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Treatment with autophagic inhibitors enhances oligonol-induced apoptotic effects in nasopharyngeal carcinoma cells

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Abstract. Although the combination of chemotherapy and radiotherapy has increased the survival rate of patients with nasopharyngeal carcinoma (NPC), certain patients do not respond well to the treatment and have a poor prognosis. Therefore, novel therapeutic drugs and strategies to improve prognosis of patients with NPC are required. As certain plant extracts can suppress the viability of cancer cells, the present study investigated whether oligonol, a polyphenolic compound primarily found in lychee fruit, exerts anticancer activities in NPC cells. MTT, ELISA and immunoblotting were performed to investigate cell survival, cytokeratin-18 fragment release, and the expression of apoptosis and autophagy markers, respectively. Oligonol decreased the viability of NPC-TW01 and NPC/HK1NPC cell lines. Oligonol increased the protein expression of several apoptosis markers, including cleaved caspase-8 and -3, cleaved PARP and cytokeratin 18 fragment. Moreover, it also increased expression of autophagy markers Beclin 1 and LC3-II, as well as LC3-II/LC3-I ratio in both NPC cell lines. Furthermore, treatment with autophagy inhibitors 3-methyladenine or LY294002 significantly increased oligonol-induced viability inhibition in NPC-TW01 cells. Combined treatment of oligonol + LY294002 reduced LC3-II expression and the LC3II/LC3I ratio while increasing cleaved caspase-8 and -3, cleaved PARP and cytokeratin 18 fragment expression in NPC-TW01 cells. These findings indicated autophagy inhibitors could enhance viability inhibition and apoptotic effects induced by oligonol in NPC cells.

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Introduction

Nasopharyngeal carcinoma (NPC) is a highly metastatic malignant tumor originating from the epithelium of the nasopharynx (1). Genetic mutations, Epstein-Barr virus infection, tobacco smoking and consumption of alcohol and salted fish are risk factors for NPC (1). In 2019, age-standardized incidence rate (ASIR) of NPC was 2.12 globally. However, certain regions have higher ASIRs of NPC than the global average, including China, Singapore and Taiwan, with rates of 5.7, 10.81 and 7.14, respectively (2,3). The survival rate of patients with NPC has been improved significantly by combining chemotherapy and radiotherapy (4). However, ~30% of patients still have a poor prognosis due to distant metastases (5), suggesting that certain patients do not receive adequate benefits from the current treatment. Thus, improving the current treatments or developing new therapeutic drugs is necessary to improve prognosis of patients with NPC.

In recent years, increasing evidence (6-9) has indicated that bioactive compounds from plants have promising anticancer properties. For example, cordycepin and fucoidan are natural compounds extracted from *Cordyceps sinensis* and brown seaweed cell wall matrix, respectively (10,11). These compounds can induce apoptosis in various cancer cell lines, including esophageal, colorectal, liver, and non-small-cell lung cancer (12-15). Similarly, curcumin extracted from *Curcuma longa* inhibits the survival of non-small-cell lung, papillary thyroid, and bone cancer cells by inducing apoptosis (16-18) *in vitro*. It also suppresses bone cancer cell growth *in vivo* (18). Additionally, curcumin can inhibit oral cancer cell survival by inducing autophagy (19). Furthermore, paclitaxel, an anti-microtubule agent extracted from *Taxus brevifolia*, is used clinically to treat multiple cancers, including ovarian, lung, and breast (20).

Apoptosis is a programmed cell death characterized by specific biological features, such as plasma membrane blebbing, apoptotic body formation, cytokeratin 18 fragment release, DNA fragmentation and poly ADP-ribose polymerase (PARP) cleavage (21-23). In addition, caspases, a family of endoproteases, are divided into initiator and effector caspases (23); they

are involved in executing apoptosis. Upon activation, initiator caspases such as caspase-8 can activate effector caspases, such as caspase-3. This leads to the cleavage of PARP, a DNA repair enzyme, ultimately contributing to apoptosis (23).

