Original Paper

Endoplasmic Reticulum Stress Promotes Autophagy and Apoptosis and Reduces Chemotherapy Resistance in Mutant p53 Lung Cancer Cells

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Key Words

Endoplasmic reticulum stress • PI3K/AKT/mTOR signaling pathway • Lung cancer • Cisplatin • Autophagy • Apoptosis • Chemotherapy resistance

Abstract

Background/Aims: Lung cancer (LC) is known to be one of the most prevalent cancers around the world. During this study, we aimed to investigate the involvement of endoplasmic reticulum stress (ERS) in autophagy, apoptosis, and chemotherapy resistance of mutant p53 LC cells. **Methods:** Immunohistochemistry was employed to help determine the p53 mutation status of cancer cells from 12 primary LC patients, who were subsequently assigned to either the mutant p53 (n = 39) or wild-type p53 group (n = 53). **Results:** Mutant p53 cells exhibited increased expression of the chaperonin protein (CHOP), glucose-regulated protein 78 (GRP78), and inositol-requiring enzyme-1α (IRE1α). The Mutant p53 cells were also found to be sensitive to chemotherapy and displayed decreased expression of PI3K, Akt, and mTOR. The mutant p53 cells were treated with tunicamycin to induce ERS and rapamycin in order to inhibit mTOR. Both agents increased the expression of CHOP, GRP78, IRE1α, LC3-II, Beclin-1, Atg7, caspase-3, caspase-12, cleaved caspase-3, cleaved caspase-12, as well as decreased cell proliferation as well as the expression levels of PI3K, Akt, and mTOR. Enhanced levels of cell apoptosis and reduced chemotherapy resistance were also detected. **Conclusion:** The findings of our study suggest that ERS promotes autophagy and apoptosis, while acting to reduce chemotherapy resistance in mutant p53 LC cells by downregulating the PI3K/Akt/mTOR signaling pathway.

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Introduction

Lung cancer (LC), is characterized by unrestrained cell growth in lung tissues [1]. As one of the most common malignant tumors worldwide, the leading risk factors involved with the occurrence of LC include both smoking as well as exposure and environmental factors

[2]. The disease manifests itself with severe impairment of both patient health and quality of life [3]. The prognosis of LC is still unsatisfying, and 5-year survival rate is just 11% [4]. Current LC treatment approaches are mainly limited to radiotherapy, chemotherapy, surgical resection, immunomodulatory therapy and targeted therapy for tumor removal [5-8]. At present, platinum-based chemotherapy represents the primary treatment approach for advanced cases of LC. As a result of this cisplatin and its analogues has become a widely used anti-tumor treatment tool for LC drug therapy. However, in recent years reports of increasing number of cisplatin-resistant LC cases have been identified [9, 10]. Tung et al. highlighted that mutant p53 may confer cisplatin resistance via upregulation of Nrf2 expression [11]. Recent evidence has revealed that the pattern and frequency of p53 mutations vary among cancer populations depending on the geographical origin. LC commonly presents with p53 gene mutations [12], with 81% of squamous cell carcinoma as well as 46% of lung adenocarcinoma patients carrying a p53 mutation. Likewise, reports have indicated there to exist a high mutation rate in hotspot regions [13]. Alterations in p53 are critical for the histological progression, malignant transformation, invasion, and metastasis in LC, regarding both *in vivo* and *in vitro* models [14, 15].

Recently, endoplasmic reticulum stress (ERS) has been highlighted due to its extensive involvement in the development and progression of LC [16]. The endoplasmic reticulum (ER) is a common organelle feature of many cells among eukaryotic cells. The ER offers a region for protein folding and modification and is responsible for the storage of intracellular stores of calcium (Ca^{2+}) [16]. Pathological and physiological stimuli, such as DNA damage, hypoxia, oxidative stress, and nutrition, can disrupt the normal ER functions, which may consequently lead to misfolded or unfolded ER proteins, and in turn cause an increase in mitochondrial Ca^{2+} , thus inducing ERS [18]. ERS triggers processes involved in cell survival and death, such as autophagy and apoptosis [19, 20]. Likewise, this notion of the action of ERS was also reported by Yu, et al, and their previous work of evidence demonstrating that the activation of ERS could promote autophagy and apoptosis and reverse chemoresistance of human small cell lung carcinoma cells by inhibiting the PI3K/AKT/mTOR pathway [21]. The phosphatidylinositol 3-kinase (PI3K)/mTOR pathway is a major target of rapamycin/protein kinase B (PI3K/mTOR/AKT) pathway plays a central role in many cancers, including LC, thyroid carcinoma, breast cancer, and cervical cancer [22-26]. Along with the downstream targets AKT and mTOR, PI3K is plays a central role for various physiological processes, such as cell growth, cell motility, cell survival, proliferation, differentiation, as well as in the progression of malignant disease [27]. The PI3K/AKT/mTOR pathway is known to be involved in human LC [28-30], however the fine mechanisms through by which ERS and the PI3K/Akt/mTOR pathway collaborate in human LC are still under investigation. The central objective of this study was to explore the effects of ERS on autophagy, apoptosis and chemotherapy resistance in mutant p53 LC cells through its regulation of the PI3K/Akt/mTOR signaling pathway.

Materials and Methods

Ethics Statement

This study was conducted with the approval by the Ethics Committee of Xiangya Hospital, Central South University. All participating study subjects were provided with, and subsequently signed written informed consent documentation. All the study experiments were performed with strict adherence with the principles of the Declaration of Helsinki.

Study Subjects

A total of 92 patients, who had previously been pathologically diagnosed with LC and admitted to Xiangya Hospital, Central South University, between January 2013 and January 2014 were recruited for the purposes of this study. All of whom were diagnosed with unresectable and locally advanced non-small cell lung cancer (NSCLC) (44 cases of adenocarcinoma and 48 cases of squamous cell carcinoma) by means of transbronchial or percutaneous lung biopsy and had never undergone any radiotherapy or chemotherapy

treatments. The relevant patient clinical data were made record of. The tumor-node-metastasis (TNM) cancer staging adopted in this study was based on the staging criteria published jointly by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) [31]. All patients in our study were at stages III~IV. The patients were grouped according to the immunohistochemistry staining of their respective tumors, namely: the mutant p53 group (39 patients) or the wild-type p53 group (5 patients). The patients in the mutant p53 and wild-type p53 groups were comparable in relation to age, sex, histological differentiation, pathological type, smoking, and lymph node metastasis.

