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# Dihydroartemisinin, an artemisinin derivative, reverses oxaliplatin resistance in human colorectal cancer cells by regulating the SIRT3/PI3K/AKT signalling pathway.

Xiaodong Shen, Chencheng Shi, Ming Lei, Rongjian Zhou, Shaoqun Liu and Chang Su

Department of Gastrointestinal Surgery, Minhang Hospital Affiliated to Fudan University, Shanghai, China.

**Keywords:** dihydroartemisinin; SIRT3; oxaliplatin; colorectal cancer.

**Abstract.** Dihydroartemisinin (DHA), a derivative of artemisinin, has been shown to act as a chemosensitizer of various cancer chemotherapeutic agents both *in vitro* and *in vivo*. However, in colorectal cancer (CRC), no study has focused on the effect of DHA on oxaliplatin (L-OHP) resistance. Our study aimed to examine the effectiveness of DHA in reversing the resistance of human CRC cells to L-OHP, as well as its underlying molecular mechanisms. LoVo cells were purchased from ATCC, while LoVo/L-OHP cells were obtained by exposing LoVo cells to progressively increasing concentrations of L-OHP. LoVo/L-OHP were treated with various concentrations of DHA, and cell apoptosis ratio and viability were assessed by flow cytometry and CCK-8. Our results showed that DHA treatment remarkably decreased the viability of LoVo/L-OHP cells and increased the apoptosis ratio. As the mechanism of action, we found that DHA enhanced the expression of Sirtuin 3 (SIRT3) and suppressed the phosphatidylinositol 3-kinase (PI3K)/AKT signalling cascade. Silencing of SIRT3 reversed the effect of DHA on cell apoptosis and viability by activating the PI3K/AKT axis in LoVo/L-OHP cells. Overall, our study found that DHA has the ability to counteract L-OHP resistance in LoVo/L-OHP cells through the modulation of the SIRT3/PI3K/AKT signalling pathway, suggesting a new research target for CRC treatment.

## **La dihidroartemisinina, un derivado de la artemisinina, revierte la resistencia al oxaliplatino en células humanas de cáncer colorrectal mediante la regulación de la vía de señalización SIRT3/PI3K/AKT.**

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**Palabras clave:** dihidroartemisinina; SIRT3; oxaliplatino; cáncer colorrectal.

**Resumen.** Se ha demostrado que la dihidroartemisinina (DHA), un derivado de la artemisinina, actúa como quimiosensibilizador de diversos agentes quimioterapéuticos contra el cáncer tanto *in vitro* como *in vivo*. Sin embargo, en el cáncer colorrectal (CCR), ningún estudio se ha centrado en el efecto del DHA sobre la resistencia al oxaliplatino (L-OHP). El objetivo de nuestro estudio era examinar la eficacia del DHA para revertir la resistencia de las células humanas de CCR al L-OHP, así como sus mecanismos moleculares subyacentes. Las células LoVo se adquirieron a ATCC, mientras que las células LoVo/L-OHP se obtuvieron exponiendo las células LoVo a concentraciones progresivamente crecientes de L-OHP. Las células LoVo/L-OHP se trataron con diversas concentraciones de DHA, y el índice de apoptosis celular y la viabilidad se evaluaron mediante citometría de flujo y CCK-8. Nuestros resultados mostraron que el tratamiento con DHA disminuía notablemente la viabilidad de las células LoVo/L-OHP y aumentaba el índice de apoptosis. Desde el punto de vista de su mecanismo de acción, se observó que el DHA potenciaba la expresión de la sirtuina 3 (SIRT3) y suprimía la cascada de señalización fosfatidilinositol 3-cinasa (PI3K)/AKT. El silenciamiento de SIRT3 revirtió el efecto del DHA sobre la apoptosis y la viabilidad celular a través de la activación del eje PI3K/AKT en las células LoVo/L-OHP. En conjunto, nuestro estudio descubrió la capacidad del DHA para contrarrestar la resistencia a L-OHP en células LoVo/L-OHP a través de la modulación de la vía de señalización SIRT3/PI3K/AKT, lo cual sugiere una nueva foco de investigación para el tratamiento del CCR.

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### **INTRODUCTION**

According to the 2020 GLOBOCAN statistics on the incidence and mortality rates of 36 types of cancer worldwide, colorectal cancer (CRC) ranks third in terms of incidence (10.0%) but second in terms of mortality rate (9.4%)<sup>1</sup>. Chemotherapy based on oxaliplatin (L-OHP) is a crucial component of the comprehensive treatment for CRC, which can prolong the survival of patients

and improve their quality of life<sup>2-4</sup>. However, primary and/or acquired resistance to L-OHP remains a significant factor limiting the effectiveness of CRC treatment<sup>5,6</sup>. Therefore, it is urgent to clarify the mechanisms of L-OHP resistance in CRC and to find corresponding resistance reversal agents or chemosensitizers.

