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Original Article

High nitric oxide-adapted head and neck cancer cell lines demonstrate altered autophagy and apoptosis

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KEYWORDS
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Abstract *Background/purpose:* Autophagy is an intracellular degradative process occurring under stressful conditions. Nitric oxide (NO), a free radical, regulates autophagy and apoptosis in several cancers. However, the effect of head and neck squamous cell carcinoma (HNSCC) cell adaptation to high nitric oxide (HNO) on autophagy remains unknown. We investigated the autophagy and apoptotic changes in the HNO-adapted HNSCC cell lines.
Materials and methods: Isogenic primary HNSCC (HN18/HN30) and metastatic (HN17/HN31) cell lines were evaluated. The cells were induced with 1, 2, 3 and 4 mM DEA-NONOate, an NO donor, for 72 h and assessed for cell viability by MTT assay. "HNO-adapted cells" were defined when the cell viability in the treatment group was <10%. The surviving cells were re-treated with HNO to confirm their adaptation. HNO-adapted cells were quantified for apoptosis using flow cytometry. Autophagic structures (autophagosomes) and proteins (LC3A/B and LC3B-II) were investigated using transmission electron and confocal microscopy, respectively.
Results: HNO-adapted concentration for HN18, HN17, HN30 and HN31 cells was 3, 2, 4 and 4 mM, respectively. The HNO-adapted HN18 cells demonstrated a significantly increased apoptotic percentage, whereas no significant apoptotic change was detected in the HNO-adapted HN17, HN30 and HN31 cells compared with the parent cells. Autophagosomes were widely observed across the HNO-adapted cells. Moreover, LC3A/B and LC3B-II proteins were increased in all HNO-adapted cells.
Conclusion: Our results demonstrate that apoptosis and/or autophagy are increased during HNO adaptation in HNSCC cell lines.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is a severe disease found worldwide.[1] Chemoresistance in HNSCC cells is associated with a low patient survival rate. Autophagy is a mechanism that facilitates the cancer cells' resistance to chemotherapy and radiation treatment.[2] Autophagy is an intracellular degradation process in response to stressful conditions, such as oxidative stress, nutrient deprivation and hypoxia. The autophagic response initiates the formation of cytosolic double-membrane vesicles called phagophores. The phagophore membrane is elongated and matures into autophagosomes that contain the degraded cellular components that conjugate with lysosomes to recycle their components.[3] Human light chain 3 (LC3) proteins, including LC3A, LC3B and LC3C isoforms, play an important role in autophagosome synthesis.[4] During autophagy, pro-LC3 is cleaved to LC3-I that is subsequently converted to LC3-II. LC3-II initiates the formation of autophagosomes containing degraded intracellular materials.[5,6] Mature autophagosomes combine with lysosomes and form autolysosomes to remove damaged proteins/organelles.[7]

Autophagic dysfunction is associated with various human pathologies, such as cancers, neurodegeneration, and metabolic diseases.[8] Autophagy exhibits dual roles in tumor promotion and suppression.[9] Autophagy prevents tumor generation by regulating excess reactive oxygen species (ROS) or reactive nitrogen species (RNS).[10] In contrast, autophagy is activated in the central part of solid tumors to promote cell survival under hypoxic conditions.[11]

Nitric oxide (NO), a potent cellular messenger, regulates autophagy in physiologic and pathologic conditions. Low NO donor concentration decreased autophagosome formation and LC3-II levels in rat primary cortical neurons and a cervical cancer cell line.[12] Moreover, NO inhibited autophagy and promoted apoptosis in hepatocellular carcinoma.[13] Our recent study found that high nitric oxide (HNO) levels altered apoptosis in HNSCC cell lines.[14] However, the effect of HNO on autophagy in HNSCC cells remains unclear. The aim of this study was to investigate the autophagic and apoptotic changes in HNO-adapted HNSCC cell lines.

Materials and methods

Cell culture

The isogenic HNSCC cell lines representing primary and metastatic sites from the same patient, which were initially established by Ensley J.,[15] were provided by Professor Silvio Gutkind. The HN18 cells were obtained from primary tongue lesions and the HN17 cells were taken from neck dissections (T2N2M0 stage). The HN30 cells were obtained from primary pharynx lesions and the HN31 cells were taken from lymph node metastases (T3N1M0 stage). The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere.

HNO-adapted cells induction

HNSCC cell lines were seeded in 96-well plates (5,000 cells/well) and incubated at 37°C for 24 h. The cells were treated with 1, 2, 3 and 4 mM DEA-NONOate (Sigma, St Louis, MO, USA), an NO donor, for 72 h. The cells in DMEM without DEA-NONOate served as "parent cells". The number of viable cells in each group was determined using the thiazolyl blue tetrazolium bromide (MTT, Sigma) assay as previously described.[14] The absorbance (Abs) of the final solution was measured at 570 nm by a microplate reader (Tecan, Männedorf, Switzerland). Cell viability (%) was calculated using the formula: cell viability (%) = (mean $Abs570_{treated\ cells}$ - mean $Abs570_{blank}$)/(mean $Abs570_{parent\ cells}$ - mean $Abs570_{blank}$) × 100. "HNO-adapted cells" were defined when the cell viability in the treatment group was <10%.

