Opa-interacting protein 5 modulates docetaxel-induced cell death via regulation of mitophagy in gastric cancer

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Abstract
Damage to mitochondria induces mitophagy, a cellular process that is gaining interest for its therapeutic relevance to a variety of human diseases. However, the mechanism underlying mitochondrial depolarization and clearance in mitophagy remains poorly understood. We previously reported that mitochondria-induced cell death was caused by knockdown of Neisseria gonorrhoeae opacity-associated-interacting protein 5 in gastric cancer. In this study, we show that Neisseria gonorrhoeae opacity-associated-interacting protein 5 loss and gain of function modulates mitophagy induced by treatment with docetaxel, a chemotherapy drug for gastric cancer. The activation of mitophagy by Neisseria gonorrhoeae opacity-associated-interacting protein 5 overexpression promoted cell survival, preventing docetaxel-induced mitochondrial clearance. Conversely, short interfering RNA–mediated knockdown of Neisseria gonorrhoeae opacity-associated-interacting protein 5 accelerated docetaxel-induced apoptosis while increasing mitochondrial depolarization, reactive oxygen species, and endoplasmic reticulum stress and decreasing adenosine triphosphate production. We also found that the mitochondrial outer membrane proteins mitofusin 2 and phosphatase and tensin homolog–induced putative kinase 1 colocalized with Neisseria gonorrhoeae opacity-associated-interacting protein 5 in mitochondria and that mitofusin 2 knockdown altered Neisseria gonorrhoeae opacity-associated-interacting protein 5 expression. These findings indicate that Neisseria gonorrhoeae opacity-associated-interacting protein 5 modulates docetaxel-induced mitophagic cell death and therefore suggest that this protein comprises a potential therapeutic target for gastric cancer treatment.

Keywords
Opa-interacting protein 5, mitophagy, docetaxel, mitochondrial depolarization, cell death

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Introduction
Neisseria gonorrhoeae opacity-associated-interacting proteins (OIPs) are outer membrane proteins linked to the invasion and survival of gonococcus in human cells.1 In particular, OIP5 expression has been shown to be upregulated in esophageal, renal, and breast cancer cells.2–4 We previously reported that OIP5 knockdown induces mitochondrial-mediated cell death and growth arrest in colorectal and gastric cancer cells via modulation of pro-apoptotic markers such as caspase and B cell lymphoma (Bcl)-2-associated X protein (Bax) in mitochondria,5 which
produce adenosine triphosphate (ATP) and are involved in cell survival and apoptosis.\textsuperscript{6} Bel-2 family proteins play an important role in regulating mitochondrial membrane integrity.\textsuperscript{7–9} In particular, the pro-apoptotic proteins Bax and Bel-2 antagonist/killer (Bak) are known to promote membrane permeability, leading to cell death.\textsuperscript{10,11}

Morphological changes in mitochondria, such as fission and fusion, are associated with cell death and mitophagy.\textsuperscript{12,13} a form of autophagy that mediates the selective clearance of damaged mitochondria\textsuperscript{14} and has been implicated as a mechanism of cell survival and death,\textsuperscript{15} including in cancer cells.\textsuperscript{16,17} While damaged mitochondria promote apoptotic cell death via the release of cytochrome c, mitochondrial clearance promotes cell survival.\textsuperscript{18} Mitochondrial outer membrane proteins including mitofusin (Mfn)1 and Mfn2 and the inner membrane protein Neisseria gonorrhoeae opacity-associated (OPA)1 are required for fusion, which modulates mitochondrial motility and function.\textsuperscript{19} In contrast, mitochondrial fission is induced by dynamin-related protein (Drp)1 and is required for mitochondrial DNA repair and metabolite regulation.\textsuperscript{20} Notably, targeting mitophagy has been proposed as an effective strategy for inhibiting tumor progression.\textsuperscript{21,22}