Chemotherapy Regimens

Lung adenocarcinoma patients were administered pemetrexed (PEM) + cisplatin (DDP) treatment: PEM 500 mg/m² on day 1 + DDP 75 mg/m² on day 1. Patients with squamous cell carcinoma received gemtuzumab (GEM) + DDP treatment: GEM 1000 mg/m² on days 1 and 8 + DDP 75 mg/m² on days 1 and 8. After 6 cycles of chemotherapy, chest computed tomography (CT) scans were conducted to assess the chemotherapy results. Patients were then classified as having complete remission (CR), partial remission (PR), stable disease (SD), or progressive disease (PD) in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST) [32]. For evaluation purposes, patients with CR + PR + SD were considered as responders, while patients with PD were considered to be non-responders.

Cell Grouping and Transfection

H322 and NCI-H157 LC cell lines with a p53 mutation were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China), and cultured in high glucose complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 u/ml penicillin, and 100 u/ml streptomycin. After digestion and passage in a 0.25% trypsin solution, the cells were incubated at 37°C with 5% CO₂ and saturated humidity. After 2 passages, the H322 as well as the NCI-H157 cells were divided into the blank group, the ERS group, the ERS + 4-phenylbutyrate (PBA) group, the rapamycin group, or the ERS + rapamycin group. The cells in the blank group received no further treatment. In the ERS group, the cells were treated with 10 mg/l tunicamycin (Batch number: Y07980200; Sigma-Aldrich Chemical Company, St Louis, MO, USA), a glucosyltransferase inhibitor, to induce ERS over a 24 h period. In the ERS + PBA group, the cells were initially treated with 10 mg/l tunicamycin, followed by treatment with 10 mg/l 4-PBA (Batch number: 200000; Sigma-Aldrich Chemical Company, St Louis, MO, USA) and a small molecular chemical chaperone, in order to terminate ERS. In the rapamycin group, the cells were treated with 10 nmol/l rapamycin (Batch number: S17098; Shanghai Zhongkang Weiye Biological Science & Technology Co., Ltd, Shanghai, China) to suppress the PI3K/AKT/mTOR signaling pathway. In the ERS + rapamycin group, the cells were initially treated with 10 mg/l tunicamycin and then with 10 nmol/l rapamycin.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA from H322 and NCI-H157 cells was isolated using a Trizol kit (Batch number:

Table 1. qRT-PCR primer sequences. Notes: qRT-PCR, quantitative reverse transcription-polymerase chain reaction; CHOP, C/EBP homologous protein; GRP78, glucose-regulated protein 78; IRE1α, inositol-requiring enzyme-1α; LC3, light chain 3; Atg5, autophagy-related gene 5; Atg7, autophagy-related gene 7; P13K, phosphatidyl inositol 3-kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse

Genes	Sequences
CHOP	F: 5'-TCTGCCTTTCGCCTTTGA - 3' R: 5'-CATTCTCCTGCTCCTTCTC-3'
GRP78	F: 5'-GTTACATTGATCTACT-3' R: 5'-CACTTCACTAGAGTTTGCTG-3'
IRE1α	F: 5'-GCGATCGACTGGTGGAACT-3' R: 5'-GTTGCTCTGGCCTCTGTC-3'
LC3-I	F: 5'-GATGTCCGACTTATTCGAGAGC - 3' R: 5'-TTGAGCTGTAAGCGCCTTCTA-3'
LC3-II	F: 5'-CATCACAGTTGGCACAACG- 3' R: 5'-AGTGAGGACTTTGGGTGTGG-3'
Atg5	F: 5'-TTGACGTTGGTAACTGACAAAGT- 3' R: 5'-TGTGATGTTCCAAGGAAGAGC- 3'
Atg7	F: 5'-GATCCGGGATTTCTTTTCACG- 3' R: 5'-CAGCAGCTTGGGTTCCTTGAT-3'
Caspase-3	F: 5'-GGTCCAGACTGATGAAGGC-3' R: 5'-TTATTAGGAAAGGACAGTGGG-3'
Caspase-12	F: 5'-AGTCCCACTCAACAACCTTCT-3' R: 5'-GAAGGTGCGCTCAATGACTG-3'
P13K	F: 5'-GGTCCAGACTGATGAAGGC-3' R: 5'-TTATTAGGAAAGGACAGTGGG-3'
Akt	F: 5'-AGTCCCACTCAACAACCTTCT-3' R: 5'-GAAGGTGCGCTCAATGACTG-3'
mTOR	F: 5'-CAGTTCGCCAGTGGACTGAAG-3' R: 5'-GCTGGTCATAGAAGCGAGAC- 3'
GAPDH	F: 5'-CCTAAGGCCAACCGTGA-3' R: 5'-AGCCAGGGCAGTAATCTC-3'

681675; Gibco Company, Grand Island, NY, USA). The isolation process was conducted in accordance with the manufacturer's instructions. The concentration and purity of RNA were determined by means of ultraviolet spectrophotometry (Model 722; Shanghai No. 3 Analytical Instruments Plant, Shanghai, China). Following the PrimeScript™ RT Reagent Kit (Batch number: LS39871; Takara Holdings Inc., Kyoto, Japan) specifications, 20 µl of reverse transcription reaction system was obtained by mixing 1000 ng of total RNA with 4 µl of 5 × PrimeScript™ Buffer, 1 µl of PrimeScript™ RT Enzyme Mix I, 1 µl of 50 µM OligodT Primer, 1 µl of 100 µM Random 6-mers, and RNase-Free dH₂O. The reaction conditions for reverse transcription process was conducted at 37°C for 30 min, followed by 85°C for 6 s. qRT-PCR was conducted using the SYBR Premix Ex Taq II Kit (Batch number: 1398; Takara Holdings Inc., Kyoto, Japan). A reaction system of 20 µl was prepared with 1 µl of RT-PCR product, 10 µl of 2 × SYBR Green I Premix Ex Taq II, 0.8 µl of 10 µM Forward Primer, 0.8 µl of 10 µM PCR Reverse Primer, 0.4 µl of 50 × ROX Reference Dye, and 7.0 µl of ddH₂O. The reaction conditions were as follows: initial denaturation at 95°C for 30 s, 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s for a total of 40 cycles. The 2^{-ΔΔCT} method was adopted to calculate the relative expression levels of target genes; the data were displayed as the relative expression. Each sample experiment was repeated three times. The reference gene for the ERS markers, included C/EBP homologous protein (CHOP), glucose-regulated protein 78 (GRP78), inositol-requiring enzyme-1α (IRE1α), LC3-I, LC3-II, autophagy-related gene 5 (Atg5), autophagy-related gene 7 (Atg7), caspase-3, caspase-12, PI3K, Akt and mTOR, was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences are listed in Table 1.