Evaluation of cell adaptability to HNO

HNSCC cell lines were cultured in 96-well plates (5,000 cells/well) for 24 h. At the first exposure, the HN18, HN17, HN30 and HN31 cells were treated with 3, 2, 4 and 4 mM HNO, respectively, for 72 h. The number of viable cells was determined using the MTT assay. The cells that survived the first exposure were collected and grown in growth media until reaching 70–80% confluence. The cells were re-treated with HNO (2^{nd} exposure) for 72 h. The viable cell number at the 2^{nd} exposure was assessed. HNO-adapted cells from the second exposure were harvested to use in subsequent experiments.

Apoptosis assessment

HNO-adapted or parent cells (2.5×10^5/well in 6-well plates) were cultured in complete medium for 24 h and then trypsinized, centrifuged at 200×*g* for 5 min, and washed twice with phosphate-buffered saline (PBS). Cells induced using 10 μM Cisplatin for 24 h served as positive control. The apoptotic cells were assessed using a FITC Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend, San Diego, CA, USA) following the manufacturer's instructions. The number of apoptotic cells was calculated in a flow cytometer using the BD Accuri™ 6 plus Software (BD BioSciences, Franklin Lakes, NJ, USA). The percentage of apoptotic cells was measured and compared between the HNO-adapted and parent cells.

Transmission electron microscopy

Cells were trypsinized, fixed with 2.5% glutaraldehyde for 24 h at 4 °C and washed with PBS three times. The cells were fixed with 2% osmium tetroxide for 30 min at room temperature. The samples were dehydrated using an ascending ethanol series (70%—100%) and embedded in propylene oxide and resin. The embedded samples were polymerized in gradient conditions: 35 °C overnight, 45 °C overnight and 70 °C for 48 h. Subsequently, the samples were sliced into 70-nm thick sections using an ultramicrotome, then stained with uranyl acetate and lead citrate for 10 min each, at room temperature. The samples were observed using a JEM-1400 Plus transmission electron microscope (TEM) (JEOL, Tokyo, Japan).

Immunofluorescence

The cells (40,000 cells/well) were cultured in 8-well chamber slides (SPL Lifesciences, Kyonggo-do, South Korea) for 48 h. The cells treated with 20 μM rapamycin (autophagy inducer) for 24 h were used as positive control. For immunofluorescent staining, the cells were fixed for 15 min in methanol at −20 °C, washed three times with ice-cold PBS, and blocked with 1% PBST-BSA for 1 h at room temperature. The cells were then incubated with 1:200 anti-human LC3A/B or 1:20,000 anti-human LC3B-II (Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight. The cells were washed with ice-cold PBS three times and then stained with 1:1,000 Alexa Fluor®-488-conjugated anti-rabbit (Cell Signaling Technology) at room temperature in the dark for 1 h. The cells were washed with ice-cold PBS three times. Finally, the nuclei were stained with 1:5,000 DAPI (BioLegend) at room temperature for 5 min. The fluorescence signal was observed using a FLUOVIEW FV3000 confocal microscope (Olympus, Tokyo, Japan).

Statistical analysis

The data are presented as means and standard error of the mean (SEM). Multiple group comparisons were performed using one-way ANOVA followed by Tukey's post hoc test.

