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Studies on the role of autophagy in oxaliplatin resistant colon cancer cells

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Studies on the role of autophagy in oxaliplatin resistant colon cancer cells

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CONTENTS

LIST OF ABBREVIATIONS

SNU-C5/OXTR OXT resistant colon cancer cell line

OXT Oxaliplatin

ROS Reactive oxygen species

NAC N-acetyl cysteine

5FU 5-fluorouracil

MMR Mismatch repair

NER Nucleotide excision repair

HMGB1 High mobility group box 1

ATG5 autophagy-related protein 5

Nrf2 nuclear factor erythroid 2-related factor 2

Abstract

We investigated the role of autophagy in SNU-C5/OXTR, oxaliplatin resistant SNU-C5 coloncancer cells. SNU-C5/OXTR cells showed low levels of autophagy. Expression of important autophagic proteins such as Atg5, Atg6, Atg7, LC3-I and LC3-II was decreased in SNU-C5/OXTR cells. The expression level of p62, an essential protein for autophagy, was also low in SNU-C5/OXTR cells. The production of intracellular reactive oxygen species (ROS) was significantly reduced in SNU-C5/OXTR cells, and the level of autophagy was further increased after treatment with the ROS inhibitor N-acetyl cysteine (NAC). These findings suggest that decreased autophagy is associated with oxaliplatin resistance in SNU-C5 cells.

Key Words: Autophagy, Oxaliplatin, SNU-C5/OXTR, Reactive oxygen species, Colon cancer

1. INTRODUCTION

Oxaliplatin (OXT) is a third-generation platinum-based anticancer agent with a 1, 2-diaminocyclohexane. It uses DNA as a target site, and the platinum atoms form a cross-link with DNA to inhibit replication and transcription, leading to apoptotic cell death[1-3]. It is widely used clinically in combination chemotherapy such as 5-fluorouracil (5FU) or leucovorin, mainly for the treatment of malignant tumors of the digestive system such as colorectal cancer, gastric cancer, pancreatic cancer, etc[4-7]. Over the last three decades, OXT-based chemotherapy has been used as the adjuvant or palliative treatment for colorectal cancer, and this approach has significantly improved clinical outcome and reduced cancer recurrence[8]. However, most patients acquire resistance to OXT after prolonged use, which limits its therapeutic efficacy for cancer[2]. OXT resistance can be caused by reducedintracellular uptake or inactivation by structural change and is associated with DNA repair such as mismatch repair (MMR) or nucleotide excision repair (NER)[1]. Cancer cell resistance is the major cause of the failure of OXT therapy[2,9]. Therefore, it is very important to break through the mechanism that causes OXT resistance in order to develop more effective treatment strategies to overcome the resistance and enhance the efficacy of OXT.

Autophagyis a cell-degrading pathway for the clearance and recycling of damaged or long-lived proteins and organelles, and plays an critical role in homeostasis by maintaining good quality of proteins and organelles, and is also a process used to eliminate pathogens[10-12]. It is usually observed when responding to cell stress, starvation, hypoxia, or DNA damage.[13] In tumor cells with defective apoptosis, autophagy allows long-term survival. Contradictorily, the defect of autophagy is associated with an increase in tumorigenesis[11].It can both promote

and inhibit tumorigenesis through several mechanisms, relying on the tumor stage[14]. Yan Shi et al. havereported that the inhibition of autophagy enhances OXT-induced cell death and reactive oxygen species (ROS) production in colon cancer cells[15]. They also concluded that OXT activates autophagy as a cellular protective response through endoplasmic reticula (ER) stress and ROS in human colon cancer cells. Daolin Tang et al. have showed that reducible high mobility group box 1 (HMGB1) protein induces Beclin1-dependent autophagy and promotes resistance to OXT[16]. Inhibition of autophagy by downregulation of beclin1 or of autophagy-related protein5 (ATG5) enhanced OTX sensitivity[17]. This finding suggests that autophagy may act as one of the mechanisms for OXT resistance.

The SNU-C5 colon cancer cells havetwo missense mutations in the p53 gene, resulting in complete loss of normal p53 function[18], and have been used in many studies, including multidrug resistance and anti-cancer drug screening[19]. OXT resistant colon cancer cell line (SNU-C5/OXTR) is derived from the parental wild-type colon cancer cell line (SNU-C5/WT) by chronic exposure to OXT[20]. There are a few studies on OXT resistance using SNU-C5/OXTR cells. Jung et al. demonstrated that the combined application of the chemosensitizer betulinic acid and OXT induces cancer cell apoptosis through the mitochondrial pathway[20]. Kang et al. showed that the nuclear factor erythroid 2-related factor 2 (Nrf2) expression is higher in SNU-C5/OXTR than in parental SNU-C5 cells[21]. Recently Lee et al. discovered that the co-treatment with OXT and melatonin increases endoplasmic reticulum stress in and apoptosis of SNU-C5/OXTR cells[22].

However, there are few studies to determine the roleof autophagy in acquiring OXT resistance in colon cancer cells. Through this study, we demonstrated that the level of autophagy in SNU-C5/OXTR cells is significantly lower than that in SNU-C5 cells, suggesting a novel approach to overcome the problematic resistance to chemotherapy in colon cancer.

2. MATERIALS AND METHODS

2.1. Reagents

Oxaliplatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-acetyl cysteine (NAC), 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), and primary antibody against actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thiazolyl blue tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Amresco LLC (Solon, OH, USA). Acridine orange was purchased from Invitrogen (Eugene, OR, USA). Primary antibody against Atg5 was purchased from Abgent (San Diego, CA, USA). The antibody against Atg6, Atg7, LC3, p62, phospho-Nrf2 were purchased from Cell signaling technology (Beverly, MA, USA). The antibody against Nrf2 and HO-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against TATA-binding protein (TBP) was purchased from Abcam (Cambridge, UK). The antibody against CuZn SOD were purchased from Biodesign International (Saco, ME, USA). All other chemicals and reagents were of analytical grade.

2.2. Cell culture

The human colon cancer cell line SNU-C5 was obtained from the Korean Cell Line Bank (Seoul, Republic of Korea) and maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2/95% air. Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, penicillin (100 units/ml), and streptomycin (100 ìg/ml). The SNU-C5/OXTR cell line was obtained from the Research Center for Resistant Cells of Chosun University (Gwangju, Republic of Korea) and sub-cultured twice per week in medium containing 7.14 ìM OXT for

more than 6 months until a stable cell line was established[21].

