BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Concomitant Induction of Apoptosis and Autophagy by Prostate Apoptosis Response-4 in Hypopharyngeal Carcinoma Cells

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Accepted for publication
October 24, 2013.

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The tumor-suppressive activity of prostate apoptosis response-4 (Par-4) has been demonstrated in a variety of human cancers. In this study, for the first time to our knowledge, we demonstrated that a higher intensity of Par-4 was significantly correlated with a better response in patients with hypopharyngeal carcinoma undergoing radiotherapy alone or concurrent chemoradiotherapy. Mechanistically, an elevated expression of Par-4 induced apoptosis of hypopharyngeal carcinoma cells and sensitized cells toward chemotherapeutic agents or X-ray irradiation. Along with apoptotic incitation, intriguingly, autophagic flux also increased on Par-4 stimulation and contributed to cell death. Moreover, the expressions of multiple common regulators involved in apoptosis and autophagy were regulated by Par-4. Taken together, our results suggested a prognostic role of Par-4 in hypopharyngeal carcinoma and showed novel activity of Par-4 in apoptosis and autophagy induction. (*Am J Pathol* 2014, 184: 418–430; <http://dx.doi.org/10.1016/j.ajpath.2013.10.012>)

Hypopharyngeal carcinoma (HPC) (*International Classification of Diseases-10* code C12 to 13) is a type of head and neck malignancy. In Taiwan, the age-adjusted incidence rate of HPC has gradually increased in the past two decades from 0.84 per 100,000 individuals in 1990 to 2.94 per 100,000 individuals in 2009. Although surgery, radiotherapy alone or in conjunction with chemoradiation, is the standard treatment for HPC, understanding the molecular mechanisms is necessary for pathophysiological clarification and survival rate improvement. For instance, including p27, cyclin D1, p53, matrix metalloproteinases 2 and 9, Twist, and miRNA has been revealed to have an association with the tumorigenesis and metastasis of HPC.^{1–7} In addition, systemic investigation by microarray analysis and chromosome aberration assessment also paves the way for delineation of HPC carcinogenesis.^{8–10} Of interest, in our previous study, we showed expression of a pro-apoptosis protein, prostate apoptosis response-4 (Par-4), in HPC cases.¹¹

Par-4 was originally identified in androgen-independent prostate cancer cells undergoing apoptosis.¹² It is ubiquitously

expressed in various tissue types, and overexpression of Par-4 sufficiently induces apoptosis in cancer cells, but not in immortalized or normal cells.¹³ Par-4 contains a leucine zipper domain at the carboxy-terminus and two putative nuclear localization signals at the N-terminus. Studies have revealed that phosphorylation of Par-4 determines its activity; for example, Thr¹⁵⁵ phosphorylation by protein kinase A is required for its pro-apoptosis function and, in contrast, modification by Akt (or protein kinase B) restrains the ability of Par-4 to reduce cell survival.^{14,15} Par-4 is able to engage in extrinsic and intrinsic apoptosis pathways.¹⁶ Intriguingly, new evidence has indicated that the detailed mechanisms by which Par-4 mediates cell death involve intracellular and extracellular action modes; moreover, intracellular Par-4 is also essential for extracellular Par-4-induced apoptosis.^{17–19} In

Supported by the Taiwan National Science Council grant NSC99-2320-B-320-014-MY2 and the Taiwan Department of Health grant DOH97-TD-I-111-TM017.

addition, through direct interaction with NF- κ B and inhibition of NF- κ B transcriptional activity, Par-4 alters *DROSHA* and miRNA expression and, hence, further attenuates *BCL-2* mRNA translation.²⁰ Interestingly, evidence has also demonstrated that Par-4 is a novel substrate of caspase 3, and a cleaved fragment of Par-4 still retains pro-apoptosis activity.²¹ Remarkably, down-regulation of *Par-4* is exhibited in renal cancers, neuroblastoma, acute lymphocytic leukemia, and endometrial cancer, as well as Ras-transformed cells.^{22–26} Consistent with these findings, Par-4 has been shown to be involved in the inhibition of transformation, partly through impingement of Akt activation or topoisomerase 1 catalytic activity.^{27–30} In addition, *Par-4*-null and *Par-4*/selective for apoptosis in cancer cells transgenic mice further support the tumor-suppressive ability of Par-4 in the inhibition of primary tumor growth and metastasis.^{18,31,32} Therefore, Par-4 is considered as an attractive target for cancer therapy development.^{16,28,31}

A non-specific catabolic process, macroautophagy (referred to herein as autophagy), is up-regulated under stress conditions. On various stresses, such as nutrient starvation, hypoxia, or unfolded protein accumulation, autophagy is induced via double-membrane vacuole (autophagosome) formation to engulf aggregate-prone proteins or damaged organelles, which is followed by fusing with lysosomes (autolysosomes) to eliminate cytoplasmic substrates for the recycling of metabolic substances.³³ Several autophagy-related proteins (Atgs) and class III phosphatidylinositol 3-kinases (alias PIK3C3 or VPS34) are required for autophagosome completion. Conversely, the activity of the autophagy machinery is negatively modulated by a master regulator of multiple signaling pathways, mammalian target of rapamycin.³⁴ Because autophagy plays a cytoprotective role for homeostasis, development, and differentiation, it is inferred to alleviate tumor cell death and attenuate cancer therapeutic efficacy.^{35,36} Paradoxically, genetic and functional analysis has also elucidated the tumor-suppressive activity of autophagy.³⁷ Particularly, emerging evidence suggests cross talk between autophagy and apoptosis, including the Beclin 1 and Bcl-2/Bcl-X_L interaction, caspase- or calpain-mediated Atgs cleavage, and linkage by a molecular hub, p62.^{38–41} Yet, the regulatory mechanisms between autophagy and apoptosis are still elusive.