Autophagy, a regulated self-digestion mechanism, is characterized by formation of double-membraned vesicles called autophagosomes that engulf cytoplasmic contents and fuse with lysosomes for degradation (24). The formation of autophagosomes is regulated by autophagy-related proteins, such as Beclin 1, LC3, and Atg12-Atg5 conjugate (25,26). Beclin 1 initiates autophagy, while LC3-II contributes to autophagosome formation, a key feature of autophagy (25-27). Additionally, a previous study showed that the knockdown of Beclin 1 or LC3 decreases autophagic activity (28), indicating that Beclin 1 and LC3 serve essential roles in activating autophagy.

Certain anticancer substances, such as cisplatin and curcumin, can simultaneously induce apoptosis and autophagy in bladder and gastric cancers, respectively (29,30). Studies show that inhibiting autophagy can enhance cisplatin or curcumin-induced apoptosis (29,30), suggesting that autophagy may play a protective role in cancer cell survival.

Oligonol, a polyphenolic compound found in lychee fruit (31), has antioxidant and anti-inflammatory properties and can alleviate sarcopenia (32-34). Additionally, previous studies have suggested that oligonol may have anticancer activity, as it induces apoptosis in breast and ovarian cancer cells (35,36). Therefore, the present study evaluated the potential anticancer effects of oligonol in NPC cells.

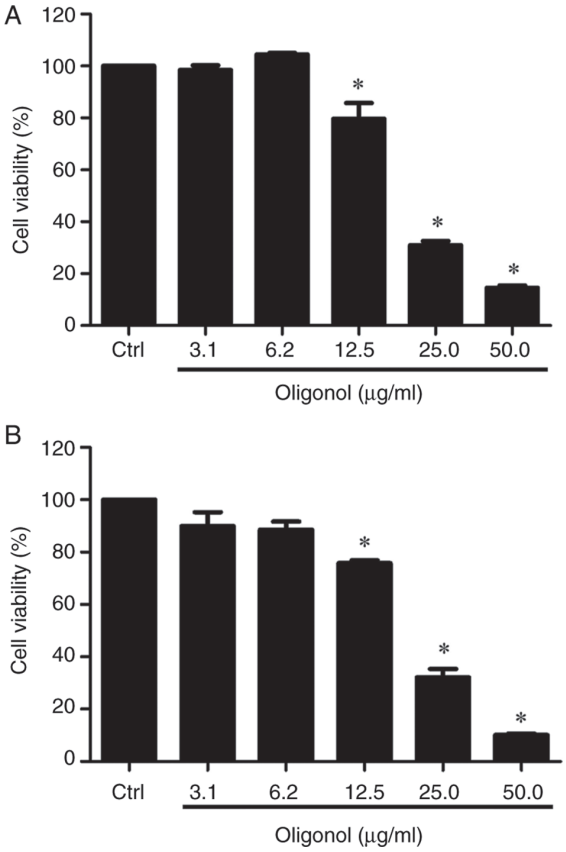

Figure 1. Oligonol inhibits viability of nasopharyngeal carcinoma cells. (A) NPC-TW01 and (B) NPC/HK1 cells were treated with oligonol for 48 h. The MTT assay was used to measure the cell viability. *P<0.05 vs. Ctrl. Ctrl, control.

Materials and methods

Materials. Oligonol was provided by Toong Yeuan Enterprise Co., Ltd. DMSO, 3-methyladenine (3-MA), LY294002 and MTT were purchased from Sigma-Aldrich (Merck KGaA). Fetal bovine serum (FBS), PBS and RPMI-1640 medium were purchased from HyClone (Cytiva).

Cell culture. NPC-TW01 cells were provided by Dr Chin-Hwa Tsai from the National Taiwan University in Taipei, Taiwan. These cells were established from moderately differentiated NPC tissue (37). The second cell line, NPC/HK1, was purchased from Quantum Biotechnology, Inc. and established from well-differentiated NPC tissues (38). Both cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS at 37˚C in a 5% CO_2 incubator.