2.3. Cell viability assay

To evaluate the sensitivity of SNU-C5 and SNU-C5/OXTR cells to OXT-induced cytotoxicity, various concentrations of OXTwas added to the medium, and the cells were incubated at 37°C for 48 h. MTT stock solution was added to each well to yield a total reaction volume of 250 ìl. After incubation for 4 h, the medium was aspirated. The formazan crystals in each well were dissolved in DMSO (150 ìl), and the absorbance at 540 nm was read on a scanning multi-well spectrophotometer[23].

2.4. Hoechst 33342 staining

Cells were stained with Hoechst 33342 cell-permeant nuclear counterstain dye (Sigma Aldrich) for 10 min, and images were acquired using a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera (Media Cybernetics, Rockville, MD, USA) to identify condensed nuclei in apoptotic cells. The apoptotic index was calculated as follows: (apoptotic cell number in treated group/total cell number in treated group)/(apoptotic cell number in control group/total cell number in control group).

2.5. Acridine orange staining

Acidic intracellular compartments were visualized by acridine orange staining. After seeding, cells were washed with phosphate buffered saline (PBS) and stained with 10 ìg/ml acridine orange (Invitrogen, Madison, WI, USA) for 15 min

at 37°C. Subsequently, the cells were washed with PBS and viewed under a laser scanning confocal microscope. Microscopic images were collected using the LSM 5 PASCAL software (Carl Zeiss, Jena, Germany). Depending on their acidity, autophagosome or autolysosomeappeared as orange or red fluorescent cytoplasmic vesicles, whereas nuclei were stained green. Alternatively, acridine orange-stained cells were trypsinized, washed with PBS, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) using the CellQuest Pro software (Becton Dickinson).

2.6. GFP- LC3 transfection and detection of GFP-LC3 puncta

Autophagy was evidenced by the formation of puncta LC3-positive structures, which are essential for the dynamic process of autophagosome formation[24]. Cells were transfected with green fluorescent protein (GFP)-tagged LC3 plasmid using lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. GFP- LC3 fluorescence was imaged using a confocal microscope equipped with the laser scanning microscope 5 PASCAL program (Carl Zeiss). The number of GFP-LC3 dots was counted within each sample.

2.7. Western blot analysis

Cell lysates were electrophoresed, and separated proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated with primary antibodies, followed by horseradish peroxidase-conjugated immunoglobulin G secondary antibodies (Pierce, Rockford, IL, USA). Protein bands were detected with an enhanced chemiluminescence Western blotting detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.8. Detection of intracellular ROS

The level of intracellular reactive oxygen species (ROS) was detected on a FACSCalibur flow cytometer. Twenty-four hours after treatment with 2 mM NAC, cells were loaded with 20 M DCF-DA for 30 min at 37℃ at the indicated times, and the supernatant was removed by suction. Cells were treated with trypsin and washed with PBS, and then the fluorescence of DCF-DA-loaded cells was measured on a flow cytometer using the CellQuest Pro software.

2.9. Statistical analysis

All measurements were made in triplicate, and all values are expressed as means ± the standard error of the mean. Results were subjected to an analysis of variance (ANOVA) followed by Tukey's post hoc test to analyze differences between conditions. $p < 0.05$ was considered significant.

3. RESULTS

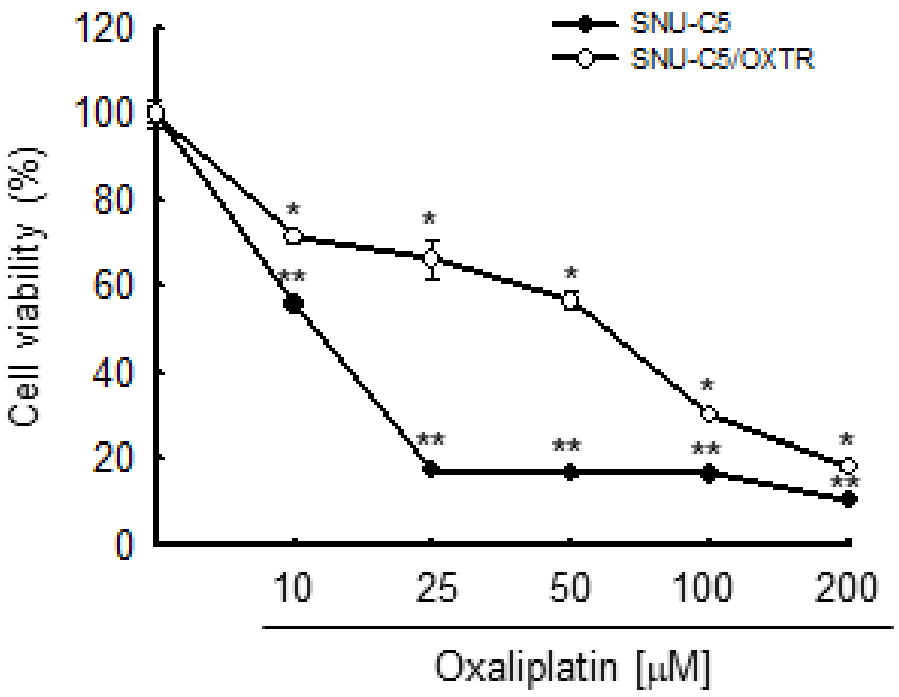
3.1. SNU-C5/OXTR cells exhibits the decreased autophagy

The cytotoxic effects of OXT in the SNU-C5 cells and their resistant variant, SNU-C5/OXTR cells were compared. The concentration of OXT that yielded 50% growth inhibition (IC_{50}) was 16 ìM in SNU-C5 cells and 74 ìM in SNU-C5/OXTR cells, in which resistance was induced by continuous culture in 7.14 ìM OXT (Figure 1A). The each IC_{50} treatment of OXT in SNU-C5 and in SNU-C5/OXTR cells induced apoptotic cell death, as shown by Hoechst 33342 staining. The treatment of 16 ìM of OXT (IC_{50} in SNU-C5) showed 6.8 of apoptotic index in SNU-C5 compared to 1.4 of apoptotic index in SNU-C5/OXTR (Figure 1B). Whereas the apoptotic cell population of 74 ìM of OXT (IC_{50} in SNU-C5/OXTR) showed 22.2 of apoptotic index in SNUC5 compared to 8.5 of apoptotic index in SNU-C5/OXTR (Figure 1B).

Accumulation of vacuolated cells, a marker of autophagy, was diminished in SNU-C5/OXTR cells relative to parent type SNU-C5 cells under light microscope (Figure 1C). The vacuoles were positively stained with the lysosome marker dye, acridine orange. As shown in Figure 1D, the number of acridine orange-stained vacuolated cells was lower in SNU-C5/OXTR cells than in SNU-C5 cells.

