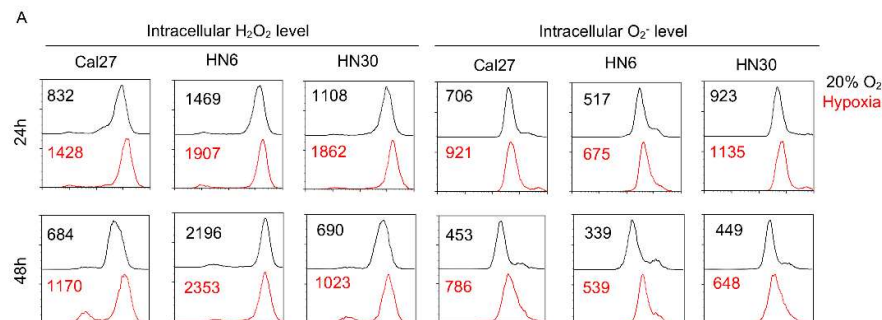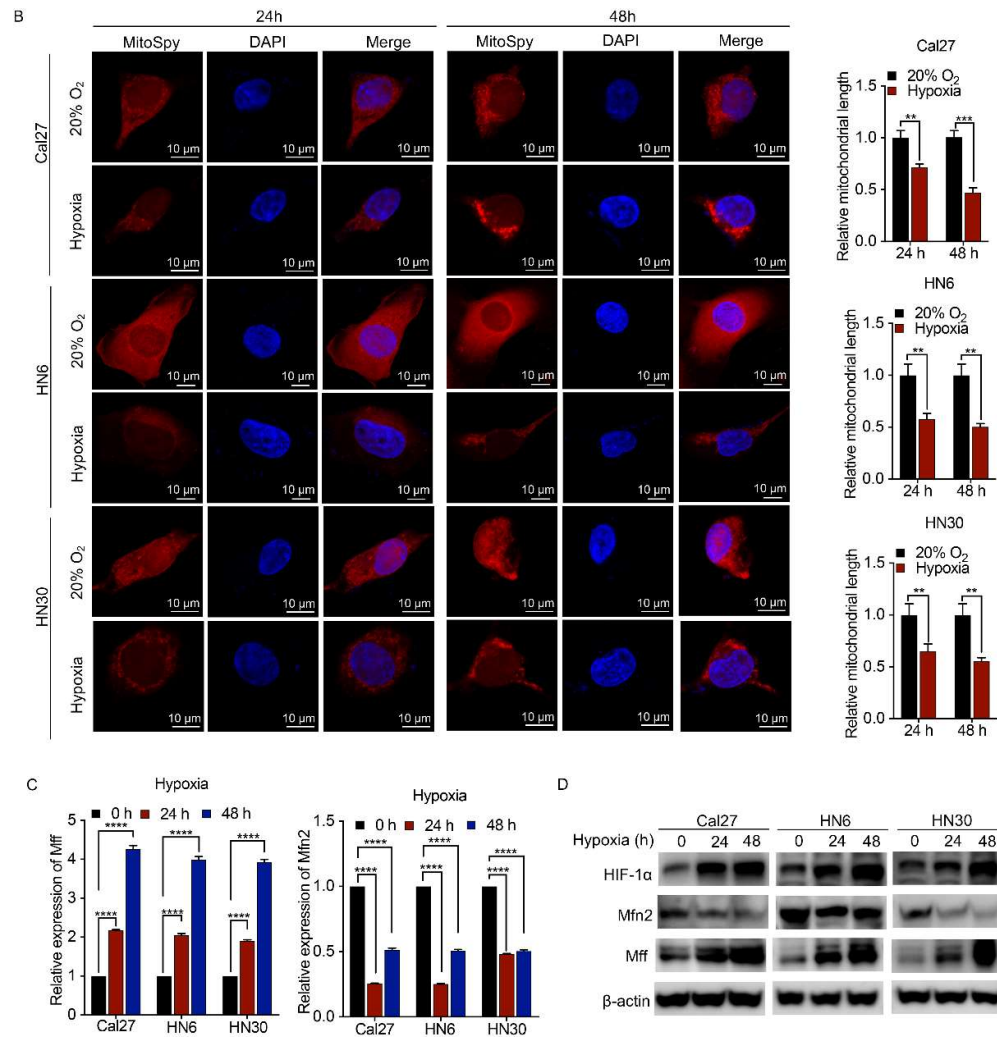
Figure 2. Hypoxia increases ROS levels and mitochondrial fission via Mff and Mfn2 in HNSCC cells

(A) The intracellular ROS levels (O_2- and H_2O_2) of Cal27, HN6, and HN30 cells after 24 h or 48 h of exposure to hypoxia were measured.

(B) The mitochondrial lengths of Cal27, HN6, and HN30 cells were measured using MitoSpy staining.

(C) The Mff and Mfn2 were screened by RT-PCR for hypoxia-induced mitochondrial fission in HNSCC cells.