MTT assay. NPC cells were seeded into a 6-well plate at a density of $3x10^5$ cells/well for 24 h. Once the cells reached 80% confluence, they were treated with oligonol at concentrations of 3.125, 6.25, 12.5, 25, and 50 μg/ml for 48 h at 37˚C with 5% CO_2. Cells not treated with oligonol were used as the control. Supernatant was removed, and 2 ml MTT reagent (0.5 mg/ml in PBS) was added to each well. After incubation at 37˚C and 5% CO_2 for 4 h, supernatant was removed, and 1 ml DMSO was added to each well to dissolve the crystals. Next, 100 μl DMSO lysate was transferred from each well to a 96-well plate. ELISA reader (BMG LABTECH) measured the optical density at 570 nm. All experiments were carried out ≥3 times independently.

ELISA. The concentration of cytokeratin 18 fragment levels in the cell culture supernatant was measured using the SimpleStep ELISA kit (cat. no. ab254515; Abcam) according to the manufacturer's instructions. Each experiment was conducted independently ≥3 times.

Immunoblotting assay. Extraction of total protein from cells and immunoblotting were performed as described previously (29). Primary antibodies for detecting PARP (cat. no. 9532; 1:1,000), cleaved PARP (cat. no. 9541; 1:1,000), caspase-8 (cat. no. 9746; 1:1,000), cleaved caspase-8 (cat. no. 9429; 1:1,000), caspase-3 (cat. no. 9662; 1:1,000), cleaved caspase-3 (cat. no. 9661; 1:1,000), Beclin 1 (cat. no. 3738; 1:1,000), LC3-I/II (cat. no. 4108; 1:1,000) and GAPDH (cat. no. 97166; 1:5,000) were purchased from Cell Signaling Technology, Inc. Horseradish peroxidase-conjugated secondary antibodies, including goat anti-rabbit (cat. no. 111-035-144; 1:5,000) and anti-mouse IgG (cat. no. 111-035-146; 1:5,000), were purchased from Jackson ImmunoResearch, Inc. Protein bands were detected using an enhanced chemiluminescence detection kit (SuperSignal™ West Pico PLUS Chemiluminescent Substrate; cat. no. 34580; Thermo Fisher Scientific, Inc.). All experiments were conducted independently ≥3 times. The intensities of protein bands were quantified using ImageJ software (version 1.43, National Institutes of Health).