Western Blotting

After one or two passages, the LC cell lines were washed three times with FBS (Batch number: 1036489; Thermo Fisher Scientific, San Jose, California, USA) and placed on ice for 30 min. Total protein was extracted and quantified using a bicinchoninic acid (BCA) protein assay kit (Batch number: 20140312; Beyotime Biotechnology Co., Shanghai, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared, and the sample volume was calculated on the basis of the relative protein concentration, followed by even mixing with SDS loading buffer at a ratio of 1:1. The mixture was heated in a boiling water bath for 5 min, and the samples were then subsequently added. Next, a 5% concentration gel and a 12% separation gel were selected for electrophoresis at 80 V and 100 V, respectively. The gels were then cut and marked by hand, and the membrane was subjected to a constant current of 200 mA electrophoretic transfer at 4°C for 2 h. After blocking with 5% nonfat dried milk for 1 h at room temperature, the membrane was then washed 3 times with Tris-Buffered Saline Tween-20 (TBST), with each wash lasting 10 min. The primary antibodies were all incubated at 4°C overnight, including CHOP (1:750; YB532; Shanghai Yu Biotechnology Co., Ltd, Shanghai, China), GRP78 (1:1000; ab21685), IRE1α (1:1000; ab37073), LC3-I (1:1000; #4249), anti-AKT (1:1000; #4685), anti-mTOR (1:1000; #2983), caspase-3 (1:1000; #9605), caspase-12 (1:1000; #2202), Atg5 (1:1500; ab10832), Atg7 (1:150000; ab5247), LC3-II (1:1000; ab48394) and rabbit anti-human β-actin polyclonal antibody (1:4000; ab5694). The membranes were then washed with TBST 3 times, with each wash lasting 10 min, followed by incubation with horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G (IgG) (Batch number: 1000000; Nanjing SunShine Biotechnology Co., Ltd, Nanjing, China) at room temperature for 1 h. The total protein was subsequently detected by means of Enhanced Chemical Luminescence (ECL) (Batch number: ml01116; Shanghai Meilian Biological Technology Co., Ltd, Shanghai, China), and scanned using the GeneSnap™ imaging system and analysis tool from the ImageJ software. Each sample experiment was performed three times. β-actin was used as the internal control, and the relative expression levels of CHOP, GRP78, IRE1α, PI3K, Akt, mTOR, caspase-3 and LC3-II/LC3-I in each group were subsequently calculated. GRP78, IRE1α, PI3K, Akt, mTOR, caspase-3, and caspase-12 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Fluorescence Staining

Autophagy was determined using GFP-LC3 fusion protein and stained with 4',6-diamidino-2-phenylindole (DAPI) for nuclei identification purposes. When the GFP-LC3 fusion protein was translocated into the membrane of the autophagosome, autophagy occurred. This translocation was represented by the visualization of multiple bright green fluorescent spots that could be observed under the fluorescence microscope. The number of spots was reflective of autophagic activity.

Flow Cytometry

After subculturing for 2 or 3 passages, LC cells were seeded into a 12-well plate with a cell concentration of 4×10^4 . After collecting the supernatant, phosphate buffer saline (PBS) was used to wash the adherent cells twice at 37°C. Then, the adherent cells were collected and digested with 0.25% trypsin (without ethylene diamine tetraacetic acid [EDTA]). The digestion was terminated by adding PBS containing 2% bovine serum albumin (BSA) at 4°C. After being mixed, the cells were placed in a centrifuge tube and centrifuged at 180 rpm for 10 min. Next, the cells were washed once again with PBS, resuspended, filtered through a 400-mesh screen, counted, and centrifuged again. The supernatant was then discarded. The cell concentration was adjusted to 1×10^6 by adding 500 μ l of cold binding buffer. Then, 5 μ l of Annexin V-fluorescein isothiocyanate (FITC) was added, and the cells were incubated under dark conditions, at 4°C for 30 min. Next, the mixture was gently oscillated with 400 μ l of binding buffer. Over a period of 1 h, quantitative analyses were conducted using a flow cytometer (FACS Aria™, Becton, Dickinson, and Company, NJ, USA).

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

PBS was used to prepare the MTT solution (Batch Number: 834008; Sigma-Aldrich Chemical Company, St Louis, MO, USA). H322 and NCI-H157 cells were first added to 96-well plates (5×10^4 cells/well), and DDP was then added to the culture for 24 h at concentrations of 0, 25, 50, 100, 200, and 400 μ mol/l. The 0 μ mol/l group was regarded as the control group, and DMEM high-glucose (Batch Number: 4832; Sigma-Aldrich Chemical Company, St Louis, MO, USA) containing no DDP was regarded as the blank group. The optical density (OD) at 570 nm was measured. The survival rate and inhibition rate of each group were calculated according to the following formula; The cell survival rate (%) = the OD value in the ERS groups – the OD value in the blank group/the OD value in the control group – the OD value in the blank group $\times$ 100%. The cell inhibition rate (%) = 1 – the cell survival rate. The half maximal inhibitory concentration (IC50) of cisplatin in each cell was calculated using SPSS 20.0 (IBM Corp. Armonk, NY, USA). Each group assay was repeated 3 times.

