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RESEARCH

A Chinese herbal Formula, Chang-Wei-Qin, Synergistically Enhances Antitumor Effect of Oxaliplatin

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Abstract Chang-Wei-Qing (CWQ), a Chinese herbal formula, has long been employed clinically to treat cancers. In this study, we investigated the synergistic effect of CWQ with oxaliplatin (OXA) on the tumor growth inhibition of orthotopic transplanted colon cancer and explored the underlying mechanism. By generating the orthotopic transplanted nude mouse model of human colon carcinoma, we found that (1) CWQ enhanced OXA-mediated tumor suppression by 4.25-fold. (2) The body weights of nude mice in CWQ group and combination group were increased. (3) The survival time of tumor-bearing nude mice was dramatically improved in CWQ and CWQ/OXA group. (4) CWQ could restore OXA-mediated deregulation of copper transporter genes, hCTR1, ATP7A and ATP7B. (5) OXA-induced drug resistance index for OXA, 5-FU, HCPT and THP were 7.59, 4.28, 5.78 and 4.50 respectively, while the reversal index by combined CWQ treatment were 6.57, 2.61, 4.97 and 3.10, respectively. Our study demonstrates that the repeated intraperitoneal injection of OXA can induce multi-drug resistance of orthotopic transplanted nude mouse model of human colon carcinoma. The CWQ treatment can alleviate OXA-triggered side effects and reverse platinum drug resistance via up-regulation of hCTR1 expression and down-regulation of ATP7A and ATP7B levels.

Keywords Colorectal cancer · Xenograft model · Changweiqing · Platinum drugs · Copper transporters

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors in the developed countries [1]. The past few years have witnessed the increased morbidity and mortality of this cancer in China [2]. The majority of deaths (approximately 90 %) associated with CRC are due to the metastasis [3], yet this process remains one of the most enigmatic aspects of cancers. Chemotherapy is one of the main treatments for advanced or metastatic colorectal cancer. Oxaliplatin (OXA) is the third generation of Platinum (Pt) cancer drugs after the Cisplatin and Carboplatin. Oxaliplatin-based chemotherapy is the standard regimens in the adjuvant setting for stage III CRC and a standard first-line treatment option for patients with metastatic CRC [4]. However, the therapeutic efficacy is limited by the emergence of primary or acquired drug resistance [5]. Therefore, identification of lead compounds or drugs reversing Pt resistance has gained great clinical significance.

Development of Pt resistance is a complex and multifactorial process that can be promoted by two major mechanisms. First, the mechanism evolved to reduced cellular transportation of Pt drugs may prevent drug accumulation in cells, and thus impairs subsequent DNA damage response. Second, cells may develop mechanism that inhibits Pt-mediated DNA damage signal delivery, consequently blocking the downstream apoptotic machinery. A series of elegant studies have shown that regulation of copper homeostasis not only play a key role in the uptake and efflux of the Pt drugs, but also modulate cell sensitivity to the cytotoxic activity of these agents [6–10]. For instance, the expression of human copper transporter 1 (hCTR1), a primary uptake transporter for copper, is

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associated with Cisplatin sensitivity in some cancer cells [11]. Other important proteins, including ATP7A and ATP7B, two copper-membrane transporting P-type ATPases, are also involved in Pt resistance and found to be overexpressed in Cisplatin-resistant tumor cells [12, 13]. It has been documented that the copper influx transporters, especially hCTR1, are involved in the cellular uptake of platinum drugs, while the other two copper transporters, ATP7A and ATP7B, import copper to Golgi apparatus which regulates the efflux of platinum drugs [6]. Hence, elevating the expression of hCTR1 and reducing the expression of ATP7A and ATP7B could be the molecular basis of reinforcing efficacy or antagonizing cellular resistance to Pt drugs.

Traditional Chinese medicine (TCM) is a popular form of complementary and alternative medicine (CAM) in China. Traditional Chinese prescriptions and formulae have been documented as effective anti-cancer drugs in the treatment of colorectal cancer [14], lung cancer [15], liver cancer [16] and breast cancer [17]. Recent studies have reported that several Chinese herbal medicines improve the curative effect of Pt drugs by sensitizing tumor cells to Pt-based chemotherapy [18–20]. Chang-Wei-Qing (CWQ), a Chinese herbal formula, consisting of Radix Astragali, Rhizoma Atractylodis Macrocephalae, Radix Codonopsis, Akebia Quinata (Thunb) Decne, Polyporus, Semen Coicis, Vitis Quinquangularis Rehd and Caulis Sargentodoxae, has also been shown to be effective in cancer treatment. Although the molecular mechanism remains unclear, some of its components, such as Radix Astragali, Radix Codonopsis and Semen Coicis, have been indeed shown to bolster immune system and enhance efficacy of chemotherapy in various human cancers [21, 22].