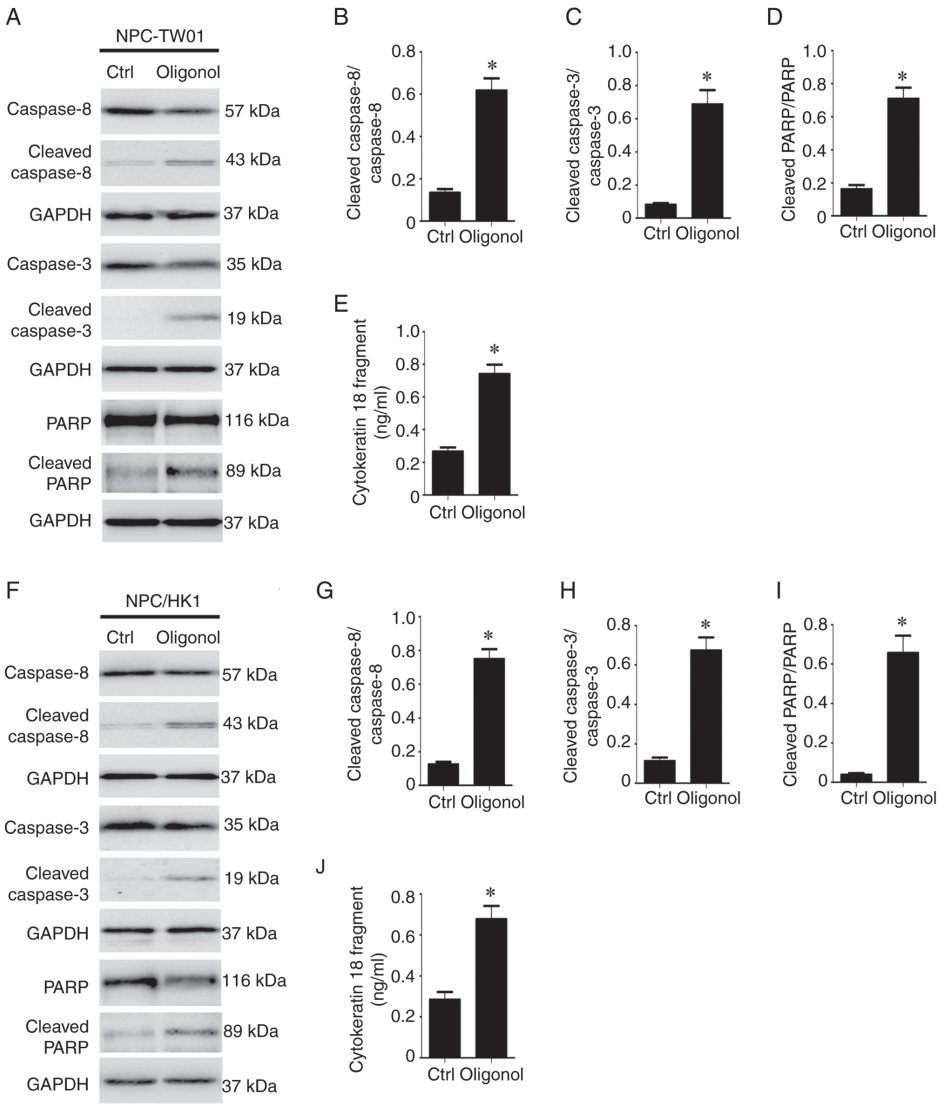

Figure 2. Oligonol induces apoptotic effects in nasopharyngeal carcinoma cells. NPC-TW01 were treated with 17 μg/ml oligonol for 48 h. (A) Protein expression of caspase-8, cleaved caspase-8, caspase-3, cleaved caspase-3, PARP, cleaved PARP and GAPDH was detected using an immunoblotting assay. The protein expression levels of cleaved caspase-8 (B) cleaved caspase-3 (C) and cleaved PARP (D) were quantified. (E) Concentration of cytokeratin 18 fragment in the cell culture supernatants was measured by ELISA. NPC/HK1 cells were treated with 17 μg/ml oligonol for 48 h. (F) Protein expression of caspase-8, cleaved caspase-8, caspase-3, cleaved caspase-3, PARP, cleaved PARP and GAPDH was detected using an immunoblotting assay. The protein expression levels of cleaved caspase-8 (G) cleaved caspase-3 (H) and cleaved PARP (I) were quantified. (J) Concentration of cytokeratin 18 fragment in the cell culture supernatants was measured by ELISA. *P<0.05 vs. Ctrl. Ctrl, control.

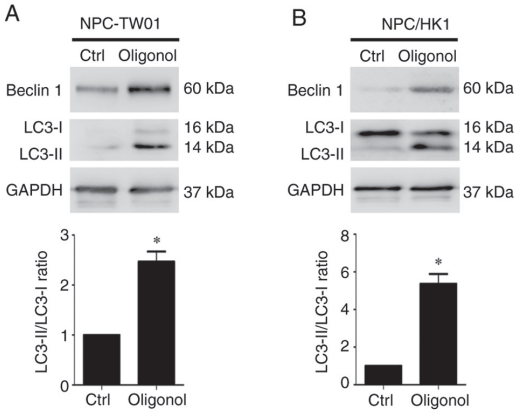

Figure 3. Oligonol induces autophagic effects in nasopharyngeal carcinoma cells. (A) NPC-TW01 and (B) NPC/HK1 cells were treated with 17 μg/ml oligonol for 48 h. Protein expression of Beclin 1, LC3-I, LC3-II and GAPDH was detected using an immunoblotting assay. *P<0.05 vs. Ctrl. Ctrl, control.

