Hyperthermia combined chemotherapy regulates energy metabolism of cancer cells under hypoxic microenvironment

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Abstract

Objective: Oral squamous cell carcinoma (OSCC) represents one of the main types of head and neck malignant tumors with high incidence and mortality as well extremely poor prognosis. Hyperthermia (HT) shows great promises for tumor therapy. However it can promote autophagy in tumor microenvironment, which is found to serve as a surviving mechanism for cancer cells. Inhibiting autophagy has been considered as an adjuvant anti-cancer strategy. The present study investigated the role of HT-induced autophagy, while attempting to combine chemotherapy and autophagy blocking with HT in OSCC cells under hypoxia and starvation microenvironment.

Materials and methods: HIF-1 α and Beclin-1 expression in tissues was determined by immunohistochemistry in 80 OSCC sample pairs. The IC₅₀ of CoCl₂, YC-1 (an inhibitor of HIF-1 α) and 3-MA (an inhibitor of autophagy) was detected by CCK-8. CoCl₂ and complete culture medium without serum were used to achieve the hypoxic and nutrient deficient microenvironment, respectively. HT was performed by heating in a 42 °C water bath. The role of HT and YC-1,3-MA on autophagy *in vitro* were assessed by qRT-PCR and Western blot, and the secretion of high mobility group box1 (HMGB1) was determined by ELISA. The migration and apoptosis rates of cells were assessed by wound healing assay and flow cytometry.

Results: We observed that HIF-1 α and Beclin1 were highly expressed in OSCC tissues, which were correlated with more advanced malignancy features. CoCl₂ could establish hypoxia microenvironment, induce HIF-1 α expression with dose-dependence as well as promote cell migration in Cal-27 and SCC-15 cells. Notably, hyperthermia and hypoxia could activate the HIF-1 α /BNIP3/Beclin1 signaling pathway and promote HMGB1 secretion, which triggered cytoprotective autophagy to counteract the hypoxia and starvation cellular stresses, as indicated by downregulation of p62 and light chain 3-II (LC3 II). Furthermore, we found that hyperthermia combined YC-1 and/or 3-MA suppressed autophagy and cell migration whereas facilitated cell apoptosis.

Conclusion: The present study demonstrated that combined use of YC-1 and 3-MA might increase death of tumor cells in physiological and hyperthermia conditions, which could be relevant with the inhibition of autophagy in OSCC tumor cells under hypoxia microenvironment *in vitro*.

Introduction

Oral squamous cell carcinoma (OSCC) is the 10th most common human malignancy worldwide and accounts for over 95% of malignant tumors in the head and neck, with high mortality [1, 2]. Despite advances in diagnosis and treatment, the 5-year survival rate of OSCC is less than 50%, and the recurrence rate is about 65%, resulting in its poor prognosis and tendency of lymph node metastasis [3]. Hypoxia is a crucial microenvironment condition for solid tumor pathophysiology and tumor metastasis, where cancer cells proliferate rapidly and form large solid tumor masses, leading to obstruction and compression of the blood vessels surrounding these masses. These abnormal blood vessels do not function sufficiently and generate poor O₂ supply to the central tumor regions, and the levels of oxygenation within the same tumor are highly variable from one area to another and can change over time [4, 5]. As an adaptive response to hypoxic stress, hypoxic tumor cells activate several survival pathways to carry out their essential biological processes compared with normal cells. The hypoxia inducible factor-1 α (HIF-1 α) is one crucial facilitator for energy adaption and oxygen metabolic stress in hypoxia and nutrition deficiency tumor environment, and functions as a general regulator of tumor aggressiveness and metastasis as well [4, 6, 7].

Hyperthermia (HT) has been successfully used in the clinical treatment of many cancers including the head and neck cancer [8] for decades, which sensitizes tumor cells to radiation by inhibiting DNA repair and increasing the

aggregation of damaged nuclear proteins [9, 10]. Previous studies have shown that initiating triggers for death in heat-shocked cells include: induction of physiological cascades; thermal protein unfolding and aggregation; necrosis that occurred at extremely elevated temperatures [14, 15]. Furthermore, hyperthermia-related elevated blood flow and vascular permeability in the heated tumor region also promote higher intratumor and peritumor drug concentrations to improve the efficacy of chemotherapy [10–12]. Therefore, hyperthermia acts as a complement of radiotherapy, chemotherapy and molecular targeted oncotherapy. However, tumor cells possess homeostatic responses to reduce heat-shock induced cell death, which involve cell cycle arrest and transient induction of the transcription of genes encoding molecular chaperones and heat shock proteins (HSPs) [13]. In short, hyperthermia induces the expression of HSPs and inhibit DNA damaged repair, whereas DNA damage, hyperthermia and HSPs evokes autophagy, which was associated with facilitated cell survival and decreased programmed cell death [13–16].

Autophagy is a cellular pathway which present only in eukaryotic cells to degrade the aged and damaged organelles, and misfolded proteins. It functions as a recycling program to provide biofuel to cells from degraded macromolecules to maintain sufficient ATP production for survival [17], and is a key component in maintaining homeostasis of cellular environment. Depending on the exact cell type and conditions, it either acts as a protagonist or an antagonist of apoptosis [18]. Autophagy also plays a crucial role in cancer pathophysiology. It is believed to prevent cancer development, but can also protect cancer cells within an already established tumor from shortage of nutrients and hypoxic conditions [18]. Furthermore, cellular damage caused by heating can be repaired and reversed by autophagy, resulting in incomplete cell necrosis [16, 19]. Researches have shown that high mobility group box-1 protein (HMGB1) regulates autophagy [20]. HMGB1 is a late inflammatory mediator associated with sepsis, malignancy, and immune disease [20], which is passively released by necrotic tissues or actively secreted by stressed cells [21]. Intracellularly, HMGB1 is involved in DNA repair, transcription and recombination as well in the regulation of apoptosis/autophagy balance. Once secreted, it participates in a variety of processes such as inflammation, proliferation, differentiation, migration, invasion and tissue regeneration [22].