Extending our previous findings,¹¹ in this study, we, for the first time to our knowledge, reported a prognostic role of Par-4 in HPC, and demonstrated a novel pro-apoptosis and autophagy activity of Par-4 through modulation of multiple common regulators, thus enhancing cell death.

Materials and Methods

Clinical Analysis

Clinical information from 93 HPC cases, which were diagnosed from January 1991 to December 1999 in Buddhist Tzu Chi General Hospital (Hualien, Taiwan), was collected,¹¹

and the patients were followed up for an additional 6 years. The relationships between Par-4 expression¹¹ and age, sex, TNM stage, treatment, and therapeutic outcome of HPC cases were analyzed using the SPSS program (SPSS, Inc., Chicago, IL). Permission to use these clinical data for research purposes was obtained from the Research Ethics Committee at Buddhist Tzu Chi General Hospital.

Cell Culture, Plasmids, and siRNA

Human hypopharyngeal carcinoma cell line FaDu (Bioresource Collection and Research Center number 60214) was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 50 μ g/mL streptomycin, and 50 U/mL penicillin at 37°C in a 5% CO₂ incubator. Par-4/pCMV6-XL6 plasmid was obtained as described previously.⁴² For microtubule-associated protein 1 light chain 3 [pGreen Fluorescent Protein (pGFP)-LC3] plasmid construction, LC3 cDNA (provided by Dr. Noboru Mizushima, Tokyo Medical and Dental University, Tokyo, Japan)⁴³ was inserted into the BglII and EcoRI sites of pEGFP-C1 expression vector (Clontech Laboratories, Inc., Mountain View, CA). Plasmid Par-4-GFP, Flag-Beclin 1, and NF- κ B-dependent luciferase reporter (NF- κ B-luc) were generously provided by Dr. Ute Preuss (University of Bonn, Bonn, Germany), Dr. Beth Levine (University of Texas Southwestern Medical Center, Dallas), and Dr. Wen-Ling Shih (National Pingtung University of Science and Technology, Pingtung, Taiwan), respectively. ON-TARGETplus SMARTpool siRNA against human *Par-4* (L-004434-00-0005) and nontargeting SMARTpool siRNA (D-001810-10⁻⁰⁵) (Thermo Fisher Scientific, Lafayette, CO) were used for RNA silencing.

Transfection and Western Blot Analysis

Briefly, cells were placed on 35-mm plates and grown to 60% to 70% confluence. Cells were then transfected with Lipofectamine 2000 reagent (Life Technologies Corporation, Carlsbad, CA) and plasmid or siRNA mixture, according to the manufacturer's instructions. After transfection, cells were harvested for Western blot analysis or direct observation by fluorescence microscope. The percentage of GFP-LC3-positive cells with GFP-LC3 dots was calculated as the number of fluorescent punctate cells/the number of GFP-LC3 expression cells. To establish a stable GFP-LC3 expression cell line, cells were transfected with pGFP-LC3 plasmid and fluorescent colonies were picked up via 800 μ g/mL G418 selection. For Western blot analysis, cell extracts were prepared as described previously.⁴² Samples were resolved on 10% to 12% SDS-polyacrylamide gels and then subjected to immunoblotting with respective antibodies: rabbit anti-Par-4 antibody (Cell Signaling Technology, Beverly, MA), rabbit anti-phosphate Par-4 (Thr163) antibody (Cell Signaling Technology), mouse anti-Akt1 antibody (Cell Signaling

Technology), mouse anti-phospho-Akt (Ser473) antibody (Cell Signaling Technology), goat anti-phospho-Bcl-2 (Ser87) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Bcl-2 antibody (Cell Signaling Technology), rabbit anti-cleaved poly (ADP-ribose) polymerase (PARP) antibody (Cell Signaling Technology), rabbit anti-cleaved caspase 3 antibody (Cell Signaling Technology), rabbit anti-Atg12 antibody (Cell Signaling Technology), rabbit anti-Atg5 antibody (Epitomics, Burlingame, CA), rabbit anti-LC3B antibody (Cell Signaling Technology), mouse anti-GFP antibody (Santa Cruz Biotechnology), rabbit anti-protein kinase C (PKC) ζ antibody (Cell Signaling Technology), and rabbit anti-p62 antibody (Cell Signaling Technology) for 1 hour. After incubation with the appropriate secondary antibody, the bands were visualized using an electrochemiluminescence system (GE Healthcare Life Sciences, Buckinghamshire, UK). The densities of the bands were measured using ImageJ software version 1.47 (NIH, Bethesda, MD), and values were normalized to the densitometric values of actin in each sample. The fold change in protein amount was then calculated for the experimental sets compared with the control.

Cell Viability Assay

Briefly, cells were incubated with 0.5 mg/mL MTT assay (Sigma-Aldrich, St. Louis, MO) in culture medium for 3 hours at 37°C, and then dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance at 540 nm was determined using a Biokinetics plate reader (Bio-Tek Instruments, Inc., Winooski, VT). The percentage of cell viability was calculated as the value of the experimental set/the value of the control.