Statistical analysis. Data are expressed as the mean ± standard error of the mean of ≥3 independent experimental repeats. The statistical analysis was performed using SPSS software (version 17.0; SPSS, Inc.). An unpaired t-test was used to compare significant differences between two groups. One-way analysis of variance and Tukey's post hoc test were used to compare significant differences between >2 groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Oligonol inhibits viability of NPC cells. NPC cell lines NPC-TW01 and NPC/HK1 were treated with oligonol, and the cell viability was measured using an MTT assay. Oligonol could significantly inhibit the viability of both NPC cell lines at 12.5, 25.0 and 50.0 μg/ml compared with the control (Fig. 1A and B). In addition, the half-maximal

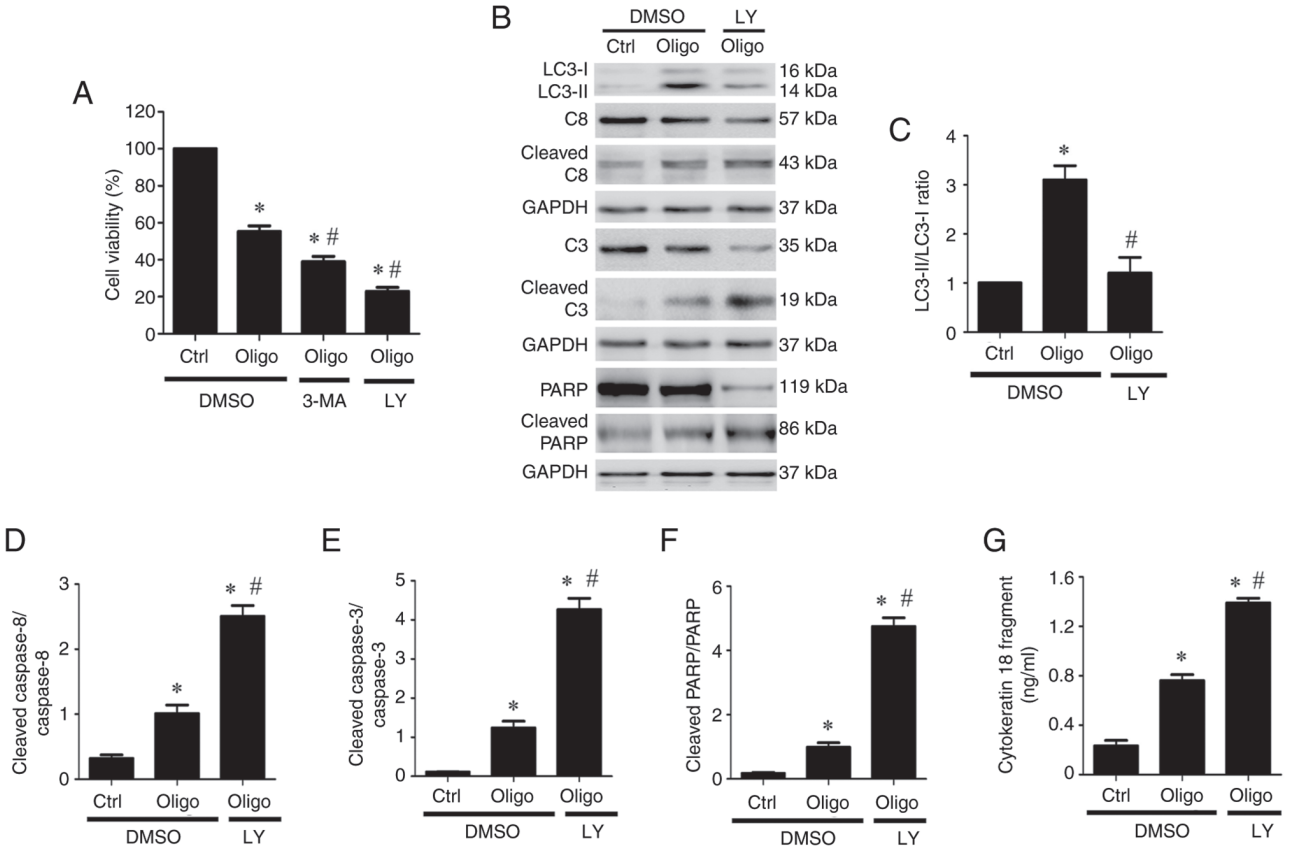

Figure 4. Inhibition of autophagic effects promotes oligonol-induced viability inhibition and apoptotic effects in nasopharyngeal cells. (A) NPC-TW01 cells were treated with 17 μg/ml Oligo and DMSO, 3-MA or LY. Cell viability was measured using MTT assay. (B) NPC-TW01 cells were treated with 17 μg/ml Oligo and DMSO or LY. Protein expression of LC3-I, LC3-II, C8, cleaved C8, C3, cleaved C3, PARP, cleaved PARP and GAPDH was examined by an immunoblotting assay. (C) Quantitative analysis of LC3-II levels normalized using LC3-I. The protein expression levels of cleaved C (D) 8 and (E) 3 and (F) cleaved PARP were quantified using ImageJ software. (G) Concentration of cytokeratin 18 fragment in cell culture supernatant was measured by ELISA. *P<0.05 vs. Ctrl+DMSO; $^{\#}$P<0.05 vs. oligo+DMSO. Ctrl, control; oligo, oligonol; 3-MA, 3-Methyladenine; LY, LY294002; C, caspase.

inhibitory concentration of oligonol in both cell lines was 17 μg/ml.

Oligonol induces apoptotic effects in NPC cells. Next, it was examined whether oligonol induces apoptotic effects in NPC cells. NPC-TW01 and NPC/HK1 cells were treated with 17 μg/ml oligonol. Immunoblotting assay was then performed to detect protein expression of apoptotic markers, such as cleaved caspase-8 and -3 and cleaved PARP (23) (Fig. 2A and F). Quantitative evaluation of immunoblots demonstrated that in both cell lines, oligonol significantly increased expression levels of cleaved caspase-8 (Fig. 2B and G), cleaved caspase-3 (Fig. 2C and H) and cleaved PARP (Fig. 2D and I) compared with the control. In addition, cytokeratin 18 is expressed in various types of epithelial cell (22). During apoptosis, it is cleaved by effector caspases and released from epithelial cells (22). Therefore, cytokeratin 18 fragments serve as a biological marker of apoptosis (22). ELISA indicated that oligonol significantly increased the release of cytokeratin 18 fragments from NPC cells compared with the control (Fig. 2E and J), suggesting that oligonol could induce apoptotic effects in NPC cells.

