All-trans Retinoic Acid combined with oxaliplatin suppressed proliferation of chemo-resistant hepatocellular carcinoma cells by inducing G2/M cell cycle arrest

Type

Research paper

Keywords

oxaliplatin, hepatocellular carcinoma, all-trans retinoic acid, chemotherapy-resistant, CylinB1

Abstract

Introduction
To investigate the effects and mechanisms of all-trans retinoic acid (ATRA) with and without oxaliplatin (OXA) on chemotherapy-resistant hepatocellular carcinoma cell lines.

Material and methods
OXA-resistant cell lines (CSQT-2-R and Hep3b-R) and subcutaneous xenograft model were used in this study. MTT assay, flow cytometry, crystal violet assay, transwell assay and western-blotting were conducted to evaluate the effects of co-treatment with ATRA and OXA on OXA-resistant HCC in vivo and in vitro. The differences between two groups were analyzed using ANOVA. All statistical tests in the study were two-sided, and statistical significance was set at $P<0.05$.

Results
We established two oxaliplatin-Resistant HCC cell lines (CSQT-2-R and Hep3b-R). The drug resistance ability can be increased up to 100% than their parental cells(CSQT-2 and Hep3b) in certain concentration of OXA. ATRA alone could not inhibited the viability of CSQT-2-R and Hep3b-R, but it can enhance the ability of OXA on apoptosis than OXA alone (75% vs 35%, $p<0.05$), which may be related to decreased p-AKT expression. Moreover, the co-treatment of two drugs arrest the cell cycle of OXA-resistant cell at G2/M phase by up-regulating CylinB1 protein.

Conclusions
ATRA combined with OXA can elicit cell cycle arrest of CSQT-2-R and Hep3b-R at G2/M phase, thereby inhibiting the proliferation of resistant HCC cell, which provides a new treatment for chemotherapy-resistant HCC.

All-trans retinoic acid combined with oxaliplatin suppressed the proliferation of chemoresistant hepatocellular carcinoma cells by inducing G2/M cell cycle arrest

Abstract

Objective: To investigate the effects and mechanisms of action of all-trans retinoic acid (ATRA) with and without oxaliplatin (OXA) on chemotherapy-resistant hepatocellular carcinoma cell lines.

Methods: OXA-resistant cell lines (CSQT-2-R and Hep3B-R) and subcutaneous xenograft models were used in this study. MTT assay, flow cytometry, crystal violet assay, transwell assay, and western blotting were used to evaluate the effects of cotreatment with ATRA and OXA on OXA-resistant HCC in vivo and in vitro. The differences between the two groups were analyzed using ANOVA. All statistical tests used in the study were two-sided, and statistical significance was defined as $P<0.05$.

Results: We generated two oxaliplatin-resistant HCC cell lines (CSQT-2-R and Hep3B-R). Drug resistance was increased up to 100% compared with that of the parental cells (CSQT-2 and Hep3B) at certain concentrations of OXA. ATRA significantly enhanced the ability of OXA to induce apoptosis and serum-stimulated cell migration compared with those in the groups treated with OXA alone, which may be related to a decrease in p-AKT expression. In a xenograft model, inhibition of the growth, weight, and volume of tumor cells achieved by cotreatment with OXA and ATRA was more pronounced than the effects of OXA treatment alone. Moreover, cotreatment with the two drugs arrested the cell cycle of OXA-resistant cells at G2/M phase by upregulating the cyclin B1 protein.

Conclusions: ATRA combined with OXA can elicit cell cycle arrest of CSQT-2-R and Hep3B-R cells at G2/M phase to inhibit the proliferation of resistant HCC cells, providing a new treatment for chemotherapy-resistant HCC.

Keywords: Hepatocellular carcinoma; chemotherapy-resistant; all-trans retinoic acid; oxaliplatin; cyclin B1

Introduction

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer, the fifth most common malignancy worldwide, and the third most common cause of cancer-related deaths [1]. Surgery offers the best potential cure for early-stage HCC, and patients with intermediate-stage HCC are treated with locoregional therapies. Patients with advanced-stage HCC can benefit from systemic treatments, and approximately half of HCC patients were reported to receive systemic therapies at a certain time during the course of the disease [2]. However, the efficacy of sorafenib [3] or lenvatinib [4] is unsatisfactory. Systemic chemotherapy is one of the few treatment alternatives, although multidrug tumor resistance is frequently observed [5,6].

