Mitochondrial dysfunction rather than mtDNA sequence mutation is responsible for the multi-drug resistance of small cell lung cancer

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Abstract. Small cell lung cancer (SCLC) accounts for ~15% of all lung cancer cases, and chemotherapy has dramatically improved the survival rate of SCLC patients. Yet, the long-term survival rate of this cancer has not improved since multi-drug resistance (MDR) may emerge after chemotherapy. Mitochondrial DNA (mtDNA) mutation-related biological processes, such as energy metabolism and reactive oxygen species (ROS) production, have been considered to be associated with tumorigenesis and drug resistance. It was hypothesized and demonstrated, in the present study, that mitochondrial dysfunction is the reason for the occurrence and progression of SCLC. mtDNA from drug sensitive and drug insensitive cell lines (H446 and H446/CDDP) was sequenced and compared with the revised Cambridge reference sequence (rCRS). The results revealed that there was no difference in the mtDNA sequence from H446 and H446/CDDP cells, but several spot mutations were observed according to that of rCRs. Further evaluation on mitochondrial function revealed that H446 cells synthesized and secreted more lactic acid and ROS compared with that of H446/CDDP cells when challenged by the same dose of cisplatin (P>0.05). In addition, examination of the mitochondrial apoptotic pathway indicated that more Bax, cleaved caspase-3 and cleaved caspase-9 were expressed in H446 cells compared with that of H446/CDDP cells when stimulated by the same dose of cisplatin (P>0.05). In conclusion, the results of the present study revealed that mtDNA mutations were responsible for the tumorigenesis of SLCL, but not associated with the drug sensitivity of SCLC cell lines. On the other hand, varied mitochondrium content-related mitochondrial dysfunction participated in the MDR of SCLC possibly by affecting the ROS-mediated mitochondrial apoptotic pathway.

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Introduction

Small cell lung cancer (SCLC) accounts for ~15% of all lung cancer cases (1,2); however, increased attention has been paid to this histotype due to its rapid growth and early dissemination to regional lymph nodes and remote organs (3). Chemotherapy has dramatically improved the survival rate of SCLC patients, yet multi-drug resistance (MDR), emerging after chemotherapy, leads to relapse and mortality (4). MDR has become the major clinical obstacle to the treatment of SCLC. New chemotherapeutics and adjustment of chemotherapy programs have not improved the long-term survival rates. Research on the mechanisms of MDR in SCLC has continuously increased since a main treatment for SCLC has not markedly evolved during the past three decades.

To date, it has not been fully elucidated how acquired MDR evolves, yet various meaningful theories and ideas have guided the direction of research efforts. Research has confirmed that MDR is caused by different mechanisms. What is more, there could be more than one mechanism involved in one type of cancer (5,6). Moreover, a theory, known as the 'Warburg effect', concerning energy metabolism reprogramming was introduced and recognized to be closely related to tumorigenesis and cancer progression. Warburg first proposed that cancer cells, different from normal cells, increase their glycolytic activity and reduce mitochondrial respiration whether there is an abundant supply of oxygen or not (7,8). Increasing evidence indicates that numerous molecular mechanisms are involved in the Warburg effect (9), and mitochondria were first to be indicated since this organelle has been so closely related to energy metabolism and cell survival. An abundance

of research indicates that mitochondrial dysfunction resulting from mutation in mitochondrial DNA (mtDNA) is responsible for tumorigenesis (10).

Mitochondrial dysfunction is also believed to provide a survival benefit to cancer cells (11). It has been reported in several cancer types such as prostate (12), kidney (13), breast (14), liver (15), colon and rectal cancer cells that mutations and/or depletion of mtDNA are the reasons for the multi-drug-resistant features (16). More importantly, chemotherapy-induced low levels of mtDNA have been proven to be related to acquired drug resistance and anti-apoptotic properties of cancer cells (17,18). A typical example was that mtDNA mutations and reduced mtDNA copy number in breast and prostate cancer patients were associated with enhanced metastasis and poor patient prognosis (12,17,18). It appears that mitochondrial dysfunction resulting from mtDNA mutation is closely associated with cancer occurrence and progression.However, there is no research clearly illustrating the possible role of mtDNA mutation in tumorigenesis and MDR of SCLC.

Thus, we investigated the sequence of mtDNA from SCLC cell lines, H446 and H446/CDDP, and compared these sequences with the revised Cambridge reference sequence (rCRS) (19) in order to ascertain whether mtDNA mutations participate in the occurrence and acquired MDR of SCLC. After that, the differences in mitochondrial function were evaluated between H446 and H446/CDDP cells. Finally, we investigated the activation of the mitochondrial apoptotic pathway in H446 and H446/CDDP cells challenged by cisplatin in order to illustrate the possible mechanism of the mitochondrial dysfunction in the MDR of SCLC.

Materials and methods

Cell lines and culture. Small cell lung cancer cell line H446 and its multi-drug-resistant cell line H446/CDDP were kindly gifted by Dr Guisheng Qian (Institute of Human Respiratory Disease, Xinqiao Hospital, Third Military Medical University). The H446 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and the MDR properties of H446/CDDP cells were induced by a low dosage of cisplatin. These cells were maintained in RPMI-1640 medium with 10% fetal calf serum (Gibco-BRL, Grand Island, NY, USA) in a humidified atmosphere containing 5% CO_2 at 37˚C. In order to maintain the MDR properties of the H446/CDDP cells, cisplatin (with a final concentration of 0.5 μg/ml) was added to the culture medium for the H446/CDDP cells.