(D) Western blot analysis was used to confirm the protein expression of HIF-1α, Mff, and Mfn2 in Cal27, HN6, and HN30 cells. Data are presented as the mean ± SD from 3 independent experiments (** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).
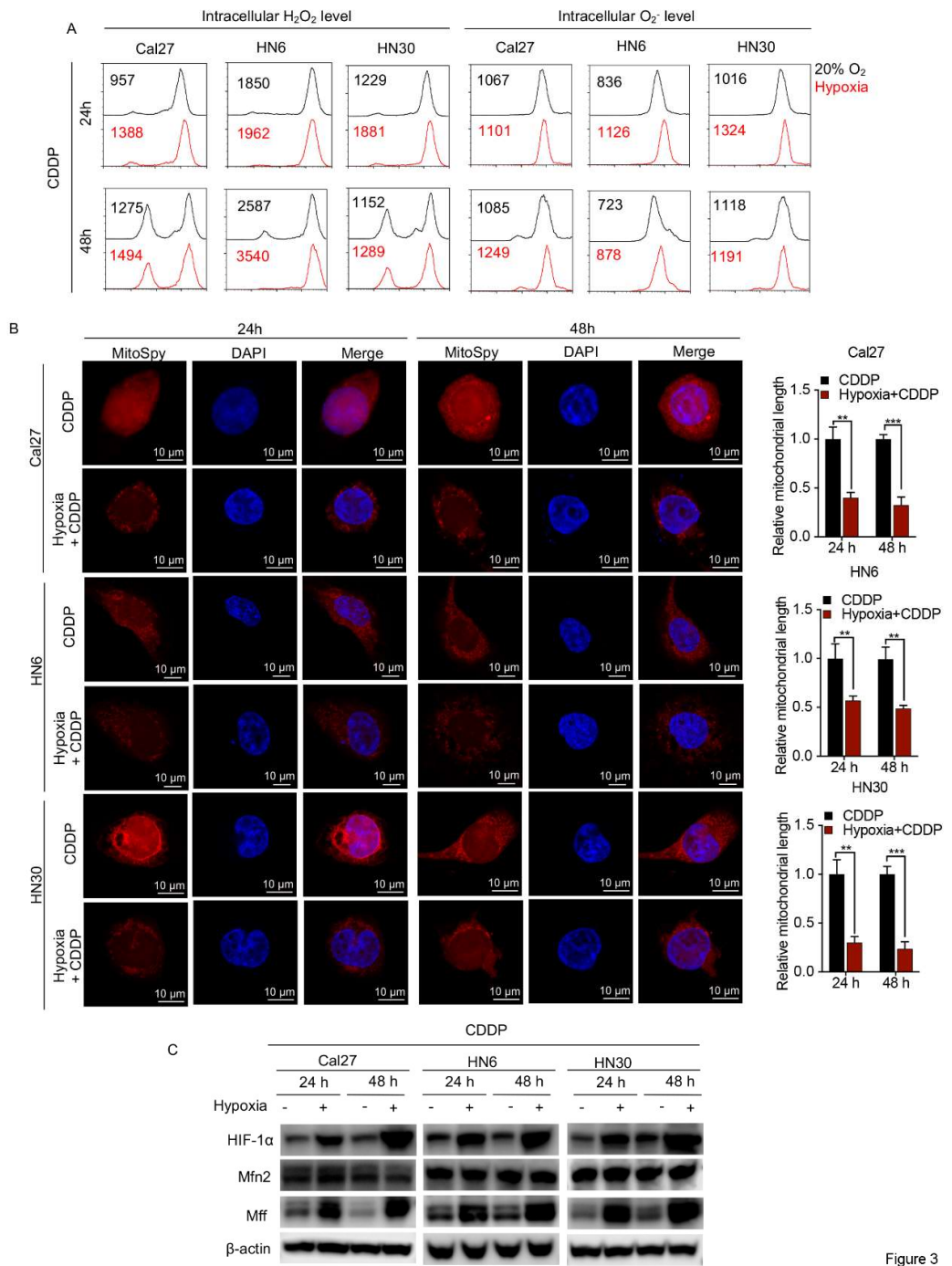
Hypoxia enhances ROS release and promotes mitochondrial fission via Mff in HNSCC cells treated with CDDP

To explore the effect of hypoxia on ROS and mitochondrial fission in HNSCC cells treated with CDDP, ROS levels and mitochondrial fission were measured. In hypoxia-CDDP-cultured HNSCC cells, the intracellular H_2O_2 levels and superoxide (O_2^-) levels were significantly higher than that in 20% O_2-CDDP-cultured cells for 24 h or 48 h (Fig. 3A). The mitochondrial fission in hypoxia-CDDP-cultured HNSCC cells were more dramatically increased than that in 20% O_2-CDDP-cultured HNSCC cells for 24 h or 48 h (Fig. 3B).

The protein level of HIF-1α significantly increased in the hypoxia-CDDP-cultured

HNSCC cells (Fig. 3C). To determine if Mff and Mfn2 proteins were still responsible for mitochondrial fission induced by hypoxia in CDDP-treated cells, Mff and Mfn2 were detected by western blotting. The post-translational level of Mff increased in hypoxia-CDDP-cultured HNSCC cells and showed a time-dependent pattern, while the post-translational level of Mfn2 was not significantly altered (Fig. 3C). Together, these results suggest that hypoxia-CDDP-cultured HNSCC cells undergo mitochondrial fission through Mff upregulation.