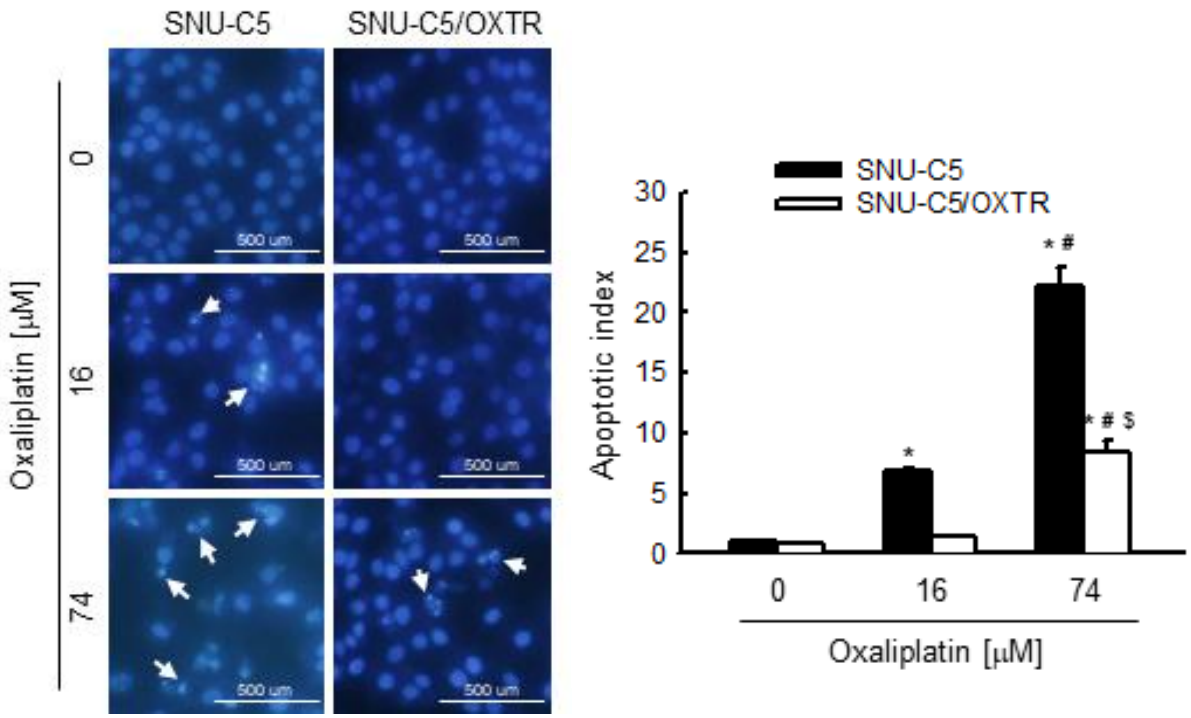
Fig. 1. SNU-C5/OXTR cells exhibits the decreased autophagy.

(A)



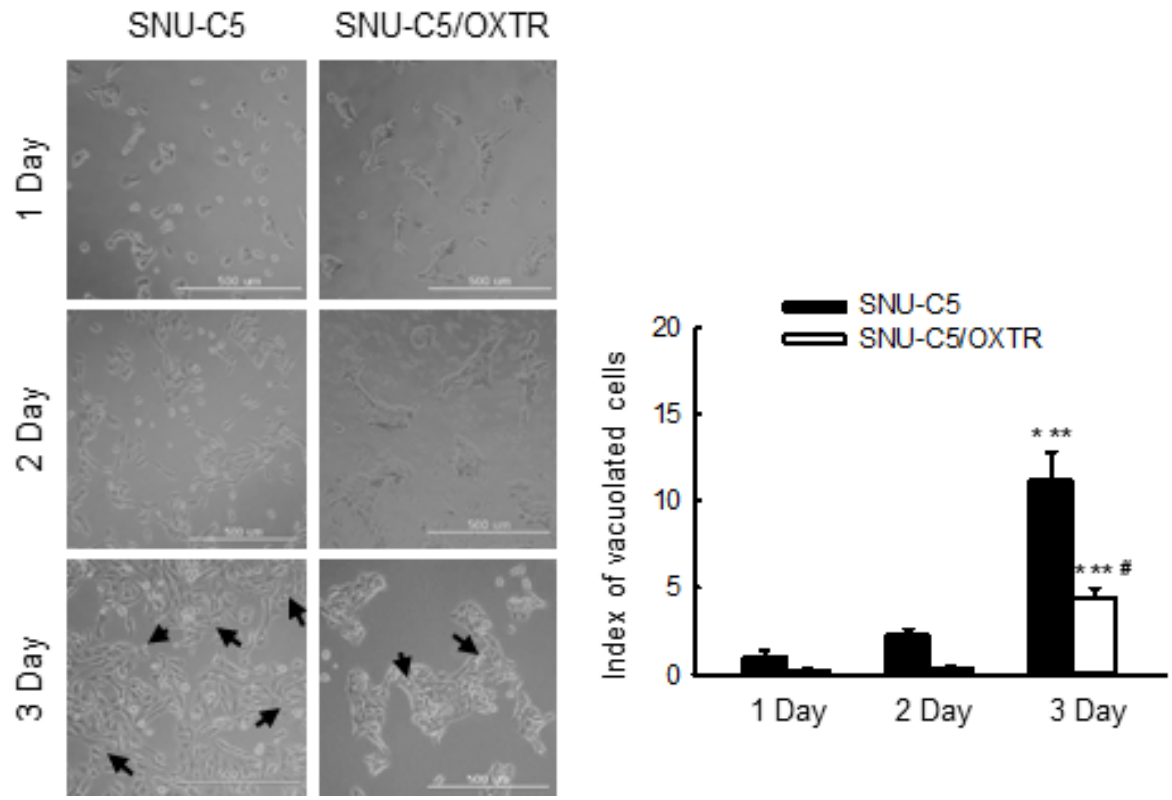
(A) Cell viability following treatment with OXT in SNU-C5 and SNU-C5/OXTR cells for 48 h was assessed using the MTT assay. *,**Significantly different from SNU-C5 or SNU-C5/OXTR control cells ($p < 0.05$).