Therefore, HSPs and autophagy are two controllers of cellular proteostasis. Under stressful cellular conditions, these two mechanisms are likely to complement each other [14]. However, whether hyperthermia-induced autophagy facilitates cell survival or accelerates cell death during the development of OSCC is still controversial. Hence, the present study aimed to investigate the relationship between hyperthermia and autophagy in hypoxia and nutrition-deficient tumor microenvironment of human OSCC. Meanwhile, the underlying mechanism was examined. This study might provide a novel promising therapeutic regiment for human OSCC.

Material And Methods

Human OSCC clinical samples

The study was approved by the Ethics Committee of Yantai Yuhuangding Hospital and written informed consent was provided by all patients. OSCC and adjacent normal tissues were obtained from 80 patients with primary OSCC, including 56 men and 24 women, aged 37–86 years, who underwent surgical resection the tumor at the Yantai Yuhuangding Hospital between August 2015 and April 2017. None of the patients had received any chemotherapy or radiotherapy before excision. All samples were confirmed by pathological examination. The histological grade and tumor stage were assigned according to the World Health Organization (WHO) [23] and the International Union against Cancer classification system [24].

Immunohistochemistry (IHC)

The expression levels of HIF-1 α and Beclin1 were analyzed by IHC. Briefly, antigen retrieval was performed by incubating the sections in 10 mM citric acid buffer (pH 6.0) at 100°C for 15 min. Subsequently, sections were dewaxed in xylene at room temperature and rehydrated in a descending ethanol series (absolute ethanol for 5 min, 95% ethanol for 5 min, 90% ethanol for 5 min and 80% ethanol for 5 min). Following three washes with PBS-Tween (0.05% Tween-20 in PBS), the sections were blocked by 5% BSA (Sangon Biotech Co., Ltd., China) in TBS for 45 min at room temperature. The sections were subsequently incubated at 4°C overnight with rabbit anti-HIF-1 α or anti-Beclin1 polyclonal antibody diluted in 3% BSA/TBS solution (1:100). The slides were washed with PBST and incubated with a HRP-conjugated goat anti-rabbit IgG secondary antibody (1:1000) at room temperature for 45 min. The slides were subsequently stained with 3,3'-diaminobenzidine tetrahydrochloride at room temperature for 10 min, and then counterstained with 0.5% Harris' hematoxylin at room temperature for 5 min. Finally, the sections were dehydrated with ethanol (80% ethanol for 5 min, 90% ethanol for 5 min, 95% ethanol for 5 min and absolute ethanol for 5 min), dried and mounted with neutral balsam. The images were screened using a microscope (magnification, x100 and x400). For scoring staining intensity, the expression level of HIF-1 α and Beclin1 in TSCC tissues were evaluated using a numerical scale (-, negative; +, weak; ++, moderate; +++, strong; Fig. 1).

Cell culture

The human OSCC cell lines Cal-27 and SCC-15 were cultured in RPMI 1640 medium (Biological Industries, Israel), supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, USA) and 100 U/ml penicillin-streptomycin (Invitrogen, Carlsbad, USA), and maintained in a humidified atmosphere at 37 °C with 5% CO₂. The following reagents were used in cell culture: 3-Methyladenine (3-MA) (Selleck, USA), 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) (Sigma, USA), Cobalt chloride (CoCl₂) (Solarbio, Beijing, China), which were dissolved with DMSO (Selleck, USA).

Cell cytotoxicity and cell viability assay

CCK-8 assay (Sangon, Shanghai, China) was used to detect the cytotoxic effect of different drug treatment on cancer cells and the 50% inhibitory concentration (IC₅₀) of the drug was calculated. OSCC cells were planted into 96-well plates a density of 5×10^3 cells/well, supplied with 100 μ L complete growth medium. After 24 h incubation, cells were exposed to CoCl₂, YC-1, and 3-MA at the concentrations of 25 μ M to 200 μ M, 10 μ M to 100 μ M, 0.5 μ M to 150 μ M, respectively. Untreated cells were used as control. At each time point, the cells were washed and incubated with 100 μ L RPMI 1640 plus 10 μ L CCK-8 solution at 37°C for 3 h. Subsequently, the absorbance was measured at 450 nm with a microplate reader (BioTek Instruments, Inc., USA). Each experiment was performed at least in triplicate. Dose response curves were established to determine the IC₅₀ values for CoCl₂, YC-1, and 3-MA in the two OSCC cell lines.

Establishment of hypoxic environment and heat treatment

Hypoxic environment was established by exposing cells to serum-free medium with IC₅₀ of CoCl₂. Hyperthermia treatment was performed by partially submerging cell culture flask in a thermostatically controlled circulating water bath (Shanghai Yiheng Scientific Instrument Co, LTD, China). Cells were treated at 42 ± 0.1 °C for 60 min and cool-down to 37 °C in less than 5 min.

RNA extraction and qRT-PCR

Total RNA from cells was extracted with Trizol reagent (Sangon, Shanghai, China). cDNA was synthesized by using PrimeScript™ RT Master Mix Kit (Takara, Japan). qRT-PCR was performed on a StepOne™ Real-Time PCR System

(Applied Biosystems, USA) with a SYBR Premix Ex Taq Kit (TaKaRa, Japan). According to the manufacturer's instructions, the PCRs were conducted at 95 °C for 30 s, followed by 40 cycles of 95 °C for 3 s, and 60 °C for 30 s. All reactions were performed in triplicate. $2^{-\Delta\Delta CT}$ method was applied to calculate the relative fold change of gene expression. All results were normalized to GAPDH. Primer sequences (Sangon, Shanghai, China) were listed below: HIF-1 α : up:5'-AGTTCCGCAAGCCCTGAAAGC-3', down:5'-GCAGTGGTAGTGGTGGCATTAGC-3'; Beclin1: up:5'-ATCTAAGGAGCTGCCGTTATAC-3', down:5'-CTCCTCAGAGTTAACTGGGT-3'; BNIP3: up:5'-AGGGCTCCTGGGTAGAACT-3'; down:5'-CTCCATTATAAATAGAAACCGAGGC-3'; GAPDH: up:5'-GCCACATCGCTCAGACACCA-3'; down:5'-TTCCCGTTCTCAGCCTTGAC-3'; LC3II: up:5'-GTCAGCGTCTCCACACCAATCTC-3'; down:5'-TCCTGGGAGGCATAGACCATGTAC-3'; SQSTM1 (P62): up:5'-TGATTGAGTCCCTCTCCAGATGC-3'; down:5'-CCGCTCCGATGTCATAGTTCTTGG-3'.