TEM Data

For transmission electron microscopy (TEM) examination of autophagic vacuoles, cell pellets were collected and resuspended in glutaraldehyde solution (2.5% glutaraldehyde and 1% tannic acid in 0.1 mol/L cacodylate buffer). After washing with 5% sucrose in 0.1 mol/L cacodylate buffer, cells were postfixed with 1% osmium tetroxide in 0.1 mol/L cacodylate buffer for 1 hour at 4°C. Samples were dehydrated and embedded with 100% Spurr's resin for 2 hours at room temperature, and polymerized for 1 hour at 60°C. Ultrathin sections were made and stained with 2% uranyl acetate and lead citrate. The sections were visualized using TEM (HITACHI H-7500; Hitachi High-Technologies Corp., Tokyo, Japan) at an accelerating voltage of 80 kV.

Flow Cytometry

To quantify the turnover of GFP-LC3, cells were transfected with pGFP-LC3 plasmid alone, or with either Par-4/pCMV6-XL6 or Flag-Becclin 1 plasmid in the presence or absence of autophagy inhibitors. At 48 hours after

transfection, 1×10^4 GFP-LC3-expressing cells in each sample were harvested and analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA), as described previously.⁴⁴ For quantification, the relative fluorescence intensity (percentage) was calculated by dividing the level of GFP-LC3 of each sample/the level of the mock control in the respective experimental set. For quantification of p62, cells were transfected with or without Par-4/pCMV6-XL6 plasmid, and posttransfection after 24 or 48 hours, cells were harvested and fixed with 4% formalin for 15 minutes at room temperature, then permeabilized with 90% cold methanol/PBS for 30 minutes. Subsequently, cells were immunostained using mouse anti-p62 antibody (EMD Millipore Corporation, Billerica, MA), followed by incubation with Alexa Fluor 546 goat anti-mouse IgG (Life Technologies Corporation). A total of 1×10^4 cells were then collected and analyzed using a FACScan flow cytometer, as previously described. The relative fluorescence intensity (percentage) was calculated by dividing the level of fluorescence of overexpressing Par-4 cells/the level of the mock control in the respective experiment. To measure the percentage of cells undergoing apoptosis and autophagy, Par-4 transiently transfected cells were treated with or without apoptotic stimulus in the presence or absence of an autophagy inhibitor. Cells were then fixed and permeabilized using BD Cytotfix/Cytoperm solution (BD Biosciences) for 30 minutes at room temperature. After washing, cells were incubated with phycoerythrin-conjugated rabbit anti-LC3B antibody (Cell Signaling Technology) and fluorescein isothiocyanate-conjugated rabbit anti-active caspase 3 antibody (BD Biosciences) for 1 hour at 4°C. Subsequently, 1×10^4 cells were harvested using a FACScan flow cytometer and further analyzed with CELLQuest version 3.0.1 and ModFitLT version 2.0 software.

MDC Staining

Briefly, cells were labeled with 50 μ mol/L monodansylcadaverine (MDC) reagent (Sigma-Aldrich) for 15 minutes. After washing with PBS, the fluorescence signal was immediately observed by fluorescence microscope with an excitation wavelength of 360 nm and an emission wavelength of 457 nm. Autolysosomal distribution was quantified as the proportion of cells with predominant perinuclear localization of fluorescent vesicles.

CHX Treatment

For protein *de novo* synthesis, cells were pretreated with 40 μ mol/L cycloheximide (CHX) for 3 hours, then transfected with or without Par-4/pCMV6-XL6 plasmid in the absence of CHX (0 hours). At 24 hours after transfection, cell lysates were prepared and subjected to Western blot analysis using a primary antibody corresponding to Par-4, cleaved PARP, LC3B, or tubulin. The densities of the bands were measured using ImageJ software version 1.47, and values were normalized to the densitometric values of tubulin in each sample.

The fold change in protein amount was then calculated for the experimental sets compared with the vector control (0 hours).

Co-Immunoprecipitation

Cells were transfected with Par-4/pCMV6-XL6 or pGFP-LC3 plasmid, or cotransfected with both. At 48 hours after transfection, cells were harvested and lysed in lysis buffer. After preclearing with protein A–Sepharose beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), cell lysates were incubated with antibody beads overnight at 4°C. The immunocomplex was precipitated using protein A–Sepharose and washed three times in lysis buffer. The immunoprecipitates were resuspended in sample buffer and subjected to Western blot analysis. The density of the band was measured using ImageJ software version 1.47. For whole cell extracts, values were normalized to the densitometric values of actin in each sample. The fold change in protein amount was calculated for the experimental set compared with the vector control.

NF- κ B Luciferase Reporter Assay

FaDu cells were cotransfected with both NF- κ B-luc and pRL-TK vector (Promega, Madison, WI) plus either Par-4/pCMV6-XL6 or Par-4–GFP expression plasmid. At 48 hours after transfection, the luciferase activity of each sample was measured using the Dual Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions, and calculated by dividing the value of luciferase activity/the value of *Renilla* luciferase activity. The relative luciferase activity was represented as the fold of activation via the experimental set over the NF- κ B–dependent luciferase reporter alone.