(B)



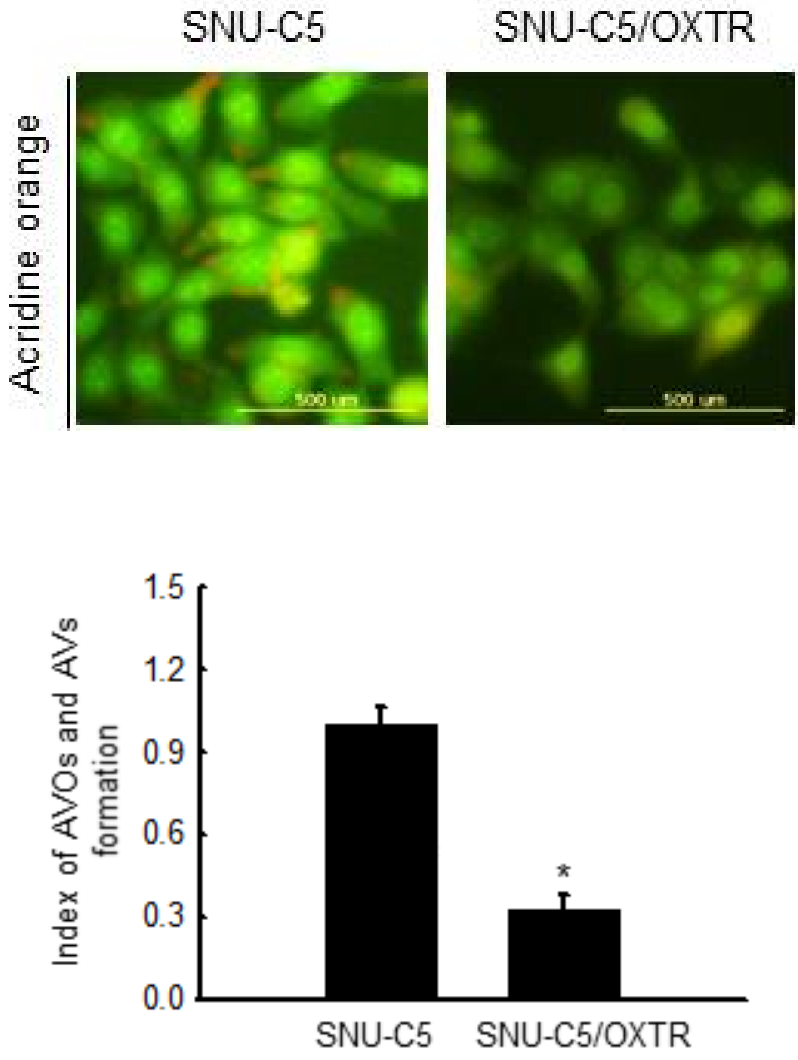
(B) In with SNU-C5, or SNU-C5/OXTR cells, apoptotic body formation (arrows) was observed by fluorescence microscopy after Hoechst 33342 staining. *Significantly different from SNU-C5 or SNU-C5/OXTR control cells ($p< 0.05$); #Significantly different from 16 ìM OXT treated SNU-C5 or SNU-C5/OXTR cells ($p < 0.05$). $Significantly different from 74 ìM OXT treated SNU-C5 cells ($p< 0.05$).

(C)



(C) After 16 h of culture, the cells were imaged with an optical microscope every day for 3 days, and the vacuolated cells/twenty cells were quantified. Arrow indicates vacuolated cell. Cells were then stained with acridine orange.

(D)



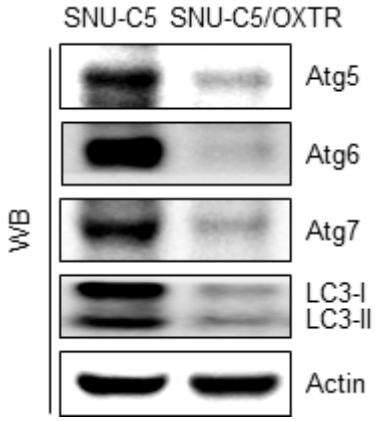
(D) The cells were imaged by fluorescence microscopy and the acridine orange-stained cells/twenty cells were quantified. The AO-Red fluorescence indicates the formation of acidic vesicular organelles (AVOs) and autolysosome vacuoles (AVs), resulting from autophagy induction, and the AO-Green fluorescence indicates AO staining of DNA/RNA in cells.

3.2 Oxaliplatin reduces the expression of autophagy-related proteins

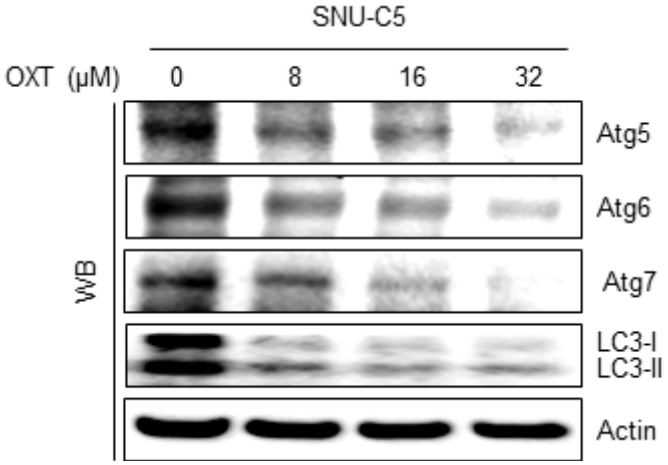
Autophagy is also characterized by the formation of autophagosome, which is dependent on recruitment of Atg proteins. Expression level of Atg proteins gives main information about the autophagic state of a cell. The expression of Atg5, Atg6, Atg7, and LC-II, which are well-characterized hallmark of autophagy, was lower level in SNU-C5/OXTR cells than in SNU-C5 cells (Figure 2A). The p62 is an essential protein for autophagy, which targets packaging and delivery proteins for autophagicdigestion. This protein has been identified as a crossroad of apoptosis, autophagy, and cancer[25]. The p62 expression level in SNU-C5/OXTR cells was lower compared to SNU-C5 cells (Figure 2B). In addition,to evaluate OXTR directly induced the autophagy, we examined protein levels of autophagy-related proteins atvarious concentration of OXT in SNU-C5 cells. As shown in Figure 2C and 2D, the expression of Atg5, Atg6, Atg7, LC-II, and p62 were decreased in a dose dependent manner. These results showed that OXT can affect to reduce autophagy in SNU-C5 cells.

Fig. 2. Oxaliplatin reduces autophagy-related proteins.

(A)

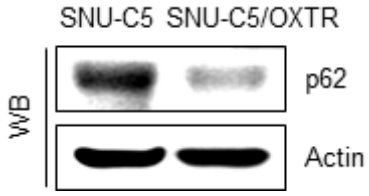


(C)

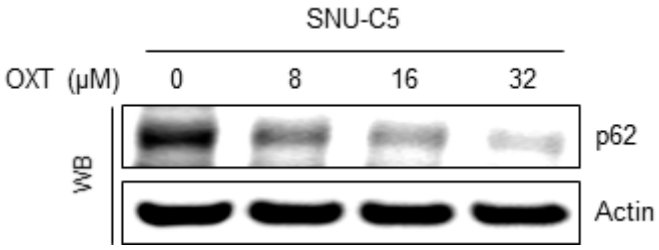


(A and C) Cells were harvested, and levels of Atg5, Atg6, Atg7, and LC3 were assessed by Western blotting.