Figure 3. Hypoxia enhances ROS levels and mitochondrial fission via Mff in HNSCC cells treated with CDDP

(A) The intracellular ROS levels (O_2^- and H_2O_2) of hypoxia-CDDP-cultured HNSCC cells were measured by flow cytometric analysis.

(B) Cal27, HN6, and HN30 cells stained with MitoSpy to evaluate mitochondrial length by confocal microscopy.

(C) Western blot analysis was used to confirm the protein expression of HIF-1α, Mff, and Mfn2 in Cal27, HN6, and HN30 cells. Data are presented as the mean ± SD from 3 independent experiments (** $P < 0.01$, *** $P < 0.001$).

Hypoxia-induced HIF-1α facilitates Mff expression level in HNSCC

To examine whether Mff accounts for hypoxia-induced chemosensitivity and malignant transformation in HNSCC, the expression levels of HIF-1α, Mff, and Mfn2 were detected in HNSCC tissues. The mRNA expression level of Mff was upregulated more in CDDP-sensitive HNSCC patients than in CDDP-resistant HNSCC patients, while Mfn2 expression was not significantly altered (Fig. 4A). Further analysis revealed that HIF-1α expression was correlated with the Mff levels ($r = 0.9456$, $P < 0.0001$) but not correlated with Mfn2 (Fig. 4B). Moreover, both Mff and Mfn2 expression were significantly upregulated in HNSCC tissues (Fig. 4C). In addition, Mff was correlated with the extracapsular spread of metastatic lymph nodes, while Mfn2 was related to tumor size, prior radiotherapy, and pathological differentiation (Fig. 4D). Together, these data revealed that hypoxia-induced mitochondrial fission events in HNSCC are correlated with chemosensitivity.

In the present study, a significantly positive correlation between HIF-1α and Mff was observed. This result indicated that HIF-1α may interact and bind directly with Mff in HNSCC cells. To verify this hypothesis, co-IP, luciferase analysis, and immunofluorescence staining were performed. The endogenous interaction between HIF-1α and Mff was confirmed by Co-IP (Fig. 4E). Moreover, hypoxia-induced HIF-1α dramatically increased the Mff transcriptional activity in 293T cells, and the knockdown of HIF-1α partly decreased Mff activity (Fig. 4F). In addition, immunofluorescent staining showed that Mff was localized to the cytoplasm and that part of HIF-1α was also localized to the cytoplasm (Fig. 4G). These results suggest that HIF-1α binds directly and regulates Mff in the cytoplasm of HNSCC cells.
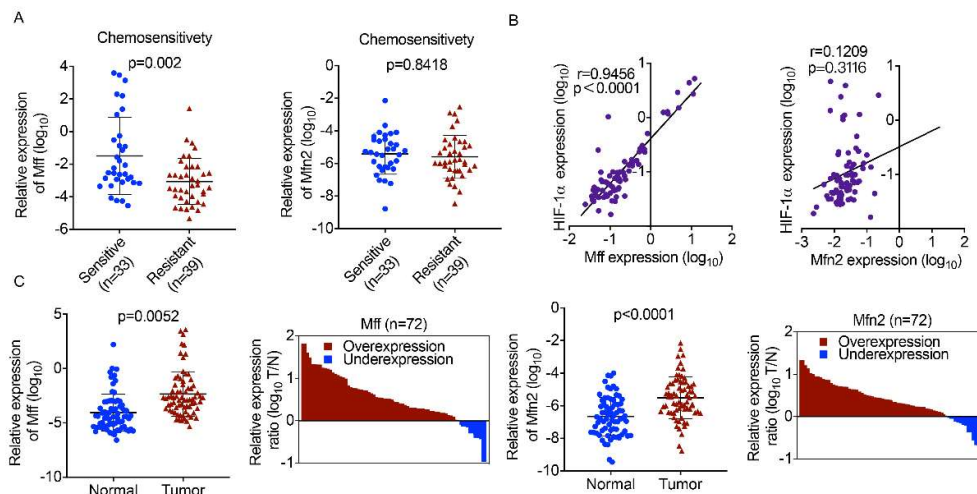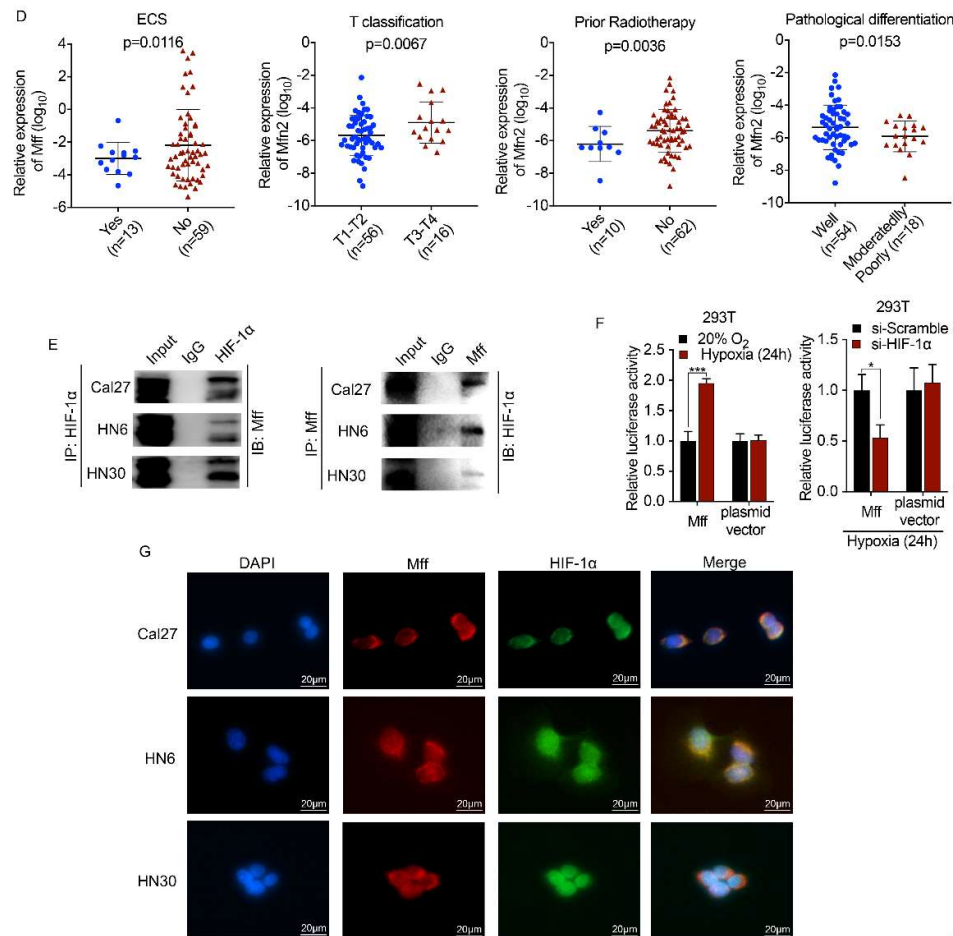
Figure 4