Previous studies have demonstrated that the mechanisms of resistance to platinum-based chemotherapy drugs may be re-

lated to the abnormal regulation of signaling networks within tumor cells caused by the sustained action of drugs. For example, abnormalities in signalling pathways such as PI3K/AKT and NF- $\kappa$ B give the cells strong anti-apoptotic abilities, leading to resistance to chemotherapy drugs<sup>7,8</sup>. Sirtuin 3 (Sirt3) plays a crucial role in controlling physiological and pathological processes, such as regulating inflammation, oxidative stress, cell proliferation, differentiation, and apoptosis. This protein belongs to the sirtuin protein family, known for its diverse biological functions<sup>9-11</sup>. Research has found that SIRT3 can cause mutant p53 to be deacetylated, leading to cancer cell apoptosis and increased sensitivity to chemotherapeutic drugs<sup>12</sup>. Other evidence also suggested that SIRT3 could improve disease conditions by regulating apoptosis and utilizing the PI3K/Akt pathway in various diseases<sup>13,14</sup>. Dihydroartemisinin (DHA) is a derivative of artemisinin, and there is evidence that DHA can act as a chemosensitizer for various cancer chemotherapeutic drugs both *in vitro* and *in vivo*<sup>15</sup>. The latest research has found that DHA can alleviate acute and chronic pain by increasing the activity of SIRT3<sup>16</sup>. However, in CRC, no study has focused on the effect of DHA on L-OHP resistance through the SIRT3/PI3K/AKT axis.

Based on previous studies, we hypothesize that DHA may affect the resistance of human CRC cells to L-OHP by regulating the SIRT3/PI3K/AKT axis. Our study aimed to examine the effectiveness of DHA in reversing the resistance of human colorectal cancer cells to L-OHP, as well as its underlying molecular mechanisms.

## METHOD

### Cell culture and induction of L-OHP resistance

Human CRC cells (LoVo, not resistant to L-OHP) were obtained from the American Type Culture Collection (CCL-229, ATCC) in Manassas, Virginia. The human L-OHP-

resistant CRC cells (LoVo/L-OHP, resistant to L-OHP) were obtained by exposing LoVo cells to progressively increased concentrations of L-OHP<sup>17</sup>. The initial concentration of L-OHP was 5  $\mu$ M, and cells were exposed to L-OHP for two days in each cycle, followed by recovery in L-OHP-free culture medium. The same concentration of L-OHP was repeated for three cycles. After completing three cycles at the same concentration of L-OHP stimulation, the dose was escalated. This procedure was repeated until the final concentration of L-OHP reached 70  $\mu$ M. Only cells that remained resistant to L-OHP after being cultured in an L-OHP-free medium for at least six months were used for subsequent experiments.

The LoVo/L-OHP and LoVo cells were cultured at 37°C and 5% CO<sub>2</sub> in RPMI-1640 medium (Thermo Fisher Scientific, Inc., MA, USA) that was supplemented with 10% Gibco® fetal bovine serum (FBS) and 100  $\mu$ g/mL penicillin-streptomycin. Cells were treated with PI3K inhibitor LY294002 (1 $\mu$ M) for 24 hours to inhibit PI3K signaling.

### Preparation of Dihydroartemisinin Solution

A stock solution of dihydroartemisinin (obtained from Puyi Biological, Nanjing, China) was prepared by dissolving it in a small quantity of dimethyl sulfoxide (DMSO, procured from Sigma-Aldrich, USA). The resulting stock solution was formulated to have a concentration of either 100 or 300 nmol/L, which was sonicated and stored at 4°C. In subsequent experiments, all cell samples were treated with 30  $\mu$ M L-OHP or an equivalent amount phosphate-buffered saline (PBS) for treatment.

### Cell transfection

The siRNA targeting the SIRT3 gene (si-SIRT3#1, #2, #3) and the negative control (NC) were procured from GeneChem Corp. for cell transfection experiments.

According to the manufacturer's instructions, the LoVo/L-OHP cells were transfected with 5 nM of the aforementioned si-RNA or negative controls. Transfection was carried out by using the lipofectamine 3000 (Invitrogen) reagent, following the prescribed protocol, while incubating the cells in a serum-free Opti-MEM medium. After 48 hours of transfection, the transfection efficiency was evaluated through RT-qPCR.