5-bromo-2'deoxyuridine (BrdU) Cell Proliferation Assay

H322 and NCI-H157 cells were selected and seeded into a 24-well plate at 5×10^4 cells/well. DDP at a concentration of 200 μ mol/l was added and incubated for 24 h. Next, 10 μ M BrdU was added to each well and incubated for 4 h. Then, 4% pre-cooled paraformaldehyde was added for 20 min to fix the cells, and the cells were washed 3 times with PBS. The cells were then permeabilized with 0.2% Triton X-100 for 10 min and washed with PBS 3 times. BrdU antibody was diluted at a ratio of 1: 100, and 300 μ l was added to each well and incubated at 4°C overnight. After washing with PBS 3 times, TRITC-labeled fluorescent second antibody was added, and the samples were then baked in an oven at 37°C. DAPI (0.5 μ g/ml) was added to stain the nuclei, distilled water was used to rinse off the PBS, glycerol was used for mounting, and the number of positive cells was counted under an inverted microscope. The assay was repeated three times for each group.

Cloning Assay

After subculturing for 2 or 3 generations, the LC cells were incubated in a 6-well plate at a concentration of 3×10^4 cells/ml. There were 300 cells per well with 3 duplicate wells for each group. When the cells began to adhere, 100 μ mol/l DDP was added, and the cells were subsequently cultured for 5 days. The adherent cells were then washed 3 times with PBS, fixed in 4% paraformaldehyde and stained with crystal violet for 10 min. After rinsing with water, microscopy was used to observe colony formation. The plating efficiency (%) = colony formation number/seeded cell number $\times$ 100%.

Statistical Analysis

A minimum of three repeats were conducted for each experiment under identical conditions. SPSS 20.0 (IBM Corp. Armonk, NY, USA) was used for statistical analyses. All data were expressed as the mean $\pm$ standard deviation. Continuous data were analyzed using the independent-sample *t*-test method. Comparisons between groups were analyzed by analysis of variance (ANOVA) or by means of *t*-test. Categorical data were presented as percentage or ratio. *P* < 0.05 was considered to be statistically significant.

Results

Correlation of Mutant p53 and Wild-Type p53 with Clinical Characteristics in NSCLC patients

p53 mutation status was connected to the NSCLC differentiation, TNM stage, and lymph node metastasis (all $P < 0.05$) however, no significant correlation was detected in regard to sex, smoking or pathological type (all $P > 0.05$, Table 2)

Mutant p53 Cells Had Increased Expression Levels of CHOP, GRP78 and IRE1α and Decreased Expression Levels of PI3K, Akt and mTOR and Were Sensitive to Chemotherapy

Compared with the wild-type p53 LC cells, mutant p53 LC cells displayed higher mRNA and protein levels of CHOP, GRP78, and IRE1α and lower expression levels of PI3K/Akt/mTOR signaling pathway-related proteins (all $P < 0.05$, Fig. 1). In addition, among 102 advanced NSCLC patients who underwent chemotherapy, there were 39 patients with mutant p53 as well as 53 patients with wild-type p53. After 6 weeks of chemotherapy, the patients with mutant p53, chemotherapy was confirmed to be effective (CR + PR = 100%) in 51 patients,

Table 2. Comparisons of the clinical characteristics in subjects between the mutant and wild-type p53 groups. Note: NSCLC, non-small cell lung cancer

	Number	Mutant p53 type	Wild-type p53 type	Survival rate (%)	P
Gender					
Male	55	21	34	38.18	0.391
Female	37	18	19	48.65	
Age					
≤ 60	48	18	30	37.50	0.321
> 60	44	23	21	47.73	
Histological type					
Squamous cell carcinoma	53	21	32	45.28	0.513
Adenocarcinoma	39	15	24	38.46	
Differentiation					
High-differentiated	29	11	18	20.69	0.014
Moderately-differentiated	21	10	11	48.39	
Low-differentiated	11	18	14	56.25	
Smoking					
Yes	22	11	11	37.93	0.258
No	17	17	17	50.00	
TNM stage					
I + II	15	14	1	30.43	0.034
III + IV	33	25	8	54.35	
Lymph node metastasis					
Yes	49	26	23	53.06	0.035
No	43	13	30	30.23

Fig. 1. mRNA and protein levels of CHOP, GRP78, IRE1α, PI3K, AKT and mTOR in the mutant and wild-type p53 groups. Note: A. Diagram of protein expression levels of CHOP, GRP78, IRE1α, PI3K, AKT and mTOR detected by Western blotting; B. Relative protein expression levels of CHOP, GRP78, IRE1α, PI3K, AKT and mTOR in the mutant and wild-type p53 groups; C. Relative mRNA expression levels of CHOP, GRP78, IRE1α, PI3K, AKT and mTOR in the mutant and wild-type p53 groups; * $P < 0.05$, indicates a comparison with P53-wild-type group; CHOP, C/EBP homologous protein; GRP78, glucose-regulated protein 78; IRE1α, inositol-requiring enzyme-1α; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin.

Table 3. Evaluation of chemotherapy efficacy in patients with p53 mutation and p53 wild-type. Note: The efficacy is divided into complete remission (CR), partial remission (PR), stable disease (SD) and progressive disease (PD). CR + PR + SD is considered responders and PD as non-responders

Groups	CR + PR + SD	PD	P
p53 mutant	31	31	0.047
p53 wild	8	22

Fig. 2. mRNA and protein expression levels of CHOP, GRP78 and IRE1 α in mutant p53 LC cells in each group. Note: A. Protein levels of CHOP, GRP78 and IRE1 α in mutant p53 LC cells detected by Western blotting; B. Protein levels of CHOP, GRP78 and IRE1 α in mutant p53 H322 cells; C. mRNA expression levels of CHOP, GRP78 and IRE1 α in mutant p53 H322 cells; D. Protein levels of CHOP, GRP78 and IRE1 α in mutant p53 NCI-H157 cells; E. mRNA expression levels of CHOP, GRP78 and IRE1 α in mutant p53 NCI-H157 cells; * $P < 0.05$, represents a comparison with the blank group; # $P < 0.05$, represents a comparison with the ERS group; ERS, endoplasmic reticulum stress; CHOP, C/EBP homologous protein; GRP78, glucose-regulated protein 78; IRE1 α , inositol-requiring enzyme-1 α .