Western blot

Cells were collected and lysed in RIPA buffer (Beyotime, Shanghai, China). Total protein was quantified using the BCA kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Subsequently, the proteins were separated by 10%-12% SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Inc.), which were blocked with 10% nonfat milk and incubated with primary and secondary antibodies. Afterward, the protein bands were visualized with an ECL detection kit (Millipore, Burlington, MA) and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc). The following antibodies were used: Beclin-1 (Cat. no.: NB500-249, Selleck, USA, 1:1000), HIF-1 α (Cat. no.: BF8002, BI, Israel, 1:1000), BNIP3 (Cat. no.: D221876, 1:1000), LC3B (Cat.no.: D163557, 1:1500), SQSTM1/p62 (Cat. no.: sc-48402, 1:1500), mouse anti- β -actin monoclonal antibody (Cat. no.: MA1-744, 1:3000), HRP-conjugated goat anti-mouse IgG antibody (Cat. no.: 31430, 1:3000) and HRP-conjugated goat anti-rabbit IgG antibody (Cat. no.: 314600, 1:3000). Antibodies were all obtained from Invitrogen, USA unless otherwise stated.

Extracellular HMGB1 measurement

HMGB1 is passively released by necrotic tissues or actively secreted by stressed cells. To measure extracellular HMGB1, supernatants of cell culture were collected and centrifuged at 8000 $\times$ g for 5 min and immediately analyzed by enzyme-linked immunosorbent assay (ELISA) (Solarbio, Beijing, China) according to the manufacturer's instructions.

Cell migration assay

Cells were seeded in 6-well plates and cultivated until 100% confluence. In the serum-free RPIM1640 medium, 100 μ M CoCl₂ was added to simulate a hypoxia environment and cells were treated separately with 50 μ M 3-MA and/or 25 μ M YC-1 combined with heat treatment for 1 hour in a 42 °C water bath. Then the cells were scraped with a 200 μ l of pipette tip and washed with PBS for three times. At 0 and 24 h after incubation in serum-free medium, the images of wound healing were captured using an inverted microscope (magnification, $\times$ 400). The area of each wound was quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.). The cell migration rate (%) was calculated as follows: [(Area of wound at 0 h - Area of wound at 24 h) / Area of wound at 0 h] $\times$ 100%.

Flow cytometry analysis of apoptosis

Cell apoptosis were detected by flow cytometry using the FITC-AnnexinV/PI Apoptosis Assay Kit (BD, USA) following manufacturer's instructions. Briefly, the cells (5×10^5 cells/well) that treated with chemotherapy (YC-1 and 3-MA) and hyperthermia (42 °C heat treatment) were harvested and centrifuged at 500 $\times$ g for 5 min at room temperature, then washed twice with PBS and resuspended in 500 μ l binding buffer solution at a density of 1×10^5 cells/ml. The cells

were subsequently stained with 5 µl FITC Annexia V and 5 µl PI using at room temperature for 15 min in the darkness. Apoptotic cells were analyzed using a CytoFLEX flow cytometer and CytExpert software (version 2.0; Beckman Coulter, Inc.) within 1 h.

Enzyme-linked immunosorbent assay (ELISA)

The levels of HMGB1 in chemotherapy (YC-1 and 3-MA) and hyperthermia (42 °C heat treatment)-treated Cal-27 and SCC-15 cells that were in hypoxia and normoxia condition were determined using ELISA kits (Ilerite Biotechnology Co, China), in line with the manufacture’s protocol.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). Data were presented as the mean ± standard deviation, comparisons between groups were performed by using Student's t-test or a one-way ANOVA, followed by a Tukey's post hoc test for multiple comparisons. A χ^2 test was used to determine the association between the expression levels of HIF-1α, Beclin1 respectively and OSCC clinical and histopathological features. P < 0.05 was considered statistically significant difference.

Results

1. HIF-1α and Beclin1 expression levels increased in OSCC tissues

The clinical significance of HIF-1α and Beclin1 in OSCC patients was evaluated by analyzing the expression level of HIF-1α and Beclin1 in 80 OSCC tissues by scoring of IHC (Fig. 1). HIF-1α mainly located in the cell nucleus while Beclin1 mainly located in the cell membrane and cytoplasm with a small amount in the cell nucleus. In this study, we observed that HIF-1α and Beclin1 were expressed both in cancer tissue and adjacent normal tissues. By multiplying the score of staining intensity and percentage of positive cells of each tissues section, we found that HIF-1α and Beclin1 were both highly expressed in OSCC tissues compared with normal tissues (P<0.05, Table 1). In addition, the high expression level of HIF-1α and Beclin1 was associated with poor cell differentiation, lymph node metastasis, advanced pathological TNM stage, and large tumor size (P<0.05, Table 2), but was not correlated with gender or age.

Table 1 The expression difference of HIF-1α and Beclin1 in OSCC tissues and paracancer tissues (n, %)

Group	Number	HIF-α expression		χ ²	P value	Beclin1 expression		χ ²	P value
		negative	positive			negative	positive		
Cancer tissues	80	23 (28.75%)	57 (71.25%)	25.42	P<0.01	29 (36.25%)	51 (63.75%)	13.23	P<0.05
Paracancer tissues	80	49 (61.25%)	21 (26.25%)			52 (65.00%)	28 (35.00%)		

Table 2. Association between HIF-1α, Beclin1 expression and the clinical and histopathological features of patients with oral squamous cell carcinoma (n=80).