Phospho-Kinase Array Analysis

Briefly, FaDu cells were transfected with or without Par-4/pCMV6-XL6 plasmid, and at 48 hours after transfection, diluent cell lysates were prepared and subjected to a protein kinase array using a Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The array membranes were washed, followed by incubation with a mixture of biotinylated detection antibodies and streptavidin–horseradish peroxidase antibodies. Phospho-kinase signals were then detected using a chemiluminescent detection reagent, and the density of each spot was further measured using ImageJ software version 1.47. The fold change of each phosphorylated protein was calculated as the value of the experimental set compared with the value of the vector control.

Statistical Analysis

Statistical differences were analyzed using the paired *t*-test and were considered to be significant at $P < 0.05$.

Figure 1 The intensity of Par-4 significantly correlates with the overall survival of patients with HPC undergoing radiotherapy alone or CCRT. Forty-five patients who underwent radiotherapy or CCRT treatment were included for analysis. **A:** Representative results for Par-4 showed nuclear-positive staining (arrowheads) by immunohistochemistry.¹¹ Original magnification, $\times 400$. The overall (average) protein intensity was evaluated by pathologists, according to the percentage of signal accumulation in the nuclei, and scored as follows: 0, no staining signal; 1, weak ($< 10\%$); 2, moderate (10% to 50%); 3, strong ($> 50\%$). **B:** The survival function of Par-4 intensity and the *P* value were calculated after adjusting for the American Joint Committee on Cancer stage and the expression percentage of Par-4.¹¹ Cum, cumulative.

Results

The Intensity of Par-4 Is Significantly Correlated with Overall Survival of Patients with HPC Undergoing Radiotherapy Alone or CCRT

We previously showed expression of Par-4 in half of tested HPC specimens.¹¹ In the current study, we assessed the significance of Par-4 in HPC. Clinical information from 93 patients with HPC, including 47 Par-4–negative and 46 Par-4–positive cases, was collected to examine the relationships with expression percentage and staining intensity of Par-4. For all patients, Par-4 was unrelated to age, sex, TNM classification, or clinical stage (data not shown). We further divided these patients into two sets according to treatment. In 48 patients undergoing surgery, there was no correlation between

Figure 2 Overexpression of Par-4 results in HPC cell apoptosis and sensitizes cells toward the apoptotic agent. **A:** Cells were transiently transfected with 0.5, 1, and 2 μ g Par-4 expression plasmid or 1.5 μ g Par-4-GFP plasmid. At 48 hours after transfection, cell lysates were harvested and subjected to Western blot analysis using primary antibodies against the indicated proteins. In addition, transfection with pcDNA3 (**left panel**) or pEGFP-N1 (**right panel**) served as an empty control. **B:** For the sensitization assay, FaDu cells were transiently transfected with 2 μ g Par-4 expression plasmid, and at 24 hours after transfection, cells were treated with or without a sublethal dose ($<LD_{50}$) of 1 μ g/mL cisplatin or 1.5 μ mol/L 5-Fu for an additional 48 hours. On the other hand, after transfection for 48 hours, cells were irradiated with or without 6 Gy X-ray for 24 hours. Cell lysates were harvested and subjected to Western blot analysis using primary antibodies against the indicated proteins. In addition, transfection with pcDNA3 served as an empty control. **C:** For the knockdown assay, FaDu cells were transfected with 150 pmol siRNA targeting Par-4 in the presence of 5 μ g/mL cisplatin, 2 μ mol/L 5-Fu, or 6 Gy X-ray irradiation. Cell lysates were then collected and subjected to Western blot analysis for Par-4, cleaved PARP, cleaved caspase 3, or actin. All Western blot analysis data shown are representative of three independent experiments. The protein expression fold change was calculated as described in *Materials and Methods*.

Par-4 and treatment outcome (data not shown). However, in contrast to 15 Par-4-negative cases, in the radiotherapy alone or concurrent chemoradiotherapy (CCRT) set, the intensity of Par-4 in 30 Par-4-positive patients revealed a statistically significant and positive correlation with overall survival after adjustment for American Joint Committee on Cancer stage and the expression percentage of Par-4 (Figure 1). Moreover, by grouping analysis, it was also better than separate analysis for each mode of treatment as the result of no statistical significance in CCRT ($P = 0.060$, $N = 34$) or few case number in radiotherapy ($P = 0.003$, $N = 11$) (data not shown). Consequently, this result apparently suggests a prognostic role of Par-4 in patients with HPC who undergo radiotherapy alone or in conjunction with chemoradiation for treatment.

Elevated Expression of Par-4 Directly Induces HPC Cell Apoptosis and Enhances the Cell Sensitivity toward Apoptotic Stimuli

Our previous studies suggested an apoptotic threshold of Par-4, which is associated with the incitement of apoptosis.^{11,42,45}