Oxaliplatin (OXA) is a third-generation platinum-derived chemotherapeutic agent that triggers cell death by inducing the formation of platinum-DNA adducts and appears to block DNA replication more effectively than other platinum compounds [7]. OXA has been used to treat colorectal and gastric cancers and has antitumor activity against HCC [8-10]. OXA-based combined treatments have recently demonstrated a promising antitumor activity in patients with HCC [11]; however, these treatments can eventually result in tumor resistance despite the initial response. Multiple mechanisms related to resistance to platinum drugs have been extensively studied, including drug accumulation deficiencies, intracellular detoxification by conjugation with glutathione, reduced DNA-platinum adduct formation, and alterations in the transport proteins and downstream signaling influencing cell death pathways. However, chemotherapy resistance to OXA in HCC is incompletely understood.

All-trans retinoic acid (ATRA) is a compound related to vitamin A that acts via

retinoic acid receptors (RARs) and rexinoid receptors (RXRs). ATRA plays an important role in a number of physiological processes, including vision, tissue maintenance, differentiation, and embryonic development. ATRA can also act as an inhibitor of carcinogenesis by blocking the proliferation of activated or transformed cells via three mechanisms: induction of apoptosis, arrest of further growth of abnormal cells, and induction of differentiation of abnormal cells back to normal cells [12, 13]. A previous study showed that ATRA has been successfully used in the therapy of human acute promyelocytic leukemia and cured 70–80% of patients in combination with anthracyclins [14]. However, the effects of ATRA on solid tumors, especially HCC, remain poorly investigated.

To the best of our knowledge, this is the first study that explored the anti-HCC effects of combined treatment with ATRA and OXA in vivo and in vitro, providing a novel and effective chemotherapeutic approach for patients with chemotherapy-resistant HCC. The study was approved by the Ethics Committee of the Eastern Hepatobiliary Surgery Hospital.

Materials and methods

Cell culture

Hep3B cells were obtained from the Cell Bank of the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences, Shanghai, China). The HCC cell line CSQT-2 was derived from the portal vein thrombus of HCC tumor and established in our laboratory. These cell lines were cultured in DMEM supplemented with 10% (v/v) FBS, 100 IU/mL penicillin, and 50 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

Generation of OXA-resistant cells

OXA-resistant Hep3B-R and CSQT-2-R cells were established by incubating Hep3B

and CSQT-2 cells with an ascending gradient of OXA concentrations from 0.5 to 24 μM. Briefly, the cells were initially incubated in the medium containing 0.5 μM OXA for 1 week and subsequently in the medium containing 1 μM OXA for 1 week; the concentration of OXA in the medium was gradually increased to reach 24 μM.

Cell viability analysis

MTT assay was used to detect cell viability [15]. Hep3B-R and CSQT-2-R cells were plated in triplicate at a density of 3,000 cells/well in a volume of 100 μL in 96-well microliter plates and incubated overnight. On the following day, various concentrations of ATRA (0 μmol/L, 5 μmol/L, and 10 μmol/L) were added to the combination groups; three days later, various concentrations of OXA (0 μmol/L, 50 μmol/L, and 100 μmol/L) were added to each group to obtain 9 subgroups treated with various combinations. The cells were then incubated with MTT at room temperature. After 4 h, an appropriate amount of DMSO (Hebei Bio-high Technology Deve Co., Ltd., Hebei, China) was added and mixed on a shaker for 15 min. The optical density values at 570 and 630 nm were measured using a multifunctional microplate reader (Synergy Neo, BioTek, USA), and results were calculated.

Apoptosis analysis

Flow cytometry was used to quantify apoptosis using an Annexin V-FITC apoptosis detection kit according to the manufacturer's protocol (BD Biosciences, CA, USA). Briefly, the cells were harvested and suspended in binding buffer. Each sample was then incubated with 100 μL of binding buffer, 5 μL of Annexin V-FITC, and 5 μL of propidium iodide (PI) for 15 min in the dark.

Cell cycle analysis

The cells were harvested, washed with phosphate buffer solution (PBS), and fixed in 75% alcohol for 12 h at 4°C. After washing in cold PBS, the cells were resuspended

in 0.5 mL of staining buffer with 25 μL of PI and 10 μL of RNase A for 30 min at 37°C. The samples were then subjected to FACS analysis.