Evaluation of drug resistance. The sensitivity of H446 and H446/CDDP cells to cisplatin was evaluated by a microculture tetrazolium (MTT) assay. Briefly, the cells were seeded in 96-well plates at a density of $1x10^4$ cells/well. The cells were treated with 5 μg/ml cisplatin for 24, 48 and 72 h when grown to confluency. The supernatant was discarded at corresponding time-points, and 20 μl MTT (5 mg/ml, dissolved in PBS and filtered through a 0.22-mm membrane) was added into each well and incubated for 4 h at 37˚C. Finally, the absorption values were determined at 492 nm on an automated Bio-Rad 550 microtiter plate reader.

Extraction of genomic DNA. Genomic DNAs were extracted from the H446 and H446/CDDP cells in a logarithmic growth phase according to the genomic DNA extraction kit instructions (Tiangen Biotechnology Co., Beijing, China). Optical density values of A260 and A280 were determined by using an ultraviolet spectrophotometer in order to evaluate the purity of the DNAs.

Amplification and sequencing of mtDNA. mtDNAs were amplified by using 26 primers (sequences shown in Table I) according to previous research (12). To amplify mtDNA in the genomic DNA samples, 1 μg of diluted genomic DNA was mixed with paired primers (primers were provided by AuGCT Biotechnology Co., Beijing, China). Along with genomic sequences, Premix Taq version 2.0 (Takara Biotechnology Co., Dalian, China) was added into a 20-μl total volume. The mtDNA was amplified under the following conditions: 35 cycles, and the cycling conditions were as follows: 95˚C for 30 sec; 54˚C for 30 sec; and 72˚C for 1 min. After that, the PCR products were purified and sequenced by ABI Prism 3700 sequencing machine.

Mitochondrial ultrastructure examination. Electron microscopy was performed on H446 and H446/CDDP cells in order to investigate whether the difference of mitochondrial structure was related to the varied sensitivity of H446 and H446/CDDP cells to cisplatin. Cell mass was collected by centrifugal method, and 1 μm thick sections were stained to locate significant areas for electron microscopic examination. Ultrathin sections were stained with uranyl acetate, and mitochondria were examined under a TEM-100CX electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan) after the cell mass was post-fixed in osmium tetroxide for 1 h, dehydrated in alcohol, and embedded in epoxy resin.

Mitochondrial staining. MitoTracker probe (Life Technologies Corporation, USA) was used to dye mitochondria in the H446 and H446/CDDP cells, and the procedure was carried out according to the manufacturer's instructions. Briefly, medium was removed from the H446 and H446/CDDP cells in logarithmic growth phase, and the cells were washed 3 times with phosphate-buffered saline (PBS; Life Technologies Corporation). The cells were then incubated with warmed (37˚C) staining solution containing MitoTracker probe (100 nM) for 30 min. After that, the cells were washed again with PBS and examined with an inverted fluorescence microscope (DMI6000B; Leica, Germany) within half an hour.

Detection of lactic acid secretion. In order to determine the metabolic difference of H446 and H446/CDDP cells, the lactic acid content in the cultural medium with or without 5 μg/ml cisplatin stimulation was detected with a lactic acid assay kit (Jiancheng Bioengineering Institute, Nanjing, China) at different time-points. The procedure was carried out according to the manufacturer's instructions, and the data were collected and are expressed as mean ± standard deviation.

Reactive oxygen species (ROS) detection. In order to evaluate the function of mitochondria in the H446 and H446/CDDP cells, ROS levels in the cells with or without cisplatin stimu-

Table I. Sequences of primers for the mtDNAs used in the present research.