With the same therapy techniques failed (PD) in 8 patients. Among the patients with wild-type p53, chemotherapy was effective in 31 patients and failed in 22 patients. These results indicated that patients with mutant p53 LC were particularly sensitive to chemotherapy in comparison to patients with wild-type p53 LC ($P = 0.047$, Table 3).

ERS Alters the Levels of CHOP, GRP78, IRE1 α , PI3K, AKT and mTOR in Mutant p53 LC Cells

Compared with the blank group, the mRNA and protein expression levels of CHOP, GRP78, and IRE1 α in the ERS and ERS + rapamycin groups were significantly elevated in H322 and NCI-H157 cells (all $P < 0.05$). The ERS + 4-PBA and rapamycin groups exhibited significantly lower mRNA and protein expression levels of CHOP, GRP78, and IRE1 α compared with those in the ERS and ERS + rapamycin groups (all $P < 0.05$). No statistical significance was detected in our comparisons between the ERS + 4-PBA and rapamycin groups with the blank group (all $P > 0.05$). No significant difference was discovered when comparing the ERS and ERS + rapamycin groups with the blank group ($P > 0.05$, Fig. 2).

Fig. 3. mRNA and protein expression levels of PI3K, AKT and mTOR in mutant p53 LC cells in each group. Note: A. Protein levels of PI3K, AKT and mTOR in H322 and NCI-H157 cell lines detected by Western blotting; B. Relative protein expression levels of PI3K, AKT and mTOR in the mutant p53 H322 cell line; C. Relative mRNA expression levels of PI3K, AKT and mTOR in the mutant p53 H322 cell line; D. Relative protein expression of PI3K, AKT and mTOR in the mutant p53 NCI-H157 cell line; E. Relative mRNA expression of PI3K, AKT and mTOR in the mutant p53 NCI-H157 cell line; * $P < 0.05$, represents a comparison with the blank group; # $P < 0.05$, represents a comparison with the ERS group; LC, lung cancer; ERS, endoplasmic reticulum stress; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin.

How compared with those in the blank group, the mRNA and protein expression of PI3K, AKT and mTOR in the ERS and ERS + rapamycin groups were displayed notably reduced levels ($P < 0.05$). The detected expressions of PI3K and AKT were not significantly different in the rapamycin group ($P > 0.05$), but the mTOR expression was decreased notably ($P < 0.05$). The ERS + 4-PBA group had no significant differences regarding the mRNA and protein expression levels of PI3K, AKT and mTOR ($P < 0.05$). Compared with those in the ERS group, the ERS + 4-PBA group had increased mRNA and protein expression levels of PI3K, AKT and mTOR, while the rapamycin group displayed elevated levels of mRNA and protein expressions of both PI3K and AKT (all $P < 0.05$), with the exception of mTOR ($P > 0.05$); the ERS + rapamycin group had decreased mTOR expression (all $P < 0.05$). No significant differences in the PI3K and AKT expression levels were detected ($P > 0.05$) (Fig. 3).

ERS Alters the Levels of Autophagic Cytokines (LC3-II/LC3-I, Atg5, and Atg7) in Mutant p53 LC Cells

Compared the levels observed in the blank group (Fig. 4), the levels of LC3-II/LC3-I, Atg5, and Atg7 in the mutant p53 LC cells in the ERS, rapamycin and ERS + rapamycin groups

Fig. 4. mRNA and protein expression levels of the autophagy factors LC3-II/LC3-I, Atg5, and Atg7 in mutant p53 LC cells in each group. **A.** Protein levels of LC3-I, LC3-II, Atg5, and Atg7 in the H322 and NCI-H157 cell lines for the five groups detected by Western blotting; **B.** Relative protein expression levels of LC3-II/LC3-I, Atg5 and Atg7 in the mutant p53 H322 cell line for the five groups; **C.** Relative mRNA expression levels of LC3-II/LC3-I, Atg5, and Atg7 in the mutant p53 H322 cell line; **D.** Relative protein expression levels of LC3-II/LC3-I, Atg5, and Atg7 in the mutant p53 NCI-H157 cell line; **E.** Relative mRNA expression levels of LC3-II/LC3-I, Atg5, and Atg7 in the mutant p53 NCI-H157 cell line; * $P < 0.05$, represents a comparison with the blank group; # $P < 0.05$, represents a comparison with the ERS group; & $P < 0.05$, represents a comparison with the rapamycin group; LC, lung cancer; ERS, endoplasmic reticulum stress; LC3, light chain 3; Atg5, autophagy-related gene 5; Atg7, autophagy-related gene 7.

all displayed significantly upregulated rates (all $P < 0.05$). No significant difference was found between the ERS + 4-PBA group (all $P > 0.05$). Compared with those in the ERS group, significant increases in the levels of LC3-II/LC3-I, Atg5, and Atg7 were detected in the ERS + 4-PBA group (all $P < 0.05$); no significant differences were observed in the rapamycin group (all $P > 0.05$), however, significantly increased levels of LC3-II/LC3-I, Atg5, and Atg7 were observed in the ERS + rapamycin group (all $P < 0.05$).

ERS Increases the Number of Autophagosomes Formed in Mutant p53 LC Cells

As displays in Fig. 5, the autophagosome formation could be observed by fluorescence microscopy after GFP-LC3 fusion proteins were translocated in mutant p53 LC cells. Compared with the blank group, autophagosome formation was significantly elevated in the ERS, rapamycin, and ERS + rapamycin groups (all $P < 0.05$). However, autophagosome formation in the ERS + 4-PBA group no significant difference was confirmed in relation to

Fig. 5. Number of autophagosomes formed in mutant p53 LC cells in each group. Note: Blue represents the nucleus; Green represents LC3 fusion protein-marked autophagosomes; Scale bar = 10 μ m; Red arrows indicate autophagosomes; * $P < 0.05$, represents a comparison with the blank group; # $P < 0.05$, represents a comparison with the ERS group; & $P < 0.05$, represents a comparison with the rapamycin group; LC, lung cancer; ERS, endoplasmic reticulum stress.

the blank group (all $P > 0.05$). Compared with that in the ERS group, autophagosome formation was exhibited no decreased levels in the ERS + 4-PBA group (all $P < 0.05$); no significant differences were observed in the rapamycin group (all $P > 0.05$), however autophagosome formation was increased in the ERS + rapamycin group (all $P < 0.05$).