Oligonol induces autophagic effects in NPC cells. As some natural extracts can simultaneously induce apoptosis and autophagy in cancer cells (39,40), it was investigated whether oligonol triggers autophagy in NPC cells. Beclin 1 and LC3-II were used as the autophagy markers in this study. Oligonol significantly induced protein expression of Beclin 1 and LC3-II (Fig. 3A and B). The conversion of LC3-I to LC3-II is a reliable marker for autophagy activation (41). LC3-II/LC3-I ratio was significantly increased by oligonol treatment compared with the control in both NPC cell lines (Fig. 3A and B), suggesting that oligonol also induced autophagic effects in NPC cells.

Inhibition of autophagic effects promotes oligonol-induced viability inhibition and apoptotic effects in NPC cells. Because autophagy contributes to cancer cell survival or death (42-44), autophagy inhibitors 3-MA and LY294002 (45) were used to investigate the effect of oligonol-induced autophagic effects on NPC cell viability. Treatment with 3-MA or LY294002 increased the viability inhibition induced by oligonol in NPC cells (Fig. 4A). Since previous studies indicate that blocking autophagy enhances the induction of apoptotic effects (29,46), the present study examined protein expression levels of LC3-II and apoptotic markers (cleaved caspase-8 and -3 and cleaved PARP) in NPC cells after combination treatment with oligonol and LY294002. LY294002 could suppress the oligonol-induced LC3-II expression (Fig. 4B) and increase in LC3-II/LC3-I ratio (Fig. 4C), indicating that the autophagy

inhibitor, LY294002, inhibited oligonol-induced autophagy activation. Cotreatment of oligonol with LY294002 could significantly enhance the protein expression of cleaved caspase-8 (Fig. 4D), cleaved caspase-3 (Fig. 4E) and cleaved PARP (Fig. 4F) compared with oligonol alone. Additionally, cotreatment of oligonol with LY294002 significantly increased release of cytokeratin 18 fragments, another apoptotic marker, compared with oligonol alone (Fig. 4G). These findings suggested that inhibition of oligonol-induced autophagic effects by autophagy inhibitors enhanced viability inhibition and apoptotic effects in NPC cells.