Crystal violet assay

Hep3B-R and CSQT-2-R cells were plated in triplicate at a density of 1,000 cells/well in 6-well plates. The combination groups were pretreated using ATRA, and ATRA and OXA were added to each group 3 days later. To measure cell growth, 1 mL of 0.1% crystal violet solution was added to each well; crystal violet was carefully removed, and 10% acetic acid was added to solubilize the stain. The absorbance of each well was measured at 570 nm vs 630 nm.

Transwell assay

For detection of migration, Hep3B-R and CSQT-2-R cells (treated with vehicle control, 10 μM ATRA, 50 μM OXA, or a combination of ATRA and OXA) were placed into the upper chamber, and DMEM containing serum was added to the lower chamber. The combination treatment group was treated with ATRA for 2 days followed by treatment with OXA for 6 h. Subsequently, migrated cells in the lower chamber were stained with 0.1% crystal violet (Sigma, MO, USA). A microscope (Olympus, Tokyo, Japan) was used to detect migrated cells in at least three random fields of view.

Western blotting

Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked in PBS containing 0.1% Tween 20 (PBS-T) and 5% skim milk at room temperature for 2 h, followed by incubation with primary antibodies at 4°C overnight. HRP-conjugated IgG (KPL, dilution 1:3,000, Gaithersburg, MD) was used as a secondary antibody. β-Actin antibody (Sigma-Aldrich; 1:2,000 dilution) was used as an internal control. Antibodies to p-Akt (Ser473; 1:1,000 dilution), Akt

(1:1,000 dilution), and cyclin B1 (1:1,000 dilution) were purchased from Cell Signaling Technology, Inc. NF-kB (1:1,000 dilution) was purchased from Abcam. The bands were visualized by enhanced chemiluminescence (ECL).

Subcutaneous xenograft model

Briefly, 5×10^6 CSQT-2-R and Hep3B-R cells were suspended in 100 μL of serum-free DMEM and subcutaneously injected into male nude mice (age: 5-6 weeks). When tumor diameter was >5 mm, 24 mice were randomly divided into the 0.1% DMSO treatment group, 10 μM ATRA treatment group, 100 μM OXA treatment group, and combined treatment group (10 μM ATRA plus 100 μM OXA). The drugs were injected intraperitoneally once every three days. Tumor size, volume (length*width^2/2), and weight were recorded. All procedures were performed according to the SIBS Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee, Shanghai Institutes for Biological Sciences.

Statistical analysis

All experiments were performed in triplicate with at least three replicates for each sample. Data are expressed as the mean ± standard error of the mean (SEM).Statistical analysis was performed using SPSS software (version 17.0, SPSS Inc., Chicago, IL). The differences between two groups were evaluated using ANOVA. All statistical tests were two-sided, and statistical significance was defined as $P<0.05$.

Results

Generation of oxaliplatin-resistant HCC cells

Comparison with parental cells indicated that CSQT-2-R and Hep3B-R cells were elongated and fusiform and manifested dyscohesive features (Figure 1A). The results

of MTT assay indicated that proliferation-suppressive effect of OXA was significantly greater in parental cells (Figure 1B). Flow cytometry experiments were performed to confirm the generation of OXA-resistant cells. The results showed that newly generated resistant cells were less sensitive to OXA than parental cells (Figure 1D and 1E). In addition, lower expression of Bax protein and higher expression of NF-κB protein were detected in resistant cells (Figure 1C).

Combined *OXA and ATRA treatment suppressed the growth of CSQT-2-R and Hep3B-R cells in vitro*

MTT assay was performed to detect combined effect of ATRA and OXA on the proliferation of OXA-resistant cells. As shown in Figure 2A, the growth of OXA-resistant cells in the control and ATRA groups was similar; however, addition of OXA alone or a combination of OXA and ATRA significantly decreased cell activity, and the effect of the combination treatment was especially pronounced. The results of the crystal violet assay showed that combined treatment with the two drugs effectively inhibited the proliferation of resistant cells (Figure 2B and 2C). Additionally, we evaluated the effect of the two drugs on the migration of HCC cells using a transwell assay. The data of Figure 2D and 2E indicate that OXA treatment of CSQT-2-R or Hep3B-R cells significantly suppressed serum-stimulated migration of the cells compared with that in the control group. Interestingly, the addition of ATRA significantly increased the inhibitory effect of OXA on CSQT-2-R and Hep3B-R cells.