To expound on the clinical role of Par-4 in HPC (Figure 1), we examined whether augmentative expression of Par-4 confers irradiation- or chemotherapeutic agent-provoked apoptosis. In HPC cells, an increase in cleaved caspase 3 and cleaved PARP was obviously displayed on overexpression of Par-4 or GFP-tagged Par-4 (Figure 2, A and B). In addition, the expression levels of phosphorylated Akt (Ser473), a Par-4 antagonist,¹⁵ and a Par-4 downstream target, Bcl-2,²⁰ were also decreased (Figure 2A). Moreover, in contrast to moderate induction of apoptosis by cisplatin, fluorouracil (5-Fu), or X-ray irradiation lower than the lethal dose ($<LD_{50}$) concentration, additional Par-4 conduction remarkably enhanced the sensitivity of HPC cells, leading to more caspase 3 and PARP cleavage, as well as cell death (Figures 2B and 3A). Although expression of endogenous Par-4 was partly induced by an apoptotic agent, apoptotic agent-induced apoptosis and cell death was lower than that in combination with exogenous Par-4 overexpression (Figures 2B and 3A). Thus, these results indicated that Par-4 itself and the expression amount (intensity) correlated with pro-apoptosis activity. Accordingly, by using siRNA targeting *Par-4*, HPC

Figure 3 Overexpression of Par-4 confines HPC cell viability. **A** and **B**: FaDu cells were treated under the same experimental conditions as described in Figure 2, B and C, respectively. In addition, in **A**, all experiments were performed in autophagy inhibitor-free (3-MA) circumstances. Cells were then subjected to a cell viability assay. The percentage of cell viability was calculated as described in *Materials and Methods* and is shown as the means $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

cells apparently showed resistance to apoptosis and attenuated cell death when encountering a higher dosage ($>LD_{50}$) of cisplatin, 5-Fu, or X-ray irradiation (Figures 2C and 3B). In conclusion, these data suggest that an increase of Par-4 sufficiently induces apoptosis and contributes to cell death by apoptotic irritants.

Overexpression of Par-4 Promotes Autophagic Flux in HPC Cells

Apart from the Par-4 pro-apoptosis activity, we also found up-regulation of autophagy by Par-4 stimulation. Via TEM examination, HPC cells manifested membranous autophagic vacuoles through exogenous Par-4 delivery (Figure 4A). In the autophagosome synthesis, in particular, the cytoplasmic localization of LC3-I changes to the lipidated form (LC3-II) through conjugation with phosphatidylethanolamine and is recruited to the autophagosomal membrane. Therefore, monitoring the intracellular localization of LC3 or conversion from LC3-I to LC3-II is widely used as a marker for autophagosome measurement.^{33,43} Because of the LC3 property, to validate autophagy activation by Par-4, we introduced GFP-LC3 reporter to HPC cells; on the other hand, we also established a stably expressing GFP-LC3 cell line for analysis. Compared with the diffusion pattern of GFP-LC3 in the vector control, on overexpression of Par-4, the number of cytoplasmic GFP-LC3 puncta was distinctly increased (Figure 4B). Because autophagy is a dynamic

process, after maturation, autophagosomes fuse with lysosomes (autolysosomes), resulting in autophagosome content and LC3-II degradation.^{33,35} Thus, accumulation of GFP-LC3 puncta is possible from a substantial increase in autophagic flux or impeded autolysosome formation. To clarify this issue, Par-4 and GFP-LC3-cotransfected HPC cells were treated with a phosphatidylinositol 3-kinase inhibitor, 3-methyladenine (3-MA), or bafilomycin A1 (BafA 1), which prevents the late phase of autophagy by inhibiting fusion between autophagosomes and lysosomes. In contrast to untreated cells, fluorescence images and quantification of GFP-LC3 dots revealed that overexpression of Par-4 caused an autophagosome increase (Figure 4, B and C), but also significantly enhanced autophagy processing because of a reduced number of fluorescent punctate cells by 3-MA inhibition and, reversely, augmentation after BafA 1 treatment (Figure 4C).

Fluorescence of GFP quenches at the low pH inside lysosomes, but GFP is still stable; accordingly, free GFP can be generated by partial degradation of GFP-LC3 in autolysosomes.⁴⁶ In agreement with these characteristics, in GFP-LC3-expressing HPC cells without 3-MA or BafA 1 treatment, additional Par-4 delivery apparently resulted in a fluorescence intensity decrease relative to the control, yet this was reversed in the presence of autophagy inhibitors (Figure 5A). Moreover, in contrast to the vector control or 3-MA inhibition, accrual conversion from LC3-I to LC3-II (Figure 2A and Supplemental Figure S1), along with a

Figure 4 Elevated expression of Par-4 facilitates autophagy in HPC cells. **A:** Representative TEM microphotograph of FaDu cells with Par-4 transfection or pcDNA3 empty control. The **arrows** indicate autophagic vacuoles. In addition, cell lysates were harvested and subjected to Western blot analysis for Par-4 or actin. **B:** GFP-LC3 puncta in FaDu cells with GFP-LC3 and an ascending concentration (0.5, 1, or 1.5 μ g) of Par-4 cotransfection, or FaDu cells stably expressing GFP-LC3 with Par-4 or Beclin 1 transfection, were examined by fluorescence microscope. In addition, transfection with pcDNA3 served as an empty control. **C:** Representative fluorescence images of GFP-LC3 puncta in FaDu cells with GFP-LC3 transfection alone, or plus either Par-4 or Beclin 1 cotransfection in the presence or absence of 5 mmol/L 3-MA or 5 nmol/L BafA 1. Serum starvation and pcDNA3 transfection were used as an autophagy inducer and empty control, respectively. The percentage of GFP-LC3-positive cells with GFP-LC3 dots was calculated as described in *Materials and Methods* and is shown as the means $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

reduced GFP-LC3 amount, increased free GFP fragments (Figure 6B) and predominant perinuclear autolysosomes dots (Figure 5B),⁴⁷ which further underlined the Par-4 pro-autophagic flux activity. Taken together, these results reveal that augmented expression of Par-4 evidently activates the autophagy machine in HPC cells.