Figure 4. HIF-1α promotes Mff expression level in HNSCC

(A) The level of Mff expression was significantly upregulated in HNSCC patients with CDDP sensitivity.

(B) The remarkably positive association between Mff expression and HIF-1α expression was found by expression correlation analysis.

(C) Both Mff and Mfn2 expression were upregulated more in HNSCC tissues than in normal tissues.

(D) The correlation between clinical parameters and Mff and Mfn2 was determined by correlation analysis.

(E) The interaction between Mff and HIF-1α was determined via a co-immunoprecipitation assay.

(F) Hypoxia-induced HIF-1α considerably increased the luciferase activity of Mff, and pretreatment with si-HIF-1α partly inhibited this effect.

(G) The subcellular localization of Mff and HIF-1α in the Cal27, HN6, and HN30 cells was assessed by immunofluorescence staining. Data are presented as the mean ± SD from 3 independent experiments (* P < 0.05, *** P < 0.001).

ROS promote Mff upregulation via HIF-1α in hypoxic-CDDP-cultured HNSCC cells

In the present study, the findings showed that hypoxia increases ROS and HIF-1α levels in HNSCC cells. To determine the effect of hypoxia-induced ROS on mitochondrial fission, H_2O_2 was treated exogenously to 20% O_2-cultured HNSCC cells and the free radical scavenger NAC was applied to hypoxic-CDDP-cultured HNSCC cells.

Hypoxia-induced ROS levels and mitochondrial fission were attenuated by NAC (1 mM, 6 h)[8] (Fig. 5A, B). The increase in HIF-1α and Mff expression by hypoxia was restored by NAC treatment (Fig. 5C). Next, exogenous H_2O_2 (20 μM, 6 h)[8] promoted