Combined treatment with *OXA and ATRA induced cell cycle arrest in CSQT-2-R or Hep3B-R cells*

To further explore the mechanism of OXA and ATRA resistance in HCC cells, we performed flow cytometry assays to determine whether OXA and ATRA influence cell

cycle progression. In the control and ATRA groups, the percentages of the cells in G1, S, or G2/M phases were unchanged. However, treatment with OXA and ATRA significantly increased the number of the cells in G2/M phase and significantly decreased the number of the cells in the S phase compared with those in the OXA group (Figure 3A-3C).

The results of flow cytometry demonstrated that combined treatment with OXA and ATRA significantly increased the accumulation of HCC cells in G2/M phase. To confirm this result, we detected the expression of related proteins in the G2/M phase. Treatment with ATRA and OXA induced a considerable increase in the expression of cyclin B1 compared with that in the control and ATRA groups (Figure 3D).

Combined treatment with OXA and ATRA inhibited AKT phosphorylation

AKT is a very important regulator of cell survival and migration and is frequently hyperactivated in human cancers [16, 17]. Phosphorylation is essential for catalytic activity of AKT; thus, we examined the level of p-AKT in OXA-resistant and treated cells. As shown in Figure 3E, the levels of p-AKT in Hep3B-R and CSQT-2-R cells were not altered by treatment with ATRA or OXA alone compared with that in the control group. However, combined treatment with both drugs dramatically decreased the expression of p-AKT. Furthermore, we detected the changes in the expression of p-AKT in vivo. As expected, the combination of ATRA and OXA significantly decreased the expression of p-AKT (Figure 4D).

Combined treatment with OXA and ATRA suppressed tumor growth in nude mice

A xenograft model was generated to investigate the in vivo effects of combined treatment on xenograft tumors. As shown in Figure 4A-4C, treatment with OXA alone or cotreatment with OXA and ATRA significantly inhibited the growth, weight, and volume of the tumor cells. In particular, combined treatment had a more pronounced

inhibitory effect (Figure 4A-4C). Xenograft tumors from the four groups were harvested for western blotting analysis to detect the expression of cyclin B1 protein. The results of western blotting showed an increase in the expression of cyclin B1 in the xenograft tumors treated with ATRA and OXA compared with that in the control and single drug treatment groups (Figure 4D), which was consistent with the in vitro observations.

Discussion

HCC is the third most common cause of death from cancer worldwide. Chemotherapy is one of current treatment modalities and is mainly used to reduce tumor recurrence and prolong survival in unresectable HCC. However, HCC is typically resistant to most commonly used chemotherapy drugs, and efficacy of chemotherapy remains poor due to multidrug resistance. OXA remains one of the most effective agents of numerous evaluated chemotherapeutic formulations. Recently, an international, multicenter, open-label, randomized phase III study of the FOLFOX4 regimen versus doxorubicin in Asiatic patients provided the foundation for application of OXA [18]. Several clinical trials have shown that OXA represents a viable and reasonable chemotherapeutic option in advanced HCC [19-21]; however, high drug resistance and side effect rates greatly limit clinical application of chemotherapy. Therefore, addressing chemotherapy resistance is very urgent and important. Several studies have explored the efficacy of OXA in combination with other drugs, such as sorafenib, ramucirumab, and ADI-PEG 20 [12-24]. However, the results of these clinical studies were unsatisfactory, and there are no in-depth studies of the molecular mechanisms.

ATRA is a cancer re-differentiation agent. ATRA alone or in combination is an important therapeutic agent that has antitumor effects on a variety of cancers,

including HCC [12, 25]. It is also reported that ATRA was related to autophagy. Wang [26] found that ATRA induced autophagy-relevant 7 (ATG7) and autophagy participated in its cytotoxicity on HCC cells and AFP interfere with the induction of ATG7 and autophagy through forming complex with RAR. This is probably one of the areas we will focus on in the future. An increasing number of studies have shown that ATRA combined with other chemotherapy agents can significantly inhibit the growth of tumors; however, there is no known approach to address chemoresistant tumors. Based on these studies, we tested the idea of using a combination of OXA and ATRA to achieve an antitumor effect on HCC.