Primer pair no.	Primer no.	Sequence 5'-3'	3' combined location	Length (bp)	Overlapping length of two-way sequencing (bp)
1	1F	CTCCTCAAAGCAATACACTG	611	840	202
	1R	TGCTAAATCCACCTTCGACC	1411		
2	2F	CGATCAACCTCACCACCTCT	1245	802	204
	2R	TGGACAACCAGCTATCACCA	2007		
3	3F	GGACTAACCCCTATACCTTCTGC	1854	860	196
	3R	GGCAGGTCAATTTCACTGGT	2669		
4	4F	AAATCTTACCCCGCCTGTTT	2499	887	208
	4R	AGGAATGCCATTGCGATTAG	3346		
5	5F	TACTTCACAAAGCGCCTTCC	3169	832	215
	5R	ATGAAGAATAGGGCGAAGGG	3961		
6	6F	TGGCTCCTTTAACCTCTCCA	3796	898	203
	6R	AAGGATTATGGATGCGGTTG	4854		
7	7F	ACTAATTAATCCCCTGGCCC	4485	975	207
	7R	CCTGGGGTGGGTTTTGTATG	5420		
8	8F	CTAACCGGCTTTTTGCCC	5255	814	201
	8R	ACCTAGAAGGTTGCCTGGCT	6031		
9	9F	GAGGCCTAACCCCTGTCTTT	5855	827	214
	9R	ATTCCGAAGCCTGGTAGGAT	6642		
10	10F	CTCTTCGTCTGATCCGTCCT	6469	886	211
	10R	AGCGAAGGCTTCTCAAATCA	7315		
11	11F	ACGCCAAAATCCATTTCACT	7148	987	205
	11F	CGGGAATTGCATCTGTTTTT	8095		
12	12F	ACGAGTACACCGACTACGGC	7937	900	196
	12R	TGGGTGGTTGGTGTAAATGA	8797		
13	13F	TTTCCCCCTCTATTGATCCC	8621	816	214
	13R	GTGGCCTTGGTATGTGCTTT	9397		
14	14F	CCCACCAATCACATGCCTAT	9230	940	205
	14R	TGTAGCCGTTGAGTTGTGGT	10130		
15-1	15-1F	CTTCTATTGATGAGGGTCTT	9991	670	218
	15-1R	GGTGTTGAGGGTTATGAGA	10622		
15-2	15-2F	AAGGATTAGACTGAACCGAA	10404	627	135
	15-2R	CTGATTGTGAGGGGTAGGA	10992		
15-3	15-3F	CAACCACCCACAGCCTAA	10857	693	198
	15-3R	TTGAGAATGAGTGTGAGGCG	11512		
17	17F	TCACTCTCACTGCCCAAGAA	11314	802	196
	17R	GGAGAATGGGGGATAGGTGT	12076		
18	18F	TATCACTCTCCTACTTACAG	11948	866	166
	18R	AGAAGGTTATAATTCCTACG	12772		
19	19F	AAACAACCCAGCTCTCCCTAA	12571	977	242
	19R	TCGATGATGTGGTCTTTGGA	13507		
20	20F	ACATCTGTACCCACGCCTTC	13338	970	207
	20R	AGAGGGGTCAGGGTTCATTC	14268		
21	21F	GCATAATTAAACTTTACTTC	14000	938	206
	21R	AGAATATTGAGGCGCCATTG	14998		
22	22F	TGAAACTTCGGCTCACTCCT	14856	1,162	180
	22R	AGCTTTGGGTGCTAATGGTG	15978		
23	23F	TCATTGGACAAGTAGCATCC	15811	765	190
	23R	GAGTGGTTAATAGGGTGATAG	5		
24-1	24-1F	CATTATCCCGCACAAGAGTG	16419	420	160
	24-1R	TGGAAAGTGGCTGTGCAGACAT	250		
24-2	24-2F	CTTTGATTCCTGCCTCATCC	132	540	100
	24-2R	TAGAAAGGCTAGGACCAAACCT	652		

Table II. mtDNA mutations and corresponding changes of amino acids.

Cell lines	mtDNA NA type	mtDNA mutations	Influenced proteins	Amino acid changes	tRNA	rRNA
H446	G	G6366A	CO1	V155I	tRNA-Cys	
H446/CDDP		A10086G	ND3	N10D		
		A13105G	ND5	I257V		
		A15311G	cytB	I189V		
		A15824G	cytB	T360A		
		G5773A				

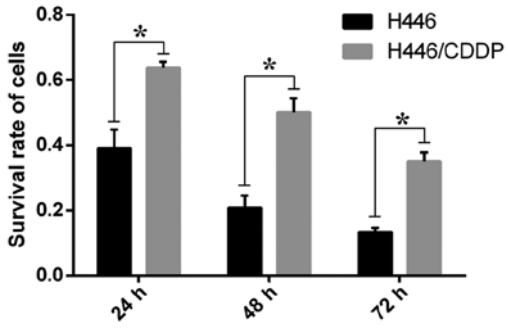

Figure 1. Sensitivity of H446 and H446/CDDP cells to cisplatin was assessed by MTT assay. Cells were treated with 5 μg/ml cisplatin for 24, 48 and 72 h. Data are expressed as means ± SD of three independent experiments. *P<0.01.

lation were detected with DCFH-DA (Beyotime Institute of Biotechnology, China). Briefly, the cells were seeded in laser confocal Petri dishes at a density of 1x10^{4}cells/well and incubated for 48 h. Then, 500 μl of DCFH-DA working liquid (1:1,500 diluted in RPMI-1640 medium and filtered through a 0.22-mm membrane) was added into each plate. Inverted confocal microscope (FV1000 IX81; Olympus) was used to capture the fluorescence intensity every 5 min a time since cells were challenged by cisplatin. Finally, fluorescence intensity in the images were digitalized and analyzed by Image-Pro Plus software.

Analysis of mitochondrial membrane potential (MMP). tetrachloro-tetraethylbenzimidazol carbocyanine iodide (JC-1; Beyotime Institute of Biotechnology) is a mitochondrial-specific cationic dye. It is a monomer when the mitochondrial membrane potential is <120 mV and emits a green light (540 nm) following excitation by blue light (490 nm). When JC-1 is converted to J-aggregates, a red light (590 nm) is emitted following excitation by green light (540 nm) (20). In brief, H446 and H446/CDDP cells were plated at a density of 2x10^5 cells/well in a 24-well plate, and cells were incubated with 5 μM JC-1 for 30 min after stimulation by cisplatin for 24 h in dark at room temperature. Finally, fluorescence was captured with an inverted fluorescence microscope (DMI6000B; Leica), and changes in the fluorescence intensity ratio between the measurements at wavelengths of 590 nm (red) and 540 nm (green) were used to evaluate the MMP.