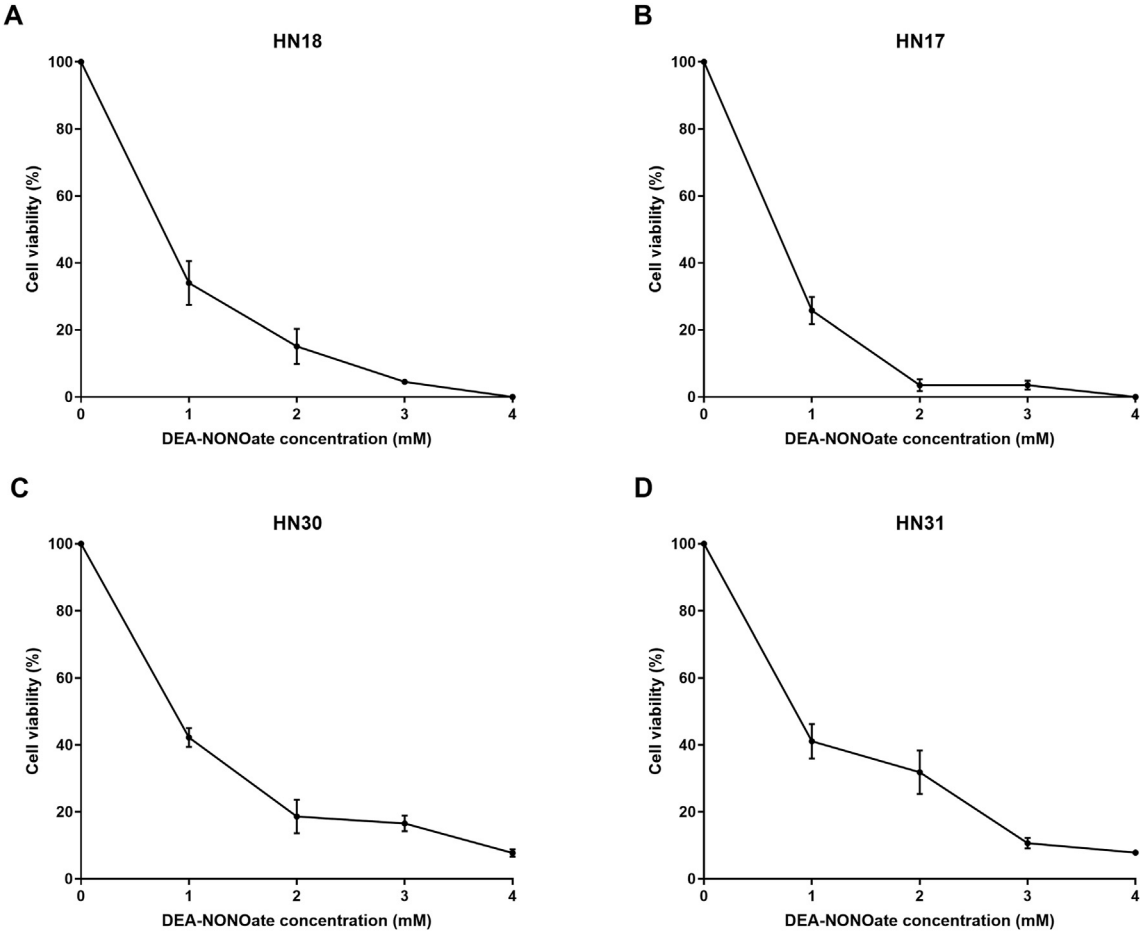

Figure 1 Effect of DEA-NONOate on HNSCC cell viability. HN18 (A), HN17 (B), HN30 (C) and HN31 (D) cells were treated with 0, 1, 2, 3 and 4 mM DEA-NONOate for 72 h. Cell viability was assessed using MTT assay. Graphs represent mean ± SEM of percent cell viability (n = 3).

Table 1 HNO concentration for adapting the HNSCC cell lines.

Cell lines	HNO concentration (mM)	Cell viability (%) (Mean ± SEM)
HN18	3	4.52 ± 0.54
HN17	2	3.52 ± 1.80
HN30	4	7.72 ± 1.00
HN31	4	7.82 ± 0.25

HNO, high nitric oxide.

Statistical analyses were performed using GraphPad Prism 7.04 software (GraphPad Software, La Jolla, CA, USA). $P \leq 0.05$ was considered significant.

Results

Effect of DEA-NONOate on HNSCC cell viability

DEA-NONOate-induced HNSCC cells demonstrated decreased viability in a dose-dependent manner (Fig. 1). The HNO concentration for adapting the HN18, HN17, HN30 and HN31 cells was 3, 2, 4 and 4 mM, respectively (Table 1).

Evaluation of HNSCC cell adaptability to HNO

The cell viability between the 1st and 2nd HNO exposures were compared. All cell lines adapted to HNO as demonstrated by significantly increased viability at the 2nd exposure compared with that of the 1st exposure ($P < 0.05$) (Fig. 2).

Apoptotic assessment in HNO-adapted cells

The percentage of apoptosis was significantly increased in the HNO-adapted HN18 cells compared with the parent cells ($P < 0.05$) (Fig. 3A). However, there was no significant difference in apoptosis in the HNO-adapted HN17, HN30, and HN31 cells compared with the parent groups ($P > 0.05$) (Fig. 3B–D).

Autophagy evaluation in HNO-adapted cells

Autophagy in the HNO-adapted cells was characterized by the appearance of intracellular structures including phagophores, membrane elongation, autophagosomes and autolysosomes. Phagophores and membrane elongation were observed at the early stage of autophagy, whereas autophagosomes and autolysosomes were seen at the late stage. Autophagosomes were widely present in all HNO-