Docetaxel is a chemotherapy drug used to treat gastric cancer that acts by regulating microtubule assembly and depolymerization.\textsuperscript{23,24} However, acquired resistance to docetaxel, which may involve autophagy, is a significant challenge in gastric cancer treatment.\textsuperscript{25,26} It was recently reported that the activation of forkhead box M1 transcription factor by mitogen-activated protein kinase (MAPK) signaling contributes to docetaxel resistance in gastric cancer, along with upregulation of mitotic centromere-associated kinesin, which alters microtubule dynamics.\textsuperscript{27} In addition, activation of C-X-C chemokine receptor type 4, which involves MAPK and nuclear factor (NF)-κB signaling, has also been implicated in docetaxel sensitivity and resistance in gastric cancer.\textsuperscript{28} Conversely, high-mobility group box–associated autophagy has been shown to protect against docetaxel-induced cell death in lung adenocarcinoma.\textsuperscript{29}

To clarify the mechanistic basis for docetaxel resistance in gastric cancer, we investigated the localization of OIP5 in mitochondria and its interaction with the mitochondrial outer membrane proteins Mfn2, phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1). We also examined the levels of docetaxel-induced mitophagy in the context of OIP5 expression level in gastric cancer cell lines. Our results suggest that targeting OIP5 and the mitophagy pathway could be an effective strategy for overcoming docetaxel resistance in gastric cancer patients.

**Materials and methods**

**Cell culture**

AGS, SNU-216, NCI-N87, SNU-620, SNU-638, SNU-668, and MKN-74 stomach cancer cell lines were purchased from the Korean Cell Line Bank (Cancer Research Center, Seoul National University, Seoul, Korea) and grown in Roswell Park Memorial Institute (RPMI)1640 medium supplemented with 5% fetal bovine serum (FBS) and 100 μg/mL penicillin and streptomycin (all from Gibco, Grand Island, NY, USA). Cells (1 × 10\(^5\)/well) were seeded on 24-well culture plates and grown at 37°C in a humidified atmosphere of 5% CO\(_2\)/95% air.

**Cell viability assay**

Cell viability was assessed with the water-soluble tetrazolium salt (WST)-1 assay (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Briefly, 10 μL of WST-1 reagent was added to each well of a 96-well plate (1 × 10\(^3\) cells/well). After incubation for 1 h, the conversion of WST-1 reagent into chromogenic formazan was evaluated with a spectrophotometer ( Molecular Devices, Sunnyvale, CA, USA). On day 1, after seeding, cells were treated with varying doses of docetaxel (2.5, 5, and 10 μM; LKT Laboratories, St. Paul, MN, USA) for numerous times (4, 8, 16, and 24 h). The autophagy inhibitors 3-methyladenine (3-MA; 5 mM) and chloroquine (CQ; 20 μM; both from Sigma, St. Louis, MO, USA) were added to serum-free medium for 24 h on day 1 after seeding.

**Cell transfection**

AGS and NCI-N87 cells (1 × 10\(^5\) cells/well) grown on 24-well plate were transfected with double-stranded short interfering (si)RNAs (30 nmol/mL) targeting OIP5, Mfn2, PINK1, and Parkin (Bioneer, Seoul, Korea) for 24 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The medium was then replaced with RPMI1640 medium containing 10% FBS for 24 h. After recovery, cell viability was evaluated with the WST-1 assay. The pCMV-sports6 control and pCMV-sports6 OIP5 overexpression vectors were obtained from Korea Human Gene Bank (Medical Genomics Research Center, Korea Research Institute of Bioscience & Biotechnology, Daejeon, Korea). The vectors were transfected into AGS cells as described above.