Western blot analysis. In order to confirm the role of mitochondrial dysfunction in MDR of H446/CDDP cells, activation of the mitochondrial apoptotic pathway was evaluated by western blot analysis. Briefly, H446 and H446/CDDP cells with or without stimulation of cisplatin for 24 h were collected by centrifugation, and the total protein was extracted by a repeated freezing and thawing method. Equal amounts of protein from each group were separated on 12% dodecyl sulfate, sodium salt (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). The membranes were soaked in blocking buffer {5% skimmed milk melted in TBS-T [25 mM Tris (pH 7.6), 138 mM NaCl and 0.05% Tween-20]} for 2 h and then probed with Bax, cleaved caspase-3, cleaved caspase-9 and β-actin (1:1,000-1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies overnight at 4˚C. After that, the membranes were further incubated with anti-rabbit IgG HRP-conjugated secondary antibody (1:5,000). Finally, immune-reactive signals were detected using an ECL detection system (Amersham Pharmacia Biotech).

Statistical analysis. Statistical analysis was performed with SPSS 17.0. Numerical variables are expressed as means ± SD. Statistical differences between the experimental groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Sensitivity of the H446 and H446/CDDP cells to cisplatin. The survival rate of the control group was taken as 1, and the results from the different time groups were compared with the control in order to calculate the relative survival rates. The survival rates of the H446 and H446/CDDP cells treated with 5 μg/ml cisplatin were significantly different (P<0.01) at all three time-points (Fig. 1). The difference in the survival rate between H446 and H446/CDDP cells increased with a decreasing p-value as the time of stimulation was prolonged.

Sequencing results of mtDNA from the H446 and H446/CDDP cells. Amplified mtDNAs of the H446 and H446/CDDP cells were sequenced and compared with rCRS. The mtDNA sequences of the H446 and H446/CDDP cells belonged to haploid type G (Table II), and several specific base mutations were detected in the mtDNA sequence of both H446 and