Variables	Total number Of Patients (n)	HIF-1a Positive (n)	HIF-1a Positive Rate (%)	χ^2	P-value	Beclin1 Positive (n)	Beclin1 Positive Rate (%)	χ^2	P-value
sex									
Male	56	39	69.6%	0.06926	0.966	39	69.64%	1.372	0.5036
Female	24	16	66.67%			19	79.17%		
Age, years									
≥60	55	47	85.45%	1.064	0.5874	43	78.18%	0.9503	0.6218
<60	25	19	76%			17	68%		
Tumor size, cm									
≥1.5	53	43	81.13%	6.661	0.0358	42	79.25%	10.87	0.0044
<1.5	27	14	51.85%			11	40.74%		
TNM stage									
I or II	24	14	58.3%	8.54	0.015	11	45.83%	8.412	0.015
III or IV	56	49	87.5%			44	78.57%		
Differentiation									
Well	29	13	44.83%	8.137	0.0171	12	41.38%	6.572	0.0374
Moderate/poor	51	39	76.47%			36	70.59%		
Lymph node metastasis									
Positive	47	39	82.98%	33.71	0.001	33	70.21%	6.729	0.0346
negative	33	7	21.21%			14	42.42%		

2. Establishment of hypoxic microenvironment

Low oxygen-induced hypoxia is the optimal hypoxia model. However, induction of chemical hypoxic conditions using CoCl_2 allows the researcher to open the culture container many times while maintaining a stable level of HIF-1. The IC50 values for CoCl_2 in Cal-27 and SCC-15 cells were 108.3 μM and 99.68 μM , respectively, as determined by CCK-8 cell viability assay. Therefore we used 100 μM CoCl_2 in free-serum RPIM 1640 to achieve the hypoxia and starvation microenvironment for the subsequent experiments (Table 3, Figure 2). Our results showed that compared with the untreated control group, stable mRNA and protein expression of HIF-1 α could be induced in Cal-27 and SCC-15 cells in a dose-dependent manner by CoCl_2 under normoxia, as measured by qRT-PCR and Western blot respectively, which demonstrated the successful establishment of hypoxic tumor microenvironment (Fig. 3A-C). Compared with control group, Cal-27 and SCC-15 cells both presented significant augment in cell migration under hypoxia microenvironment ($P < 0.05$, Fig. 7A, B). This result suggested that hypoxia microenvironment facilitated HIF-1 α expression and promoted cell migration.

Table 3 Cell viability after 24 h of CoCl_2 was detected by CCK-8 assay

group	5uM	25uM	50uM	100uM	150uM	200uM
Cal-27	79.767±1.673	74.170±1.307	67.193±1.376	56.533±1.785	40.83±0.753	21.753±0.420
Sc-15	81.777±1.724	73.05±1.197	67.687±0.556	55.280±1.997	34.053±0.523	16.153±2.077

3. Hyperthermia induced autophagy of OSCC cells in hypoxic microenvironment

To clarify the relationship between hypoxia, hyperthermia and autophagy, we tested proteins related to autophagy signaling pathway by Western blot and qRT-PCR in Cal-27 and SCC-15 cells, and found that HIF-1 α and BNIP3 were hardly expressed in the untreated control group and “HT” group (hyperthermia treatment alone), and there were no statistical difference in the expression of HIF-1 α and BNIP3 in HT group compared the control group ($P > 0.05$) (Fig.

4A, B and C). This indicated that HIF-1α and BNIP3 were not expressed in abundance under normal oxygen condition, and hyperthermia could not induce the expression of HIF-1α and BNIP3 in normoxia condition.

The expression of autophagy related genes such as Beclin1 and LC3II were higher in “HT” group compared the control group ($P<0.05$) (Fig. 4A, B and C), showing that Beclin1 and LC3II were expressed under constant oxygen condition and hyperthermia promoted Beclin1 and LC3II expression. In addition, the expression of P62 protein sharply decreased in “HT” group compared the control group ($P<0.05$) (Fig. 4A, B and C). Taken together, the results showed that hyperthermia could induce autophagy under normal oxygen condition.

However, the expression level of HIF-1α, BNIP3, Beclin1, LC3II significantly increased in the “Hy” group (hypoxia treatment alone) and “HT+Hy” group (combined hypoxia and hyperthermia treatment) compared the control group and “HT” group ($P<0.05$). In contrary, the expression of P62 sharply decreased in the “Hy” group and “HT+Hy” group compared to the control group and “HT” group ($P<0.05$) (Fig. 4A, B and C). What's more, we found that compared with the “Hy” group, the expression of HIF-1α, BNIP3, Beclin1, LC3II were significantly higher, whereas that of p62 proteins were remarkably lower in the “HT+Hy” group ($P<0.05$) (Fig. 4A, B and C).

4. Hyperthermia combined chemotherapy inhibited autophagy

To further investigate the correlation between hyperthermia induced HIF-1a/BNIP3/Beclin1 autophagy signaling pathway in hypoxia and starvation tumor microenvironment, YC-1 was used to suppress the HIF-1a-mediated autophagy pathway, and 3-MA was used for direct inhibition of autophagy. First, the IC50 of YC-1 and 3-MA were assessed by CCK-8. As shown in Fig. 2B and Table 4, the IC50 values for YC-1 in Cal-27 and SCC-15 cells were 24.37 μM and 34.72 μM, respectively. We used 20 μM for Cal-27 and 30 μM for SCC-15 cells to conduct following experiments. The IC50 values for 3-MA in Cal-27 and SCC-15 cells were 35.77 μM and 30.89 μM, respectively (Fig. 2C, Table 5), therefore we used 35 μM (Cal-27 cell) and 30 μM (SCC-15 cell) in the subsequent experiments.