ERS Increases the Levels of Apoptotic Cytokines in Mutant p53 LC Cells

As shown in Fig. 6, compared with the blank group, the levels of caspase-3, caspase-12, cleaved caspase-3 and cleaved caspase-12 in mutant p53 LC cells in the ERS, rapamycin and ERS + rapamycin groups were observed to have displayed marked increases (all $P < 0.05$), but no significant difference was detected in the ERS + 4-PBA group ($P > 0.05$). Compared with those in the ERS group, the levels of caspase-3, caspase-12, cleaved caspase-3 and cleaved caspase-12 were significantly decrease in the ERS + 4-PBA group (all $P < 0.05$); no significant differences were found in the rapamycin group (all $P > 0.05$), but the levels of caspase-3, caspase-12, cleaved caspase-3 and cleaved caspase-12 were increased in the ERS + rapamycin group (all $P < 0.05$).

ERS Increases the Apoptosis Rate in Mutant p53 LC Cells

In regard to the H322 cells, the apoptosis rates in the blank, ERS, ERS + 4-PBA, rapamycin, and ERS + rapamycin groups were 3.36%, 17.65%, 4.53%, 18.41%, and 26.34%, respectively.

Fig. 6. Relative protein and mRNA levels of caspase-3 and caspase-12 in mutant p53 LC cells in each group. Note: A. Relative protein levels of caspase-3, caspase-12, cleaved caspase-3 and cleaved caspase-12 in the H322 and NCI-H157 cells for the five groups detected by Western blotting; B. Relative protein expression levels of caspase-3, caspase-12, cleaved caspase-3 and cleaved caspase-12 in the mutant p53 H322 cell line; C. Relative mRNA expression levels of caspase-3 and caspase-12 in the mutant p53 H322 cell line; D. Relative protein expression levels of caspase-3, caspase-12, cleaved caspase-3 and cleaved caspase-12 in the mutant p53 NCI-H157 cell line; E. Relative mRNA expression levels of caspase-3 and caspase-12 in the mutant p53 NCI-H157 cell line; * $P < 0.05$, represents a comparison with the blank group; # $P < 0.05$, represents a comparison with the ERS group; & $P < 0.05$, represents a comparison with the rapamycin group; LC, lung cancer; ERS, endoplasmic reticulum stress.

The ERS + rapamycin group had the highest apoptosis rate. In NCI-H157 cells, the apoptosis rates in the blank, ERS, ERS + 4-PBA, rapamycin, and ERS + rapamycin groups were 5.18%, 16.75%, 5.43%, 17.41%, and 28.14%, respectively. Compared with those in the blank group, the apoptosis rates of the mutant p53 LC cells in the ERS group, the rapamycin group and the ERS + rapamycin group all displayed significant increases (all $P < 0.05$). The apoptosis

Fig. 7. Apoptosis rates in mutant p53 LC cells induced by ERS in each group. Note: A. Cell apoptosis in the mutant p53 H322 cell line for the five groups detected by flow cytometry; B. Cell apoptosis rate in the mutant p53 NCI-H157 cell line for the five groups; * $P < 0.05$, represents a comparison with the blank group; # $P < 0.05$, represents a comparison with the ERS group; & represents a comparison with the rapamycin group; LC, lung cancer; ERS, endoplasmic reticulum stress.

rate in the ERS + 4-PBA group was not significantly different from that in the blank group ($P > 0.05$). Compared with the ERS group, the apoptosis rate was significantly decreased in the ERS + 4-PBA group (all $P < 0.05$); no significant difference was found in the rapamycin group (all $P > 0.05$), but the apoptosis rate was increased in the ERS + rapamycin group (all $P < 0.05$) (Fig. 7).

p53 LC Cells Undergoing ERS have Reduced DDP Drug Resistance

Compared to observations made in the blank group, the cell inhibition rate in the ERS group was significantly upregulated when treated with DDP at concentrations of 25, 50, 100, 200, and 400 $\mu\text{mol/l}$ (all $P < 0.05$). The inhibition rate in the ERS + 4-PBA group was not significantly different when compared with the blank group (all $P > 0.05$). With

Table 4. Effects of different concentrations of cisplatin on the inhibition rate of mutant p53 H322 LC cells in the blank, the ERS, the ERS + 4-PBS, the Rapamycin and the ERS + Rapamycin groups respectively. Notes: * represents the comparison with the blank group ($P < 0.05$); # represents the comparison with the ERS group ($P < 0.05$); ERS, endoplasmic reticulum stress; 4-PBA, phenylbutyrate acid

Concentration of cisplatin	25 $\mu\text{mol/l}$	50 $\mu\text{mol/l}$	100 $\mu\text{mol/l}$	200 $\mu\text{mol/l}$	400 $\mu\text{mol/l}$
blank	8.06 $\pm$ 1.62	15.52 $\pm$ 1.89	23.14 $\pm$ 2.21	32.01 $\pm$ 3.94	35.67 $\pm$ 2.95
ERS	18.52 $\pm$ 4.56*	26.55 $\pm$ 3.38*	34.62 $\pm$ 2.24*	43.81 $\pm$ 3.16*	46.38 $\pm$ 2.61*
ERS + 4-PBA	8.31 $\pm$ 1.95#	16.87 $\pm$ 1.62#	24.91 $\pm$ 2.77#	33.52 $\pm$ 4.33#	34.71 $\pm$ 3.7
Rapamycin	17.46 $\pm$ 1.26*	25.32 $\pm$ 2.89*	34.62 $\pm$ 3.12*	43.07 $\pm$ 6.74*	45.28 $\pm$ 2.91
ERS + Rapamycin	56.71 $\pm$ 1.33#	61.29 $\pm$ 1.47#	73.88 $\pm$ 2.71#	80.26 $\pm$ 3.97#	89.91 $\pm$ 3.1