Our previous clinical study revealed that CWQ exerted a synergistic effect with Pt drugs in gastrointestinal cancer. This synergistic effect may be due to the reversal of the Pt resistance, yet the exact mechanism remains to be determined [23, 24]. In this study, we aim to demonstrate the cooperative tumor suppressive effects of CWQ and OXA in vivo through establishing the orthotopic transplanted nude mouse model of human colon carcinoma and to further elaborate the molecular basis of CWQ-mediated reversal of drug resistance.

Materials and Methods

Cell Culture and Reagents

Four to six-weeks-old specific pathogen-free (SPF) BALB/c nu/nu nude mice, 16–18 g in body weight, were purchased from Shanghai Public Health Center Laboratory Animal Center with license for SCXK (Shanghai) 2005–0,001, and housed separately in the barrier system with clean laminar flow (SPF level) at room temperature (25±1) °C and relative humidity 40~60 %. Human colon cancer HCT116 cells were purchased from Cell Bank, Chinese Academy of Sciences of Life Sciences, Cell Resource Center.

Preparation of CWQ

All crude drugs of CWQ were purchased from a local herbal medicine market (Shanghai, China). Components have been identified and provided by the Department of Pharmacy of Putuo Hospital, Shanghai University of TCM. Briefly, Radix Astragali, Rhizoma Atractylodis Macrocephalae, Radix Codonopsis, Akebia Quinata (Thunb) Decne, Polyporus, Semen Coicis, Vitis Quinquangularis Rehd and Caulis Sargentodoxae were mixed at a ratio of 10:5:5:8:8:10:10:10. The herb mixture was decocted twice with 3,000 ml water for 1 h each time, and the decoction was filtrated and stored at 4 °C for use. Decoction liquid was prepared for animal administration at 1.33 g/ml. Preparation of CWQ was under the standard quality control defined by the Chinese State Food and Drug Administration (SFDA).

Animals and Xenograft Models

Human colon cancer HCT116 cells in the exponential growth phase were prepared by concentrating the cell suspension of 1×10^7/ml. 2×10^6 cells in 0.2 mL were inoculated subcutaneously into the right forelimb of nude mice for 7 to 14 days when the subcutaneous tumor grew to 1 to 1.5 cm in diameter. Mice with apparent tumor growth but without epidermal ulceration were selected and the tumor was cut into 2×2 mm in size for further experiments. 32 nude mice were preoperative fasting for 12 h followed by intraperitoneal injection of 2.5 % amyl barbital sodium (35 mg/kg). After routine disinfection of the skin, ventral midline incision was made to open the abdominal cavity. Fresh tumor blocks were inserted into a concave niche made by inward push with a blunt object at the end of cecum. Medical OB glue was dropped on the surface of tumors and spread to the cecal wall before the abdomen was sutured. After transplantation of the tumors, the nude mice were fasting for six hours before fed regular diet.

Drug administration

The tumor-bearing nude mice were randomly divided into 4 groups (18 mice per group) after 7 days: control, OXA, CWQ and OXA/CWQ groups. The control group was treated by gastric irrigation with physiological saline and intraperitoneal injection of water; The OXA group was treated by gastric irrigation with physiological saline and intraperitoneal injection of OXA; The CWQ group was treated by gastric irrigation with CWQ and intraperitoneal injection of water; The combination group was treated by gastric irrigation with CWQ and intraperitoneal injection of OXA. The human clinical dosage of CWQ was used for nude mice, while the dosage

of OXA was determined by the maximal tolerated dose of nude mice. The same dosage of OXA or CWQ was administered in OXA, CWQ and OXA/CWQ groups. Each group was divided into two subgroups: eight mice were sacrificed and the tumors were collected for immunohistochemistry, western blot, PCR and assessment of drug resistance index; the body weight and survival time of the rest ten mice were recorded and analyzed.