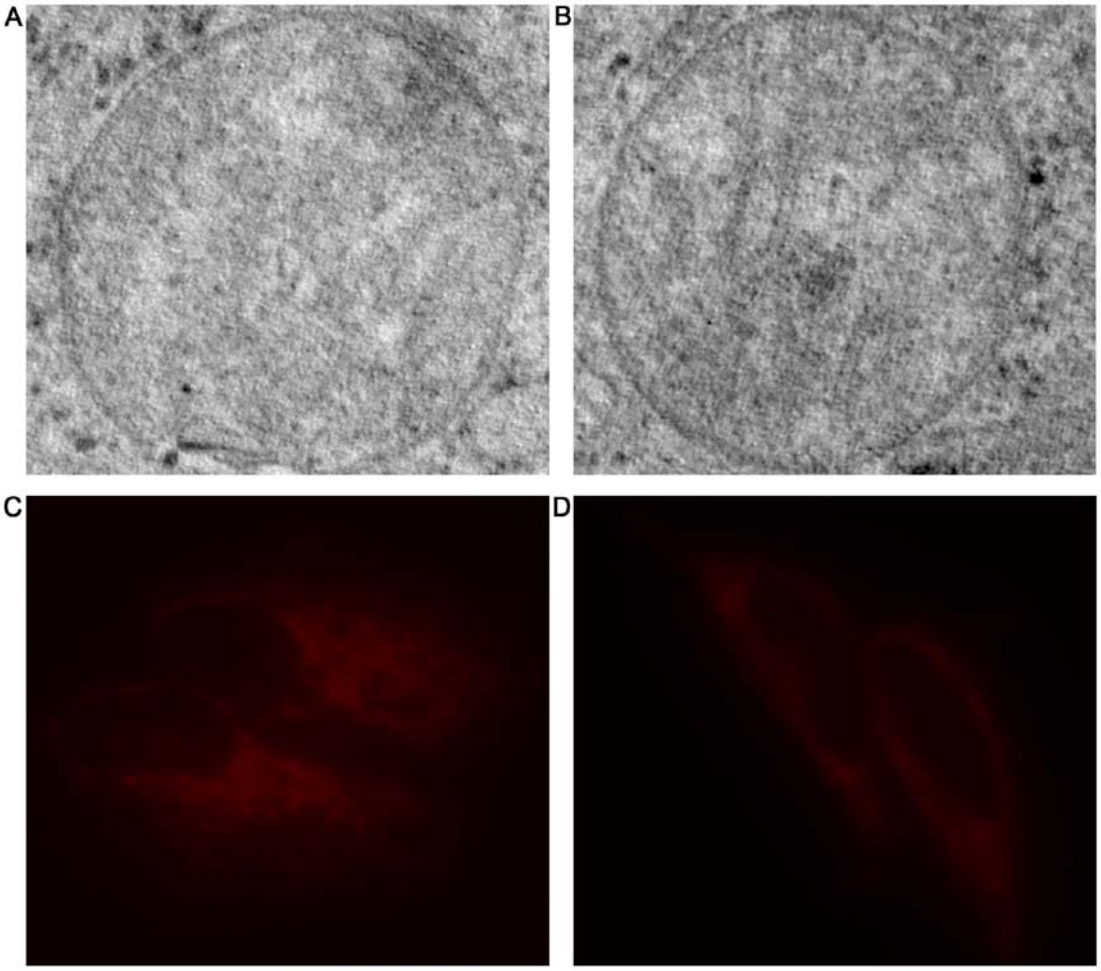

Figure 2. Mitochondrial ultrastructure of H446 (A) and H446/CDDP (B) cells was examined by transmission electron microscopy. Mitochondria in H446 (C) and H446/CDDP (D) cells were stained by mitoTracker probe; fluorescence intensity represents the content of mitochondria.

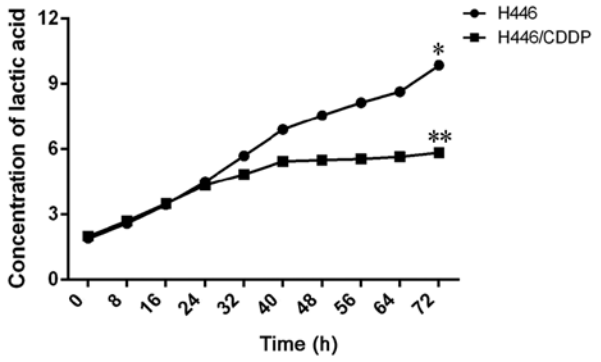

Figure 3. Concentration of lactic acid in the culture supernatant of the H446 and H446/CDDP cells. *P vs. **P<0.01.

H446/CDDP cells, which could be related to types of amino acid protein in the mitochondrial respiratory chain and the secondary structure of tRNAs and rRNAs. It was also found that the mtDNA sequence of the H446 cell line was the same as that of the H446/CDDP cells.

Ultrastructure and content of mitochondria in the H446 and H446/CDDP cells. Since there was no difference in the sequences of mtDNA in the H446 and H446/CDDP cells, we explored the structural and functional differences of mitochondria in these two cell lines. Results from electron microscopic observation (Fig. 2A and B) revealed no difference in the ultrastructure of mitochondria in the H446 and H446/CDDP cells. Then, we compared the number of mitochondria in these two cell lines. Mitochondrial staining results revealed that the fluorescence of mitochondria in the H446 cell line (Fig. 2C) was much stronger than that of the H446/CDDP cells (Fig. 2D).

Lactic acid generation in the H446 and H446/CDDP cells treated with cisplatin. Concentration of lactic acid in the culture supernatant was detected in order to evaluate the metabolic difference in the H446 and H446/CDDP cells. The concentration of lactic acid in the cultural medium from the H446 and H446/CDDP cells increased at about the same speed when stimulated by cisplatin (Fig. 3). However, the generating speed of lactic acid in the H446 cells accelerated 24 h after stimulation and the concentration of lactic acid in the H446 cells was extremely higher than that in the H446/CDDP cells 72 h after of cisplatin treatment (P<0.01).

ROS generation in the H446 and H446/CDDP cells treated with cisplatin. ROS generation in the H446 and H446/CDDP