### Cell Counting Kit-8 (CCK-8)

LoVo, LoVo/L-OHP, and LoVo/L-OHP+DHA (100nmol/L or 300nmol/L) cells were treated with different concentrations of OHP (5 $\mu$ M-70 $\mu$ M)<sup>17,18</sup>. Cells were cultured into 96-well plates at 5 $\times$ 10<sup>3</sup> cells/well density. After incubating the cells with CCK-8 reagent (10 $\mu$ L, Sangon) for 2 hours, the absorbance at 450 nm was determined on a microplate reader (Thermo Fisher Scientific, MA, USA). The half-maximal inhibitory concentration (IC<sub>50</sub>) of L-OHP was determined in LoVo and LoVo/L-OHP cells<sup>19</sup>.

### Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was used to determine the expression of PI3K, SIRT3 and AKT in the cells. We utilized the RNAiso plus kit (procured from Takara, Japan) to obtain RNA from cells and subsequently performed reverse transcription into cDNA using the Prime-Script™ one-step qRT-PCR kit (obtained from Takara, Dalian, China). For RT-qPCR, we employed the SYBR Premix ExTaq (TaKaRa) and the ABI PRISM7300 Sequence Detection System (from Applied Biosystems). Primer sequences are shown in Table 1. GAPDH served as an internal control. The mRNA expressions were calculated by the 2<sup>- $\Delta\Delta$ Ct</sup> method.

### Western blot

Total proteins were extracted from the cell lysates and quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific).

**Table 1**  
The sequences of all constructs.

Name	Sequence
SIRT3	Forward 5'-CCCCAAGCCCTTTTTCACCTT-3'
	Reverse 5'-CGACACTCTCTCAAGCCCA-3'
PI3K	Forward 5'-TCTAAACCCTGCTCATC-3'
	Reverse 5'-CTTGCCGTAATCATCCC-3'
AKT	Forward 5'-CTACAACCAGGACCATGAGAAG-3'
	Reverse 5'-ACACGATACCGGCAAAGAAG-3'
GAPDH	Forward 5'-CACCCACTCCTCCACCTTTG-3'
	Reverse 5'-CCACCACCCTGTTGCTGTAG-3'

Then, 20  $\mu$ g of the extracted proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The PVDF membranes were then incubated with a primary antibody at 4°C overnight. After washing, the membranes were incubated with Goat Anti-Rabbit IgG H&L secondary antibody (ab96899, 1/1000) at 37°C for 45 minutes. The primary antibodies included the SIRT3 antibody ab217319, 1/1000, Abcam), PI3K antibody (ab140307, 1/2000, Abcam), AKT antibody (ab8805, 1/1000, Abcam), bcl-2 antibody (ab32124, 1/1000, Abcam), Bax antibody (ab32503, 1/1000, Abcam), cleaved caspase-3 antibody (ab32042, 1/5000, Abcam), P-gb (ab202976, 1/1000, Abcam), GAPDH antibody (ab8245, 1/1000, Mybiosource), and GAPDH was served as an internal control. We utilized the Pierce ECL Western Blotting Substrate to visualize the protein bands, which was procured from Pierce in Shanghai, China. The membranes were developed and imaged using a chemiluminescence imaging system.

### Flow cytometry for cell apoptosis

Cells were incubated at 37°C with 5% CO<sub>2</sub>. After the incubation of 24 h, LoVo/L-OHP cells were collected into 1.5 mL tubes with annexin-FITC and propidium iodide (PI) reagents, followed by incubation for 10 min. To detect cell apoptosis, 200  $\mu$ L PI was supplemented with 1 mL phosphate buffer

solution (PBS) into the flow tube. Then, the apoptotic cells were measured using flow cytometry (Beckman Coulter).

### Statistical analysis

The data is presented as mean  $\pm$  SD, and we utilized one-way ANOVA followed by a Tukey post hoc test for statistical comparisons. For significance, a p-value less than 0.05 was considered.