(B)



(D)

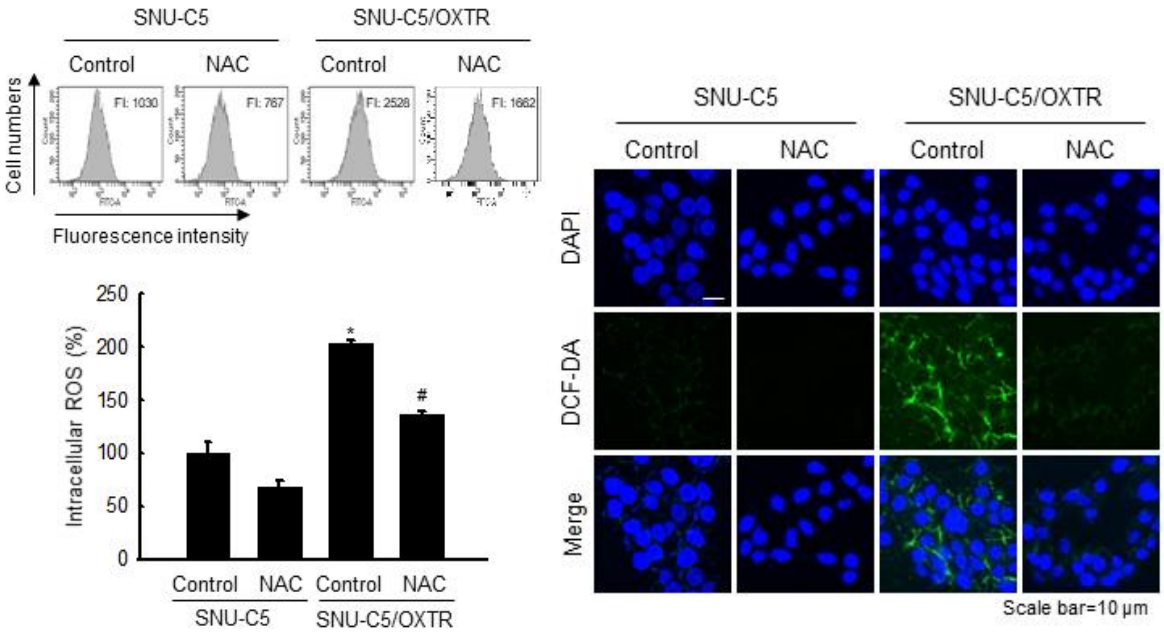


(B and D) Cells were lysed and the level of P62 protein was analyzed by Western blotting.

3.3 Intracellular ROS level and antioxidant system is higher in SNU-C5/OXTR cells than in SNU-C5 cells.

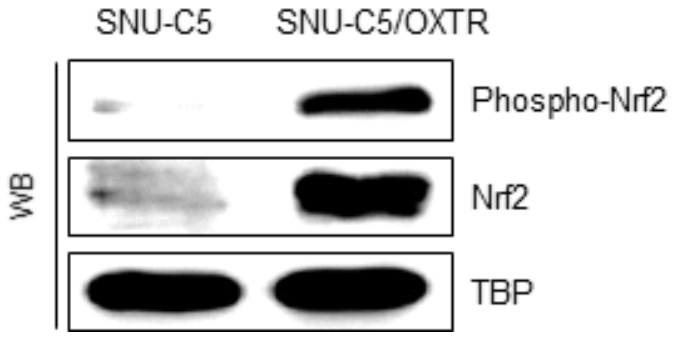
To evaluate the relationship between the ROS and OXT-resistant cells, ROS levels were detected by flow cytometry and confocal microscopy after staining of DCF-DA. The ROS levels were higher in SNU-C5/OXTR cells than in SNU-C5 cells, however, an antioxidant N-acetyl cysteine (NAC) attenuated their ROS levels (Figure 3A and 3B). The main transcription factor of antioxidant enzymes, nuclear Nrf2 expression and its active form of Nrf2 (phospho-Nrf2) was higher in SNU-C5/OXTR cells than in SNU-C5 cells (Figure 3C), leading to higher expression of its target protein HO-1 and CuZn SOD in SNU-C5/OXTR cells (Figure 3D).

Fig. 3. Intracellular ROS level and antioxidant system is higher in SNU-C5/OXTR cells than in SNU-C5 cells.



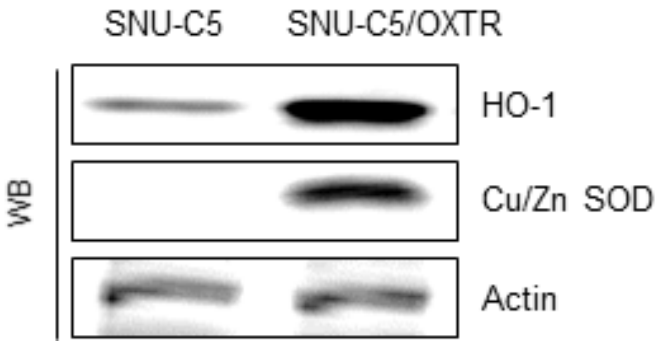
Cells were treated with 2 mM NAC for 24h. ROS levels were assessed by (A) flow cytometry and (B) Confocal microscope after DCF-DA staining. DAPI staining was used to determine the number of nuclei and to assess gross cell morphology. *Significantly different from SNU-C5 control cells ($p < 0.05$); #Significantly different from SNU-C5/OXTR control cells ($p < 0.05$).

(C)



(C) Cells were harvested, and levels of phospho-Nrf2, Nrf2, TBP were assessed by Western blotting. TBP is nuclear loading control.