Table 4 Cell viability after 24 h of YC-1 was detected by CCK-8 assay

group	5uM	10uM	20uM	50uM	80uM	100uM
Cal-27	66.747±1.272	53.797±1.146	41.930±2.763	29.257±0.882	21.627±0.332	10.810±0.357
Scc-15	79.333±0.757	69.320±1.995	58.123±1.595	48.487±1.147	28.710±2.933	16.237±2.011

Table 5 Cell viability after 24 h of 3-MA was detected by CCK-8 assay

group	2.5uM	5uM	25uM	50uM	80uM	100uM
Cal-27	95.107±2.633	84.607±0.618	76.137±1.146	57.297±1.679	36.533±1.676	24.997±1.061
Scc-15	87.203±2.038	79.683±1.337	68.630±0.726	48.720±2.623	40.37±1.776	29.367±1.606

Through PCR and Western blot analysis we found that the expression level of HIF-1α and BNIP3 were not statistically different in “HT+Hy” group vs. “HT+Hy+3-MA” group and “HT+Hy+YC-1” group vs. “HT+Hy+YC-1+3-MA” group ($P<0.05$) (Fig. 5A, B and C). Both HIF-1α and BNIP3 expressions were lower in the “HT+Hy+YC-1” group than in the “HT+Hy” group and “HT+Hy+3-MA” group, and were even lower in the “HT+Hy+YC-1+3-MA” group ($P<0.05$) (Fig. 5A, B and C). Reduced Beclin1 and LC3II expression levels were observed in the following groups: HT+Hy+YC-1+3-MA < HT+Hy+3-MA < HT+Hy+YC-1 < HT+Hy ($P<0.05$) (Fig. 5A, B and C). Conversely, increased expression level of P62 were observed in the following groups: HT+Hy+YC-1+3-MA > HT+Hy+3-MA > HT+Hy+YC-1 > HT+Hy ($P<0.05$) (Fig. 5A, B and C).

These data indicated hyperthermia induced autophagy through activating HIF-1 α /BNIP3/Beclin1 signaling pathway. 3-MA had a stronger effect in inhibiting autophagy than YC-1, and combined use of 3-MA and YC-1 provided further inhibition of autophagy in OSCC cells. Therefore, we concluded that hyperthermia might not only induce autophagy through activating HIF-1 α /BNIP3/Beclin1 signaling pathway, but also involve other pathways in hypoxia and starvation microenvironment. Moreover, hyperthermia combined with chemotherapy inhibits autophagy.

5. Hyperthermia combined chemotherapy inhibited the secretion of HMGB1

HMGB1 was translocated from the nucleus to the cytoplasm and secreted or passively released through the permeabilized plasma membrane of succumbing/dead cells. We measured the extracellular HMGB1 protein by ELISA, and found that compared with the control group, the secretion of HMGB1 significantly increased with hypoxia or hyperthermia treatment alone and combined in Cal-27 and SCC-15 cells, with protein level in HT+Hy group > HT group > Hy group > control group ($P < 0.05$, Fig. 6), suggesting that hyperthermia and hypoxia promoted HMGB1 secretion under normal oxygen conditions, and that hyperthermia was stronger than hypoxia in promoting HMGB1 secretion. On the contrary, addition of 3-MA and YC-1 significantly reduced the secretion of HMGB1, with protein level in HT+Hy+YC-1+3-MA group < HT+Hy+3-MA group < HT+Hy+YC-1 group < HT+Hy group ($P < 0.05$, Fig. 6). Based on the above experimental data, we reached a conclusion that hyperthermia and hypoxia might facilitate HMGB1 secretion in starvation tumor microenvironment, and the use of chemotherapy drugs to could also inhibit the secretion of HMGB1 in addition to autophagy inhibition.

6. Inhibition of autophagy reduced tumor cell migration in hypoxia microenvironment

In order to determine the effect of autophagy and hypoxia on Cal-27 and SCC-15 cell migration, we performed a wound healing assay in Cal-27 and SCC-15 cells and the results showed compared with untreated control cells, cell mobility increased in “Hy” group and decreased in “HT” group ($P < 0.05$, Fig. 7A and B), which demonstrated hypoxia might promote cell migration while hyperthermia inhibit cell migration. In addition, “HT+Hy” group had worse migratory ability than the “Hy” group had ($P < 0.05$) ($P < 0.05$, Fig. 7A and B), which demonstrated that hyperthermia could inhibit cell migration whether in normoxia or hypoxia conditions. Compared with “HT+Hy” group, the cell migration of “HT+Hy+3-MA” group, “HT+Hy+YC-1” group and “HT+Hy+YC-1+3-MA” group were all significantly reduced ($P < 0.05$, Fig. 7A and B) and “HT+Hy+YC-1+3-MA” group had the strongest inhibition of cell migration, which indicated that inhibiting autophagy might reduce tumor cell migration in hypoxia microenvironment. However, there was no significant difference in migration between “HT+Hy+3-MA” group and “HT+Hy+YC-1” group ($P < 0.05$, Fig. 7A and B).

7. Inhibition of autophagy enhanced hyperthermia-induced apoptosis in OSCC cells

Apoptosis was investigated using flow cytometry analysis. In terms of early cell apoptosis, compared “Con” group, the cell apoptosis rate of Cal-27 and SCC-15 were increased in “HT” group and “HT+Hy” group while the group of “HT” was significantly higher than the group of “HT+Hy” ($P < 0.05$, Fig. 8A, B and Ca-b), and take “HT+Hy” group as a contrast, early cell apoptosis were significantly enhanced in group of “HT+Hy+YC-1”, “HT+Hy+3-MA” and “HT+Hy+YC-1+3-MA” ($P < 0.05$, Fig. 8A, B and Ca-b), with there was no significant change in “Hy” group compared “Con” group and in “HT+Hy+3-MA” group vs “HT+Hy+YC-1” group ($P > 0.05$, Fig. 8A, B and Ca-b). Moreover, when talking about late cell apoptosis, compared “Con” group, the cell apoptosis rate of Cal-27 and SCC-15 were decreased in “HT” group and “Hy” group ($P < 0.05$, Fig. 8A, B and Cc-d) as well there was no statistical change in “HT+Hy” group ($P > 0.05$, Fig. 8A, B and Cc-d); However, the apoptosis rate of “HT+Hy” group was enhanced that compared with “Hy” group in two cell lines, and compared with “HT” group, which promote cell late apoptosis in Cal-27 cells. besides,

compared with “HT+Hy” group, “HT+Hy+YC-1” group and “HT+Hy+3-MA” , “HT+Hy+YC-1+3-MA” group obviously promoted late cell apoptosis ($P>0.05$, Fig. 8A, B and Cc-d). In conclusion, We found that compared with the control group, the cell apoptosis rate increased in “HT” group and decreased in “Hy” group ($P<0.05$, Fig. 8A, B and Ce-f). Meanwhile, the cell apoptosis rate of “HT+Hy” group was higher than “Hy” group ($P<0.05$, Fig. 8A,B and Ce-f), which supported the conclusion that hyperthermia significantly promoted tumor cell apoptosis at normal oxygen concentration and at hypoxia condition, while hypoxia inhibited cell apoptosis in nutrition deficiency microenvironment. In addition, YC-1 and 3-MA both facilitated cell apoptosis ($P<0.05$, Fig. 8A,B and Ce-f) in approximately equivalent scale ($P>0.05$) and combined use of both drugs had the strongest effect on cell apoptosis ($P<0.05$, Fig. 8A,B and Ce-f). The results revealed that hyperthermia combined with chemotherapy promoted OSCC cell apoptosis under hypoxia and nutrition deficiency microenvironment *in vitro*. In general, the results from the two cell lines (Cal-27 and SCC-15) showed a similar tendency.