Assessment of Transplanted Tumor Growth, Nude Mouse Body Weight and Survival Time

Observed and recorded nude mouse abdomens, and nude mouse activity and eating situation weekly after operation. Mice were sacrificed after 4-week drug administration followed by anatomical analysis of the tumor and the surrounding intestinal wall. Tissues were fixed by 10 % formalin and paraffin-embedded followed by HE staining. Tumor weight was recorded to determine the tumor growth inhibition rate. The inhibition rate (%)=[(average tumor weight of control group - average tumor weight of drug administered groups) / the average tumor weight of control group] x 100 %. The body weight was recorded each week for 6 weeks. The survival time was determined from the beginning of drug administration to the death of the nude mice.

MTT Assay for Cell Viability

Primary cultured HCT116 cell suspensions were seeded at 1×10^4 per well in 96-well plate. Once the cells adhered to the plate, the culture medium was replaced by fresh medium containing titrated concentrations of OXA, 5-FU, HCPT or THP as indicated in the figure. After 48-h culture at 37 °C in a 5 % CO_2 humidified atmosphere, MTT solution was added to each well at the final concentration of 1 mg/ml for 4 h. The absorbance of samples was measured at 570 nm using an enzyme-linked immunoassay instrument. Resistance index (Resistance factor, RF) and reverse index (Reversal index, RI) were calculated. Resistance index=IC50 of drug-resistant cells / IC50 of drug-sensitive cells. Reversal index= IC50 of reversal agent-treated resistant cells / IC50 of resistant cells.

Immunohistochemistry

Briefly, tumor tissue was fixed with 10 % formalin for 24–48 h, dehydrated through 70 %, 80 %, 95 % and 100 % alcohol, cleared with xylene and embedded in a paraffin block. Sectioned the paraffin-embedded tissue block at 4 μm thickness and transferred the sections onto glass slides which were subsequently deparaffinized, rehydrated and blocked before incubated with the primary antibodies, anti-hCTR1, anti-ATP7A and anti-ATP7B (purchased from Boster Bioengineering Co. Ltd., Wuhan, Hubei, China), at room temperature for 1 h. After incubation with the biotinylated secondary antibody and substrate solution, the tissue sections were observed under microscopy.

Quantitative Fluorescence PCR Analysis

About 1 μg mRNAs were used as template for reverse transcription. The reaction condition was as follows: 25 °C for 10 min, 40 °C for 60 min and 70 °C for 10 min. The obtained cDNAs were stored at −20 °C. The primers and probes of hCTR1, ATP7A, ATP7B and GAPDH were designed and synthesized by Shanghai ShanJing Biotechnology Company (Sequences are shown in Table 1). Fluorescence quantitative polymerase chain reaction (qPCR) system contained a total volume of 50 μl including 25 μl 2×PCR buffer, 1.2 μl Primers (25 pmol/μl), 0.3 μl probe, 1 μl template (cDNA) and 22.5 μl DEPC water. qPCR amplification condition was 94 °C for 20 s, 60 °C for 30 s for 40 cycles. The results were analyzed using the ABI Prism 7,300 SDS package.

Western Blot Analysis

Cell lysates were run on a 10 % SDS-PAGE and transferred to a PVDF membrane which was blocked for overnight. The membrane was sequentially incubated with the primary antibodies for 1.5 h and the secondary antibodies for 1.5 h. The western blots were developed by chemiluminescence and analyzed using the UVP gel image processing system Labworks 4.6 software.

Table 1 The hCTR1, ATP7A, ATP7B, and GAPDH primer and probe sequences for qPCR reaction

Gene	Forward primers	Reverse primers
hCTR1	GCTGGGACGGGATACTTTCTC	AATGGCAGTGCTCTGTGATGTC
ATP7A	CACCATCGCATTTGCCTACTC	GGGTTCACTTTGGCTCTCTCAA
ATP7B	AAAGCCCGACCTAGAGAGATATGAG	CCAATGTGCACGCTGACTTG
GAPDH	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA

The probes are modified with 5-end FAM and 3-end TAMRA

Statistical Analysis

Quantitative data are expressed as the mean±standard deviation (SD). Statistical differences were evaluated by unpaired Student's *t*-test using statistical SPSS 15.0 software. A value of $p<0.05$ was considered statistically significant.

Results

CWQ Inhibited Tumor Growth in vivo

To test whether CWQ administration can suppress tumor growth in vivo, we generated human colorectal cancer xenograft nude mouse model as described in the *Materials and Methods* section. These nude mice were supplemented with physiological saline, OXA, CWQ or both drugs for 4 weeks before sacrificed for evaluating the in vivo anti-tumor effect of CWQ. As shown in Fig. 1, by determining the tumor weight, we found that either OXA or CWQ treatment could inhibit tumor growth in vivo. Remarkably, CWQ could cooperate with OXA in tumor growth inhibition and resulted in a 53.6 % decrease in tumor weight compared to the control mice. Additionally, through calculating the tumor inhibition rate, we found that combined administration of CWQ and OXA presented more than four-fold tumor suppressive effect over OXA treatment alone.