mitochondrial fission (Supplementary Fig. 2A). In addition, H_2O_2 treatment increased HIF-1α and Mff content in 20% O_2-cultured HNSCC cells (Supplementary Fig. 2B). These results showed that hypoxia-elicited ROS regulate the Mff protein level via HIF-1α to promote mitochondrial fission.
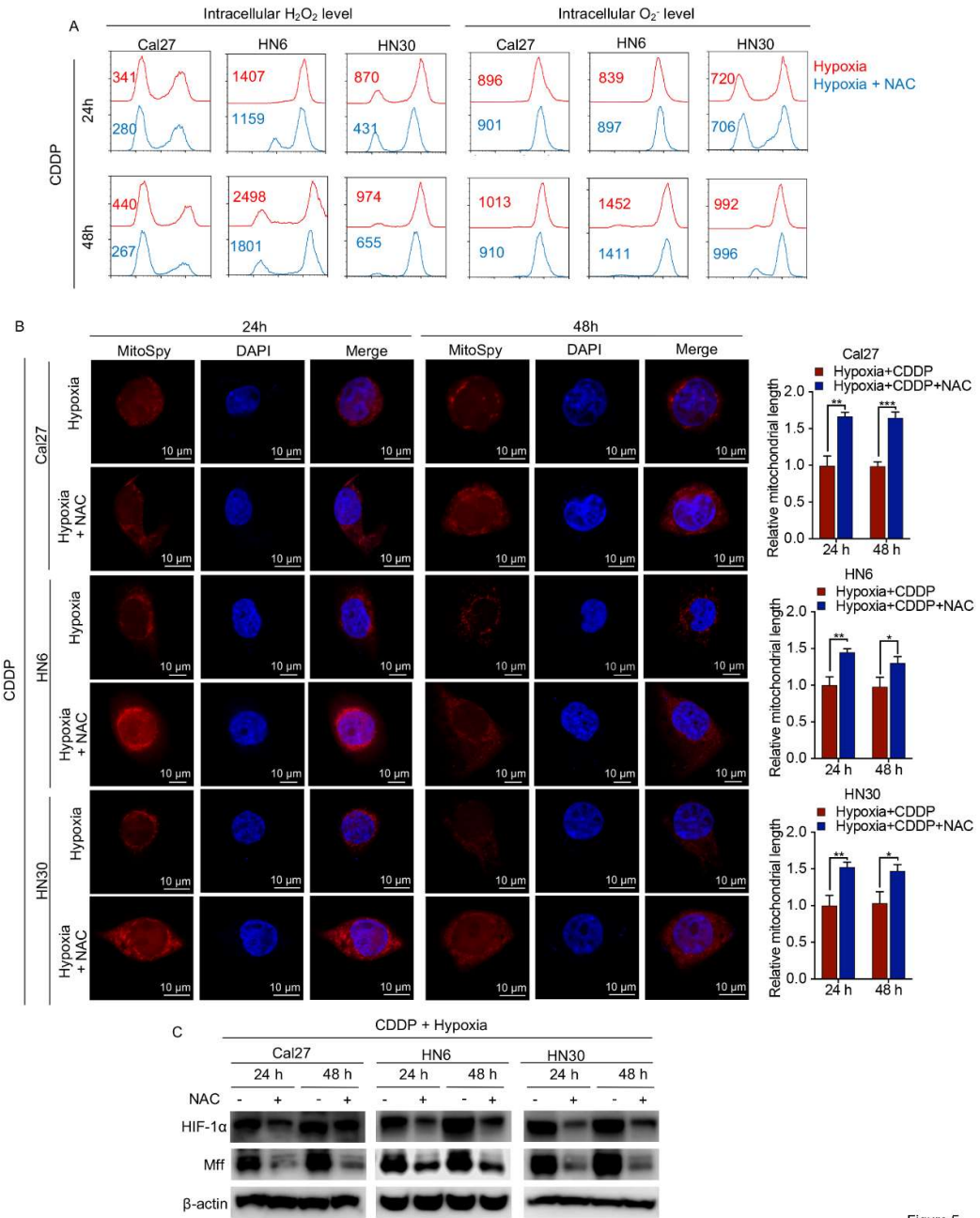

Figure 5. NAC attenuated hypoxia-upregulated HIF-1α and Mff
(A-B) NAC decreased hypoxia-induced ROS and mitochondrial fission in HNSCC cells treated with CDDP.
(C) Western blot analysis was used to determine the protein expression of HIF-1α and Mff in hypoxia-CDDP-cultured HNSCC cells treated with NAC. Data are presented as the mean ± SD from 3 independent experiments (* P < 0.05, ** P < 0.01, *** P < 0.001).

Knockdown of Mff suppresses hypoxia-induced mitochondrial fission and CDDP chemosensitivity in HNSCC cells