**Isolation of genomic DNA, total RNA, and protein**

Total RNA from gastric cancer cells (2 × 10\(^6\) cells/well) grown in a 100-mm cell culture dish and gastric cancer tissue samples (approximately 50–100 mg) was prepared using TRIZol reagent (Invitrogen) according to the manufacturer’s protocols. Cells were lysed on ice for 30 min in radioimmunoprecipitation assay (RIPA) buffer consisting of 50 mM/L Tris-HCl (pH 7.4), 150 mM/L NaCl, 1% (v/v) Nonidet (N) P-40, 0.25% sodium deoxycholate, 1 mM/L phenylmethylsulfonylfluoride, 1 mM/L sodium orthovanadate, and 1×
protease inhibitor cocktail (Sigma). Lysates were passed through an 18-gauge needle and centrifuged. The protein content in the supernatant was analyzed with the Pierce Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Measurement of mitochondrial ATP levels
Mitochondrial ATP levels were measured with the mitochondrial ToxGlo assay (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Briefly, AGS, SNU-216, NCI-N87, and MKN-74 cells (2 × 10^5/well) were grown on six-well plates (Corning Inc., Corning, NY, USA) for 24 h and then treated for 0, 4, 8, 16, and 24 h with docetaxel (5 μM). The cells were harvested and re-seeded at 2 × 10^4 cells/well in Nunc clear-bottom 96-well culture plates (Sigma). Cells were separated by centrifugation at 200g for 10 min, and 50μL of fresh medium containing 10μM of glucose or galactose was added to each well. Plates were incubated at 37°C for 90 min. A 100-μL volume of assay solution was added to each well followed by incubation at room temperature for 30 min. ATP luminescence was measured using a luminometer (Molecular Devices).

Quantitative polymerase chain reaction and western blotting
OIP5, PINK1, Parkin, and Mfn2 expression levels were measured by quantitative polymerase chain reaction (qPCR) using complementary DNA (cDNA) synthesized from 5 μg of total RNA with a reverse transcription kit (Promega). Briefly, 1 μL of cDNA was used as a template and duplicate reactions were prepared for each sample with the ABI Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and gene-specific primers. The amplification was carried out on an ABI Step One Plus instrument (Applied Biosystems). RNA quantity was normalized to β-actin level, and gene expression was quantified according to the 2^(-ΔΔCt) method.

For western blotting, gastric cancer cells were solubilized in RIPA lysis buffer and protein concentration was measured with the bicinchoninic acid assay. Equal amounts of protein (20–50 μg) were separated by 10%–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), which was blocked by incubation for 1 h with 5% skimmed milk in phosphate-buffered saline (PBS) or Tris-buffered saline (TBS) with 5% powdered milk and 1% Triton X-100 (PBST and TBST, respectively) followed by overnight incubation at 4°C with primary antibodies diluted in 1× PBST or TBST. Antibodies against the following proteins were used: β-actin, Bcl-2-interacting protein (Bnip)3, Bax, autophagy protein (Atg)5, Mfn1 and Mfn2, Drp1, activating transcription factor (ATF)4, glucose-regulated protein (GRP)78, and CCAAT-enhancer-binding protein homologous protein (CHOP) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000); light chain (LC)3B and Beclin-1 (both from Sigma; 1:1000); cleaved caspase-3, -9, poly (ADP-ribose) polymerase (PARP), phosphorylated (p-)Drp1, and p-eIF2 (1:700; Cell Signaling Technology, Danvers, MA, USA); voltage-dependent anion channel (VDAC)1, and PINK1 (Abcam, Cambridge, MA, USA); and OIP5 (Proteintech, Rosemont, IL, USA). The membrane was washed three times with PBST or TBST. Secondary antibodies diluted in PBST or TBST were added to the cells for 40 min at room temperature. Horseradish peroxidase (HRP) anti-rabbit and anti-mouse IgG (Sigma; 1:6000) were used as secondary antibodies. The membrane was washed six times with PBST or TBST for 1 h, and protein bands were visualized with the Clarity Western ECL kit (Bio-Rad, Hercules, CA, USA).