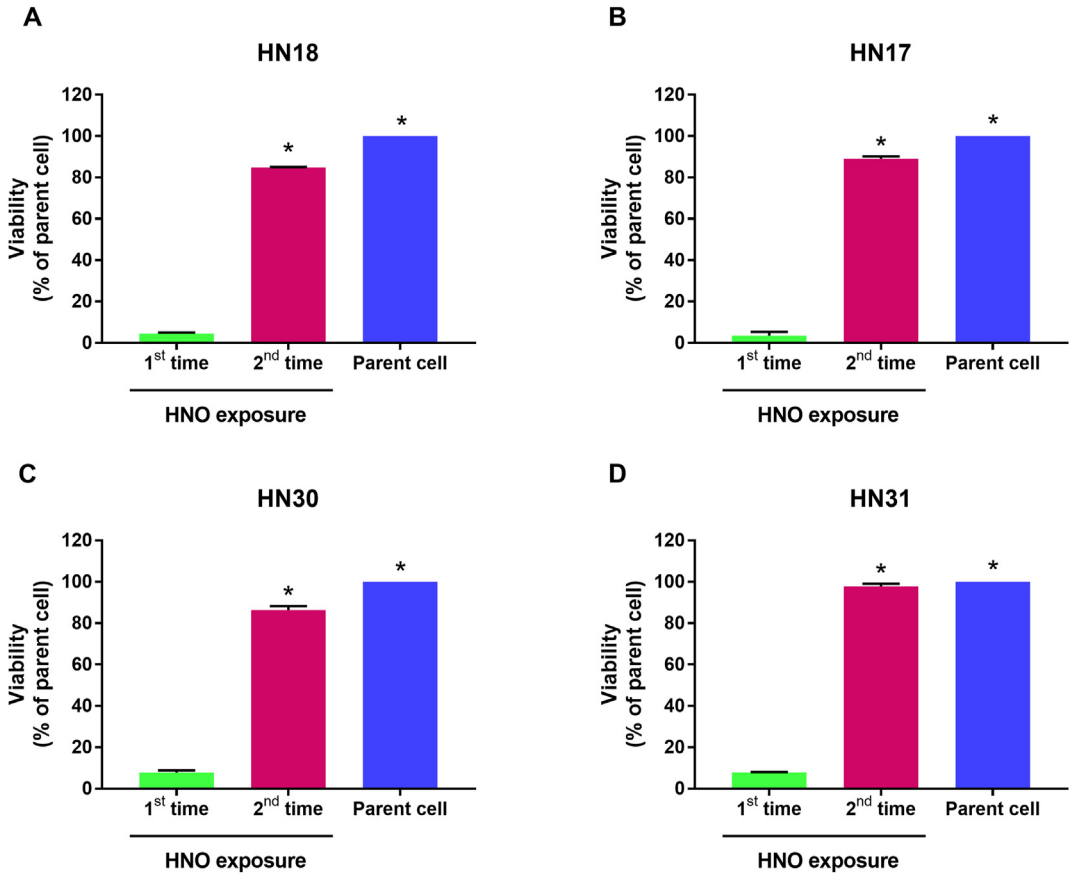

Figure 2 Evaluation of HNSCC cell adaptability to HNO. HN18 (A), HN17 (B), HN30 (C) and HN31 (D) cells were exposed to HNO at each time point. MTT assay was used to evaluate cell viability after treatment. Bars represent mean ± SEM of percent cell viability (n = 3). *: $P < 0.05$ vs 1st HNO exposure.

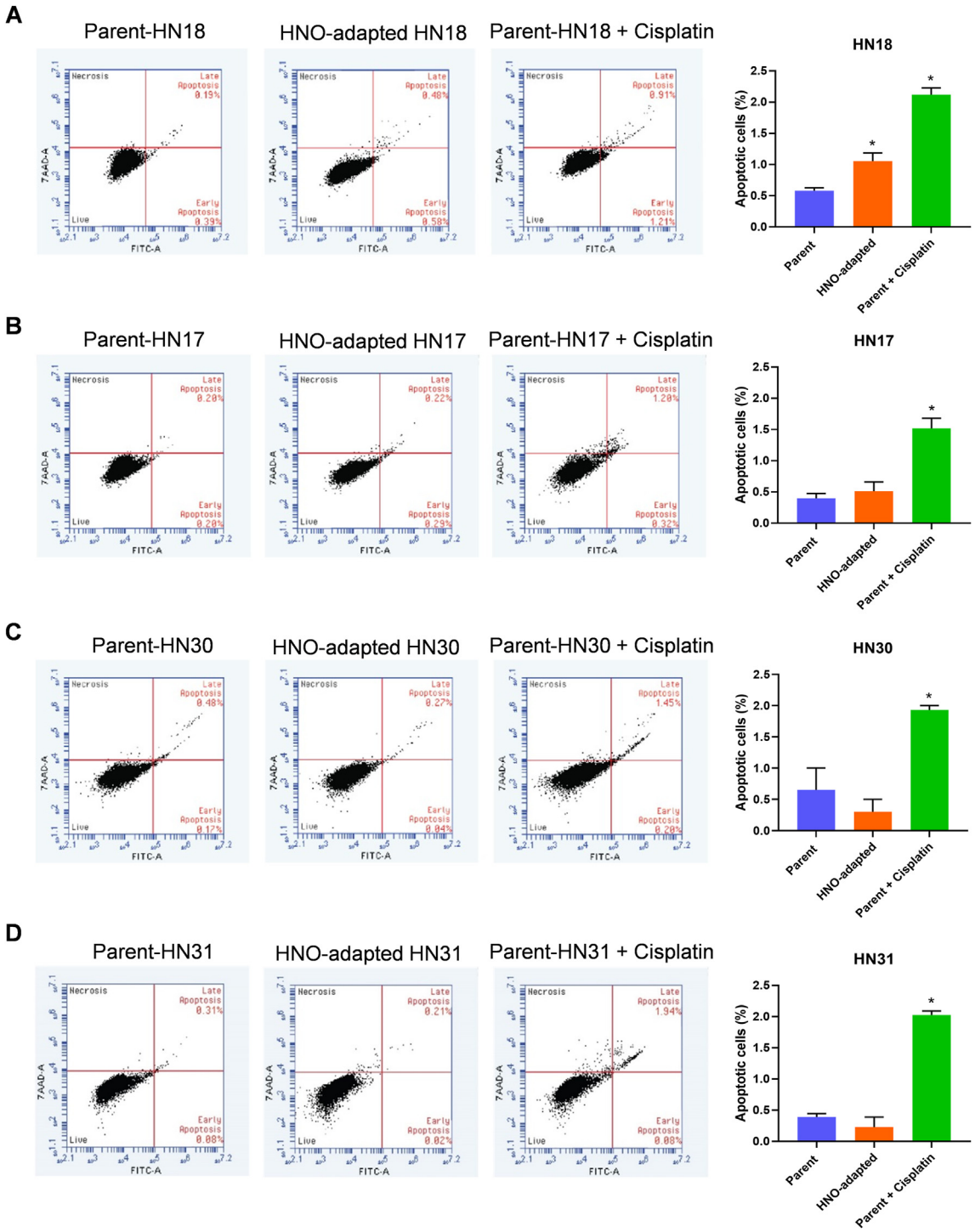

Figure 3 Apoptotic assessment in HNO-adapted cells. HNO-adapted HN18 (A), HN17 (B), HN30 (C) and HN31 (D) cells were evaluated for apoptosis using flow cytometry. Cisplatin-treated parent cells were used as a positive control. Scatter-plot diagrams depict cell status: live, early/late apoptosis and necrosis. Bars represent mean ± SEM of percent apoptotic (early + late) cell (n = 3). *: $P < 0.05$ vs. parent cell.