In the present study, a stepwise increase in the dosages of OXA was used to generate OXA-resistant cells (CSQT-2-R and Hep3B-R). Comparison with the corresponding parental cells indicated that OXA-induced resistant cells manifested morphological and molecular changes. Furthermore, the results of western blotting showed that downregulation of Bax was associated with enhanced apoptosis [27], and activation of NF-κB increased resistance to chemotherapy [28]. Generation of OXA-resistant HCC cells enabled to explore chemosensitivity of these cells to cotreatment with ATRA and OXA. The data indicated that treatment with a combination of ATRA with OXA inhibited the growth of OXA-resistant HCC cells in vitro and in vivo compared with that achieved by single drug treatments. This result is very similar to the data of previous studies, which showed that ATRA enhanced the role of cisplatin in chemotherapy of liver cancer [29]. In addition to chemosensitivity, we investigated the effects of combined treatment on cell migration, and the results of the present study indicated that a combination of the two drugs significantly reduced the migration of OXA-resistant HCC cells.

Uncontrolled proliferation is a hallmark of tumor cells. Moreover, aberrant cell

cycle accounts for dysregulated cell growth, which ultimately leads to the formation of tumors [30]. Normal cell cycle progression depends on the activities of cyclins/cyclin-dependent kinases (CDKs) [31], and different stages of the cell cycle are linked to different cyclins. Cyclin B1 protein is involved in the regulation of the cell cycle and controls the S-G2 progression and mitosis. [32]. In agreement with the data of a previous study [33], the results of the present study indicated that ATRA greatly promoted the accumulation of OXA-resistant HCC cells in G2/M phase, which may indicate a mechanism of checkpoint repair after a failure.

AKT is a proto-oncogene that has become a major subject of various medical studies. AKT can trigger a cascade of responses, from cell growth and proliferation to survival and motility, driving tumor progression [34]. Therefore, the present study examined the expression of p-AKT in chemoresistant Hep3B-R and CSQT-2-R cell lines treated with ATRA and OXA. The results of western blotting demonstrated that the expression of p-AKT was dramatically inhibited both in vitro and in vivo after cotreatment with ATRA and OXA compared with that detected after monotherapy, which was consistent with inhibition of proliferation and migration of chemoresistant HCC cells.

Conclusion

Characteristics of OXA-resistant HCC cells were different from those of parental cells. ATRA potentiated the chemotherapeutic effect of OXA on chemoresistant HCC cells by arresting the cell cycle in G2/M phase, which may represent the mechanism of inhibition of the proliferation of chemoresistant HCC cells.

Conflict of interest

No potential conflicts of interest were disclosed.

Funding

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Authors' contribution

Juxian Sun, Jie Shi and Shuqun Cheng conceived of the study, and provided administrative support; Chang Liu contributed to investigation; Zongtao Chai contributed to methodology and data procession; Lei Guo provided materials and samples, and participated in data analysis and validation; Chongde Lu participated in data collection and analysis; All authors read and approved the final manuscript and consented to publish this manuscript.