Co-immunoprecipitation
AGS and NCI-N87 cells (2 × 10^5/well) grown on 100-mm cell culture dishes were lysed in buffer containing 50mM of Tris-HCl (pH 7.5), 250mM of NaCl, 5mM of ethylenediaminetetraacetic acid (EDTA), 0.5% (v/v) NP-40, and protease inhibitor cocktail. Lysates were incubated with anti-OIP5 and anti-Mfn2 antibodies at 4°C for 16h. Protein A/G PLUS agarose (Santa Cruz Biotechnology) was used to pull down immunocomplexes, which were washed three times with a solution of 50mM of Tris-HCl (pH 7.5), 250mM of NaCl, 5mM of EDTA, and 0.5% (v/v) NP-40. Immunoprecipitated proteins were resolved by 12% SDS-PAGE.

Immunofluorescence
AGS and NCI-N87 cells grown on coverslips were fixed for 15 min with 4% paraformaldehyde and processed for immunofluorescence microscopy. The coverslips were mounted on slides, which were incubated in blocking buffer consisting of 1% bovine serum albumin in PBS for 30 min at 37°C, followed by overnight incubation at 4°C with antibodies against OIP5, Mfn2, and PINK1 (1:200 dilution in blocking buffer). Cells were then labeled with Alexa Fluor 555–conjugated goat anti-rabbit and anti-mouse and Alexa Fluor 488–conjugated goat anti-rabbit and anti-mouse antibodies. Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). Samples were visualized with a LSM 510 META confocal microscope (Zeiss, Wetzlar, Germany).

Immunohistochemical analysis of human gastric normal/cancer tissue samples
Microarrays of human gastric normal and tumor tissues were purchased from SuperBioChips (Seoul, Korea). Tissue sections were deparaffinized with xylene and
Mitochondria and cytosol isolation

Mitochondria and cytosol were isolated from AGS, SNU-216, NCI-N87, and MKN-74 cells (2 × 10^6) grown on a 100-mm plate using a Mitochondria Isolation kit for cultured cells (Pierce, Rockford, IL, USA) according to the manufacturer’s protocol. Briefly, cells were washed twice with cold PBS, scraped in 1 mL of cold PBS, and centrifuged at 850g for 2 min. The pellets were resuspended in cold mitochondrial isolation reagent A containing protease inhibitor cocktail, vortexed, and incubated on ice for 2 min. Mitochondria isolation reagent B and C were added to the samples, which were centrifuged at 700g for 10 min at 4°C to obtain the cytosolic fraction. Mitochondria were isolated by re-centrifugation at 3000g for 15 min followed by 12,000g for 5 min at 4°C.

Statistical analysis

Each experiment was performed at least three times. Data from one representative experiment are shown. Quantitative data are presented as mean ± standard deviation; in vivo data are expressed as mean ± standard error of the mean. Statistical analysis was performed using SAS 9.2 software (SAS Institute, Cary, NC, USA). The Student’s t test was used to compare the means of quantitative data between groups, and p < 0.05 was considered to be statistically significant.

Results

Upregulation of OIP5 in gastric cancer is associated with docetaxel-induced cell death and autophagy induction

To characterize OIP5 expression at different stages of gastric cancer development, we examined normal and cancerous tissue samples from 55 gastric cancer patients by immunohistochemistry (Figure 1(a)). OIP5 immunoreactivity was approximately twofold higher in tumor stages I and II (74% and 53%, respectively) than in stages III and IV (35% and 23%, respectively). Notably, NCI-N87 and MKN-74 cells exhibited higher OIP5 expression levels than other gastric cancer cell lines, as determined by western blotting (Figure 1(b)). Moreover, the viability of these cells was reduced by docetaxel treatment in a dose- and time-dependent manner, as determined by Annexin V staining/flow cytometry and western blot analysis of cleaved caspase-3 and -9, PARP, Bax, and Bnip3 expression (Figure 1(c) and (d) and Supplementary Figure 1(a)). These results indicate that elevated expression of OIP5 in gastric cancer cells results in reduced levels of docetaxel-induced cell death.