Also, we checked the mouse body weight in response to drug treatment. We found that mice supplemented with OXA lost weight after 4 weeks compared to the control group, which was probably due to the side effect of OXA. Interestingly, CWQ administration counteracted the weight loss caused by OXA treatment, which implies that CWQ may alleviate the side effect OXA. Particularly, the average gained weight of the nude mice supplemented with CWQ alone was much more than that of the other three groups ($P<0.05$), indicating that CWQ might also cause metabolic changes in these xenograft mouse models (Fig. 2).

CWQ Extended Survival of Tumor Xenograft Nude Mice

Next, we wondered whether CWQ administration could prolonged the life span of these nude mice harboring tumor xenograft. As shown in Fig. 3, the median survival time of the CWQ and the CWQ/OXA groups was longer than that of the control and OXA groups ($P<0.05$), suggesting a tumor suppressive function of CWQ. In consistency with the former data (Fig. 1), combination of CWQ and OXA treatment also dramatically increased the life span compared to the other three groups ($P<0.05$). Further, we noticed that OXA treatment did not extended the nude mouse life span probably due to drug resistance and/or side effects, while the combined treatment of OXA and CWQ showed synergistic effect on extension of survival time (Fig. 3), which indicates that CWQ may abrogate OXA resistance and/or side effects. It is noteworthy that CWQ administration results in tumor growth inhibition and life span extension to a greater extent than OXA (Figs. 1 and 3), thus CWQ could be a more potent anticancer drug which is worth further investigation.

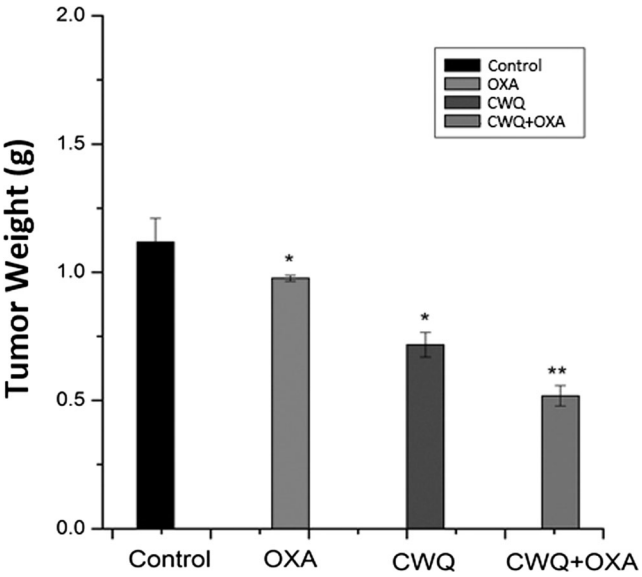

Fig. 1 The effect of CWQ on tumor growth in vivo. Note : * $P<0.05$, ** $P<0.01$, compared to the control group