Discussion

OSCC is the most common solid tumor in head and neck, and a growing body of evidence indicates that the microenvironment of solid tumor with hypoxia, low PH, poor nutrition, poor perfusion and abnormally high interstitial fluid pressure change the biological behavior of tumor, which significantly reduces the effect of radiotherapy and chemotherapy and promotes tumor cell proliferation, invasion, and migration [25, 26]. The adaptation and survival of tumor cells in heterogeneous microenvironment requires the coordination of complex pathways and mechanisms, such as hypoxia induction factor 1 (HIF-1), unfolded protein reaction (UPR), rapamycin (mTOR) and autophagy [27]. Hypoxia is a crucial microenvironment condition for solid tumor pathophysiology, including tumor proliferation, invasion, and metastasis, and HIF-1 α is a key molecule that is highly expressed under hypoxia. In general, under the condition of normoxia, the biosynthesis of mitochondrial respiration and anabolism is facilitated by oncoprotein *MYC*, allowing cancer cells to proliferate under conditions of adequate oxygen and nutrition [28, 29]. But under hypoxia, energy metabolism is regulated by HIF-1 rather than *MYC*, HIF-1 is a powerful mediator of carbohydrate reprogramming from oxidative phosphorylation to glycolytic metabolism in hypoxic reactions by regulating oxygen transport (angiogenesis) and oxygen consumption (glycolysis metabolism) [30]. And contrary to *MYC*, HIF-1 strongly inhibits mitochondrial respiration and biogenesis [31]. Metabolic pathway changes of tumor cells during hypoxia or malnutrition are considered to be a feature of cancer cells, namely metabolic reprogramming [32]. In addition, when tumor cells are exposed to stressful microenvironment, especially low PH, low oxygen and nutrient deficiency, autophagy is activated to cycle cellular metabolic substrate to meet their high metabolism and energy demand, and to suppress the body's inflammatory response induced by tumor to prevent the cytotoxicity accumulation and to promote tumor cell survival. Therefore, autophagy constitutes a way to prolong the survival of tumor cells [25, 26, 33, 34].

Ribeiro *et al.* had investigated 93 OSCC samples for HIF-1 α expression, demonstrating that metastatic lymph nodes and intratumoral regions of corresponding primary tumors expressed HIF-1 α at a high frequency [35]. Therefore, HIF-1 α is considered to be a potential prognostic marker of many cancers, including OSCC[36]. The formation of HIF-1 α is oxygen-dependent. At the tumor margin, blood vessels grow to provide sufficient nutrients and oxygen, allowing the synthesis and rapid ubiquitin-mediated degradation within 10 min of HIF-1 α under normoxia. In contrast, HIF-1 α protein activity is prolonged under hypoxia [28]. Therefore, one important consequence of hypoxia is the induction of HIF-1 α , which activates a series of downstream genes that facilitate tumor cell survive in hypoxia microenvironment, such as autophagy-related genes Beclin1 [37]. In this study, we detected the expression level of HIF-1 α and Beclin1 in 80 pairs of OSCC tissues and adjacent normal tissue, and our results suggested that HIF-1 α and Beclin1 were both highly expressed in OSCC tissues compared with normal tissues and were significantly associated with large tumor

size, advanced TNM grade, high pathological grade and lymphocytic infiltration. This finding is consistent with previous research results.

Literatures have reported that autophagy is a self-degrading process and plays an indispensable role in sustaining cellular homeostasis under stress, which act as a housekeeper to clear damaged organelles such as fragmented mitochondria, to remove misfolded proteins and to recycle cellular components [38, 39]. The role of autophagy in cancer is most dramatic and dynamic. In normal cells, autophagy inhibits tumor occurrence, however, in hypoxia and nutrient deficiency tumor microenvironment, autophagy promotes tumor cell survival as an alternative energy supply pathway besides the “Warburg effect”. Autophagy initiation occurs under stress conditions, such as nutrient and energy deficiency, hypoxia, reactive oxygen species (ROS), protein aggregation and production of damaged organelles [40, 41]. As is well known, the conversion of cytosolic LC3-I to LC3-II, which binds autophagic vacuoles, is the primary hallmark of autophagy. In addition, the protein p62 (also known as SQSTM1) has been reported to interact with the autophagic effector protein LC3 and to be degraded through an autophagy-lysosome pathway [42]. Therefore, activation of autophagic flux leads to decrease in p62 and LC3-I levels and an increase in LC3-II level. Our research showed that OSCC cells Cal-27 and SCC-15 also underwent autophagy under hypoxia condition. In physiological conditions, Beclin-1 and Bcl-2 form a complex compound that results in inhibiting the activation of the autophagy pathway. It has been confirmed that BNIP3 is the target molecule of HIF-1 α . Under hypoxia conditions, the expression of HIF-1 α significantly augments, which upregulates BNIP3, and BNIP3 interacts with Bcl-2 or Bcl-XL and ultimately forms heterodimer, which will prevent the binding of Bcl-2 to Beclin-1, thereby the released Beclin-1 will activate the autophagy pathway [43]. As a result, HIF-1 α /BNIP3/Beclin-1 signaling pathway is an important way of inducing autophagy under hypoxic conditions.