## RESULTS

### DHA inhibited the proliferation of LoVo/L-OHP cells and induced their apoptosis

We first treated LoVo/L-OHP cells with different concentrations (100 nmol/L or 300 nmol/L) of DHA then used the CCK-8 assay to detect the IC<sub>50</sub> of all cells. As shown in Fig. 1-A, the IC<sub>50</sub> of LoVo/L-OHP cells to L-OHP remarkably declined ( $p < 0.05$ ) after DHA treatment. In the next experiment, we treated LoVo/L-OHP cells with 30  $\mu$ M L-OHP or an equal amount of phosphate-buffered saline (PBS) and investigated the changes in cell viability of LoVo/L-OHP cells after DHA treatment. Our findings demonstrated a notable decline in cell viability of LoVo/L-OHP cells following DHA treatment ( $p < 0.05$ , as depicted in Fig. 1-B). Moreover, flow cytometry analysis substantiated a marked rise in apoptotic cells in LoVo/L-OHP cells when treated with 300 nmol/L DHA ( $p < 0.05$ , as illustrated in Fig. 1-C). Furthermore, compared with the LoVo/L-OHP group, the expression of cleaved caspase-3 protein and the Bax/Bcl-2 ratio in the L-OHP +300 nmol/L DHA and L-OHP +100nmol/L DHA groups were remarkably increased, while the expression of P-gb protein was markedly decreased ( $p < 0.05$ , Fig. 1-D). In the subsequent experiments, cells were treated with 300 nmol/L of DHA.

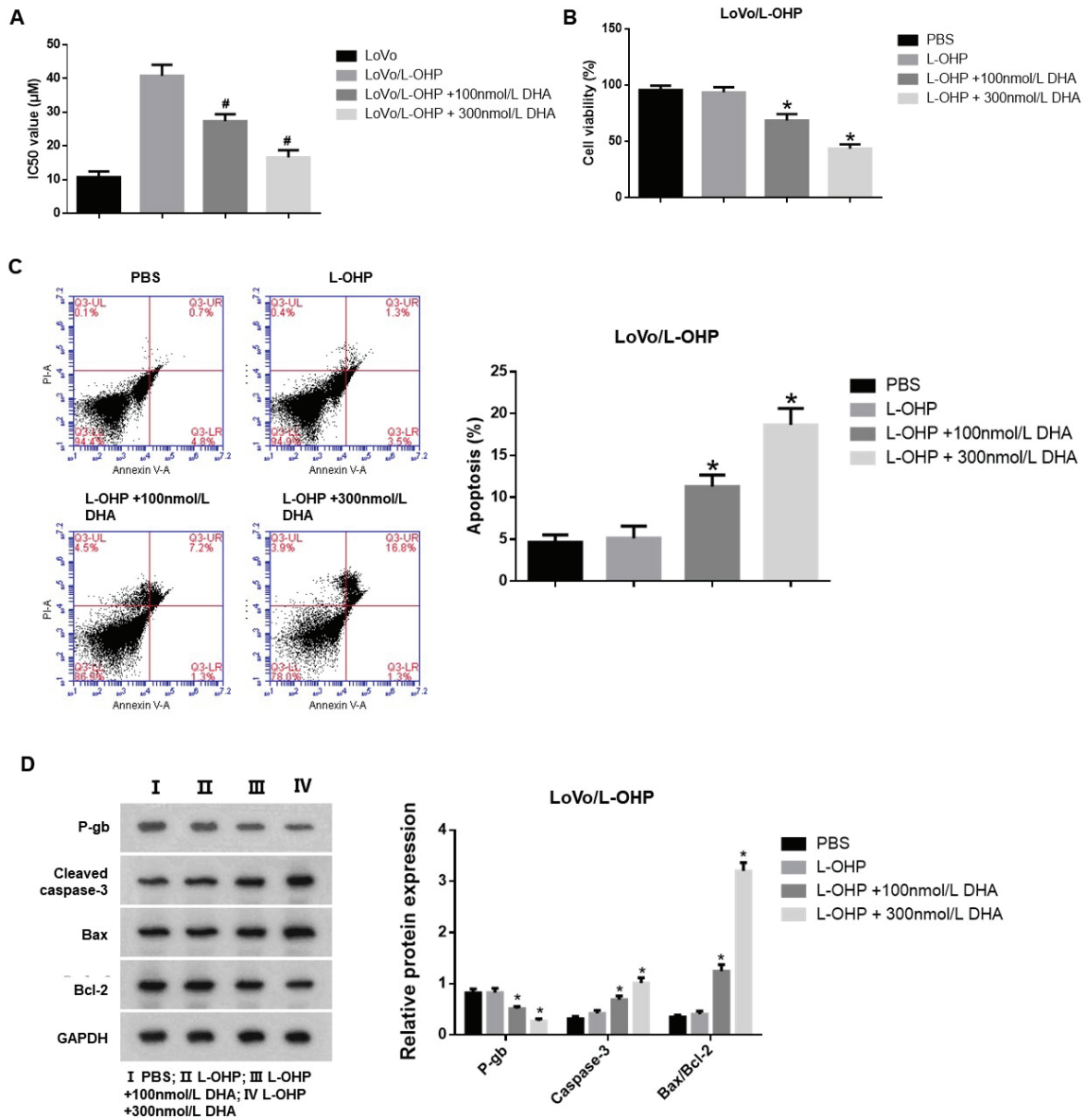
### The expression of SIRT3, PI3K, and AKT in LoVo/L-OHP cells after DHA treatment