(D)



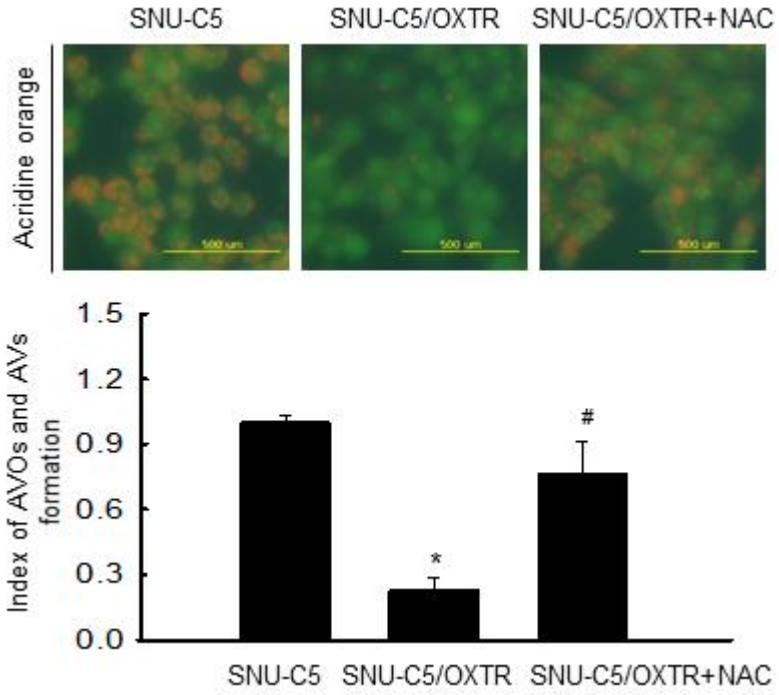
(D) HO-1, Cu/Zn SOD, Actin were also assessed by Western blotting. Actin is total protein loading control.

3.4. ROS regulate autophagy in SNU-C5/OXTR cells

Based on several lines of evidence from basic research, ROS are considered to be key regulators of autophagy[26,27]. After addition of NAC, the level of FI by acridine orange stainingin SNU-C5/OXT cells was recovered up to untreated SNU-C5 cells (Figure 4A and 4B). These results suggest that ROS is related toautophagy in SNU-C5/OXTR cells.

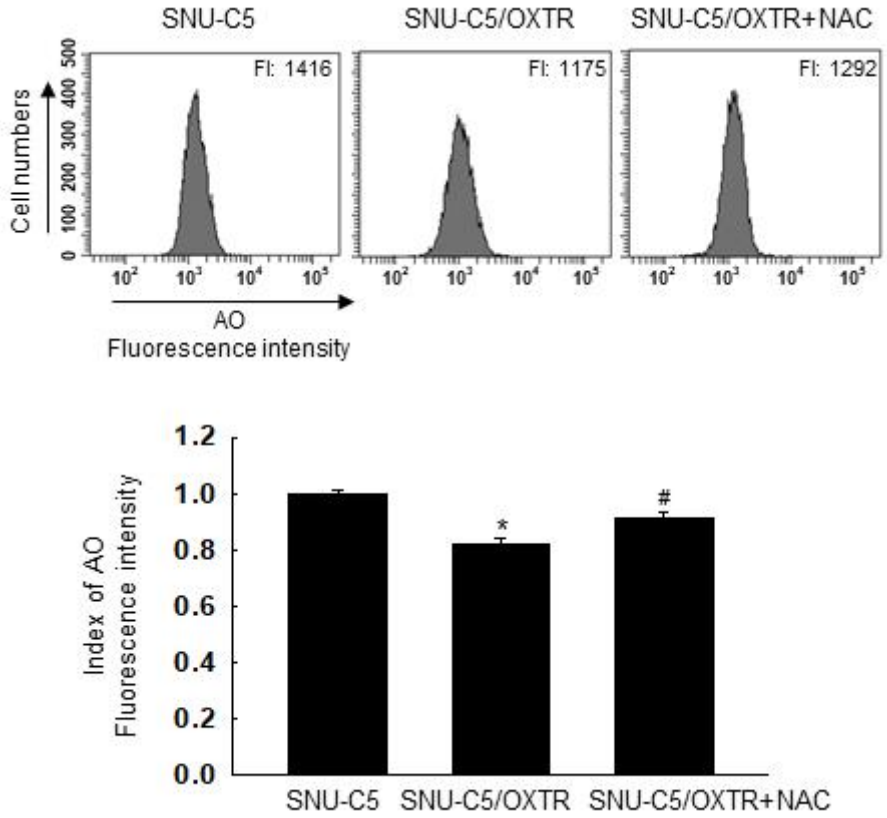
Fig. 4. ROS regulate autophagy in SNU-C5/OXTR cells

(A)



(A) The cells were imaged by fluorescence microscopy and the acridine orange-stained cells/twenty cells were quantified. The AO-Red fluorescence indicates the formation of acidic vesicular organelles (AVOs) and autolysosome vacuoles (AVs), resulting from autophagy induction, and the AO-Green fluorescence indicates AO staining of DNA/RNA in cells. *Significantly different from SNU-C5 control cells ($p < 0.05$). #Significantly different from SNU-C5/OXTR control cells ($p < 0.05$).

(B)



(B) The acridine orange-stained cells were assessed by flow cytometry. FI: Fluorescence intensity. *Significantly different from SNU-C5 control cells ($p < 0.05$). #Significantly different from SNU-C5/OXTR control cells ($p < 0.05$).

4. DISCUSSION

OXT is a important platinum drug that is used for colorectal cancer treatment. Unfortunately, major cause of treatment failure is intrinsic or acquired resistance to OXT[13]. Autophagy, a critical process required to maintain cellular homeostasis, is activated in cancers and its inhibition can lead to either increased cell death or increased cell survival[28]. Suppression of autophagy enhanced cell death induced by OXT in hepatocellular carcinoma[29]. However, there has been little study on the role autophagy in OXT resistance arising in colon cancer cells after prolonged OXT treatment. To investigate the function of autophagy in this study we used a oxaliplatin-resistant SNU-C5 colon cancer cell line, SNU-C5/OXTR.

During process of autophagy, double-membraned vesicles called autophagosome form in the cytoplasm. These vesicles then fuse with lysosomes to form autolysosome[30]. Acridine orange is a fluorescence dye that stains acidic vacuoles such as autophagosome, autolysosome in living cells[31]. The results of this study showed that there were fewer vacuoles in SNU-C5/OXT cells than in SNU-C5 cells, indicating a lower level of autophagy in SNU-C5/OXT cells.

The formation of autophagosome involves initiation, nucleation, elongation, and recycling, each of which depends on specific proteins. LC3 is conjugated to phosphatidylethanolamine through an enzymatic cascade involving Atg3, Atg7, and the Atg5 - Atg12 complex; the resultant lipid-conjugated form is targeted to the autophagosome membrane[32]. Thus, the level of Atg5, and LC3 can be used as markers of autophagy. Our results showed that SNU-C5/OXT cells were observed lower expression level of Atg5, Atg6, Atg7, LC3-I and LC3-II and lower level of p62 than SNUC5 cells. Based on these findings, we preliminarily assume that the OXT chemo-resistance of SNU-C5/OXT cells is related to attenuation of

autophagy.