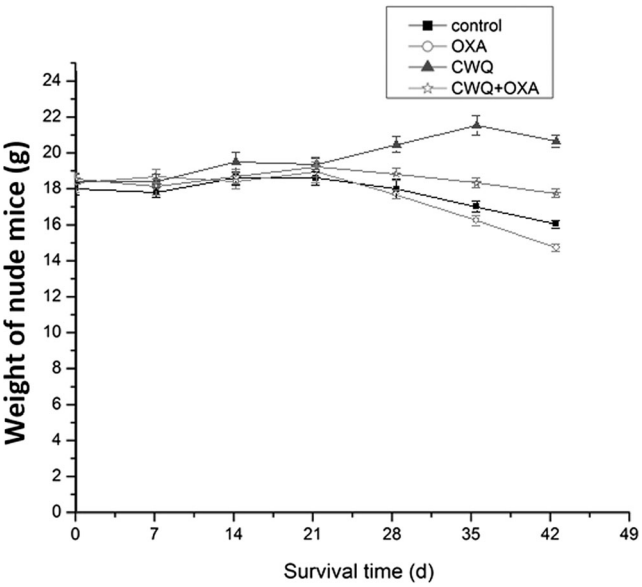

Fig. 2 The effect of CWQ on the body weight of xenograft mice

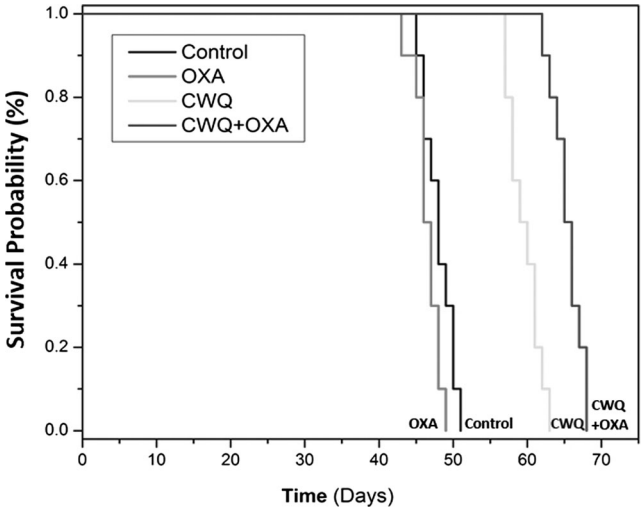

Fig. 3 The effect of CWQ on the survival of xenograft mice

CWQ Restored hCTR1, ATP7 and ATP7B mRNA Expression in Platinum-Resistant Tumor Cells.

Next, we asked how CWQ reversed tumor cell resistance to OXA. It has been shown that reduced expression of hCTR1 and elevated expression of ATP7A and ATP7B are associated with platinum resistance in tumors [11, 25]. Hence, we performed qPCR to assess the mRNA expression levels of these genes in the tumor xenografts. Indeed, we found that OXA treatment reduced hCTR1 mRNA expression (Fig. 4a) but increased ATP7A (Fig. 4b) and ATP7B (Fig. 4c) mRNA levels. Nevertheless, CWQ treatment exerted opposite effects on expression of these genes. Moreover, addition of CWQ could rescue OXA-mediated abnormal expression of hCTR1, ATP7A and ATP7B (Fig. 4).

CWQ Restored hCTR1, ATP7 and ATP7B Protein Expression in Platinum-Resistant Tumor Cells

Further, we also tested whether CWQ rescued hCTR1, ATP7 and ATP7B expression at protein level. As shown in Fig. 6, immunohistochemical analysis revealed that the protein level of hCTR1 was inhibited (Fig. 5a) whereas ATP7A (Fig. 5b) and ATP7B (Fig. 5c) protein levels were elevated in the OXA-treated tumors. Consistently, addition of CWQ rescued the aberrant expression of these three proteins caused by OXA supplementation (Fig. 5).

To confirm our observation that CWQ restored hCTR1, ATP7 and ATP7B protein expression, we also conducted western blotting to determine protein level. As shown in the Fig. 6, hCTR1 expression decreased in the OXA-treated tumors while was restored to normal level in tumors treated with both OXA and CWQ. Conversely, ATP7A and ATP7B expression elevated in the OXA-treated tumors, which was significantly rescued in double drug-treated tumors (Fig. 6). The right penal of Fig. 6 shows the quantitative protein level of western blotting normalized by β-actin expression.

CWQ Reversed OXA-Triggered Multi-Drug Resistance in HCT116 Cells

Although we found that CWQ worked together with OXA to inhibit tumor growth in vivo by reversing platinum resistance, it remained elusive whether CWQ could also boost the chemotherapeutic effects of other anti-cancer drugs. To test this idea, we isolated and cultured the HCT116 cells from tumor xenograft and treated these cells with different anti-cancer drugs including OXA, 5-FU, HCPT and THP. Intriguingly, we found that the primary tumor cells from OXA-treated nude mice developed multi-drug resistance, in addition to OXA itself, compared to those cells from the control group (Fig. 7), whereas, CWQ administration did not confer drug resistance on tumor cells. It was found that CWQ treatment significantly reversed multi-drug resistance caused by OXA (Fig. 7): resistance indexes of OXA, 5-FU, HCPT and THP in OXA-treated tumor cells were 7.59, 4.28, 5.78 and 4.50, respectively; reversal indexes by addition of CWQ were 6.57, 2.61, 4.97 and 3.10, respectively.