To further investigate the expression of SIRT3, PI3K, and AKT in LoVo/L-OHP cells

after DHA treatment, we examined the expression of SIRT3, PI3K, and AKT in each group of cells using WB and PCR. As shown in Figs 2 A-B, our results indicated a significant decrease in SIRT3 expression level, whereas PI3K and AKT expression levels were significantly elevated in LoVo/L-OHP cells compared to the LoVo group ( $p < 0.05$ ). After administering L-OHP to LoVo/L-OHP cells and subsequently treating them with 300 nmol/L DHA, we observed a noteworthy upsurge in the protein and mRNA levels of SIRT3 as compared to the LoVo/L-OHP+L-OHP group ( $p < 0.05$ ). Furthermore, we observed a reduction in expression levels of PI3K and AKT in cells following DHA treatment ( $p < 0.05$ ).

### Silencing SIRT3 promoted L-OHP resistance in LoVo/L-OHP cells after DHA treatment through the PI3K/AKT pathway

To validate the role of the SIRT3/PI3K/AKT signalling pathway in L-OHP resistance of LoVo/L-OHP cells after DHA treatment, we transfected cells with si-SIRT3#1, si-SIRT3#2, si-SIRT3#3, and si-NC and measured the knockdown efficiency of SIRT3. As shown in Figs. 3 A-B, in LoVo/L-OHP cells after DHA treatment, si-SIRT3#1, si-SIRT3#2, and si-SIRT3#3 all markedly reduced the expression of SIRT3, and we selected si-SIRT3#2 with the highest knockdown efficiency for subsequent experiments. In addition, we also used the PI3K inhibitor LY294002 to inhibit the PI3K/AKT pathway and divided the cells into groups: control (LoVo/L-OHP+L-OHP+DHA), si-NC (control+si-NC), si-SIRT3 (control+si-SIRT3), and si-SIRT3+LY294002 (control+si-SIRT3+LY294002). As shown in Fig. 3-C, compared with the si-NC group, silencing SIRT3 remarkably increased cell viability and reduced the proportion of cell apoptosis ( $p < 0.05$ ). However, the addition of the PI3K agonist LY294002 reversed this effect. Compared with the si-SIRT3 group, the si-SIRT3+LY294002 group had significantly reduced cell viability and increased apoptosis (Fig. 3-D).