ROS, as byproducts of oxygen metabolism, is a group of highly reactive ions and molecules. Their role is described in various pathophysiological situations. Cancer cells have been shown to have increased ROS levels compared to normal cells. Elevated levels of ROS in cancer cells are known to contribute to biochemical and molecular changes required for cancer initiation, progression and resistance to chemotherapy[33]. Regulation of autophagy is determined by intensity and duration of ROS exposure and cell type[34]. ROS are associated with both chemo-sensitization and chemo-resistance.[33] In our study, the ROS inhibitor NAC significantly increased the level of acridine orange staining in SNU-C5/OTX cells. This finding suggest that ROS may be a critical regulator of reduced autophagic activity in SNU-C5/OTX cells.

In conclusion, the results of this study show that low level of autophagy is related to OXT resistance in SNU-C5/OXT cells. This finding presents the role of autophagy in mechanism of chemo-resistance and can be the basis for proposing a novel approach to overcoming resistance. In order to gain better awareness in this field, more extensive and systematic research should be conducted in the future.

5. REFERENCES

1 Alcindor, T. & Beauger, N. Oxaliplatin: a review in the era of molecularly targeted therapy. *Curr Oncol* **18**, 18-25, doi:10.3747/co.v18i1.708 (2011).

2 Huang, H. *et al.*Inhibition of PGE2/EP4 receptor signaling enhances oxaliplatin efficacy in resistant colon cancer cells through modulation of oxidative stress. *Sci Rep* **9**, 4954, doi:10.1038/s41598-019-40848-4 (2019).

3 Kelland, L. The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer* **7**, 573-584, doi:10.1038/nrc2167 (2007).

4 Kornmann, M., Fakler, H., Butzer, U., Beger, H. G. & Link, K. H. Oxaliplatin exerts potent in vitro cytotoxicity in colorectal and pancreatic cancer cell lines and liver metastases. *Anticancer Res* **20**, 3259-3264 (2000).

5 Lin, L. *et al.*ATXN2L upregulated by epidermal growth factor promotes gastric cancer cell invasiveness and oxaliplatin resistance. *Cell Death Dis* **10**, 173, doi:10.1038/s41419-019-1362-2 (2019).

6 Shiragami, R. *et al.* Enhancedantitumor activity of cerulenin combined with oxaliplatin in human colon cancer cells. *Int J Oncol* **43**, 431-438, doi:10.3892/ijo.2013.1978 (2013).

7 Wang, Y., Zhang, D., Li, Y. & Fang, F. MiR-138 Suppresses the PDK1 Expression to Decrease the Oxaliplatin Resistance of Colorectal Cancer. *Onco Targets Ther* **13**, 3607-3618, doi:10.2147/OTT.S242929 (2020).

8 Andre, T. *et al.*Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *New Engl J Med* **350**, 2343-2351, doi:DOI 10.1056/NEJMoa032709 (2004).

9 Holohan, C., Van Schaeybroeck, S., Longley, D. B. & Johnston, P. G. Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer* **13**, 714-726,

doi:10.1038/nrc3599 (2013).

10 Colombo, M. I. Autophagy: a pathogen driven process. *IUBMB Life* **59**, 238-242, doi:10.1080/15216540701230503 (2007).

11 Mathew, R., Karantza-Wadsworth, V. & White, E. Role of autophagy in cancer. *Nat Rev Cancer* **7**, 961-967, doi:10.1038/nrc2254 (2007).

12 Levine, B. & Klionsky, D. J. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell* **6**, 463-477, doi:10.1016/s1534-5807(04)00099-1 (2004).

13 Martinez-Balibrea, E. *et al.* Tumor-Related Molecular Mechanisms of Oxaliplatin Resistance. *Mol Cancer Ther* **14**, 1767-1776, doi:10.1158/1535-7163.MCT-14-0636 (2015).

14 Liu, E. Y. & Ryan, K. M. Autophagy and cancer--issues we need to digest. *J Cell Sci* **125**, 2349-2358, doi:10.1242/jcs.093708 (2012).

15 Shi, Y. *et al.*Autophagy protects against oxaliplatin-induced cell death via ER stress and ROS in Caco-2 cells. *PLoS One* **7**, e51076, doi:10.1371/journal.pone.0051076 (2012).

16 Tang, D. *et al.*HMGB1 release and redox regulates autophagy and apoptosis in cancer cells. *Oncogene* **29**, 5299-5310, doi:10.1038/onc.2010.261 (2010).

17 Selvakumaran, M., Amaravadi, R. K., Vasilevskaya, I. A. & O'Dwyer, P. J. Autophagy inhibition sensitizes colon cancer cells to antiangiogenic and cytotoxic therapy. *Clin Cancer Res* **19**, 2995-3007, doi:10.1158/1078-0432.CCR-12-1542 (2013).

18 Rand, A. *et al.*p53 functional loss in a colon cancer cell line with two missense mutations (218leu and 248trp) on separate alleles. *Cancer Lett* **98**, 183-191 (1996).

19 Ku, J. L. & Park, J. G. Biology of SNU cell lines. *Cancer Res Treat* **37**,

1-19, doi:10.4143/crt.2005.37.1.1 (2005).

20 Jung, G. R. *et al.*Effect of betulinic acid on anticancer drug-resistant colon cancer cells. *Basic Clin Pharmacol Toxicol* **101**, 277-285, doi:10.1111/j.1742-7843.2007.00115.x (2007).

21 Kang, K. A. *et al.*Interaction of DNA demethylase and histone methyltransferase upregulates Nrf2 in 5-fluorouracil-resistant colon cancer cells. *Oncotarget* **7**, 40594-40620, doi:10.18632/oncotarget.9745 (2016).

22 Lee, J. H., Yoon, Y. M., Han, Y. S., Yun, C. W. & Lee, S. H. Melatonin Promotes Apoptosis of Oxaliplatin-resistant Colorectal Cancer Cells Through Inhibition of Cellular Prion Protein. *Anticancer Res* **38**, 1993-2000, doi:10.21873/anticanres.12437 (2018).

23 Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D. & Mitchell, J. B. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* **47**, 936-942 (1987).

24 Steinmetz, T. D. *et al.*TFG is required for autophagy flux and to prevent endoplasmic reticulum stress in CH12 B lymphoma cells. *Autophagy*, 1-19, doi:10.1080/15548627.2020.1821546 (2020).

25 Koustas, E. *et al.*Autophagy-related Proteins as a Prognostic Factor of Patients With Colorectal Cancer. *Am J Clin Oncol-Canc* **42**, 767-776, doi:10.1097/Coc.0000000000000592 (2019).