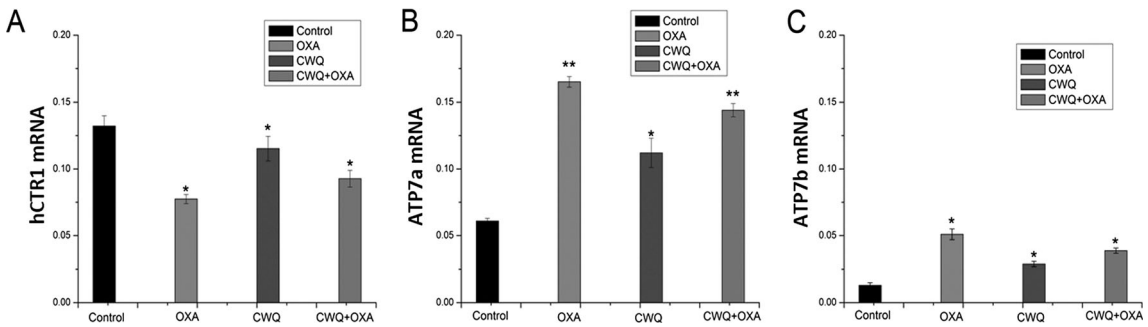

Fig. 4 CWQ restores hCTR1, ATP7A and ATP7B mRNA expression in tumor tissues. **a** hCTR1 expression in response to drug treatment. **b** ATP7A expression in response to drug treatment. **c** ATP7B expression in response to drug treatment. Note: * $P<0.05$, ** $P<0.01$, compared to the Control group

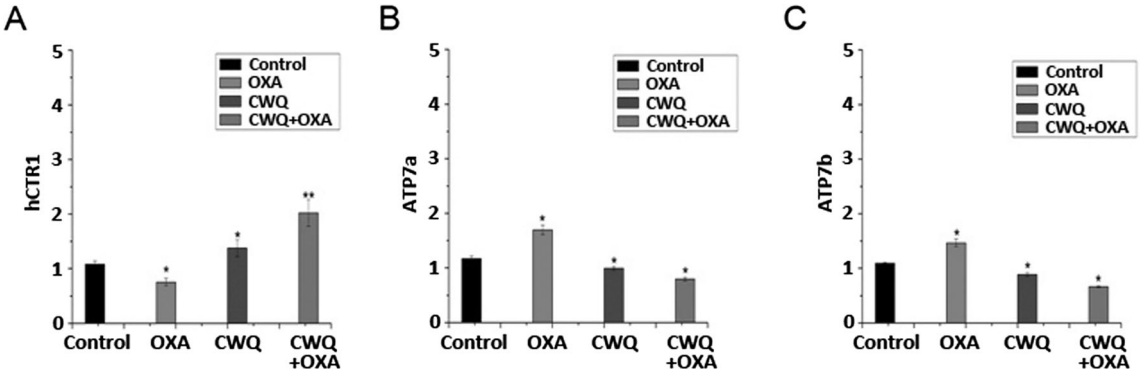

Fig. 5 CWQ restores hCTR1, ATP7A and ATP7B protein expression determined by immunohistochemistry. **a** Quantitative analysis of immunohistochemistry staining for hCTR1. **b** Quantitative analysis of immunohistochemistry staining for ATP7A. **c** Quantitative analysis of immunohistochemistry staining for ATP7b. Note: * $P<0.05$, ** $P<0.01$, compared to the Control group

Discussion

Recently, several studies showed that CWQ can prevent cancer development through inhibiting invasion or reversing drug resistance in colorectal carcinoma cells [20, 26, 27], but it remains unclear whether CWQ can do so in vivo. In this study, through generating the human colorectal tumor xenograft mouse model, we demonstrate for the first time that Chang-Wei-Qing (CWQ) enhances OXA-mediated colorectal tumor growth inhibition via restoring cell sensitivity to platinum drugs in vivo. More importantly, we found that single CWQ treatment is sufficient to reduce tumor weight (Fig. 1) and prolong survival time of the tumor-bearing mice (Fig. 3) to a greater extent than single OXA treatment in vivo. Together with our previous data showing that CWQ can inhibit colorectal tumor cell metastasis in vitro [26], the study demonstrates an underappreciated tumor suppressive function of CWQ in colorectal cancer therapy. Also, we found that OXA administration reduced mouse body weight probably due to its side effect, whereas CWQ alleviated OXA-caused side effects by increasing mouse body weight (Fig. 2). Notably, we did not found any side effects of CWQ on the xenograft mice, because these mice showed better body weight and survival compared to the control or OXA-treated mice (Figs. 2 and 3). Together with the fact that CWQ is a commonly used Chinese herbal medicine, we believe that CWQ could be a non-toxic, or at least less-toxic, medicine for cancer treatment.