Previous researches have demonstrated that mild hyperthermia could directly damage proteins and organelles and thus trigger cytoprotective autophagy to tolerate the cellular stresses and prolong the survival of cancer cells [44–46]. Literatures reported that heat stress induced autophagy in several types of cancer cells, such as hepatocellular carcinoma cells (SMMC7721 and Huh7), cervical cancer cell (HeLa) and lung cancer cell (A549 cell) [28, 42]. Our experimental data also indicated that hyperthermia could induce autophagy in both hypoxia and normoxic starvation microenvironments, and that autophagy was further enhanced in hypoxia condition. This might stem from the following reasons: HT-induced protein denaturation and aggregation results in the up regulation of HSPs, which are reported to up-regulate the autophagy mediator Beclin-1 [14]. Moreover, Hyperthermia can induce oxidative stress in cells and can further augment the generation of ROS [47]. ROS is a known inducer of autophagy and apoptosis. It has been reported that ROS acted on the complex formed by Beclin-1 and anti-apoptotic Bcl-2 homologs such as Bcl-2 and Bcl-xL. Moreover, this complex repressed the pro-autophagic activity of Beclin-1 and ROS could induce the dissociation of autophagy molecules Beclin 1 and Bcl-2, thus activating the Beclin1-induced autophagy pathway, increasing the expression of LC3-II, thereby initiating autophagy-associated pathways [48, 49]. In addition, ROS is also reported to upregulate the activity of HIF1- α , and some scholars have also shown that the expression and activity of HIF-1 α is not only induced in response to limited oxygen supply, but it is also regulated through related signaling pathways, including the extracellular signal-regulated kinase (ERK) [50] and the protein kinase B (AKT) [51] pathway, and hyperthermia promoted the expression of HIF-1 α by activating phosphatidylinositol 3-kinase (PI3K) PI3K/AKT and mitogen-activated protein kinase (MAPK) MAPK/ERK signal pathway [52] while relative study have demonstrated that heat treatment can cause hypoxia in the local tissue and increase HIF-1 α expression levels, which can induce the over-proliferation of any residual tumors [51]. In conclusion, hyperthermia and hypoxia induced the activation of HIF-1 α , which furthermore facilitates the activation of HIF1- α /BNIP3/Beclin1 autophagy signaling pathway. In our study, the expressions of Beclin1 and LC3-II significantly increased when cells were treated with hypoxia alone and increased even further when exposed to hyperthermia, which supported the above mechanism.

Namely, HIF1- α /BNIP3/Beclin1 autophagy signaling pathway was activated under hypoxia conditions and hyperthermia further enhanced the activation of the pathway by secreting ROS. The endothelial cells of the tumor microvessels proliferate and are more sensitive to heat than normal cells. Therefore, the temperature change caused by hyperthermia in the tumor area leads to apoptosis and necrosis of tumor cells. Hyperthermia can enhance the expression of apoptotic genes, such as *p53*, thereby impeding the cell cycle, inhibiting tumor cell proliferation, and leading to tumor cell apoptosis [53]. And our research results demonstrated that cell migration was inhibited and cell apoptosis rate was significantly augmented when untreated or hypoxia-treated cells were exposed to hyperthermia. Existing literatures show that HMGB1 release occurs passively as cell permeability breaks down upon necrosis [54] and late stage of apoptosis [55]. And in our experimental results, the secretion of HMGB1 was upregulated by hyperthermia. Nevertheless, during tumor development and cancer therapy, HMGB1 has been reported to play paradoxical roles in promoting both cell survival and death by regulating multiple signaling pathways. It has been demonstrated HMGB1 increases pro-survival autophagy in a Beclin1-dependent way during chemotherapy [56]. Moreover, it has been demonstrated ATG5-mediated autophagy pathway promoted the secretion of HMGB1 in starvation and lipopolysaccharide (LPS) treatment, and ROS signaling was required in this process [57]. What's more, several of the secondary messengers, such as cytosolic free calcium and ROS can regulate HMGB1 secretion [56]. We also demonstrated that both hyperthermia and hypoxia facilitated the secretion of HMGB1. Furthermore, a previous research has confirmed that secreted HMGB1 activated receptors for advanced glycation end products and Toll-like receptor-4 and induced autophagy in skeletal muscle [58].

Recently, more and more researchers have paid their attention to the manipulation of autophagy to enhance the efficacy of cancer therapy. YC-1 is a guanylate cyclase-activator and inhibitor of HIF-1 α [59] while 3-MA is a key drug in studying autophagy, which can block autophagy [60]. Therefore we used YC-1 and 3-MA to examine the effect on autophagy. In the present study, we found the use of YC-1 significantly downregulated the expression of HIF-1 α induced by CoCl₂, although the exact mechanism is uncertain. We observed that inhibition of HIF-1 α significantly suppressed BNIP3 expression and the administration of YC-1 and 3-MA alone or in combination significantly downregulated the expression of autophagy related genes LC3II, Beclin1 and HMGB1, and increased the expression level of P62 when exposed to mild hyperthermia, hypoxia and nutrition deficiency microenvironment. Furthermore, we found that YC-1 and 3-MA treatment suppressed cell migration and increased cell apoptosis in Cal-27 and SCC-15 cell lines, which suggested that hyperthermia and hypoxia induced a protective effect of autophagy by activating HIF-1 α /BNIP3/Beclin1 pathway and by stimulating the secretion of HMGB1, and autophagy act as a survival mechanism to alleviate hyperthermia and hypoxia injury. But even more importantly, this result could be reversed by the use of autophagy inhibitor and by blocking HIF-1 α . In view of clinical application, when thermochemotherapy was used in combination, HT-related elevated blood perfusion also supported higher intra- and peritumoral drug concentrations, changed the tumor microenvironment to improve the efficacy of chemotherapy, while chemotherapy could effectively inhibit HT-induced autophagy to increase cell apoptosis. Autophagy and apoptosis are often inseparable and highly interactional. Research found that specifically blocking autophagy enabled ROS to increase significantly in malignant tumor cells. Thus, autophagy can aggravate the apoptosis of tumor cells [61]. Previous study shows that both apoptosis and autophagy are activated in response to metabolic stress [62], and accumulating evidence reveals that autophagy and apoptosis can cooperate, antagonize or assist each other, thus influencing the differential the fate of the cell [63].