26 Guan, Y. *et al.*Effects of PP2A/Nrf2 on experimental diabetes mellitus-related cardiomyopathy by regulation of autophagy and apoptosis through ROS dependent pathway. *Cell Signal* **62**, 109339, doi:10.1016/j.cellsig.2019.06.004 (2019).

27 Xue, D. F., Pan, S. T., Huang, G. & Qiu, J. X. ROS enhances the cytotoxicity of cisplatin by inducing apoptosis and autophagy in tongue squamous cell carcinoma cells. *Int J Biochem Cell Biol* **122**, 105732,

doi:10.1016/j.biocel.2020.105732 (2020).

28 Eskelinen, E. L. The dual role of autophagy in cancer. *Curr Opin Pharmacol* **11**, 294-300, doi:10.1016/j.coph.2011.03.009 (2011).

29 Ding, Z. B. *et al.*Autophagy activation in hepatocellular carcinoma contributes to the tolerance of oxaliplatin via reactive oxygen species modulation. *Clin Cancer Res* **17**, 6229-6238, doi:10.1158/1078-0432.CCR-11-0816 (2011).

30 Yorimitsu, T. & Klionsky, D. J. Autophagy: molecular machinery for self-eating. *Cell Death Differ* **12 Suppl 2**, 1542-1552, doi:10.1038/sj.cdd.4401765 (2005).

31 Fan, C., Wang, W., Zhao, B., Zhang, S. & Miao, J. Chloroquine inhibits cell growth and induces cell death in A549 lung cancer cells. *Bioorg Med Chem* **14**, 3218-3222, doi:10.1016/j.bmc.2005.12.035 (2006).

32 Kabeya, Y. *et al.*LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* **19**, 5720-5728, doi:10.1093/emboj/19.21.5720 (2000).

33 Galadari, S., Rahman, A., Pallichankandy, S. & Thayyullathil, F. Reactive oxygen species and cancer paradox: To promote or to suppress? *Free Radic Biol Med* **104**, 144-164, doi:10.1016/j.freeradbiomed.2017.01.004 (2017).

34 Chen, Y., McMillan-Ward, E., Kong, J., Israels, S. J. & Gibson, S. B. Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. *Cell Death Differ* **15**, 171-182, doi:10.1038/sj.cdd.4402233 (2008).

6. Figure legends

Fig. 1. SNU-C5/OXTR cells exhibits the decreased autophagy. (A) Cell viability following treatment with OXT in SNU-C5 and SNU-C5/OXTR cells for 48 h was assessed using the MTT assay. *,**Significantly different from SNU-C5 or SNU-C5/OXTR control cells ($p < 0.05$). (B) In with SNU-C5, or SNU-C5/OXTR cells, apoptotic body formation (arrows) was observed by fluorescence microscopy after Hoechst 33342 staining. *Significantly different from SNU-C5 or SNU-C5/OXTR control cells ($p< 0.05$); #Significantly different from 16 ìM OXT treated SNU-C5 or SNU-C5/OXTR cells ($p < 0.05$). $Significantly different from 74 ìM OXT treated SNU-C5 cells ($p< 0.05$). (C) After 16 h of culture, the cells were imaged with an optical microscope every day for 3 days, and the vacuolated cells/twenty cells were quantified. Arrow indicates vacuolated cell. Cells were then stained with acridine orange. (D) The cells were imaged by fluorescence microscopy and the acridine orange-stained cells/twenty cells were quantified. The AO-Red fluorescence indicates the formation of acidic vesicular organelles (AVOs) and autolysosome vacuoles (AVs), resulting from autophagy induction, and the AO-Green fluorescence indicates AO staining of DNA/RNA in cells.

Fig. 2. Oxaliplatin reduces autophagy-related proteins. (A and C) Cells were harvested, and levels of Atg5, Atg6, Atg7, and LC3 were assessed by Western blotting. (B and D) Cells were lysed and the level of P62 protein was analyzed by Western blotting.

Fig. 3. Intracellular ROS level and antioxidant system is higher in SNU-C5/OXTR cells than in SNU-C5 cells. Cells were treated with 2 mM NAC for 24h. ROS levels were assessed by (A) flow cytometry and (B) Confocal

microscope after DCF-DA staining. DAPI staining was used to determine the number of nuclei and to assess gross cell morphology. *Significantly different from SNU-C5 control cells ($p < 0.05$); #Significantly different from SNU-C5/OXTR control cells ($p < 0.05$). (C) Cells were harvested, and levels of phospho-Nrf2, Nrf2, TBP were assessed by Western blotting. TBP is nuclear loading control. (D) HO-1, Cu/Zn SOD, Actin were also assessed by Western blotting. Actin is total protein loading control.

Fig. 4. ROS regulate autophagy in SNU-C5/OXTR cells (A)The cells were imaged by fluorescence microscopy and the acridine orange-stained cells/twenty cells were quantified. The AO-Red fluorescence indicates the formation of acidic vesicular organelles (AVOs) and autolysosome vacuoles (AVs), resulting from autophagy induction, and the AO-Green fluorescence indicates AO staining of DNA/RNA in cells. *Significantly different from SNU-C5 control cells ($p < 0.05$). #Significantly different from SNU-C5/OXTR control cells ($p < 0.05$). (B) The acridine orange-stained cells were assessed by flow cytometry. FI: Fluorescence intensity. *Significantly different from SNU-C5 control cells ($p < 0.05$). #Significantly different from SNU-C5/OXTR control cells ($p < 0.05$).

7. 국문 초록

옥살리플라틴 내성을 가진 대장암 세포인 SNU-C5/OTXR 세포에서 자가 포식의 역할에 대해 연구했다. SNU-C5/OTXR 세포에서 자가 포식에 중요한 역할을 하는 Atg5, Atg6, Atg7, LC3-I 그리고 LC3-II의 발현이 감소함을 확인하였다. 또한 자가 포식에서 필수적인 단백질인 p62의 발현량도 감소함을 밝혔다. 한편 SNU-C5/OTXR 세포에서 Reactive oxygen speces 즉 활성 산소가 유의하게 감소하였다. 활성 산소의 억제제인 N-acetyl cysteine을 처리한 후 자가 포식은 증가하였다. 결론적으로 SNU-C5/OXTR 세포에서 자가 포식의 감소는 옥살리플라틴 내성과 관련이 있을 것으로 추정할 수 있었다. 이런 소견은 옥살리플라틴 항암제 내성의 기전에서 자가 포식의 역할을 보여주는 것이다. 향후 추가적인 연구가 필요할 것으로 생각된다.