A previous in vitro study has shown that CWQ can reverse drug-resistance of colorectal cancer cells by sequestering YB-1 in the cytoplasm and as thus reducing the expression of YB-1 target gene, MDR1 (multidrug resistance protein 1) [27]. MDR1 is an important protein of the cell membrane that pumps many foreign substances, including chemotherapeutic drugs, out of cells, therefore triggering drug resistance. While in this study, to explore the specific molecular basis of Pt resistance, we examined the expression of copper transporting-related genes which are highly associated with Pt drug sensitivity [11–13]. Our findings provide an additional mechanism of reversing drug resistance by CWQ. Interestingly, MDR1-mediated extrusion of chemical compounds is ATP-dependent [28], thus this newly identified mechanism may be also responsible for MDR1-induced drug resistance, which would be of great importance to be elucidated in our future study. Additionally, our further data show that CWQ can reverse multi-drug resistance caused by OXA treatment. This intriguing observation may also be partially due to repression of MDR1 by CWQ. In sum, our study indicates that use of the Chinese herbal medicine, CWQ, can be a potent and promising therapeutic strategy in treatment of human colorectal cancer.

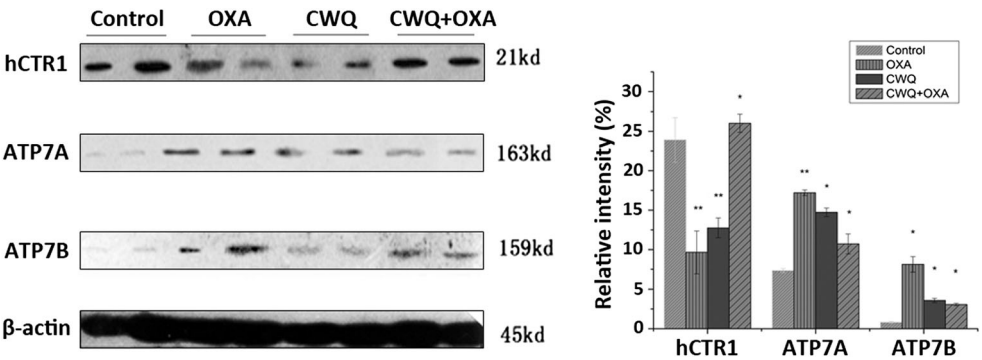

Fig. 6 CWQ restores hCTR1, ATP7A and ATP7B protein expression determined by western blotting. Left penal shows western blots of hCTR1, ATP7A and ATP7B; right penal shows quantification of western blotting signal. Note: * $P<0.05$, ** $P<0.01$, compared to the Control group