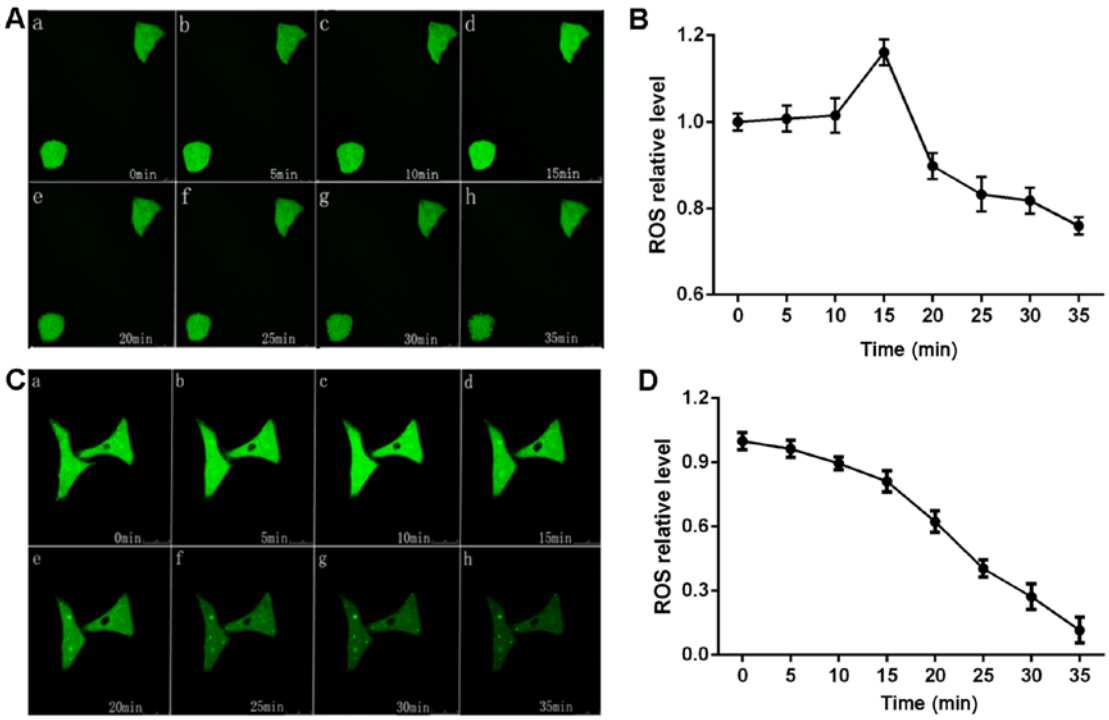

Figure 4. Trends for ROS generation in the (A and B) H446 and (C and D) H446/CDDP cells following treatment with cisplatin.

cells was detected so as to evaluate the ability of these two cell lines to cope with oxidative stress induced by cisplatin. The ROS content in the H446 cells increased and peaked at 15 min after being stimulated by cisplatin, and then the ROS content slowly decreased (Fig. 4). Meanwhile, H446 cells became shrunken after being stimulated and it was most obvious 35 min after treatment of cisplatin. On the other hand, ROS in the H446/CDDP cells decreased at a relatively stable speed when stimulated with cisplatin and there was no shrinking by this cell line upon stimulation with cisplatin.

Depolarization of the MMP in the H446 and H446/CDDP cells. ROS generation is closely related to depolarization of the MMP which may further lead to cell apoptosis. Depolarization of MMP in H446 and H446/CDDP cells stimulated by cisplatin was evaluated by cationic dye JC-1. The green light became stronger in both the H446 and H446/CDDP cells challenged by cisplatin (Fig. 5). But much stronger green light against red light was detected in the H446 cells compared with that of the H446/CDDP cells, which meant that reduction in the MMP in the H446 cell line was much more obvious than that of the H446/CDDP cells stimulated by 5 μg/ml cisplatin.

Expression of the mitochondrial apoptosis pathway in the H446 and H446/CDDP cells treated with cisplatin. Expression of the mitochondrial apoptosis pathway in the H446 and H446/CDDP cells treated with cisplatin was evaluated by western blotting. Expression of Bax, cleaved caspase-3 and cleaved caspase-9 was increased in both the H446 and H446/CDDP cells when challenged by cisplatin (Fig. 6). However, expression levels of these molecules in the H446/CDDP cells were much lower than levels in the H446 cells ($P<0.05$).

Discussion

Mitochondria are intracellular semi-autonomous organelles, which possess a self-owned genome and participate in energy metabolism and homeostasis (21). Human mtDNA is a 16.6-kb double-stranded, circular DNA molecule encoding for 13 complex polypeptides belonging to the mitochondrial respiratory chain, 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) (21). Since mtDNA is essential for cells, the accumulation of mtDNA mutations and/or reset of mtDNA copy number are believed to affect energy production, cell proliferation as well as cell apoptosis (21,22).

It was first investigated, in the present study, whether mtDNA mutations are associated with the tumorigenesis of SCLC and the MDR phenotype of H446/CDDP cells. mtDNAs from H446 and H446/CDDP cells were amplified, sequenced and compared with rCRS. The results revealed that several spot mutations existed in the mtDNA of SCLC cell lines, which led to changes of amino acids in mtDNA-encoded proteins and base-shifts in rRNA. Astonishingly, the sequences of mtDNA in the H446 cells were the same as that of the MDR cell line H446/CDDP, which meant that mtDNA mutation in sequences was not related to the MDR characteristic of the H446/CDDP cells. Research indicates that both mutations in mtDNA sequences and low mtDNA copy number are closely related to the dysfunction of mitochondria. A reduced mtDNA content could result in impaired mitochondrial respiration and depolarization of the mitochondrial membrane (23). We then detected the mitochondrial content in the H446 and H446/CDDP cells, and found that the fluorescence representing the content of mitochondria in the H446 cells was much stronger than that of H446/CDDP cells. This result was consistent with previous reports indicating that varied mtDNA

