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Reverse effect of curcumin on CDDP-induced drug-resistance via Keap1/p62-Nrf2 signaling in A549/CDDP cell

Jie Shen[1,2,3,4,#], Ya-Juan Chen[2,#], Yuan-Wei Jia[1,2,3], Wen-Ying Zhao[5], Guang-Hai Chen[2], Ding-Feng Liu[2], Yun-Yu Chen[2,4], Chao Zhang[2,4], Xiao-Ping Liu[2,4✉]

[1]*Anhui Provincial Center for Drug Clinical Evaluation, Yijishan Hospital, Wannan Medical College, Wuhu, Anhui 241001, China*

[2]*Pharmacy School, Wannan Medical College, Wuhu, Anhui 241002, China*

[3]*Department of Clinical Pharmacy, Yijishan Hospital, Wannan Medical College, Wuhu, Anhui 241001, China*

[4]*Anhui Provincial Engineering Research Center for Polysaccharides Drugs, Wannan Medical College, Wuhu, Anhui 241001, China*

[5]*Oncology Department, Yijishan Hospital, Wannan Medical College, Wuhu, Anhui 241001, China*

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ABSTRACT

Objective: To assess the effect of curcumin on CDDP-induced drug resistance and explore the underlying molecular mechanism through Nrf2 system and autophagy pathway.

Methods: A drug-resistant cell model was established by exposing A549/CDDP cell to 2 μg/mL CDDP. A549/CDDP cell was treated with 20 μg/mL CDDP and 10 μM curcumin. The cell viability and apoptosis level, the signals of Keap1/P62-Nrf2 and autophagy pathway were analyzed.

Results: CDDP induction promoted drug-resistant phenotype in A549/CDDP cell and activated autophagy as well as Nrf2 signals in A549/CDDP cell. Meanwhile, curcumin combination attenuated autophagy and Nrf2 activation induced by CDDP, and reversed the drug-resistant phenotype. Notably, curcumin combination augmented Keap1 transcription. Furthermore, Keap1 ablation with short hairpin RNAs hampered the efficacy of curcumin, suggesting Keap1 played a crucial role on reversal effect of curcumin.

Conclusions: The present findings demonstrate that CDDP promotes abnormal activation of Nrf2 pathway and autophagy, leading to drug resistance of A549/CDDP cell. Curcumin attenuates this process and combat drug-resistance through its potent activation on Keap1 transcription, which is essential for interplay between oxidative stress induced Nrf2 activation and autophagy/apoptosis switch.

1. Introduction

Chemoresistance proves a tough obstacle to CDDP cisplatin (CDDP) based regimen, the backbone of chemotherapy for human tumors including lung cancer [1,2]. Inspiringly, the ongoing development of natural polyphenols as novel chemosensitizers has opened up new opportunities to combat chemoresistance for us [3]. Curcumin, a natural polyphenolic compound derived from spice turmeric (*Curcuma longa*) and favored by Asian as dietary ingredients, had been demonstrated to enhance chemotherapy efficacy through regulating nuclear factor erythroid 2-related factor 2 (Nrf2) activation [3,4], which regulates reactive oxygen species (ROS) to response to cellular redox by activating transcription of antioxidant genes, including phase II detoxifying enzymes and other stress response proteins.

Recently, this polyphenol has also been revealed to induce autophagic cell death [5–7]. Moreover, both autophagy and Nrf2 activation were essential for cancer cells to survive against oxidative stress and to promote chemoresistance [8,9]. Importantly, Nrf2-ARE system activation had been associated with deregulation of autophagy induced by oxidative stress [10,11].

First author: Jie Shen, Department of Clinical Pharmacy, Yijishan Hospital, Wannan Medical College, Wuhu, Anhui 241001, China.
Tel: +86 553 5738200.
E-mail: jie_shen23@aliyun.com
✉Corresponding author: Xiao-Ping Liu, Pharmacy School, Wannan Medical College, Wuhu, Anhui 241002, China.
Tel: +86 553 3932601
E-mail: lxpwmc@126.com

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[#] Jie Shen and Ya-Juan Chen contributed equally to this work.

Curcumin might sensitize CDDP through repression of Nrf2-ARE system activation, and its relation with autophagy has also been revealed. Therefore, we hypothesized that curcumin might reverse chemoresistance by regulating cell responses to oxidative stress and autophagy. In our study, we employed CDDP-induced human lung adenocarcinoma cell lines (A549/CDDP) as a model to investigate autophagy and Nrf2 signaling, and observe the effects of curcumin on drug-resistant phenotype.

2. Materials and methods

2.1. Cell culture

The human lung adenocarcinoma cell A549 and the CDDP-resistant clones A549/CDDP cell line were cultured at 37 °C in a 5% CO_2 and 95% air atmosphere, in DMEM (HyClone, USA) supplemented with 10% FBS (HyClone), 100 U/mL penicillin and 100 U/mL streptomycin. A549/CDDP cell was maintained with 2 μg/mL CDDP (Sigma–Aldrich, St. Louis, MO, USA). Curcumin (curcuminoid content ≥ 94%, curcumin ≥80%), and 3-Methyladenine (3-MA, M9281) were obtained from Sigma–Aldrich.