To determine whether docetaxel affects autophagy activation in gastric cancer, we analyzed the expression levels of the autophagy markers LC3B and Atg5 in docetaxel-resistant NCI-N87 and MKN-74 cells exposed to docetaxel for various lengths of time (Supplementary Figure 1(b)). Increased LC3B and Atg5 were detected in docetaxel-resistant cells NCI-N87 and MKN-74, corroborating that high-level OIP5 expression is related with increased autophagy in docetaxel-treated cells. To confirm that the LC3B expression pattern reflected autophagic processes, we investigated whether autophagy inhibitors increased docetaxel-induced cell death. Treatment of NCI-N87 and MKN-74 cells with 3-methyladenine (3-MA) and chloroquine (CQ) significantly enhanced docetaxel-induced cell death and inhibited autophagic flux, while it did not affect these parameters in docetaxel-sensitive AGS and SNU-216 cells (Figure 1(e) and (f)). In addition, overexpression of OIP5 increases LC3B levels and also restores cell viability in docetaxel-sensitive cells such as AGS (Figure 1(g) and (h)).

OIP5 overexpression protects against docetaxel-induced mitochondrial damage and activates mitophagy

To determine whether OIP5 is involved in docetaxel-induced mitochondrial damage and mitophagy, we evaluated the expression of the mitophagy markers Mfn1, Mfn2, PINK1, and OIP5 over time in cells treated with docetaxel. The docetaxel-resistant cell lines NCI-N87 and MKN-74 exhibited higher levels of Mfn2 and PINK1 than the docetaxel-sensitive cells AGS and SNU-216 (Figure 2(a) and (b)). Interestingly, OIP5, Mfn2, and PINK1 were overexpressed in NCI-N87 and MKN-74 cells, indicating that these factors attenuate docetaxel-induced cell death via mitophagy activation.

To determine whether OIP5 expression and docetaxel-induced mitochondrial cell death modulate ATP production, we measured ATP levels in docetaxel-treated gastric cancer cells. We found that ATP generation was reduced in low OIP5-expressing (low-OIP5) cells,
that observed in cells expressing high levels of OIP5 (high-OIP5), upon treatment with docetaxel for the indicated time (Figure 2(c)). To evaluate the effect of docetaxel on mtROS production, docetaxel-treated cells were stained with MitoSOX and sorted by flow cytometry 2, 4, and 6 h later. The mtROS level was threefold to fourfold higher in docetaxel-sensitive than in docetaxel-resistant cells (Figure 2(d)). To assess the involvement of mtROS production in the relationship between docetaxel-induced cell death and OIP5 expression, the levels of cell viability were measured in high- and low-OIP5 cells after docetaxel treatment, in the presence or absence of ROS scavengers such as the antioxidant N-acetylcysteine (NAC) and the mitochondria-specific antioxidant M-TEMPO, via WST-1 assay analysis. Pretreatment with NAC and M-TEMPO significantly inhibited docetaxel-mediated cell death and enhanced cell viability in low OIP5-expressing cells (AGS and SNU-216; Figure 3(a)). To further explore the mechanisms by which expression of OIP5 regulates cell viability (Figure 3(b) and (c)) through docetaxel-mediated mtROS production (Figure 3(d) and (e)), we analyzed cell viability in the presence or absence with NAC and M-TEMPO in OIP5-overexpressing (AGS) and siOIP5-knockdown (MKN-74) cells (Figure...
After docetaxel treatment, AGS cells transfected with the mock vector exhibited high levels of cell mortality (Figure 3(b)) and mtROS production (Figure 3(d)), while AGS cells transfected OIP5 overexpression vector exhibited reduced cell mortality in the presence or absence of the treatment with NAC and M-TEMPO (Figure 3(b)) and decreased mtROS generation than mock-transfected cells (Figure 3(d)). In contrast, siOIP5-knockdown MKN-74 cells resulted in increased cell mortality and mtROS production following docetaxel treatment, compared to that observed in control siRNA–treated cells (Figure 3(c) and (e)). After docetaxel treatment, MKN-74 cells transfected with the control siRNA did not much increase the levels of cell mortality (Figure 3(c)) and mtROS generation (Figure 3(e)). Interestingly, OIP5 knockdown resulted in a significant increase in basal mtROS production levels in MKN-74 cells. These results suggest that OIP5 expression may attenuate docetaxel-induced mtROS generation, thereby protecting cells from mtROS-induced cell death.