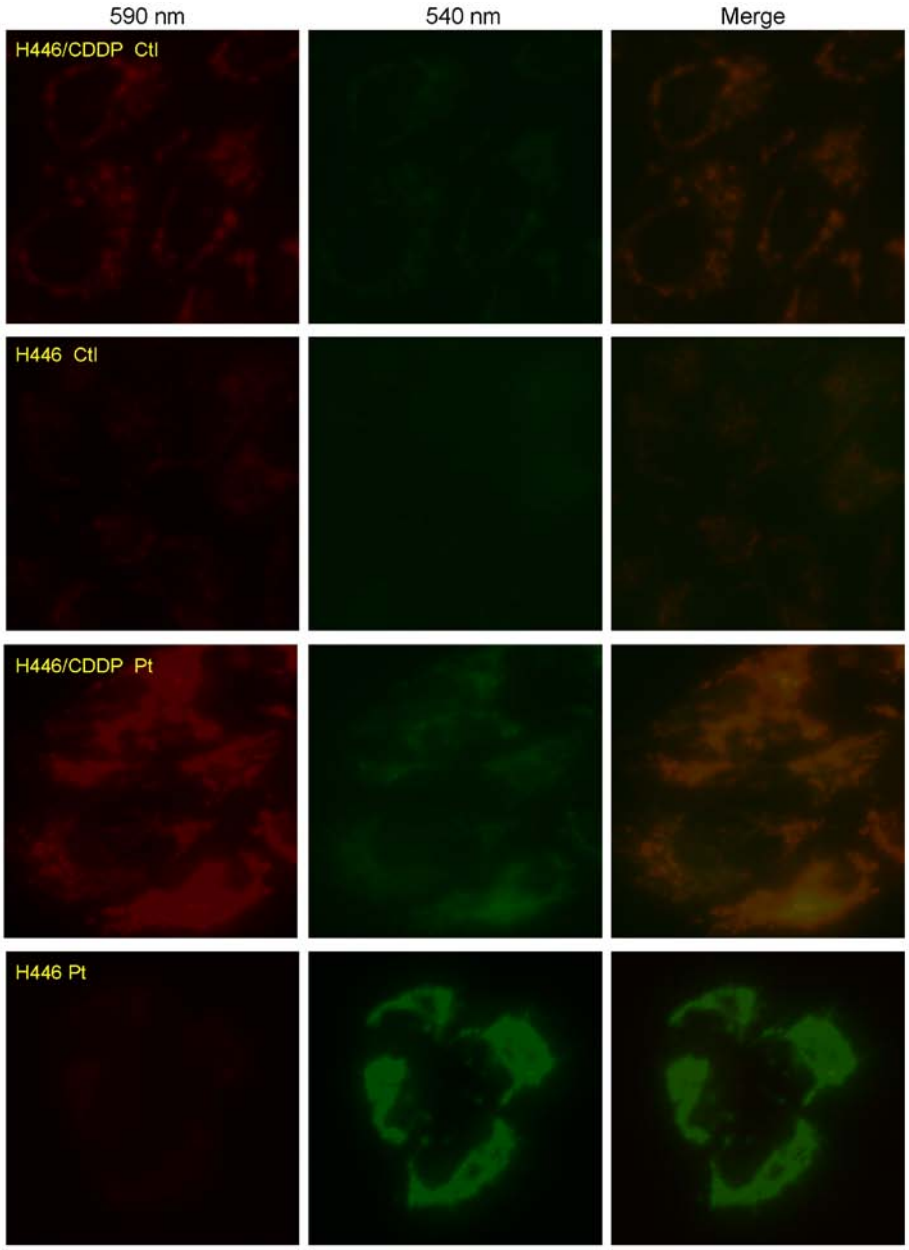

Figure 5. Depolarization of the mitochondrial membrane potential (MMP) in the H446 and H446/CDDP cells was evaluated by JC-1. Changes in the fluorescence intensity ratio between green light (540 nm) and red light (590 nm) were used to evaluate MMP.

content markedly increases the risk of lymph node metastasis and high mortality of patients with late-stage tumors (24).

Based on the findings that mutations in mtDNA sequences are responsible for the tumorigenesis of SCLC and varied mitochondrial content in single cells is related to drug sensitivity of H466 and H446/CDDP cells, we wondered how this varied content of mitochondria in the H446 and H446/CDDP cells influenced their sensitivity to cisplatin. As known, mitochondria possess many biological functions, including ATP generation, production of ROS, providing reaction sites for many biological processes and modulating cell apoptosis (25). Evidence also suggests that an energy generation-related 'Warburg effect' may be blamed for the survival benefit of cancer cells (26,27). Lactic acid is an important metabolite of glycolytic activity which could be taken as a marker of anaerobic glycolysis. Generation and secretion of lactic acid from H446 and H446/CDDP cells were evaluated in order to assess their difference in glycolytic activity. Results showed that the H446/CDDP cells generated less lactic acid than that of H446 cells when stimulated by the same dosage of cisplatin, which indicated that a stronger ability of lactic acid removal benefited cancer cell survival.

Then we wondered whether this difference in energy generation was associated with the biological behavior of H446 and H446/CDDP cells. Naito *et al* (28) reported that mtDNA depletion promotes the chemoresistance of breast cancer cells. In addition, HeLa cells lacking or without mtDNA have been confirmed to become resistant to adriamycin and photodynamics (29). More importantly, Park *et al* (30) showed that hepatoma cells deprived of mtDNA were less sensitive to hydrogen peroxide and to ROS-inducing agents and further research illustrated that this drug-resistant phenotype was associated with increased expression of antioxidant enzymes manganese superoxide dismutase and glutathione peroxidase.