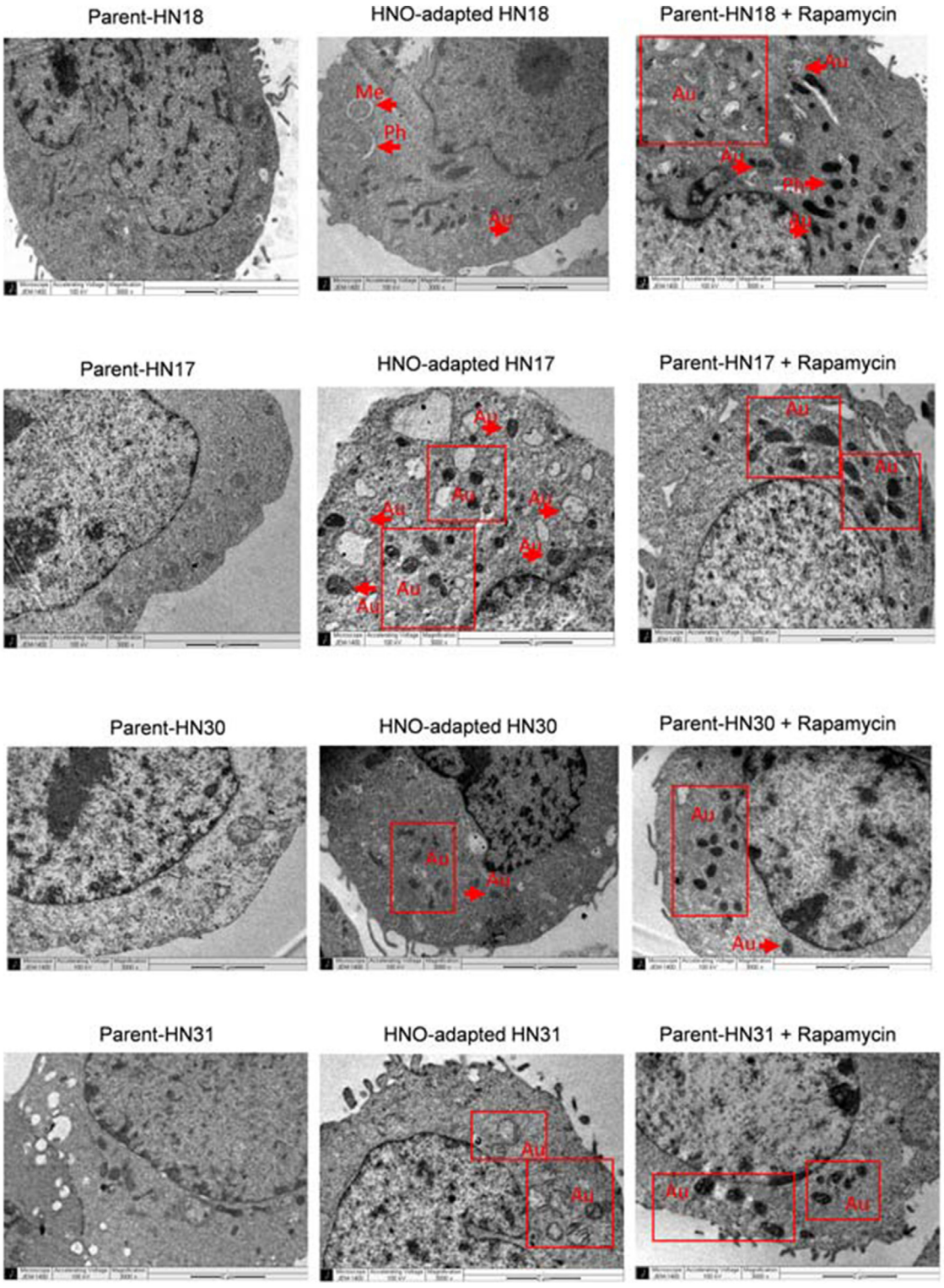

Figure 4 Autophagy evaluation in HNO-adapted cells. Parent, HNO-adapted and rapamycin-treated HN18, HN17, HN30 and HN31 cells were evaluated for their autophagy characteristics, including phagophores (Ph), membrane elongation (Me) and autophagosomes (Au) using TEM. Autophagy structures are indicated by reds arrows or grouped in a box. Rapamycin-treated cells were used as a positive control.