To evaluate the biological function of Mff knockdown in hypoxia-CDDP-cultured HNSCC cells, Cal27, HN6, and HN30 cells were stably transfected with LV-Mff and LV-NC. The protein expression level of Mff in HNSCC cells with LV-Mff was notably low (Supplementary Fig. 3A). Moreover, there was no statistically significant difference in the proportion of LV-Mff and LV-NC transfected HNSCC cells that were apoptotic in 20% O_2 for 24 h (Supplementary Fig. 3B). Sensitivity to CDDP was higher in the LV-NC transfected HNSCC cells than that in the LV-Mff transfected HNSCC cells under hypoxia for 24 h (Fig. 6A) and 48 h (Fig. 6B). Meanwhile, mitochondrial fission in hypoxia-CDDP-cultured conditions was dramatically decreased in HNSCC cells with LV-Mff for 24 or 48 h (Fig. 6C). Therefore, inhibition of hypoxia-induced mitochondrial fission could attenuate the sensitivity of hypoxic HNSCC cells to CDDP.
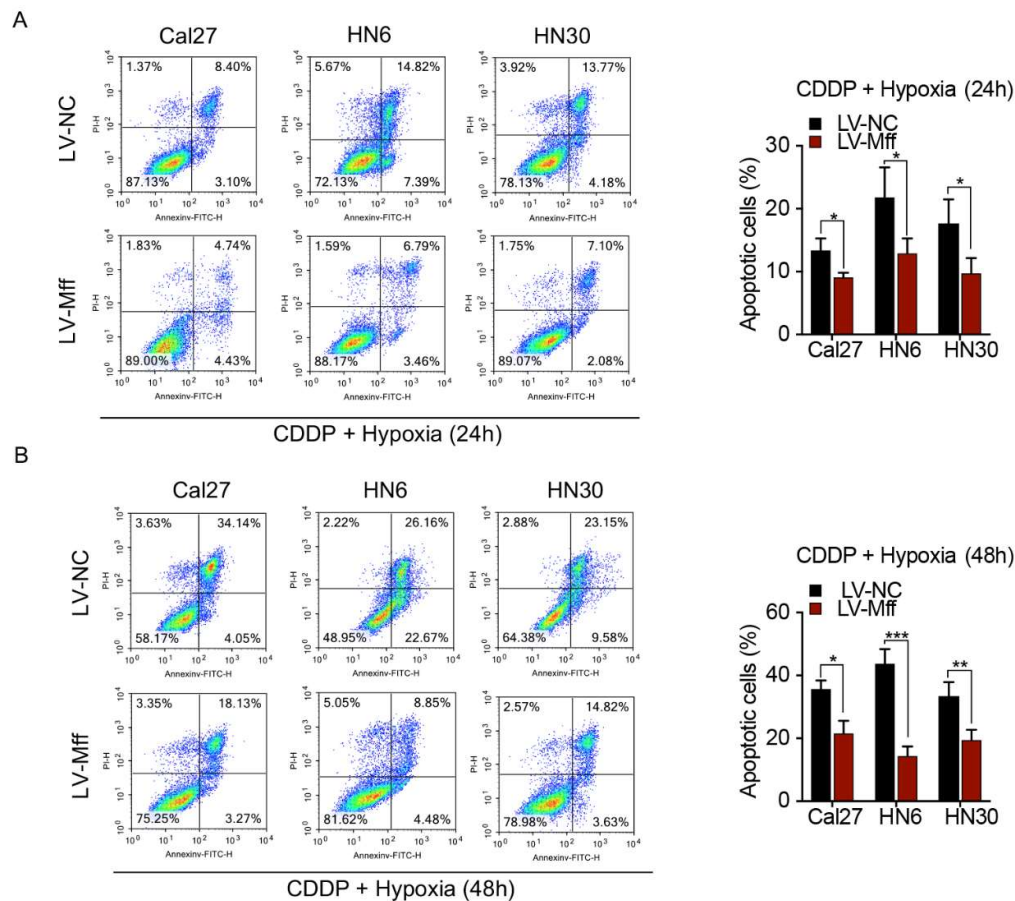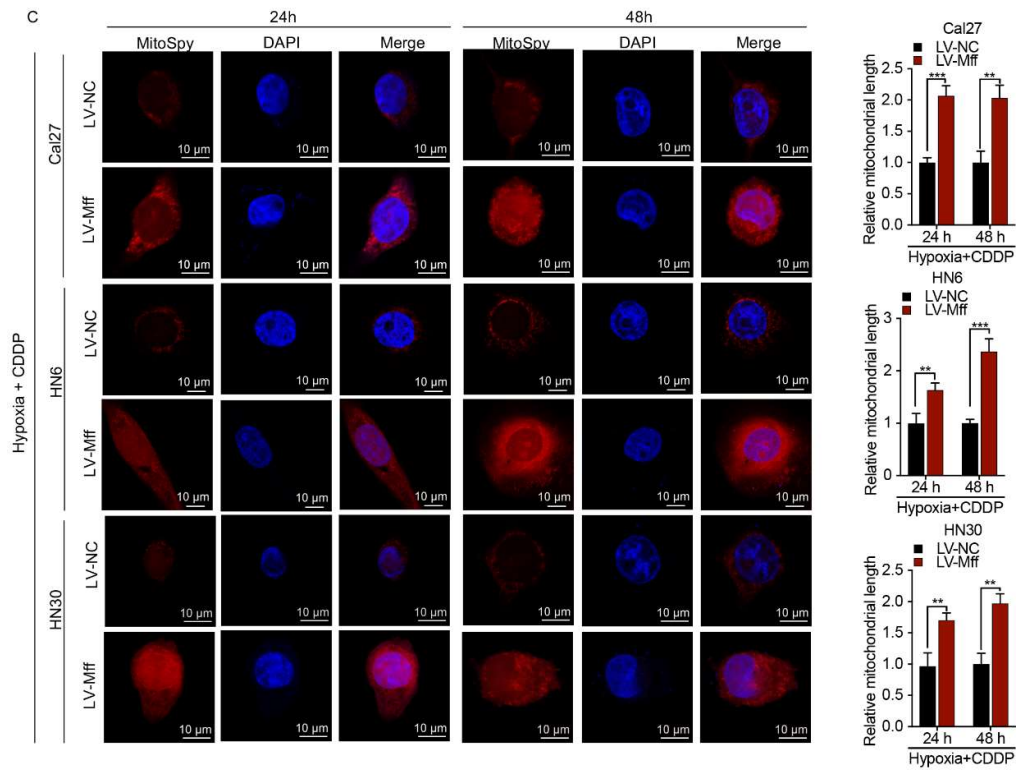
Figure 6

Figure 6. Knockdown of Mff attenuates CDDP chemosensitivity and mitochondrial fission in hypoxic conditions

(A-B) Flow cytometric analysis was used to analyze the proportion of HNSCC cells stably transfected with LV-Mff or LV-NC under hypoxic conditions.