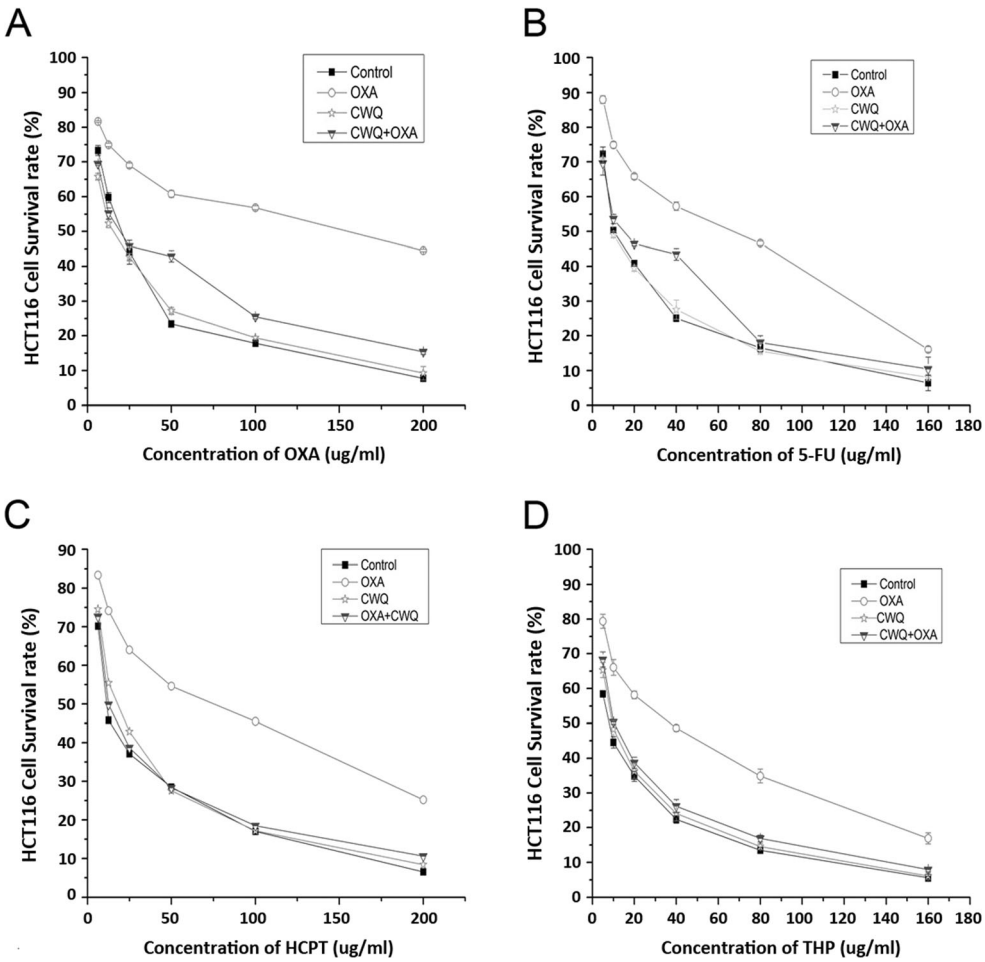

Fig. 7 CWQ Reverses OXA-triggered chemotherapy resistance. (A) CWQ reverses cell resistance to OXA. (B) CWQ reverses cell resistance to 5-FU. (C) CWQ reverses cell resistance to HCPT. (D) CWQ reverses cell resistance to THP

OXA resistance is triggered through distinct mechanisms [29], including modulation of drug transporters, drug detoxification, DNA repair, and cellular tolerance to DNA damage and apoptosis [30, 31]. Numerous studies have attempted to develop potent lead compounds or drugs for reversing platinum-resistant, however, most results are frustrating and the toxicity of these chemicals is the main disadvantage leading to failure of these studies. Therefore, the development of safe and effective agents reversing platinum resistance has gained great interests.

Accumulating evidence has shown that Chinese herbal medicines have collaborative effects in combination with traditional chemotherapy,such as controlling tumor progression [24], reducing the toxicity of chemotherapy [32], increasing the chemotherapeutic sensitivity by reversing chemotherapeutic resistance [18], enhancing the immune function and improving the quality of life in patients with tumors [33]. Each of the eight components in CWQ has been tested and characterized as a useful anti-carcinoma drug in clinical therapy in china, however, very few evidence shows synergistic effect on platinum resistance when the eight Chinese Herbs are used together according to Compatibility of Traditional Chinese Medicines.

Through both in vivo and in vitro approaches, we indeed provide evidence that CWQ administration synergistically cooperate with OXA to inhibit colon cancer formation. All our conclusions are drawn by manipulation of human colon cancer HCT116 cells, thus it would be necessary to test other human colon cancer cell lines as well. Also, it is worthwhile to examine whether CWQ combined with OXA may have synergistic effect on suppression of other human cancers. We found that OXA induced cell resistance to multiple anticancer drugs, including 5-FU, HCPT and THP, and it was of great interest that CWQ could antagonize this side effect of OXA and boost tumor suppressive function of all these three drugs. Recently, it has been found that copper cellular transportation (uptake and efflux) is critical for platinum drug accumulation in cells. Hence, several important regulators involved in copper transportation, such as hCTR1, ATP7A and ATP7B, have been extensively studied as potential targets to increase cell sensitivity to platinum-based chemotherapy. Although we show herein that CWQ can reverse cell resistance to OXA through regulating hCTR1, ATP7A and ATP7B expression and another seminal study suggested that ATP7B expression in colorectal tumors is associated with clinical outcome to oxaliplatin/5FU [34], it remains uncertain whether

expression of copper transporter gene, particularly ATP7B, is responsible for CWQ-mediated cellular sensitivity to 5-FU, HCPT and THP.

Taken together, our findings substantiate that CWQ possesses tumor suppressive function via reversing cellular resistance to OXA, 5-FU, HCPT and THP in vitro and/or in vivo. This Chinese herbal formula can be an optimal adjuvant of anti-cancer drugs in treatment of human colorectal cancer.

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Conflict of Interest The authors declare that they have no conflict of interest.

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