Apoptosis and Autophagy Are Simultaneously Activated by Par-4 by Acting on Multiple Regulators

Par-4-activated autophagy reduced cell viability, but this was partially mitigated in the presence of an autophagy inhibitor (Figure 3A and Supplemental Figure S2). To determine the relationship between apoptosis and autophagy by Par-4 induction, we examined the occurrence times of apoptosis and autophagy during Par-4 overexpression. HPC cells were pretreated with CHX to synchronize Par-4 translation, and after removal of CHX, apoptosis and autophagy were analyzed. Relative to exogenous Par-4 introduction at the beginning (0 hours), the cleaved PARP and LC3-II amounts simultaneously increased under overexpression of

Par-4 for 24 hours (Figure 7A). This result implied that Par-4 facilitated apoptosis and autophagy at the same time. To demonstrate this possibility, we further assessed these two biological phenomena via flow cytometry. Relative to the untreated vector control, a lower percentage of cells with apoptosis and/or autophagy under cisplatin, 5-Fu, or X-ray irradiation treatment was displayed. On additional Par-4 introduction, the percentages of the apoptosis or autophagy populations were obviously increased, but there was a greater distribution of cells in autophagy. Intriguingly, 15% to 30% of cells presented concomitant activation of these cellular processes (Figure 7B and Supplemental Figure S3A). Eventually, cell viability was reduced by Par-4-induced apoptosis and autophagy (Figure 3A and Supplemental Figure S2). Conversely, the percentage of cells entering apoptosis and/or autophagy was decreased in the presence of an autophagy inhibitor, and cells revealed moderate recovery of cell viability (Supplemental Figures S2B and S3B). Particularly, in contrast to lacking an inhibitor, the elevated percentage of apoptotic cells under cisplatin or 5-Fu treatment or in combination with overexpression of Par-4 was manifested after autophagy inhibitor addition (Supplemental Figure S3B);

Figure 5 Overexpressing Par-4 enhances autophagic flux in HPC cells. **A:** For quantification of the turnover of GFP-LC3, FaDu cells were transiently transfected with GFP-LC3 plasmid alone or cotransfected with either Par-4 or Beclin 1 expression plasmid in the presence or absence of 5 mmol/L 3-MA or 5 nmol/L BafA 1. Samples were then prepared, and the intensity of GFP-LC3 was measured using flow cytometry. Reduced GFP-LC3 fluorescence is indicated (arrow) in the representative histogram. The relative fluorescence intensity of each sample was calculated as described in *Materials and Methods* and is shown as the means $\pm$ SD of three independent experiments. **B:** FaDu cells were transiently transfected with Par-4 or Beclin 1 expression plasmid in the presence or absence of 5 mmol/L 3-MA. At 48 hours after transfection, autolysosomes were labeled with an autolysosome tracer, MDC reagent, and observed by fluorescence microscope. Transfection with pcDNA3 was used as an empty control. The perinuclear localization of autolysosomes is indicated (arrowheads), and the distribution percentage was quantified as described in *Materials and Methods*, and is shown as the means $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$. N, nucleus.

however, cell viability was still increased, with the exception of apoptotic agent treatment alone (Supplemental Figure S2B). Of interest, Par-4–induced cell death was not apparently changed in the presence of a pan-caspase inhibitor (data not shown). Considering the influence of apoptosis and autophagy in HPC cells, collectively, these data suggest profound effects of Par-4–induced autophagy and apoptosis on cell death. In addition, relative to apoptosis, autophagy activation by Par-4 may play a dominant role to influence cell fate (Supplemental Figure S4).

Next, we sought to identify common regulators that participate in the activation of apoptosis and autophagy by Par-4. Although the amounts of core components of autophagy involved in apoptosis, such as Atg12, Atg5, mammalian target of rapamycin, or Beclin 1, were constant regardless of the elevated expression of Par-4, the expression levels of Bcl-2 and activated Akt were decreased on overexpression of Par-4 (Figure 2A and data not shown).^{35,37,38,40} Of interest, phosphorylation of Bcl-2 at residue Ser87, which mediates autophagy stimulation,⁴⁸ was increased owing to exogenous Par-4 introduction (Figure 2A). Accumulating evidence indicates that p62 (alias sequestosome-1) acts as a signaling hub to mediate autophagy via interacting with ubiquitinated cargo and LC3.⁴¹ In addition, it has also been shown that p62 forms a ternary complex with PKC ζ and Par-4 to antagonize Par-4–mediated PKC ζ inhibition and protect cells from apoptosis through NF- κ B re-activation.^{49,50} To assess whether p62 is involved in Par-4–induced apoptosis and autophagy, overexpressing Par-4 cells were immunostained with a primary antibody against p62 and subjected to flow cytometry. Similar to the altered expression of p62 within autophagy,⁵¹ the fluorescence intensity of p62 was

obviously elevated at 24 hours relative to the mock control, but recovered at 48 hours after transfection (Figure 6A). Consistently, immunoprecipitation displayed that elimination of p62 by Par-4 further disrupted the interaction between p62 and LC3 or PKC ζ , and led to inhibition of NF- κ B activity (Figure 6, B and C). Thus, these results indicate that the behavior of p62 is involved in Par-4–activated apoptosis and autophagy. In addition, we also determined the regulatory pathways of apoptosis and autophagy. By overexpressing Par-4, the protein amounts of various phosphorylated kinases involved in apoptosis and autophagy were altered in contrast to the vector control (Figure 8 and Supplemental Table S1). Through affecting multiple molecules, collectively, these data suggest that simultaneous activation of apoptosis and autophagy by Par-4 contributes to cell death, which accounts for the prognostic role of Par-4 in HPC (Figure 1).