(C) The mitochondrial length of HNSCC cells stably transfected with LV-Mff or LV-NC under hypoxic CDDP-cultured conditions were measured using MitoSpy staining. Data are presented as the mean ± SD from 3 independent experiments (* P < 0.05, ** P < 0.01, *** P < 0.001).

Mff regulates CDDP sensitivity in HNSCC xenografts

Since Mff expression facilitates the chemosensitivity of HNSCC cells under hypoxia, we evaluated the effect of Mff on tumorigenicity in a xenograft model. For this purpose, Cal27 cells stably transfected with LV-Mff or LV-NC were injected subcutaneously into the dorsal flanks of nude mice. The results showed that Cal27 cells with a stable expression of LV-Mff had enhanced tumor growth and tumor weight in the presence of CDDP (Fig. 7A, B). Moreover, apoptosis was attenuated in CDDP-treated xenografts of Cal27 cells stably transfected with LV-Mff (Fig. 7C). Conclusively, our findings reveal that hypoxia-induced ROS promote mitochondrial fission and CDDP chemosensitivity through HIF-1α/Mff regulation in HNSCC cells (Figure 7D).
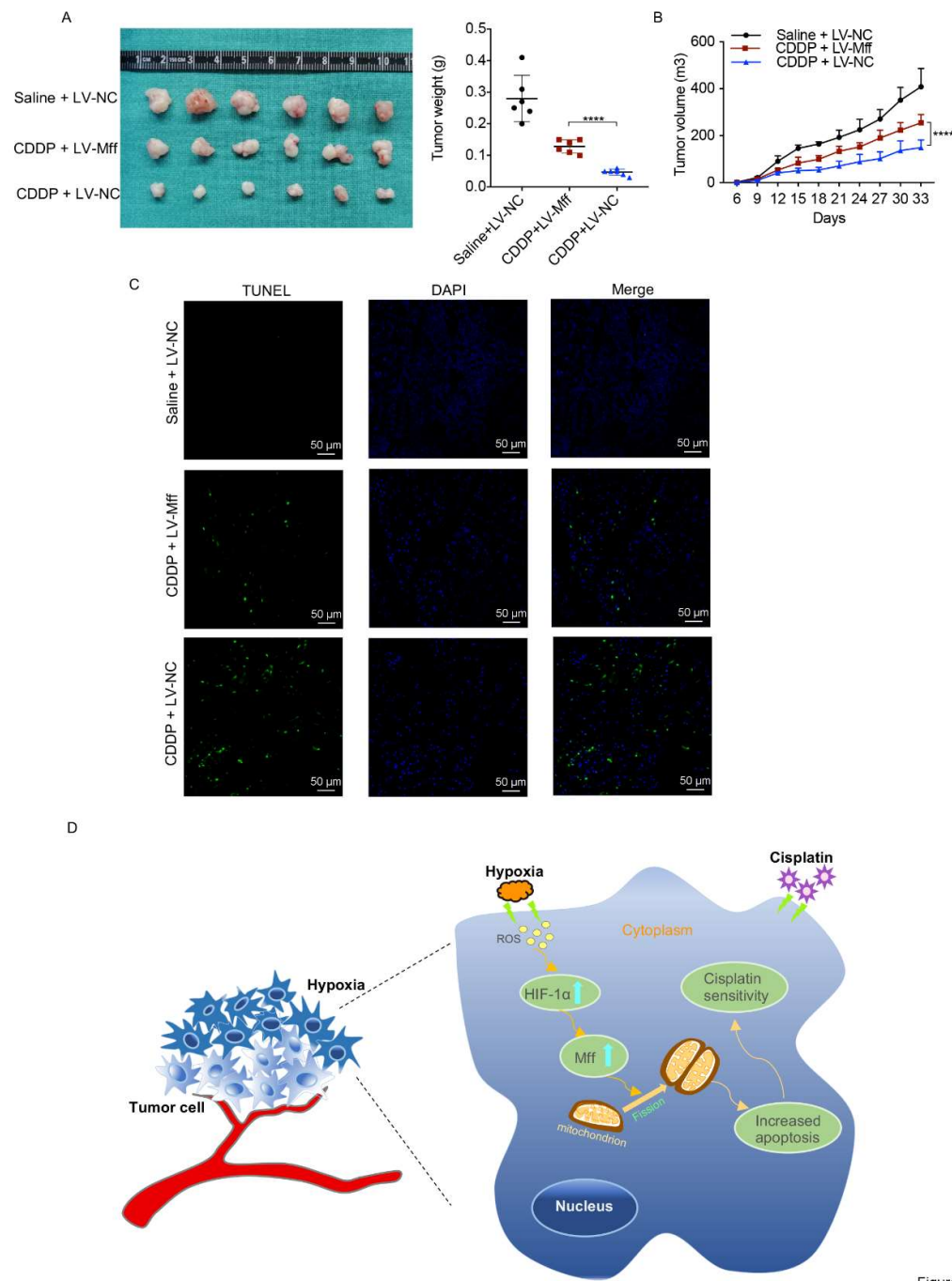
Figure 7. Mff knockdown inhibits apoptosis and CDDP sensitivity in HNSCC cells in vivo