Discussion

Plant extracts have been reported to serve a key role in inhibiting the survival of cancer cells (12-15). Oligonol is a polyphenolic compound primarily extracted from lychee fruit that suppresses cancer cell viability (35,36). However, whether it has anticancer activity in NPC cells is still unknown. Here, oligonol effectively inhibited the viability of NPC cell lines NPC-TW01 and NPC/HK-1, which suggested that oligonol exerted anticancer effects in NPC cells.

As oligonol may induce apoptosis in cancer cells (35,36), the present study investigated its effects on NPC cells. Oligonol could stimulate the expression of apoptosis markers, including cleaved caspase-8 and -3 and cleaved PARP and induce the release of cytokeratin 18 fragments, suggesting that oligonol has the ability to induce apoptotic effects in NPC cells. Since caspase-8 can be cleaved (activated) through the apoptotic death receptor pathway (23), oligonol may also have the ability to activate the death receptor pathway. Previous studies have indicated that inhibiting Akt or inducing AMP-activated protein kinase (AMPK) activation promotes expression of Fas ligand (FasL) (47,48), an inducer of the death receptor pathway. As oligonol can inhibit Akt or promote AMPK activation (49,50), oligonol may induce expression of FasL by inhibiting Akt or inducing activation of AMPK in NPC cells. This could initiate the death receptor pathway and ultimately induce the activation of caspase-8. To determine if AMPK mediates oligonol-induced FasL expression in NPC cells, an AMPK inhibitor will be combined with oligonol to treat the NPC cells. The expression of FasL will then be assessed using an immunoblotting assay.

The present study examined whether oligonol induces autophagy in NPC cells, as certain natural extracts have been reported to simultaneously induce apoptosis and autophagy in cancer cells (39,40). Oligonol induced autophagic effects in NPC cells, as evidenced by increased expression of Beclin 1 and LC3-II, two autophagy markers. According to previous studies, inhibition of autophagy results in apoptosis in lung cancer cells treated with cisplatin and endometrial cancer cells treated with paclitaxel (29,44). The present study used two autophagy inhibitors (3-MA and LY294002) to confirm the role of autophagy in oligonol-induced viability inhibition and apoptotic effects. Autophagy inhibitors enhanced oligonol-induced viability inhibition. Expression of apoptosis markers, including cleaved caspase-8 and -3 and cleaved PARP and the release of cytokeratin 18 fragments significantly increased following cotreatment of oligonol with LY294002. These findings suggested that apoptotic effects were promoted by inhibiting oligonol-induced autophagic effects, leading to an increase in NPC cell viability inhibition. Therefore, oligonol-induced autophagy may serve a cytoprotective role in NPC cells.

It is unclear how the autophagic effects induced by oligonol inhibit the apoptotic effects in NPC cells. Induction of autophagy can cause mitochondria elongation (51,52), which results in inhibition of cytochrome c release (51,52), a pro-apoptotic factor associated with caspase-8 activation (51,53), preventing apoptosis and maintaining cell viability (51). Additionally, autophagosome formation proteins p62 and LC3-II interact with caspase-8, leading to its degradation when the mature autophagosome fuses with the lysosome (51,54). Therefore, oligonol-induced autophagy may decrease expression of cleaved caspase-8, which can inhibit the enhancement of apoptotic effects induced by oligonol in NPC cells. Further experiments should confirm these possible mechanisms in the future. For example, to investigate whether caspase-8 interacts with p62 under oligonol treatment in the NPC cells, protein-protein interaction techniques, such as co-immunoprecipitation and fluorescence resonance energy transfer, will be utilized.

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Availability of data and materials

The datasets generated in the present study may be requested from the corresponding author.

Author's contributions

YTW, YCC and YYL designed the study. CHL, WCC, TJH and SJC performed experiments. YTW collected and analyzed the data. YTW and YYL wrote the manuscript. YCC and YYL revised the manuscript. YTW and YYL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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