Table 5. Effects of different concentrations of cisplatin on the inhibition rate of mutant p53 NCI-H157 LC cells in the blank, the ERS, the ERS + 4-PBS, the Rapamycin and the ERS + Rapamycin groups respectively. Notes: * represents the comparison with the blank group ($P < 0.05$); # represents the comparison with the ERS group ($P < 0.05$); ERS, endoplasmic reticulum stress; 4-PBA, phenylbutyrate acid

Group	25 $\mu\text{mol/l}$	50 $\mu\text{mol/l}$	100 $\mu\text{mol/l}$	200 $\mu\text{mol/l}$	400 $\mu\text{mol/l}$
blank	9.14 $\pm$ 1.74	16.85 $\pm$ 1.98	24.41 $\pm$ 2.21	32.01 $\pm$ 3.94	36.85 $\pm$ 2.78
ERS	19.47 $\pm$ 4.11*	27.65 $\pm$ 3.07*	35.86 $\pm$ 2.12*	43.12 $\pm$ 3.34*	47.08 $\pm$ 2.28*
ERS + 4-PBA	9.37 $\pm$ 1.84#	17.28 $\pm$ 1.56#	25.78 $\pm$ 2.8#	34.72 $\pm$ 4.19#	36.37 $\pm$ 3.39#
Rapamycin	20.54 $\pm$ 1.82*	28.23 $\pm$ 3.28*	37.22 $\pm$ 3.41*	43.37 $\pm$ 5.47*	45.58 $\pm$ 3.76*
ERS + Rapamycin	60.14 $\pm$ 1.26#	68.59 $\pm$ 1.76#	78.11 $\pm$ 2.81#	83.18 $\pm$ 3.45#	87.05 $\pm$ 3.61#

Table 6. The comparison of clone formation ability of mutant p53 H322 LC cells in the blank, the ERS, the ERS + 4-PBS, the Rapamycin and the ERS + Rapamycin groups respectively. Notes: * represents the comparison with the blank group ($P < 0.05$); # represents the comparison with the ERS group ($P < 0.05$); & represents the comparison with the Rapamycin group ($P < 0.05$); ERS, endoplasmic reticulum stress; 4-PBA, phenylbutyrate acid

Groups	The number of initial cells	The number of clone formation	The clone formation efficiency
blank	100	135.54 $\pm$ 11.44	45.18
ERS	100	105.80 $\pm$ 10.52*	35.27
ERS + 4-PBA	100	133.45 $\pm$ 8.58#	43.22
Rapamycin	100	104.67 $\pm$ 11.35*	34.89
ERS + Rapamycin	360	56.55 $\pm$ 9.12*#&	18.85

DDP treatment, the inhibition rate in mutant p53 LC cells was found to be dose-dependent. Compared with the blank group, the inhibition rate was significantly different in cells treated with 200 $\mu\text{mol/l}$ DDP ($P < 0.05$), but no significant difference was observed between cells treated with 400 $\mu\text{mol/l}$ DDP and 200 $\mu\text{mol/l}$ DDP ($P > 0.05$). The aforementioned results obtained suggested that 200 $\mu\text{mol/l}$ DDP was used as the final concentration for the clone formation assay (Table 4 & 5).

ERS Decreases the Proliferation of Mutant p53 LC Cells

In the H322 cells, after treatment with 200 $\mu\text{mol/l}$ DDP, the number of BrdU positive cells in the blank, ERS, ERS + 4-PBA, rapamycin, and ERS + rapamycin groups was 64, 34, 60, 36 and 11, respectively (Fig. 8A & C). In the NCI-H157 cells, after treatment with 200 $\mu\text{mol/l}$ DDP, the number of BrdU positive cells in the blank, ERS, ERS + 4-PBA, rapamycin, and ERS + rapamycin groups was 66, 35, 61, 35 and 10, respectively (Fig. 8B & D). Among these two cell types, the number of BrdU positive cells in the ERS, rapamycin and ERS + rapamycin groups was significantly lower than that in the blank and ERS + 4-PBA groups (all $P < 0.05$). The number of BrdU positive cells in the ERS + rapamycin group was notably lower than that in the rapamycin group, while the number of BrdU positive cells in the ERS + 4-PBA group was obviously higher than in that of the ERS group (all $P < 0.05$).

Fig. 8. Proliferation in mutant p53 LC cells induced by ERS in each group. Note: A. Cell proliferation in the mutant p53 H322 cell line detected by BrdU cell proliferation assay; Scale bar = 20 μ m; B. Cell proliferation in the mutant p53 NCI-H157 cell line detected by BrdU cell proliferation assay; C. Statistical analysis of BrdU-positive H322 cells; D: Statistical analysis of BrdU-positive NCI-H157 cells; * $P < 0.05$, represents a comparison with the blank group; # $P < 0.05$, represents a comparison with the ERS group; BrdU, 5'-bromo-2'-deoxyuridine; LC, lung cancer; ERS, endoplasmic reticulum stress.

Table 7. The comparison of clone formation in mutant p53 NCI-H157 LC cells in the blank, the ERS, the ERS + 4-PBS, the Rapamycin and the ERS + Rapamycin groups respectively. Notes: *represents the comparison with the blank group ($P < 0.05$); #represents the comparison with the ERS group ($P < 0.05$); &represents the comparison with the Rapamycin group ($P < 0.05$); ERS, endoplasmic reticulum stress; 4-PBA, phenylbutyrate acid

Groups	The number of plated cells	The number of clone formation	The clone formation efficiency
blank	300	133.87 $\pm$ 15.45	44.62
ERS	300	108.13 $\pm$ 14.70*	36.04
ERS + 4-PBA	300	132.34 $\pm$ 11.01#	43.78
Rapamycin	300	104.00 $\pm$ 13.04*	34.67
ERS + Rapamycin	300	59.21 $\pm$ 8.86*#&	19.74

ERS increases Colony Formation in H322 Cells

In the H322 cell line, after treatment with 200 μ mol/l DDP, the colony formation rates in the blank, ERS, ERS + 4-PBA, rapamycin, and ERS + rapamycin groups were 45.18%, 33.27%, 19.22%, 34.89% and 18.85%, respectively (Table 6). In the NCI-H157 cell line, after treatment with 200 μ mol/l DDP, the colony formation rates in the blank, ERS, ERS + 4-PBA, rapamycin and ERS + rapamycin groups were 44.62%, 36.04%, 19.78%, 34.67% and 19.74%, respectively (Table 7). The colony formation rates of both cell lines in the ERS group, the rapamycin group and the ERS + rapamycin group were lower than in those of the blank group and the ERS + 4-PBA group (all $P < 0.05$). The colony formation rate in the ERS + rapamycin group was lower than that of the rapamycin group ($P < 0.05$). The colony formation rate in the ERS + 4-PBA group was higher than that in the ERS group ($P < 0.05$).