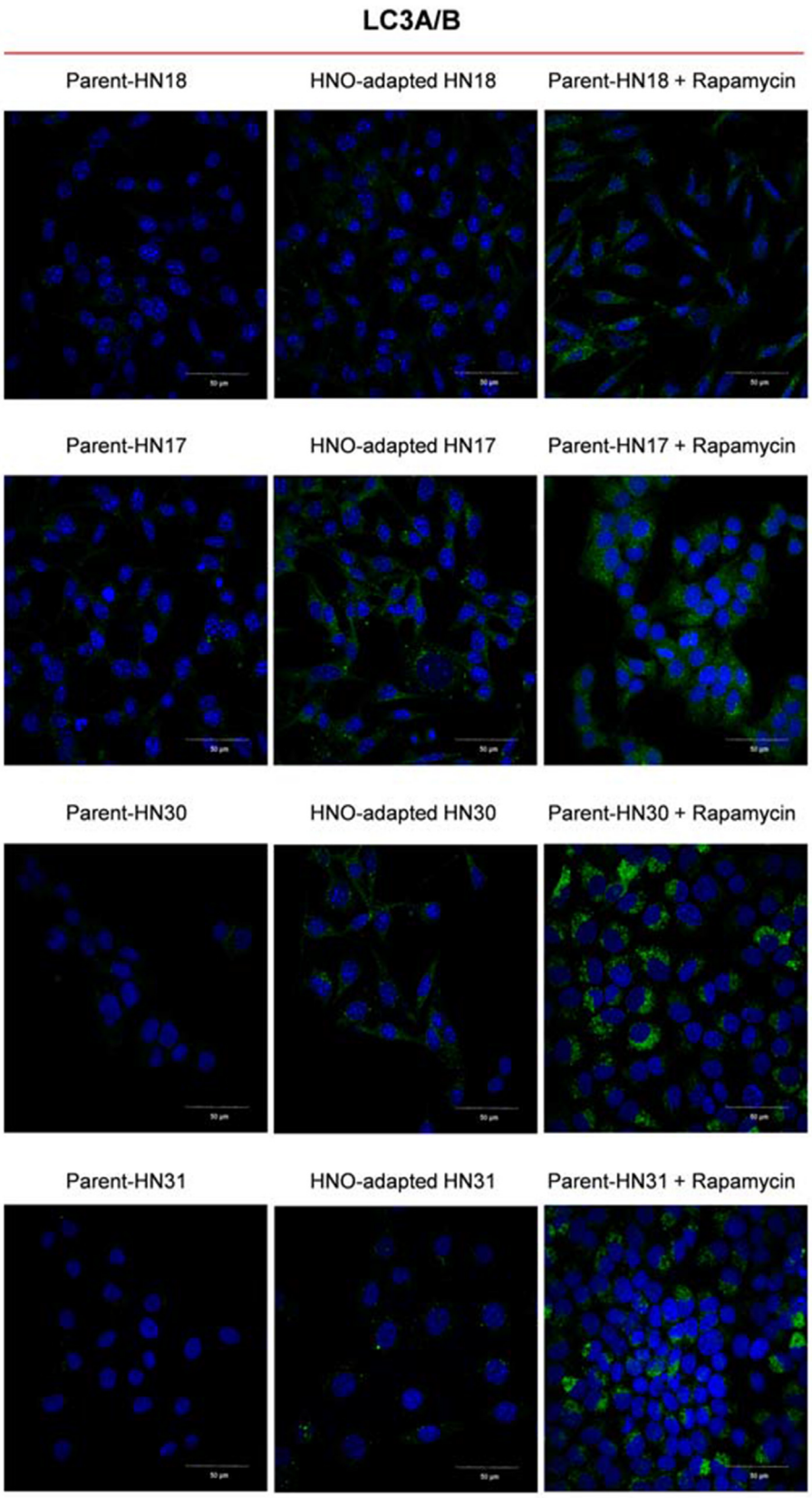

Figure 5 LC3A/B protein expression in HNO-adapted cells. Total LC3A/B was localized in parent, HNO-adapted and rapamycin-treated parent cell lines using immunocytochemistry. Rapamycin-treated parent cells were used as a positive control. Green signal intensity represents the LC3A/B expression level. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