(A) The tumor weights and volumes of Cal27 cells are shown. Nude mice bearing xenografts of Cal27 cells stably transfected with LV-NC or LV-Mff treated with saline or CDDP (n=6 per group).

(B) Tumor growth curves of Cal27 cells for each group are shown.

(C) Tumor cell apoptosis in response to CDDP was attenuated in Cal27 cells stably transfected with LV-Mff.

(D) A proposed model illustrating the regulatory role of hypoxia-induced ROS promoting mitochondrial fission and CDDP chemosensitivity via regulated HIF-1α/Mff.

Discussion

The present study shows that Mff promotes mitochondrial fission and consequently CDDP sensitivity. Through exploring the mechanism of Mff expression regulation, we found that HIF-1α binds directly to and regulates Mff in the HNSCC cell cytoplasm. Through further experiments, the results revealed that hypoxia-induced ROS increased HIF-1α/Mff levels, promoting mitochondrial fission and CDDP sensitivity (Figure 7D). Emerging evidence suggests that abnormal mitochondrial morphology is relevant in various diseases.[25, 26] Moreover, the biological mechanisms of the response of mitochondrial fission to CDDP sensitivity in malignant tumors are still unclear. Previous studies have revealed that an increase in mitochondrial fission in cancer cells enhances apoptosis and CDDP sensitivity in gastric cancers,[27] gynecological cancers,[28] and tongue squamous carcinomas.[18, 19] Due to the hypoxic microenvironment of HNSCCs, the cells were cultured under hypoxic conditions in the present study. A recent study showed that hypoxia-induced mitochondrial fission causes CDDP resistance in ovarian cancer cells.[8] In the present study, our findings indicated that hypoxia-induced mitochondrial fission promotes CDDP sensitivity in HNSCC cells. The opposite effects of tumor cell mitochondrial fission on CDDP sensitivity may be explained by differences in the cancer cells.

Carefully orchestrated interactions between mitochondrial proteins that facilitate tumor cell death remain an immediate focus of research. Mff, a key molecule that determines mitochondrial size and shape and the regulation of mitochondrial cell death, is a novel therapeutic target in cancer.[29, 30] LATS2 facilitates the apoptosis of non-small cell lung cancer by regulating Mff-induced mitochondrial fission.[31] Furthermore, Mff-induced mitochondrial fission was attributed to decreased colorectal cancer cell viability.[32] Our findings showed that Mff was a key molecule participating in mitochondrial fission, contributing to CDDP sensitivity in hypoxia-cultured HNSCC cells. Both Mff and Mfn2 expression were upregulated in HNSCC tissues, indicating that mitochondrial dynamics in tumor tissues are more active than that in normal tissues. Moreover, the expression of Mff and HIF-1α in HNSCC patients showed that hypoxia-induced mitochondrial fission events in HNSCC are correlated with chemosensitivity. Recent evidence has shown that HIF-1α regulates the participation of mitochondrial dynamics in various biological behaviors.[33, 34, 35] Under hypoxic conditions, HIF-1α modulated mitochondrial fission by direct regulation of the expression of Drp1.[36] Due to the markedly positive correlation between HIF-1α and Mff, further experiments in the present study revealed that HIF-1α participates in mitochondrial fission by regulating Mff.

Previous studies have revealed that hypoxic conditions could promote an increased level of ROS in the tumor microenvironment.[8, 11] The ROS are generated by mitochondria and other cellular elements and participate in malignant tumor biological behavior through the regulation of HIF-1α.[10, 37, 38] Hypoxia-induced mitophagy is associated with cancer aggressiveness via the interplay of ROS/HIF-1α in gastric cancer cells.[39] Our results demonstrated that hypoxia-induced ROS increases the expression of HIF-1α and Mff, while NAC attenuates these ROS effects.

In summary, we demonstrated that hypoxia-induced ROS promote mitochondrial fission and CDDP chemosensitivity via HIF-1α/Mff regulation in HNSCC cells. Moreover, Mff may serve as a new biomarker for the prediction of neoadjuvant chemosensitivity and is important for overcoming chemoresistance, which requires further exploration.

Conflict of interest
There is no any potential conflict of interest. The authors do not have commercial or other associations that might pose a conflict of interest.

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