Discussion

LC currently represents for one of the leading malignant tumors worldwide. In the present study, we demonstrated that in mutant p53 LC, ERS is highly prevalent and that the PI3K/AKT/mTOR signaling pathway is upregulated. Additionally, our findings provide evidence indicating that ERS contributes to autophagy, apoptosis and reduces chemotherapy resistance in mutant p53 LC cells through the downregulation of the PI3K/AKT/mTOR signaling pathway.

The results of our experiment highlighted that patients with mutant p53 LC have a poorer response to chemotherapy than patients with wild-type p53. Genetically, carcinogenesis involves multiple processes by which the activation of oncogenes and the inactivation of tumor suppressor genes represent central factors for tumor development and progression of the disease [33]. Tumor suppressor mutations, as reported in LC cells, may contribute to the pathogenesis of invasive cancer [12]. p53 is widely known as a tumor suppressor, while wild-type p53 has been linked with apoptosis, cell cycle arrest and DNA repair [34]. Conversely, the loss of p53 due to mutations induces various types of human cancers [35] owing to the fact that mutant p53 can cause aberrant mitosis and reduce apoptosis [14]. Mutant p53 can also promote proliferation, invasion, angiogenesis and increase genomic instability and chemoresistance [36]. Studies conducted by Wei et al. revealed that mutant p53 and poor tumor differentiation were correlated with poor prognosis in LC [37]. These findings were in concert with the results of our study, in regard to the prevalence of ERS in mutant p53 LC tissues.

Furthermore, when rapamycin was applied, LC cells exhibited elevated LC3, Atg5, Atg7, caspase-3, and caspase-12 levels; more autophagosomes; increased cell apoptosis; and decreased tumor diameters. A previous study found that over-expression of miR-144 suppressed proliferation and promoted apoptosis and autophagy in LC cells by targeting the p53-induced glycolysis and apoptosis regulator, which was consistent with our results [38]. When ERS was inhibited by 4-PBA, autophagy factors and apoptotic factors also in turn displayed reduced levels. miR-144 had reduced apoptosis and increased drug resistance. LC3-II/LC3-I, Atg7 and Atg5 are autophagy-related gene transcripts [39]. Located on the ER cytoplasmic surface, caspase-12 is a key biomarker of ERS-specific apoptosis [40, 41]. Caspase-3 is a cysteine protease, which are pro-apoptotic proteins with prominent roles in ERS-induced cell death [42, 43]. ERS is well-characterized in human LC [44, 45]. Recent evidence has suggested that ERS can initiate internal pathways that may induce cellular death [27]. The central objective of the ERS response is to limit disturbance and ultimately recover ER balance; however, in terms of intense or sustained ERS, these pathways will activate programmed cell death [46]. Calcium imbalance or aberrant protein folding in the ER result in ER stress and cell destruction responses [47, 48]. Alterations in the ER molecular chaperone levels can suppress tumor growth *in vivo* [44]. Likewise, ERS-induced apoptosis is highly dependent on the upregulation of CHOP [49]. CHOP is the best-characterized factor in the transition from ERS to cell apoptosis [50]. CHOP-knockdown cells are resistant to cell apoptosis that is usually induced by ERS [16], that can lead to various processes, including autophagy and apoptosis, which function as the regulators of cell survival and death [19, 20]. ERS can also activate the unfolded protein response, which reports have earmarked due to their role in chemotherapeutic resistance or antitumor therapy based on the stimuli and cell context [51]. GRP78, a molecular chaperone protein and a member of the heat shock protein-70 family, is critical for tumor progression and chemoresistance. GRP78 has also been shown to be negatively associated with cell survival [52]. Jiang et al. revealed that ERS upregulates GRP78 protein levels in human pancreatic cancer cells [53]. In mammalian cells, IRE1 α mediates ER stress and is both necessary and sufficient for apoptosis [54]. Shimodaira et al. demonstrated that CHOP promotes IRE1 α and autophagy, especially under ER stress conditions [55].

The PI3K/AKT/mTOR pathway is significantly decreased in mutant p53 LC cells. The PI3K/AKT/mTOR pathway is an important target due to its regulatory role in various cancers

[24, 56, 57]; it is also involved in the invasion and metastasis and progression of hepatocellular carcinoma, cervical cancer, and LC [22, 25, 58]. ERS shares a correlation with the PI3K/Akt/mTOR signaling pathway. Our experimental data revealed that increases in ERS are followed by decreases in PI3K/AKT/mTOR signaling, as well as reductions in chemoresistance; when the PI3K/AKT/mTOR signaling pathway is blocked, autophagy and apoptosis factors are increased, chemotherapy resistance is weakened, and the tumor shrinks [27, 59], which has an important function in autophagy and apoptosis regulation [2, 60, 61]. It has been reported that blocking the PI3K/AKT/mTOR cascade promotes autophagy [62] that inhibiting PI3K/Akt/mTOR signaling promotes cell apoptosis in LC [28], which is consistent with our results.

Conclusion

Our study provides new insight into the mechanism connecting ERS and the PI3K/AKT/mTOR signaling pathway in LC progression. Our findings provided evidence highlighting that ERS promotes cell autophagy and apoptosis and reduces the chemotherapeutic resistance of mutant p53 LC cells via downregulating the PI3K/AKT/mTOR signaling pathway. These results offer important information for understanding and treating mutant p53 LC.

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Disclosure Statement

The authors have declared that no competing interests exist.

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