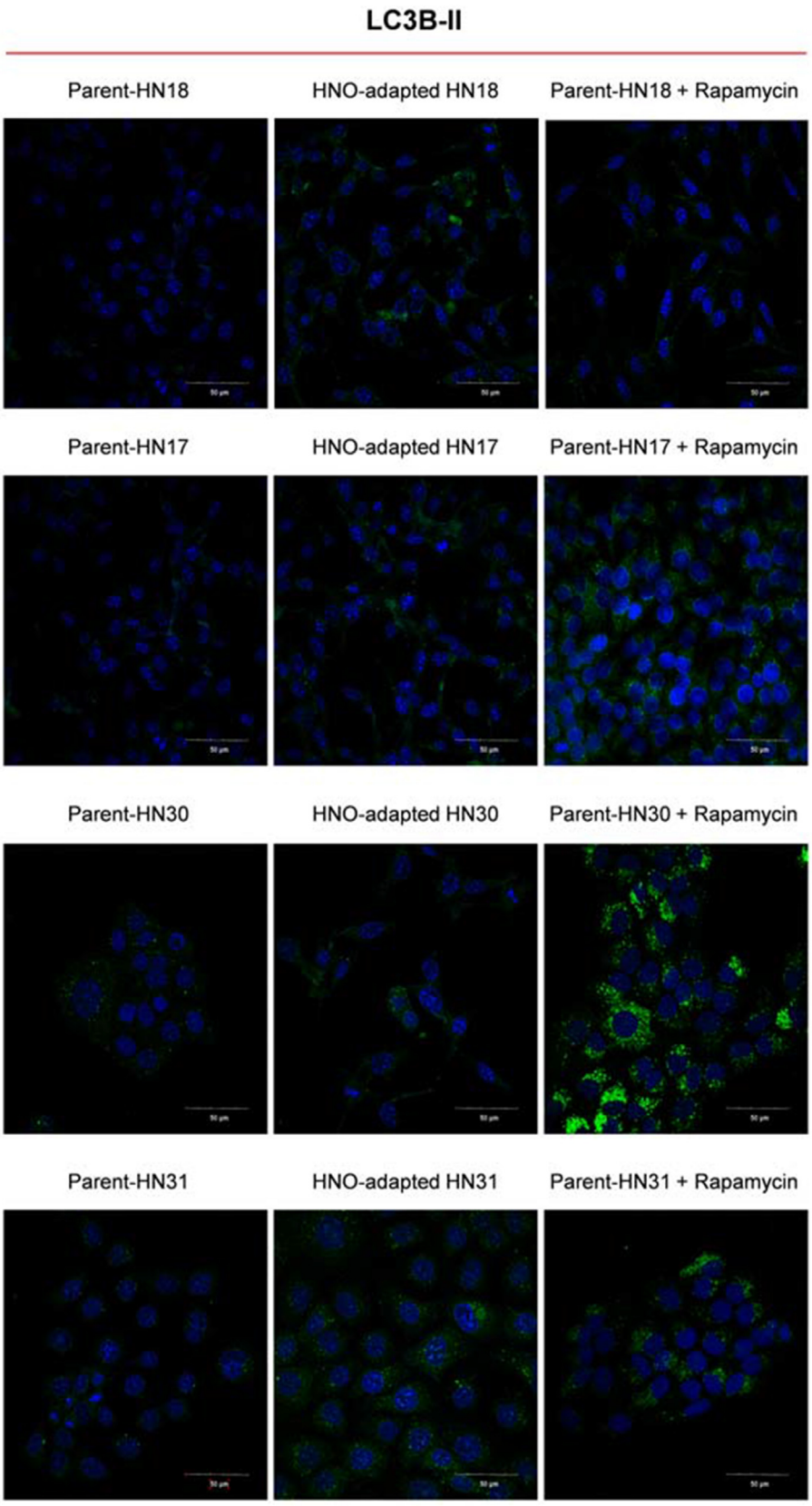

Figure 6 LC3B-II protein expression in HNO-adapted cells. LC3B-II was localized in parent, HNO-adapted and rapamycin-treated parent cell lines using immunocytochemistry. Rapamycin-treated parent cells were used as a positive control. Green signal intensity represents the LC3B-II expression level. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

adapted cell lines (Fig. 4). Moreover, phagophores and membrane elongation structures were observed in the HNO-adapted HN18 cells. In contrast, the parent cells demonstrated normal morphology.

Autophagy-associated proteins expression in HNO-adapted cells

Autophagy marker proteins (LC3A/B and LC3B-II forms) were investigated in HNO-adapted cells using a confocal microscope. The total LC3A/B level was higher in all HNO-adapted cell lines than that in the parent groups (Fig. 5). Moreover, LC3B-II (an autophagosome membrane association marker) was markedly localized in all HNO-adapted cell lines, whereas LC3B-II was rarely expressed in parent cells (Fig. 6).

Discussion

NO is a free radical molecule that exerts several biological effects on cancer cells depending on its concentration. Low NO concentration maintains cytoprotective functions, such as autophagy inhibition.[12,13] In contrast, high NO levels promote anti-tumorigenic effects, such as inducing apoptosis.[11] Our results confirmed the cytotoxic effect of NO on HNSCC cells in a dose-dependent manner. DEA-NONOate, a NO donor, has been used to investigate the HNO-adapted properties in several cancer cell types.[16,17] Our study used the DEA-NONOate induction model to demonstrate the effect of HNO on HNSCC cell adaptation. Stage 3 cell lines (HN30/HN31) exhibited relatively high HNO resistance compared with stage 2 cell lines (HN18/HN17). These data suggest that the HNO adaptation in HNSCC cells may depend on clinical staging.

Previous results showed that almost all HNO-adapted HNSCC cells demonstrated similar apoptosis characteristics compared with the parent cells.[14] Antiapoptotic pathways were activated in a long-term (65 days) HNO-treated human lung adenocarcinoma cell line.[17] However, high levels of NO induced Fas-mediated apoptosis in an ovarian cancer cell line.[18] The present study found that short-term HNO treatment (72 h) had no apoptotic effect on HN17, HN30 and HN31 cells; however, it increased apoptosis in HN18 cells compared with parent cells. The apoptotic change in HNO-adapted cancer cells may be NO exposure time- or cancer type-dependent.