**OIP5 overexpression attenuates docetaxel-induced autophagic cell death via endoplasmic reticulum stress**

Mitochondria respond to a variety of stress signals, including growth factor deficiency, hypoxia, oxidative stress, and DNA damage. To determine whether OIP5 expression regulates endoplasmic reticulum (ER) stress, we examined the expression of the ER stress markers, phosphorylated protein kinase RNA–like endoplasmic reticulum kinase (PERK), endoribonuclease inositol-requiring enzyme 1,
and ATF6, as well as that of OIP5, in cells treated with tunicamycin (ER stress inducer). The levels of the ER stress markers, but not that of OIP5, increased in a dose-dependent manner upon tunicamycin treatment (Figure 4(a)).

To evaluate whether docetaxel activates ER stress signaling in gastric cancer cells, we examined the expression of proteins in the ER stress signaling pathway in cells treated with docetaxel. Docetaxel-sensitive cells exhibited increased expression of the chaperone protein GRP78 (Bib), components of the PERK signaling pathway.

**OIP5 interacts with mitochondrial outer membrane proteins during docetaxel-induced mitophagy**

To assess the mitochondrial expression of OIP5 and mitophagy markers in docetaxel-treated AGS and NCI-N87 cells, we carried out a fractionation experiment to separate the cytosol and mitochondria (Figure 5(a) and Supplementary Figure 3), with β-actin and VDAC1 levels serving as internal controls. Western blot analysis indicated that docetaxel-resistant cells exhibited increased expression of OIP5, Mfn2, and PINK1 within the mitochondrial and cytosolic fractions, compared to that observed in docetaxel-sensitive cells. These data indicate that cells expressing high levels of OIP5 are resistant to docetaxel, an effect that is dependent on the clearance of mitochondria damaged by docetaxel-induced stress.

We subsequently examined the interaction between OIP5 and Mfn2 or PINK1 in docetaxel-treated gastric cancer cells by immunofluorescence labeling. In AGS cells, OIP5 and Mfn2 were expressed at low levels, and there was no evidence of interaction between the two proteins. Conversely, OIP5 and Mfn2 colocalized in dimethyl...
sulfoxide–treated NCI-N87 cells following docetaxel treatment, suggesting that the two proteins interacted (Figure 5(b)). We therefore postulated that OIP5 localizes to the mitochondrial outer membrane via interaction with Mfn2. Since PINK1 is also a mitochondrial outer membrane marker and a mitophagy regulator and was present at a high level along with OIP5 in the mitochondrial fraction, we screened for potential interactions between OIP5 and PINK1. While AGS cells expressed only PINK1, NCI-N87 cells expressed both PINK1 and OIP5. Moreover, docetaxel-induced autophagy enhanced the colocalization, and presumably the interaction, of these two proteins in NCI-N87 cells (Figure 5(c)).

We further examined these interactions in docetaxel-treated AGS and NCI-N87 cells via co-immunoprecipitation (co-IP) analysis. We found that the interaction between OIP5 and Mfn2 and PINK1 was stronger in docetaxel-treated cells expressing high levels, as compared to low levels, of OIP5 by precipitation with anti-OIP5 antibody (Figure 5(d)). Meanwhile, co-IP analysis using an anti-Mfn2 antibody detected stronger interactions between OIP5 and PINK1 in NCI-N87 than in AGS cells. Our findings suggest that upregulation of OIP5 might lead to interaction with and activation of the mitophagy markers Mfn2 and PINK1, thereby blocking docetaxel-induced cell death.