Discussion

Our previous studies revealed a dramatic discrepancy in the expression percentage of Par-4 between nasopharyngeal carcinoma and HPC.¹¹ In the present study, statistical analysis further indicated a significant survival function of Par-4 intensity in patients with HPC undergoing radiotherapy or chemoradiotherapy (Figure 1). Accordingly, these results suggested that Par-4 plays a prognostic role in HPC and serves as a marker to distinguish between different head and neck cancers. Mechanistically, Par-4 augmentation by exogenous Par-4 delivery efficiently resulted in apoptosis. In addition, additional Par-4 introduction also obviously sensitized HPC cells toward apoptotic stimuli.

A **B** **C**

Figure 6 p62 Is involved in coregulation of apoptosis and autophagy by Par-4. **A:** For quantification of p62, FaDu cells were transiently transfected with or without Par-4 expression plasmid for 24 or 48 hours. Cells were then immunostained with primary antibody against p62, followed by incubation with fluorochrome-conjugated secondary antibody. The fluorescence intensity was measured using flow cytometry. The relative fluorescence intensity of each sample was calculated as described in *Materials and Methods* and is shown as the means $\pm$ SD of three independent experiments. **B:** Cell lysates from the indicated transfectants were subjected to immunoprecipitation with PKC ζ or GFP antibody. The immunoprecipitates and inputs (WCE) were subjected to Western blot analysis for Par-4, p62, PKC ζ , GFP, or actin. Representative results from three independent experiments are shown. Quantification of the band and fold change of protein amount were calculated as described in *Materials and Methods*. pcDNA3 transfection was used as the empty control. **C:** FaDu cells were cotransfected with both NF- κ B-luc and pRL-TK vector, plus either Par-4 or Par-4-GFP plasmid. After transfection for 48 hours, the luciferase activity of each sample was measured and the relative luciferase activity was calculated as described in *Materials and Methods*, and is shown as the means $\pm$ SD of three independent experiments. In addition, exogenous Par-4 was also displayed using Western blot analysis. All Western blot analysis data shown are representative of three independent experiments. ** $P < 0.01$, *** $P < 0.001$.

Conversely, partial apoptosis was observed as the result of a lower increase of endogenous Par-4 by a lower concentration ($<LD_{50}$) of apoptotic agent alone (Figures 2B and 3A). In light of the apoptotic threshold of Par-4, as described previously,^{11,42,45} these data implied that the intensity (amount) of Par-4 could be used as a valuable marker to evaluate the therapeutic efficacy in patients with HPC before radiotherapy alone or in combination with chemotherapy. Along with Par-4 pro-apoptosis activity, we also observed a decrease in the amount of Bcl-2, which could be correlated with transcriptional inhibition via Par-4 and WT1 interaction or Par-4-induced abnormal miRNA expression, leading to translation attenuation.^{20,62} In addition, negative regulation of Akt activity by the Par-4/PKC ζ complex could account for the reduction of phosphorylated Akt (Ser473) on Par-4 overexpression.²⁹

Of interest, we discovered autophagy enhancement by Par-4, in addition to apoptosis incitation. Similar to starvation-induced alteration of lysosome position,⁶³ perinuclear autolysosome accumulation under overexpression of Par-4 (Figure 5B) could be ascribed to intracellular lysosomes clustering to the microtubule organizing center, eventually fusing with autophagosomes. In the autophagy

machine, p62 brings ubiquitinated protein aggregates to autophagosomes via interaction with LC3, followed by degradation of cargo and p62 in autolysosomes.³⁵ In concert with the perinuclear clustering of autolysosomes, p62 speckles around the nucleus were observed in response to exogenous Par-4 conduction (data not shown). Via protein *de novo* synthesis analysis and flow cytometry, we showed that autophagy was activated after Par-4 overexpression for 24 hours; meanwhile, the expression level of p62 was elevated and further declined at 48 hours (Figures 6A and 7A). These results consequently corroborated Par-4-induced dynamic alteration of the autophagy process. Consistently, the changed expression of p62 has also been reported in autophagy-competent/apoptosis-defective cells under metabolic stress.⁵¹ However, p62 is a target for caspase 8 cleavage^{40,41}; therefore, whether Par-4-activated apoptosis also contributes to degradation of p62 should be further clarified.