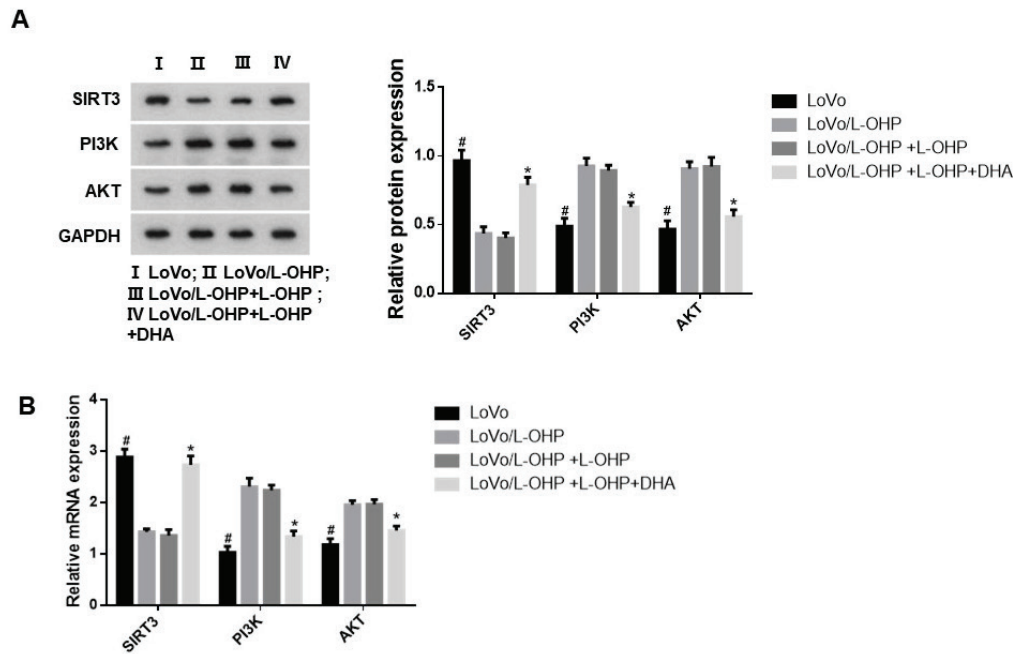


**Fig. 1.** DHA inhibited the proliferation of LoVo/L-OHP cells and induced their apoptosis. (A) The IC50 of all groups of cells were detected by CCK-8 assay. (B) Cell viability of all groups of cells was detected by CCK-8 assay. (C) The apoptosis rate of all groups of cells was analyzed by flow cytometry. (D) The expression of P-gp protein related to drug resistance and apoptosis-related proteins, cleaved caspase-3 protein, Bax, and Bcl-2, was detected by Western blot. Continuous data were compared using ANOVA. \* $p < 0.05$  compared with the L-OHP group. # $p < 0.05$  compared with the LoVo/L-OHP group.

**Effects of SIRT3 knockdown on relevant proteins in LoVo/L-OHP cells after DHA treatment**

Finally, we used WB to measure the protein expression of the SIRT3/PI3K/AKT signalling pathway proteins, the drug-resistant protein P-gb, and the apoptosis-related

proteins cleaved caspase-3, Bax and Bcl-2. As illustrated in Fig. 4, our data demonstrated that transfection with si-SIRT3 resulted in a notable decline in SIRT3 protein expression and increased protein expression of PI3K and AKT ( $p < 0.05$ ).



**Fig. 2.** The expression of SIRT3, PI3K and AKT in LoVo/L-OHP cells after DHA treatment. The SIRT3, PI3K and AKT expression in each group were measured by WB (A) and PCR (B). ANOVA was used to compare the groups. # $p < 0.05$  compared with the LoVo/L-OHP group. \* $p < 0.05$  compared with the LoVo/L-OHP+L-OHP group.

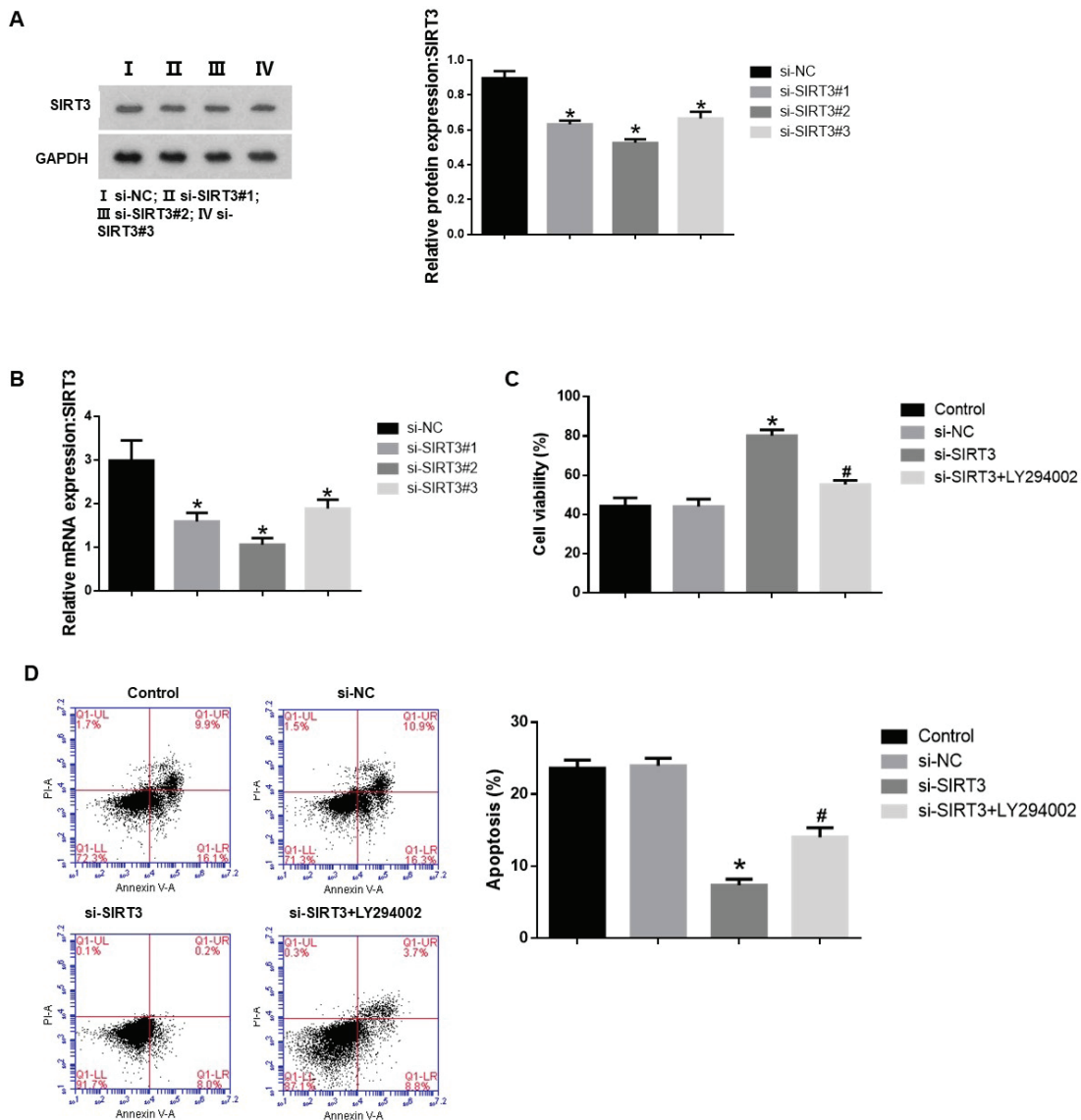
On the other hand, treatment with PI3K inhibitor LY294002 remarkably reduced the protein expression levels of PI3K and AKT ( $p < 0.05$ ). Silencing SIRT3 markedly enhanced the expression of P-gb while reducing the protein expression of cleaved caspase-3 and the ratio of Bax/Bcl-2. However, the PI3K inhibitor LY294002 reversed this effect. In the si-SIRT3+LY294002 group, the protein expression of P-gb decreased, while the expression of cleaved caspase-3 and the ratio of Bax/Bcl-2 significantly increased ( $p < 0.05$ ).