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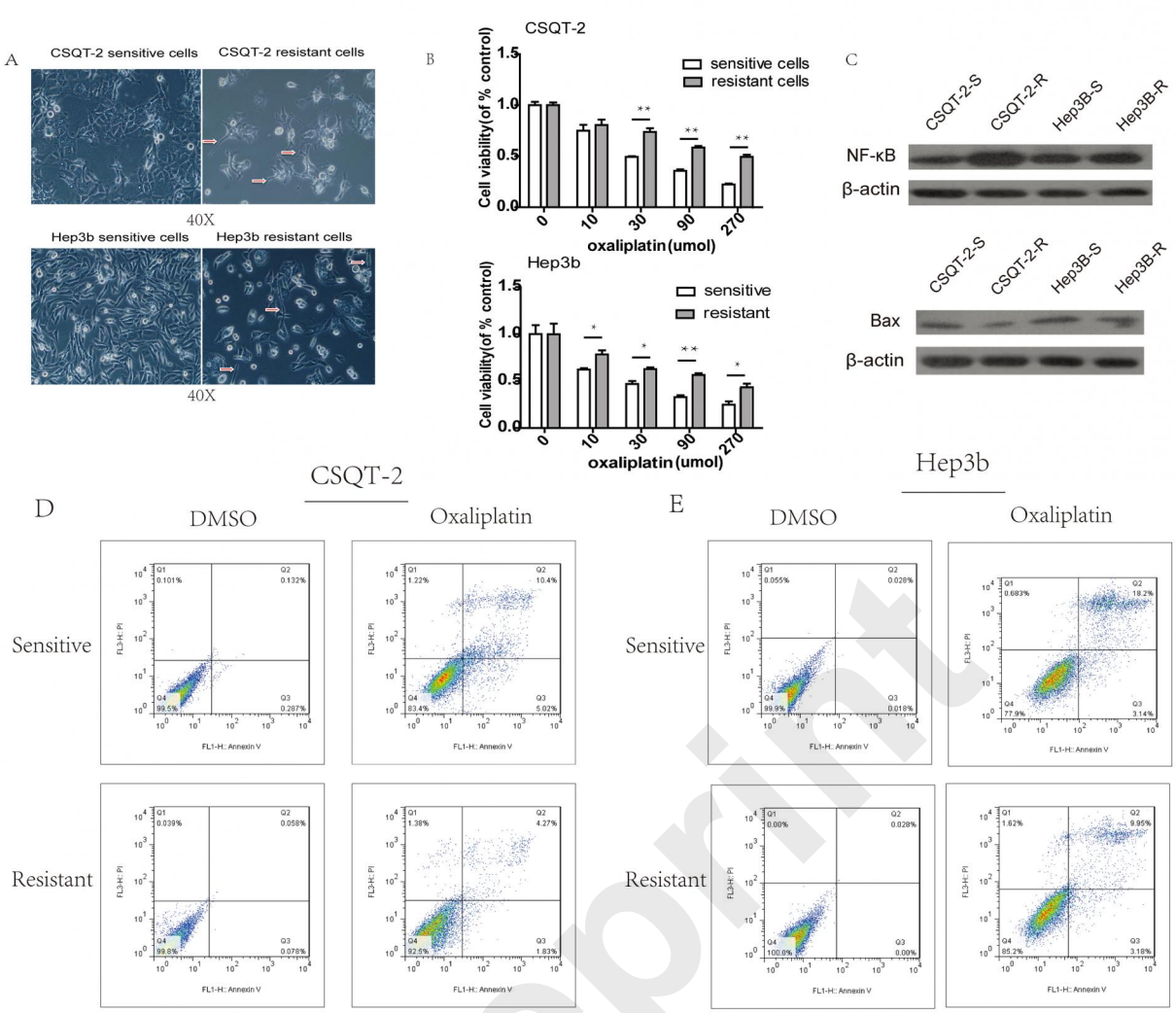
Figure Legends

Figure 1 OXA-induced resistant cells (CSQT-2-R and Hep3B-R) exhibited morphological and molecular changes. (A) Morphology changes of parental and resistant cells; (B) The cell viability of parental and resistant cell after being exposed to different concentrations of OXA ($^{*}P<0.05$,$^{**}P<0.01$); (C) Western-blotting analysis showed a higher expression of NF-κB and lower expression of Bax compared to parental cells; (D) Flow cytometry showed that OXA-induced resistance was successfully established in CSQT-2 cells; (D) OXA-induced resistance was successfully established in Hep3b cells. The percentage represents the rate of apoptosis. ATRA, all-trans retinoic acid; OXA, oxaliplatin.

Figure 2 OXA and ATRA combined treatment suppressed the growth of CSQT-2-R and Hep3b-R cells in vitro. (A) Cell growth of the indicated groups was measured by MTT assay; (B) Crystal violate results revealed that co-treatment with ATRA and OXA effectively inhibit the proliferation of CSQT-2-R cells; (C) Crystal violate results revealed that co-treatment with ATRA and OXA effectively inhibit the proliferation of Hep3b-R cells; (D) The migratory capability of CSQT-2-R treated with ATRA and OXA was examined by transwell assay; (E) The migratory capability of Hep3b-R treated with ATRA and OXA was examined by transwell assay; $^{**}P<0.01$. ATRA, all-trans retinoic acid; OXA, oxaliplatin.