In order to investigate the autophagy signaling of A549/CDDP cell exposed to CDDP induction, time course of conversion of microtubule-associated protein light-chain 3 (LC3) which is a biochemical marker for autophagy was detected. A549/CDDP cell was exposed to 20 μg/mL CDDP for 4, 12, 24, and 48 h, respectively. A549/CDDP cell were treated with different doses of curcumin for 24 h, 10 μM was identified as a low concentration of curcumin based on the cell viability assay. To clarify the effect of curcumin on CDDP induced drug-resistance, A549/CDDP cells were treated with increasing doses of CDDP for 24 h, in combination with 10 μM curcumin, and with pretreatment of autophagy inhibitor 3-MA (2 mM), respectively. For pathway study, A549/CDDP cell was treated with DMEM as control, CDDP (20 μg/mL unless otherwise stated), curcumin (10 μM unless otherwise stated), CDDP + curcumin, CDDP + 3-MA (2 mM), respectively.

2.2. Cell viability assays

The cell viability was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method. In brief, exponentially growing cells were plated in 96-well plates (1×10^4 cells per well). Then indicated concentrations of CDDP were added, and incubation proceeded for 24 h. To each well, 20 μL MTT (Beyotime Biotech, Beijing, China) was added followed by incubation for 4 h, and then 150 μL dimethyl sulfoxide (Beyotime) was added to dissolve the formazan crystals. Absorbance was measured in Multiskan Go microplate spectrophotometer (Thermo Fisher Scientific, Vantaa, Finland) at a wavelength of 570 nm. The inhibition rate was calculated as follows: $(1 - A_{exp\ group}/A_{control}) \times 100\%$.

2.3. Real time quantitative PCR determination

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription of 2 μg of RNA was done using oligo (dT) primers. Real time quantitative PCR were determinated on StepOne Plus Real time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) with 20 ng of cDNA, together with QuantiNova SYBR Green PCR Master mix (Qiagen, Germany) and specific primers (Table 1). The expression of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was measured for comparative reference. The mRNA relative quantitation was calculated using the ΔΔCt method.

2.4. Western blot analysis

Cells were washed and lysed in ice-cold radio-immunoprecipitation lysis buffer (Beyotime). Cell lysates were sonicated and centrifuged to remove debris. Protein concentrations were determined using the Protein Assay Kit (Beyotime).

For western blot analysis, protein samples (30 μg) were separated on a 10% w/v SDS-PAGE gel by electrophoresis, then transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). Membranes were incubated with the desired primary antibody overnight at 4 °C, and then with corresponding secondary antibody. Antibodies against Keap1, Nrf2, p62/SQSTM1(P62), LC3-I, LC3-II, NQO1, Lamin B, and actin were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). Immunoreactive bands were visualized using the diaminobenzene coloration method. The representative bands were determined with Chemiscope 3000 gel image system (Clinx Science Instruments, Shanghai, China) and analyzed.

2.5. Keap1 knockdown by lentivirus short hairpin RNA (shRNA)

In order to explore the role of Keap1 in synergistic effect of curcumin, cultured cells were transfected with designed shRNA for Keap1, three of four independently designed shRNAs (Table 2) against Keap1. Lentivirus shRNA targeting human Keap1 (GenBank accession NM_203500) of A549/CDDP were screened and constructed by Genechem (Shanghai, China). Knockdown with lentivirus was performed according to the manufacturer's instruction. In brief, A549/CDDP cells were seeded into 6-well plates, and infected on the following day with quantitative lentivirus granula. The cell infection rate was identified to above 80% according to green fluorescent protein fluorescence intensity observed under fluorescence microscope after 3 days of infection. Cells were harvested and whole cell lysates were prepared for western blot. For the apoptosis level assay, infected cells were disposed on combination of CDDP and curcumin for 24 h and subjected to the flow cytometry assay to determine cell apoptosis level.

Table 1
Primer sequences used in real-time PCR assays.

Gene	Sequence (5′–3′)
Nrf2	(F) ATAGCTGAGCCCAGTATC
	(R) CATGCACGTGAGTGCTCT
Keap1	(F) CTGGTATCTGAAACCCGTCTA
	(R) TGGCTTCTAATGCCCTGA
NQO1	(F) CAGTGGTTTGGAGTCCCTGCC
	(R) TCCCCGTGGATCCCTTGCAG
P62	(F) ATCGGAGGATCCGAGTGT
	(R) TGGCTGTGAGCTGCTCTT
GAPDH	(F) AGAAGGCTGGGGCTCATTTG
	(R) AGGGGCCATCCACAGTCTTC