## DISCUSSION

For researchers, the mechanism of L-OHP resistance in CRC is still elusive. Despite many molecular medicine studies to explain the resistance mechanisms of CRC, it is challenging to distinguish genuinely effective targets for regulating resistance. In addition, most existing multidrug resis-

tance reversal agents have issues such as incomplete resistance reversal, high technical requirements, and significant adverse reactions<sup>20, 21</sup>. Therefore, finding new sensitizers to combine with L-OHP to treat CRC patients is still necessary. We demonstrated that DHA could inhibit the L-OHP resistance in LoVo human colorectal cancer cells by regulating the SIRT3/PI3K/AKT axis.

Studies conducted in the past have demonstrated that DHA can hinder the growth of cancer cells and trigger apoptosis in pancreatic cancer, ovarian cancer, and colorectal cancer. An added advantage of DHA is its low toxicity towards normal tissue cells<sup>22, 23</sup>. In addition, DHA also has a killing effect on multidrug-resistant tumor cells<sup>24</sup>. In our study, we treated CRC-resistant cells LoVo/L-OHP with different concentrations of DHA and found that the viability of LoVo/L-OHP cells markedly decreased, and the proportion of apoptotic cells increased significantly after DHA treatment. This phenomenon was



**Fig. 3. Silencing SIRT3 promotes L-OHP resistance in LoVo/L-OHP cells after DHA treatment through the PI3K/AKT pathway.** The expression of SIRT3 was measured by WB (A) and PCR (B). (C) Cell viability of all groups of cells was detected by CCK-8 assay. (D) The apoptosis rate of all groups of cells was analyzed by flow cytometry. ANOVA was used to compare the groups. \* $p < 0.05$  vs si-NC group. # $p < 0.05$  vs si-SIRT3 group.

more pronounced in the group with higher concentrations of DHA, indicating that DHA can inhibit the resistance of LoVo/L-OHP to L-OHP. Similar results have been obtained in other cancers. In non-small-cell lung cancer (NSCLC), DHA can eliminate radiation resistance of NSCLC cell lines and xenograft models by inhibiting the TGF- $\beta$ , PI3K/Akt, and STAT3 signalling pathways, and enhance

the anti-tumor effect of radiotherapy<sup>25</sup>. In breast cancer, DHA regulates the STAT3/DDA1 axis to inhibit the proliferation of breast cancer tumor cells<sup>26</sup>. In addition, Yao *et al.* confirmed that DHA can effectively sensitize CRC cells to 5-fluorouracil (5-FU) by mediating ROS-induced apoptosis<sup>27</sup>. Wang *et al.* also showed that DHA inhibits the characteristics of cancer stem-like cells (CSLCs)