Figure 3 OXA and ATRA combined treatment elicit cell cycle arrest in CSQT-2-R or Hep3b-R cells. (A) The cell cycle analysis was performed in CSQT-2-R cells; (B) The cell cycle analysis was performed in Hep3b-R cells; (C) The combination of ATRA and OXA induced arrest in G2/M phase; $^{*}P<0.05$, $^{***}P<0.01$; (D) Protein expression changes of CyclinB1 in CSQT-2-R was detected by western-blotting; (E) p-AKT protein expression in CSQT-2-R was detected by western-blotting assay. ATRA, all-trans retinoic acid; OXA, oxaliplatin.

Figure 4 OXA and ATRA combined treatment suppressed tumor growth of nude mice in vivo. (A) Compared with single drug treatment, co-treatment with OXA and ATRA significantly reduced the tumor volume; (B) Xenograft images; (C) Co-treatment with OXA and ATRA significantly reduced the tumor weight; (D) An increased expression of CyclinB1 and a decreased expression of p-AKT were observed in combined treatment group. ATRA, all-trans retinoic acid; OXA, oxaliplatin.



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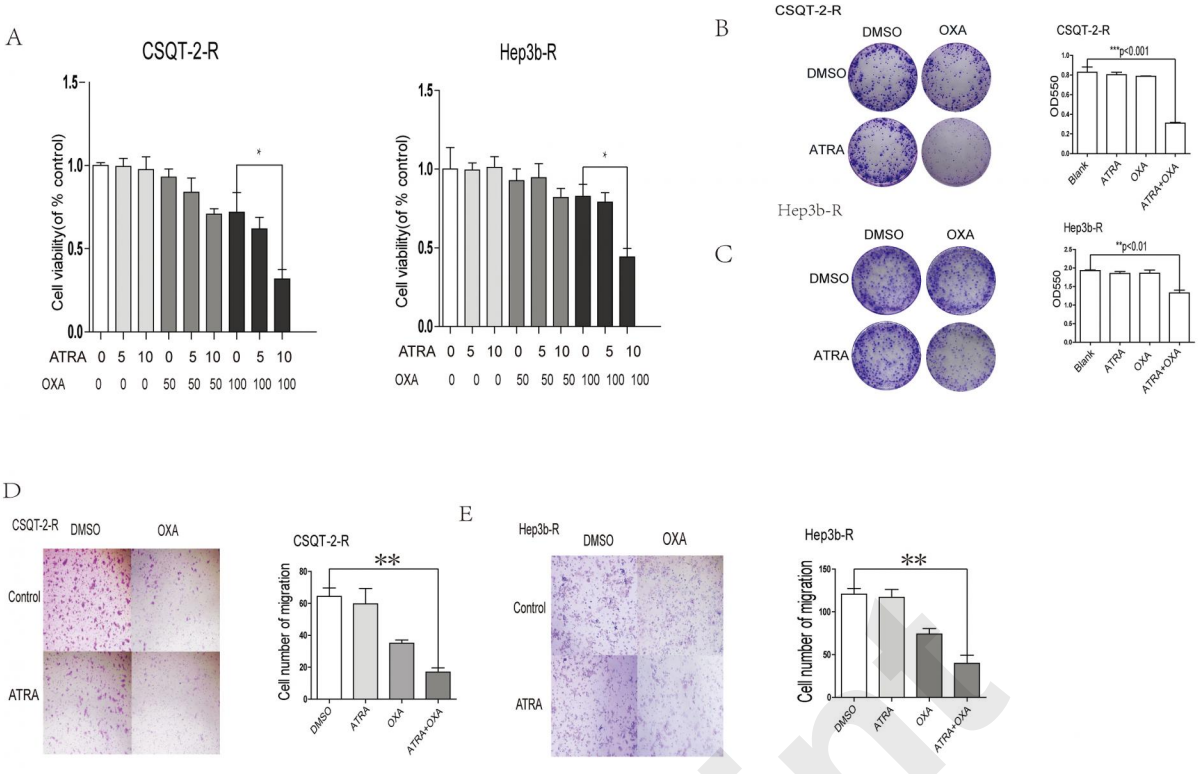

Figure 2 OXA and ATRA combinatorial treatment suppressed the growth of CSQT-2-R and Hep3b-R cells in vitro. (A) Cell growth of the indicated groups was measured by MTT assay; (B) Crystal violate results revealed that co-treatment with ATRA and OXA effectively inhibit the proliferation of CSQT-2-R cells; (C) Crystal violate results revealed that co-treatment with ATRA and OXA effectively inhibit the proliferation of Hep3b-R cells; (D) The migratory capability of CSQT-2-R treated with ATRA and OXA was examined by transwell assay; (E) The migratory capability of Hep3b-R treated with ATRA and OXA was examined by transwell assay; **P<0.01. ATRA, all-trans retinoic acid; OXA, oxaliplatin.