Autophagy is a mechanism for maintaining cancer cell survival by regulating excess ROS or RNS.[10] We investigated the autophagic characteristics in the HNO-adapted cell lines. Autophagosomes and autophagy markers (LC3A/B and LC3B-II) were increased across the HNO-adapted cell lines. Among the LC3 isoforms (LC3A, LC3B and L3C), LC3B is the most studied protein because it is associated with autophagosome development.[4] LC3B-II is an active protein that contributes to autophagosome formation.[5] These findings imply that autophagy may be a common cytoprotective process in HNO-adapted HNSCC cells.

The molecular connections between autophagy and cell death are multidirectional and unresolved.[19] Autophagy can stimulate or inhibit apoptosis. In contrast, activation of caspases during apoptosis increased or decreased autophagy. However, some evidence suggested that autophagy promoted cell death by degrading mitochondria.[20,21] Currently, the reports on the effect of NO on autophagy and apoptosis are limited. Zhang et al. suggested that low NO levels inhibited autophagy, but induced apoptosis in hepatocellular carcinoma cells.[13] In contrast, our study found that autophagy and apoptosis were increased in HNO-adapted HN18 cells. The HNSCC cell lines used in this study have variability in the epidermal growth factor signaling pathway,[15] thus, this factor might influence apoptosis and autophagy levels between the HNO-adapted cells. Collectively, the molecular mechanisms of autophagy and apoptosis in NO-induced HNSCC cell lines needs further dissection and the other related signaling pathways should be evaluated.

In conclusion, this is the first study to demonstrate the diverse autophagic and apoptotic responses in HNO-adapted HNSCC cells. HNO-adapted HN17, HN30 and HN31 cells demonstrated increased autophagy characteristics, but not apoptosis. Interestingly, autophagy and apoptosis were induced in HNO-adapted HN18 cells. The mechanism linking autophagy and apoptosis under HNO in HNSCC requires further investigation.

Conflicts of interest

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

Acknowledgments

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References

1. Guo K, Xiao W, Chen X, Zhao Z, Lin Y, Chen G. Epidemiological trends of head and neck cancer: a population-based study. *BioMed Res Int* 2021;2021:e1738932.
2. Rikiishi H. Autophagic action of new targeting agents in head and neck oncology. *Cancer Biol Ther* 2012;13:978—91.
3. Yun CW, Lee SH. The roles of autophagy in cancer. *Int J Mol Sci* 2018;19:e3466.
4. Schaaf MB, Keulers TG, Vooijs MA, Rouschop KM. LC3/GABARAP family proteins: autophagy-(un)related functions. *Faseb J* 2016;30:3961—78.
5. Hwang HJ, Kim YK. The role of LC3B in autophagy as an RNA-binding protein. *Autophagy* 2023;19:1028—30.
6. Kabeya Y, Mizushima N, Ueno T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 2000;19:5720—8.
7. Yim WW, Mizushima N. Lysosome biology in autophagy. *Cell Discov* 2020;6:e6.
8. Wirawan E, Vanden Berghe T, Lippens S, Agostinis P, Vandenabeele P. Autophagy: for better or for worse. *Cell Res* 2012;22:43—61.
9. Rosenfeldt MT, Ryan KM. The multiple roles of autophagy in cancer. *Carcinogenesis* 2011;32:955—63.
10. Filomeni G, De Zio D, Cecconi F. Oxidative stress and autophagy: the clash between damage and metabolic needs. *Cell Death Differ* 2015;22:377—88.

11. Degenhardt K, Mathew R, Beaudoin B, et al. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell* 2006;10:51—64.
12. Sarkar S, Korolchuk VI, Renna M, et al. Complex inhibitory effects of nitric oxide on autophagy. *Mol Cell* 2011;43:19—32.
13. Zhang X, Jin L, Tian Z, et al. Nitric oxide inhibits autophagy and promotes apoptosis in hepatocellular carcinoma. *Cancer Sci* 2019;110:1054—63.
14. Utispan K, Koontongkaew S. High nitric oxide adaptation in isogenic primary and metastatic head and neck cancer cells. *Anticancer Res* 2020;40:2657—65.
15. Cardinali M, Pietraszkiewicz H, Ensley JF, Robbins KC. Tyrosine phosphorylation as a marker for aberrantly regulated growth-promoting pathways in cell lines derived from head and neck malignancies. *Int J Cancer* 1995;61:98—103.
16. Aqil M, Deliu Z, Elseth KM, Shen G, Xue J, Radosevich JA. Part II-mechanism of adaptation: A549 cells adapt to high concentration of nitric oxide through bypass of cell cycle checkpoints. *Tumor Biol* 2014;35:2417—25.
17. Aqil M, Elseth KM, Vesper BJ, et al. Part I-mechanism of adaptation: high nitric oxide adapted A549 cells show enhanced DNA damage response and activation of anti-apoptotic pathways. *Tumor Biol* 2014;35:2403—15.
18. Garban HJ, Bonavida B. Nitric oxide sensitizes ovarian tumor cells to Fas-induced apoptosis. *Gynecol Oncol* 1999;73:257—64.
19. Gump JM, Thorburn A. Autophagy and apoptosis: what is the connection? *Trends Cell Biol* 2011;21:387—92.
20. Debnath J, Baehrecke EH, Kroemer G. Does autophagy contribute to cell death? *Autophagy* 2005;1:66—74.
21. Goldman SJ, Taylor R, Zhang Y, Jin S. Autophagy and the degradation of mitochondria. *Mitochondrion* 2010;10:309—15.