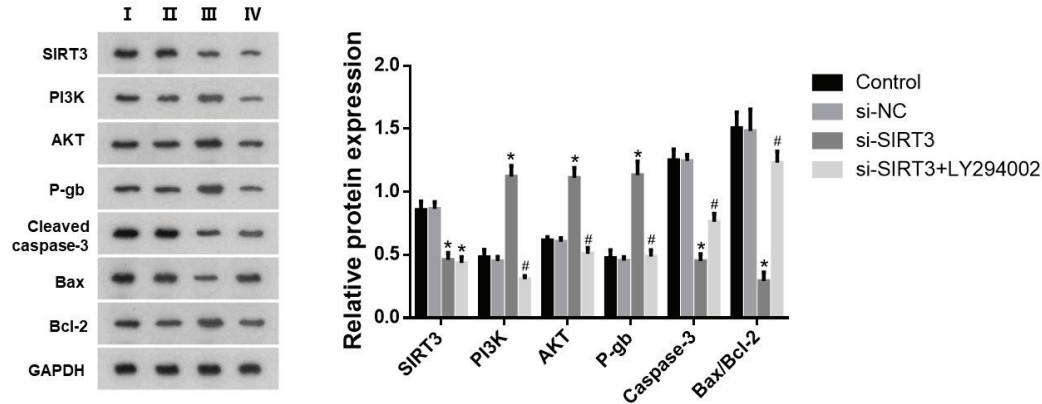


Fig. 4. Effects of SIRT3 knockdown on relevant proteins in LoVo/L-OHP cells after DHA treatment. The protein expression of SIRT3, PI3K, AKT, P-gb, cleaved caspase-3, Bax and Bcl-2 were measured by WB. ANOVA was used to compare the groups. \* $p < 0.05$  vs si-NC group. # $p < 0.05$  vs si-SIRT3 group.

in CRC through the AKT/mTOR signalling pathway<sup>28</sup>. These results suggested that DHA can be a sensitizing cancer treatment agent.

The protein and mRNA levels of SIRT3 were enhanced in LoVo/L-OHP cells after treatment with DHA, indicating that SIRT3 may be involved in the sensitization of LoVo/L-OHP by DHA. Cao *et al.* found that activation of SIRT3 inhibits cisplatin resistance in lung cancer cells and reduces the proliferation and invasion of lung cancer cells<sup>29</sup>. Another research showed that the activity of SIRT3 in colon cancer cells can be enhanced by nitric oxide synthase (NOS), which helps to promote cell apoptosis<sup>30</sup>. To study the mechanism of action, we silenced SIRT3 and found that the viability of LoVo/L-OHP cells improved, and the proportion of apoptotic cells and apoptotic proteins cleaved caspase-3 and the ratio of bax/bcl-2 decreased significantly, indicating that the knockdown of SIRT3 affected the resistance of LoVo/L-OHP cells to L-OHP after DHA treatment. Furthermore, we confirmed that silencing SIRT3 enhanced the expression of PI3K and AKT in LoVo/L-OHP cells. The aberrant activation of the PI3K/AKT pathway acts against chemotherapy-induced apoptosis by increasing the expression of anti-apoptotic genes (bcl-2 and XIAP) and reducing the expres-

sion of pro-apoptotic genes (bax)<sup>31</sup>. In our study, we inhibited the PI3K/AKT axis with LY294002 and found that the viability of LoVo/L-OHP cells decreased while apoptosis increased. This also reversed the effect of knocking down SIRT3 on the viability and apoptosis of LoVo/L-OHP cells. Zhang *et al.*<sup>32</sup> found that inhibiting the PI3K/AKT signalling pathway in CRC cells can inhibit cell proliferation and activate apoptosis, thereby reversing oxaliplatin resistance, consistent with our research results.

#### Limitation

Our research also has some limitations. Whether other genes regulate SIRT3 is not clear. We also did not perform *in vivo* experiments to confirm the apoptosis results. All these need further studies to illustrate.

Our study found that DHA could inhibit the L-OHP resistance in LoVo human colorectal cancer cells by regulating the SIRT3/PI3K/AKT axis. The results might provide a new integrated treatment approach for CRC treatment in clinical practice.

#### Ethics approval and consent to participate

The ethic approval was obtained from the Ethic Committee of The Minhang Hospital, Affiliated with Fudan University, and

written informed consent was obtained from all patients. All of the authors have consented to publish this research.

#### Availability of data and materials

The data are free to access and available upon request.

#### Competing interests

All authors declare no conflict of interest.

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#### ORCID number of authors

- Xiaodong Shen:  
0009-0000-0520-8896
- Chencheng Shi:  
0009-0008-1369-2946
- Ming Lei:  
0009-0000-8480-197X
- Rongjian Zhou:  
0009-0007-5598-0834
- Shaoqun Liu:  
0000-0002-2016-0947
- Chang Su:  
0009-0004-6481-2638

#### Authors' contributions

Each author has made an important scientific contribution to the study and assisted with drafting or revising the manuscript.

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