Intriguingly, our results indicated concomitant stimulation of apoptosis and autophagy by Par-4 consistently; the cell populations with apoptosis and autophagy were also increased on overexpression of Par-4 (Figure 7). Relative to apoptosis induction, our data further suggested a dominant role of Par-4-activated autophagy in cell viability (Figures 3A and 7B

Figure 7 Concomitant activation of apoptosis and autophagy by overexpression of Par-4. **A:** FaDu cells were pretreated with 40 $\mu\text{mol/L}$ CHX; cells were then transfected with Par-4 expression plasmid in the absence of CHX (0 hours). At 24 hours after transfection, cell lysates were prepared and subjected to Western blot analysis for Par-4, cleaved PARP, LC3B, or tubulin. In addition, transfection with pcDNA3 served as an empty control. Representative results from three independent experiments are shown. The protein expression fold change was calculated as described in *Materials and Methods*. **B:** For measurement of the cell populations with apoptosis and/or autophagy, Par-4 or pcDNA3 vector-transfected cells were treated with or without 1 $\mu\text{g/mL}$ cisplatin, 1.5 $\mu\text{mol/L}$ 5-Fu, or 6 Gy X-ray. In addition, all experiments were performed under autophagy inhibitor-free (-3-MA) conditions. Samples were then prepared for flow cytometry as described in *Materials and Methods*. The percentages of the cell population entering apoptosis and/or autophagy of each sample are shown as the means $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

and Supplemental Figures S2B, S3B, and S4). Particularly, in autophagy-defective conditions (3-MA addition), cell death was conversely enhanced under chemotherapeutic agent treatment alone (Supplemental Figure S2B), which could be due to chemical interaction. In addition, in this case, whether autophagy served as a cell survival promoter or contributor in cell death should be further investigated. Although autophagic cell death or cell death with autophagy remains uncertain,⁶⁴ emerging evidence has indicated that autophagy can contribute to apoptosis-independent cell death and suggests a tumor-suppressive activity.^{37,65,66} Therefore, in contrast to basal autophagy in HPC cells, excessive activation of autophagy by Par-4 could promote cell collapse. There are many common components and switching points, including calpain- or caspase-mediated Atgs cleavage, as well as Bcl-2 family and Beclin 1 interaction, which have been identified to establish a connection between apoptosis and autophagy.^{38–41,67–71} In

HPC cells, we did not observe cleaved forms of Atg5 and Beclin 1 under Par-4-induced apoptotic conditions (data not shown). However, we showed an increased expression of phosphorylated Bcl-2 at residue Ser87 in response to Par-4 increase (Figure 2A), which is concordant with autophagy stimulation by c-Jun N-terminal kinase 1-mediated Bcl-2 phosphorylation at residues Thr69, Ser70, and Ser87.⁴⁸ Moreover, the activated phosphorylation of several kinases involved in apoptosis and autophagy was also influenced by Par-4, including c-Jun N-terminal kinase 1 (Figure 8 and Supplemental Table S1). Together with down-regulation of Bcl-2 and activated Akt by Par-4 (Figure 2), our data indicated a complex interplay and suggested that apoptosis and autophagy were not mutually exclusive under Par-4 induction circumstances. However, whether Par-4 can modulate modifications of autophagy core components, dissociate the Bcl-2/Beclin 1 complex via enhancing BH3-only pro-apoptotic protein

Figure 8 Overexpression of Par-4 leads to alteration of the amounts of various phosphorylated kinases. FaDu cells were transiently transfected with Par-4 expression plasmid, and at 48 hours after transfection, diluent cell lysates were prepared and subjected to protein kinase array analysis. The densities of the signals of the phospho-kinases were measured and the fold change of each phosphorylated protein was calculated as described in *Materials and Methods* and is shown as the means $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

binding affinity, direct Beclin1 escape from microtubule association, or activate the autophagy-dependent immune response to regulate apoptosis and autophagy at the same time is worthy of investigation.^{71–74}

Another important connector participating in Par-4–induced apoptosis and autophagy is p62. Functionally, p62 can directly interact with multiple proteins, including mitogen-activated protein kinase kinase, extracellular signal–regulated kinase, receptor-interacting protein, tumor necrosis factor receptor-associated factor, atypical PKC, ubiquitin, and LC3, through its respective domain; thus, p62 influences NF- κ B activation, cell growth, apoptosis, and autophagy.⁴¹ In addition, degradation of caspase 8 within autophagy and cleavage of p62 by caspase 8 during apoptosis also establish a complicated connection between apoptosis and autophagy.^{40,41,67} Evidence has revealed that accumulation of p62 in autophagy-defective tumor cells can induce reactive oxygen species and genome damage and alter NF- κ B signaling, thereby enhancing tumorigenesis.⁵¹ Remarkably, our data indicated that Par-4–mediated elimination of p62 interfered with p62 binding to PKC ζ and LC3, respectively, and led to repression of NF- κ B transcriptional activity (Figure 6), consistent with the inhibition of NF- κ B via limiting PKC ζ activity by Par-4,^{49,50} and confined tumor cell survival through down-regulation of tumor-prone protein p62 within apoptosis and autophagy.

In summary, to our knowledge, this is the first study to indicate novel pro-apoptosis and autophagy activity of Par-4 to support a prognostic role in HPC survival. Furthermore, our data suggest that it is of benefit to develop Par-4 as a marker for therapeutic strategies or efficacy evaluation. In addition, inducing *Par-4* expression in combination with radiotherapy or chemoradiotherapy could be a desirable management strategy for cancer therapy.

Acknowledgments

We thank Dr. Chia-Chin Liu and Li-Ting Lin for technical assistance in the TEM and X-ray irradiation experiments, respectively, and Dr. Cheng-Yung Lee for clinical statistical analysis.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.10.012>.

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