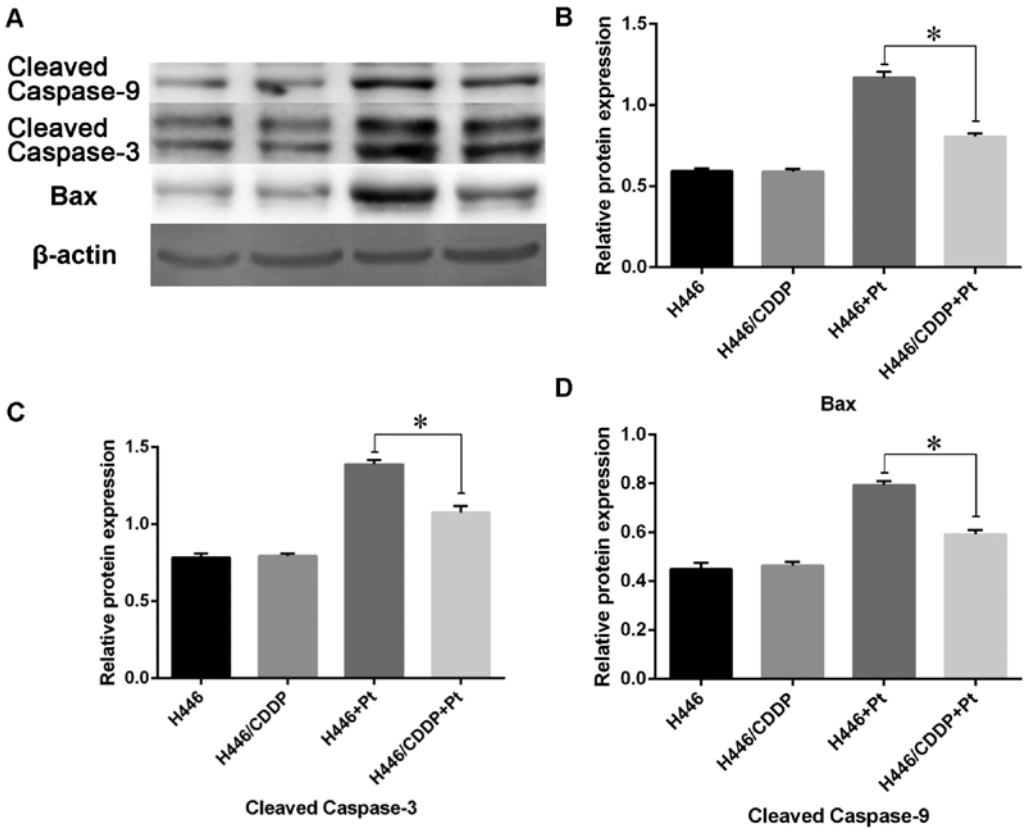

Figure 6. Evaluation of apoptotic regulators in the H446 and H446/CDDP cells stimulated by cisplatin. (A) After protein quantitation, western blot analysis was performed to investigate the contents of Bax, cleaved caspase-3 and cleaved caspase-9. (B-D) The ratios of these proteins vs. β-actin in three independent experiments were obtained by densitometric scanning using an image analysis system. *P<0.05.

Based on this knowledge, we investigated the production of ROS in H446 and H446/CDDP cells stimulated by cisplatin, and the results showed that more ROS were produced in the H446 cells when challenged by cisplatin compared with that of the H446/CDDP cells. Our findings indicated an upgraded antioxidant capacity in H446/CDDP cells which was also consistent with previous research illustrating that the insensitivity of cancer cells to chemotherapeutic drugs paclitaxel and cisplatin is associated with increased antioxidant expression levels (31,32).

Varied mitochondrial content, difference in lactic acid secretion and ROS generation were confirmed to be related to the discriminative sensitivity of H446 and H446/CDDP cells to cisplatin. While the mechanisms of how those differences participate in the MDR of SCLC remain unknown. As we know, mitochondria are crucial centers of metabolism and ROS production (33). An excess of ROS induces mitochondrial inner membrane permeabilization leading to mitochondrial depolarization, swelling, cytochrome *c* release and subsequent apoptotic death (34). We further evaluated the activation of the mitochondrial apoptotic pathway in H446 and H446/CDDP cells challenged by cisplatin. Molecules of the Bcl-2 family were evaluated since they are closely related to the mitochondrial apoptotic pathway, and their abnormal expression leads to loss of $\Delta\Psi m$, leakage of apoptogenic proteins, activation of caspase-3, and finally cell death (35). Evaluation of the mitochondrial-mediated cell apoptosis pathway showed that cell apoptosis-promoting proteins, Bax, cleaved caspase-3 and cleaved caspase-9, were increased in the H446 cells when compared with the levels in the H446/CDDP cells following stimulation by cisplatin. This indicated that abnormal generation of ROS affected the sensitivity of H446 and H446/CDDP cells to cisplatin probably by interfering with the mitochondrial apoptotic pathway.

In conclusion, mtDNAs from small cell lung cancer cell line H446 and its multi-drug-resistant cell line H446/CDDP were sequenced and compared with rCRS. The results revealed that mtDNA mutations were responsible for the tumorigenesis but not for the MDR capacity of SCLC. On the other hand, a difference in lactic acid and ROS generation confirmed that mitochondrial dysfunction participated in the MDR of SCLC. Finally, evaluation of the ROS-related mitochondrial apoptotic pathway in H446 and H446/CDDP cells challenged by cisplatin explained, at least to some extent, that mitochondrial dysfunction participated in the MDR of SCLC.

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