Conclusion

Although HT is considered to be a promising cancer treatment regimen, cellular damage caused by heating could be repaired and reversed by the production of HSPs and autophagy process in hypoxia and starving environment,

resulting in incomplete cell necrosis and attenuating the effects of HT therapy. In this study, we demonstrated that exposure to hypoxia and hyperthermia could induce autophagy in the OSCC cells Cal-27 and SCC-15. This process could be reversed by the use of autophagy inhibitor and by blocking HIF-1 α . In summary, our findings might benefit further understanding of the biological effects of thermo-chemo-therapy on cancer cells, and we believed that inhibition of autophagy might be a useful and promising therapeutic strategy to enhance the therapeutic effect of HT in hypoxia and nutrient deficiency tumor environment. In addition, further research on animal model is required in the future.

Declarations

Acknowledgements:

Author contribution:

Fan Shi participated in the design of this study and drafted the manuscript.

Qiaozhen Sun and Dan Luo carried out the study and performed the statistical analysis.

Xuexiao Zhou and Pei Shen carried out the literature search and data acquisition.

Shengzhi Wang provided assistance for data analysis and revised the manuscript.

All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest

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Figures

Figure 1

Immunohistochemistry were performed to analyze the expression level of HIF-1 α and Beclin1 in oral squamous cell carcinoma (OSCC) tissues and adjacent paracancer tissues. Magnification, $\times 200$. A, B: Normal tissue; C-F: OSCC tissues. HIF-1 α , hypoxia inducible factor-1 α ; Beclin1, a coiled-coil Bcl-2-interacting protein homologous. HIF-1 α was mainly expressed in the cell nucleus while Beclin1 mainly located in the cell membrane and cytoplasm with a small amount in the cell nucleus. The positive staining of HIF-1 α and Beclin1 showed uniform light yellow, brownish yellow or tan granules.

Figure 2

Determination of the IC₅₀ of CoCl₂, YC-1 and 3-MA by CCK-8 assay in Cal-27 and SCC-15 cells (A,B,C). Each data represented the mean ± SEM from three independent experiments. CoCl₂: Cobalt chloride; 3-MA:3-Methyladenine; YC-1: 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole.

Figure 3

CoCl₂ significantly induced the mRNA (A) and protein (B,C) expression of HIF-1α in a dose-dependent manner. Cal-27 and SCC-15 cells were co-cultured with different concentration of CoCl₂ for 24 h, then the mRNA and protein expression levels of HIF-1α were analyzed by qRT-PCR and Western blot, which were normalized to those of β-actin. Data were expressed as the mean ± SEM from three independent experiments. * P<0.05, ** P<0.01, and *** P<0.001 vs. 50 μM CoCl₂; # P<0.05, ## P<0.01, and ### P<0.001 vs. 50 μM CoCl₂.

Figure 4

Hyperthermia and hypoxia induced the expression of autophagy hallmark proteins in OSCC cells (A, B, C). Cal-27 and SCC-15 cells were cultured in hypoxia condition and were exposed to 42°C for 1h, followed by recovery at 37 °C for 24 h. The protein (A, B) and mRNA (C) levels of HIF-1α, BNIP3, Beclin1, LC3-II and p62 were analyzed by qRT-PCR and Western blot and normalized to those of β-actin. Data were expressed as the mean ± SEM from three independent experiments. a: Con; b: HT; c: Hy; d: HT+Hy. Con: control; HT: hyperthermia; Hy: hypoxia; HT+Hy: hyperthermia+hypoxia. * P<0.05 vs. Con group; # P<0.05 vs. HT group; & P<0.05 vs. Hy group

Figure 5

Addition of YC-1 and 3-MA inhibited HT-induced autophagy in hypoxia and starvation tumor microenvironment, both of which both involved downregulation of the HIF-1α/BNIP3 /Beclin1 signaling pathway. The (A,B) protein and (C) mRNA expression of HIF-1α, BNIP3, Beclin1, LC3-II and p62 in OSCC cells were measured by Western blot and qRT-PCR, which were normalized to β-actin. The results were presented as the mean ± standard deviation of three independent experiments. d: HT+Hy; e: HT+Hy+3-MA; f: HT+Hy+YC-1; g: HT+Hy+YC-1+3-MA. HT: hyperthermia; Hy: hypoxia; YC-1: 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole; 3-MA: 3-Methyladenine. * P<0.05 vs. HT+Hy group; # P<0.05 vs. HT+Hy+3-MA group; & P<0.05 vs. HT+Hy+YC-1 group; no: no significant difference (P>0.05).

Figure 6

HT combined chemotherapy (YC-1), autophagy blocking (3-MA) alone or both inhibited the secretion of HMGB1 in Cal-27 and SCC-15 cells under hypoxia and starvation microenvironment as measured by ELISA. Data were presented as mean $\pm$ SEM from three independent experiments. Con: control; HT: hyperthermia; Hy: hypoxia; YC-1: 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole; 3-MA:3-Methyladenine; There were statistically significant differences among the groups.

Figure 7

HT combined chemotherapy (YC-1), autophagy blocking (3-MA) alone or both inhibited the migration of Cal-27 and SCC-15 cells in hypoxia and starvation microenvironment, as determined by wound healing assay (A-C). Magnification, $\times 400$. Data were expressed as the mean $\pm$ SEM from three independent experiments. Con: control; HT: hyperthermia; Hy: hypoxia; YC-1: 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole; 3-MA: 3-Methyladenine; no: no significant difference ($P > 0.05$).

Figure 8

HT combined chemotherapy (YC-1), autophagy blocking (3-MA) alone or both facilitated the apoptosis of Cal-27 and SCC-15 cells in hypoxia and starvation microenvironment, as measured by flow cytometry (A-C). Data were presented as the mean $\pm$ SEM from three independent experiments. Con: control; HT: hyperthermia; Hy: hypoxia; YC-1: 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole; 3-MA: 3-Methyladenine; no: no significant difference ($P>0.05$). ns: no significant difference ($P>0.05$). There were differences between the remaining groups.

Supplementary Files

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- [Attachment1.docx](#)