**OIP5 regulates docetaxel-induced mitochondria fusion–fission**

Mitochondrial dynamics, including fusion and fission, are critical for clearance of damaged mitochondria; fission has also been shown to induce mitochondrial cell death. Here, we found that cells expressing low levels of OIP5 had higher rates of docetaxel-induced mitochondrial autophagic cell death; moreover, the phosphorylation of Drp1—an important factor in mitochondrial fission—was increased in docetaxel-sensitive gastric cancer cells (Supplementary Figure 2) and was associated with higher rates of mitochondrial cell death and mtROS production. We therefore examined the involvement of Drp1 in mitochondrial dynamics using mitochondrial division inhibitor (Mdivi)-1, a specific inhibitor of Drp1, along with...
docetaxel in cells transfected with OIP5-specific siRNA. Docetaxel treatment resulted in increased phosphorylation of Drp1 but decreased expression of PINK1 and Parkin in AGS and SNU-216 cells expressing low levels of OIP5, effects that were reversed by Mdivi-1 treatment (Figure 6(a) and Supplementary Figure 4(a)). In contrast, NCI-N87 and MKN-74 cells overexpressing OIP5 and transfected with a control siRNA showed increased PINK1 and Parkin expression upon docetaxel treatment, which was also reversed by Mdivi-1 treatment. Last, cells transfected with OIP5 siRNA showed increased phosphorylation of Drp1 upon docetaxel treatment, and this effect was suppressed by treatment with Mdivi-1 (Figure 6(b) and Supplementary Figure 4(b)). Results from OIP5 gain- and loss-of-function experiments suggest that OIP5 is a novel modulator of docetaxel-induced mitophagy in gastric cancer cells.

A recent study found that ROS production by damaged mitochondria activates mitophagy and mitochondrial degradation and triggers Parkin/PINK1 pathway–dependent mitophagy by inducing mitochondrial recruitment of Parkin. We therefore isolated cells stained with MitoSOX dye by fluorescence-activated cell sorting (FACS) to determine the effects of docetaxel and Mdivi-1 treatment on
AGS cells overexpressing OIP5. In control-transfected cells, fluorescence intensity was increased by docetaxel treatment, and this effect was abrogated by Mdivi-1 (Figure 6(c) and Supplementary Figure 5). Similar observations were made in NCI-N87 cells. These findings suggest that cells expressing low levels of OIP5 produce high levels of mtROS from damaged mitochondria but that inhibiting mitophagy can block mtROS-induced cell death. We also found that AGS cell viability was decreased by Parkin or PINK1 knockdown, relative to that of NCI-N87 cells expressing high levels of OIP5, while docetaxel treatment further increased the rate of cell death (Figure 6(d) and (e)). Interestingly, a greater decrease in NCI-N87 cell viability was observed upon Parkin or PINK1 knockdown combined with docetaxel treatment. Subsequent western blot analysis revealed that in NCI-N87 and AGS cells transfected with Parkin-specific siRNA and treated with docetaxel, OIP5 and Mfn2 were downregulated to a greater extent than in cells subjected to Parkin or PINK1 knockdown only. Conversely, transfection with the PINK1-specific siRNA combined with docetaxel treatment had no effect on OIP5 or Mfn2 expression. Thus, OIP5 may regulate mitophagy by modulating the expression of Mfn2, Parkin, and PINK1.

Discussion

OIP5 is overexpressed in various human malignancies, including breast, kidney, brain, esophagus, and colorectal cancer.2–5 The results of this study suggest that OIP5 overexpression provides a protective effect against cellular stress and docetaxel resistance in gastric cancer cells. We found that OIP5 primarily localizes to the mitochondrial outer membrane, along with Mfn2 and PINK1, in these cells and that OIP5 knockdown leads to mitochondria-mediated cell death. OIP5 overexpression also inhibited cell death via mitophagy, by clearing mitochondria...
damaged by docetaxel treatment. These results indicate that mitophagy, a mitochondria-specific form of autophagy, plays an important role in cell survival and death in docetaxel-treated gastric cancer cells.

Mitophagy is a process that involves the selective targeting of dysfunctional mitochondria for degradation at the autophagosome through interactions of key regulator molecules at the outer mitochondrial membrane, but how the functional mechanism is not entirely elucidated. In recent report, mitochondrial depolarization plays an important role in activating Parkin-dependent mitophagy by stabilizing PINK1 kinase at the outer mitochondria membrane. Mitochondrial membrane depolarization also promotes proteolytic cleavage and degradation of the fusion protein such as Opal thereby reducing the size of mitochondria for the uptake of dysfunctional mitochondria. The localization of the Parkin E3 ubiquitin ligase to the mitochondria is regulated by the PINK1 which is serine/threonine kinase that undergoes voltage-dependent recruitment leading to proteolysis at the inner mitochondrial membrane in healthy mitochondria. However, the localization of Parkin accumulates at the outer mitochondrial membrane in response to mitochondrial depolarization.

Recent evidence demonstrates that PINK1 phosphorylation of ubiquitin on serine 65 is required to recruit Parkin to mitochondria. Many of mitochondrial proteins have been identified as Parkin substrates at the outer mitochondrial membrane including Vdac1 and Mfn2, and the most characterization of Parkin substrates is markedly altered by Parkin activity. Specific targets such as Mfn2 are phosphorylated by PINK1 at the outer mitochondrial membrane, and Mfn2 has been shown to selectively recruit Parkin to damaged mitochondria. However, the wide range of mitochondrial substrates that are ubiquitinated and then phosphorylated by PINK1 suggests that Mfn2 may be only one of many receptors for Parkin at the mitochondria. In our study, we show that OIP5 interacts with mitochondrial outer membrane proteins during docetaxel-induced mitophagy, suggesting that the localization and expression of OIP5 increases in the activation of mitophagy and cell viability may restore in docetaxel-induced gastric cancer cell death.

Targeting mitophagy has recently attracted interest as a potential strategy for cancer treatment. The mitochondrial outer membrane proteins PINK1 and Parkin repair damaged mitochondria via the mitophagy pathway and regulate cell death and survival via interactions with Bcl-2 family proteins. In melanoma, docetaxel-induced cell death is caspase dependent and mediated by mitochondrial depolarization via altered mitochondrial membrane potential along with pro-apoptotic Bcl-2 family proteins including Bax. Bcl-2 proteins also regulate mitochondrial dynamics by altering the permeability of the mitochondrial outer membrane via assembly of Bax/Bak. Bcl-2 also targets Raf-1 to the mitochondrial outer membrane to protect cells from various stressors. Notably, OIP5 was shown to interact with Raf1 in lung cancer cells. Interestingly, gastric cancer cells expressing low levels of OIP5 had higher rates of Bax-dependent apoptosis upon docetaxel treatment, while OIP5 overexpression reversed this effect by restoring mitochondrial networks.

Mitochondria are an important source of ROS in mammalian cells. Normally, proper levels of ROS may function as signals to promote cell proliferation, whereas an excessive increase of ROS could induce cell death. Recent report have demonstrated that mitochondrial fission plays an important role in ROS production of cardiovascular cells. However, we still do not know precisely how mitochondrial dynamics and functional mechanism can be integrated into those ROS-related signaling pathways involved in cell survival. Docetaxel treatment increased the level of ROS in mitochondria as well as mitochondrial permeability; it was previously reported that docetaxel-mediated NF-kB signaling enhances mitochondrial cell death and that docetaxel induces ER stress via phosphoinositide 3-kinase–Akt or c-Jun N-terminal kinase signaling in various cancers. The ER stress modulator PERK plays an important role in apoptosis resulting from ER stress mediated by ROS from damaged mitochondria. Our results indicate that AGS cells expressing low levels of OIP5 have increased rates of cell death via docetaxel-induced ER stress but that this effect can be overcome by OIP5 overexpression.

In conclusion, our findings indicate that OIP5 interacts with PINK1 to modulate the mitophagy pathway in docetaxel-induced gastric cancer and that this molecule therefore comprises a potential molecular target for gastric cancer therapy.

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Supplementary material
Supplementary material is available for this article online

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