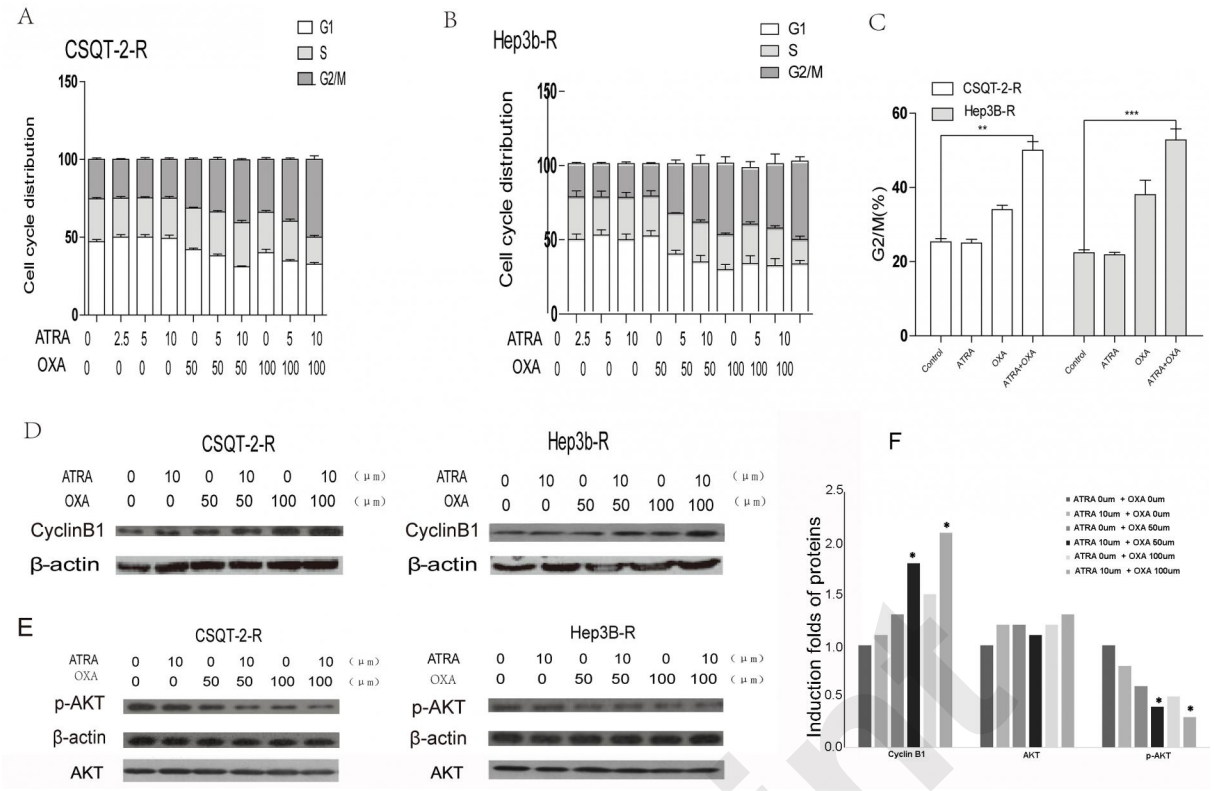

Figure 3 OXA and ATRA combined treatment elicit cell cycle arrest in CSQT-2-R or Hep3b-R cells. (A) The cell cycle analysis was performed in CSQT-2-R cells; (B) The cell cycle analysis was performed in Hep3b-R cells; (C) The combination of ATRA and OXA induced arrest in G2/M phase; *P<0.05,***P<0.01; (D) Protein expression changes of CyclinB1 in CSQT-2-R was detected by western-blotting; (E) p-AKT protein expression in CSQT-2-R was detected by western-blotting assay. ATRA, all-trans retinoic acid; OXA, oxaliplatin.

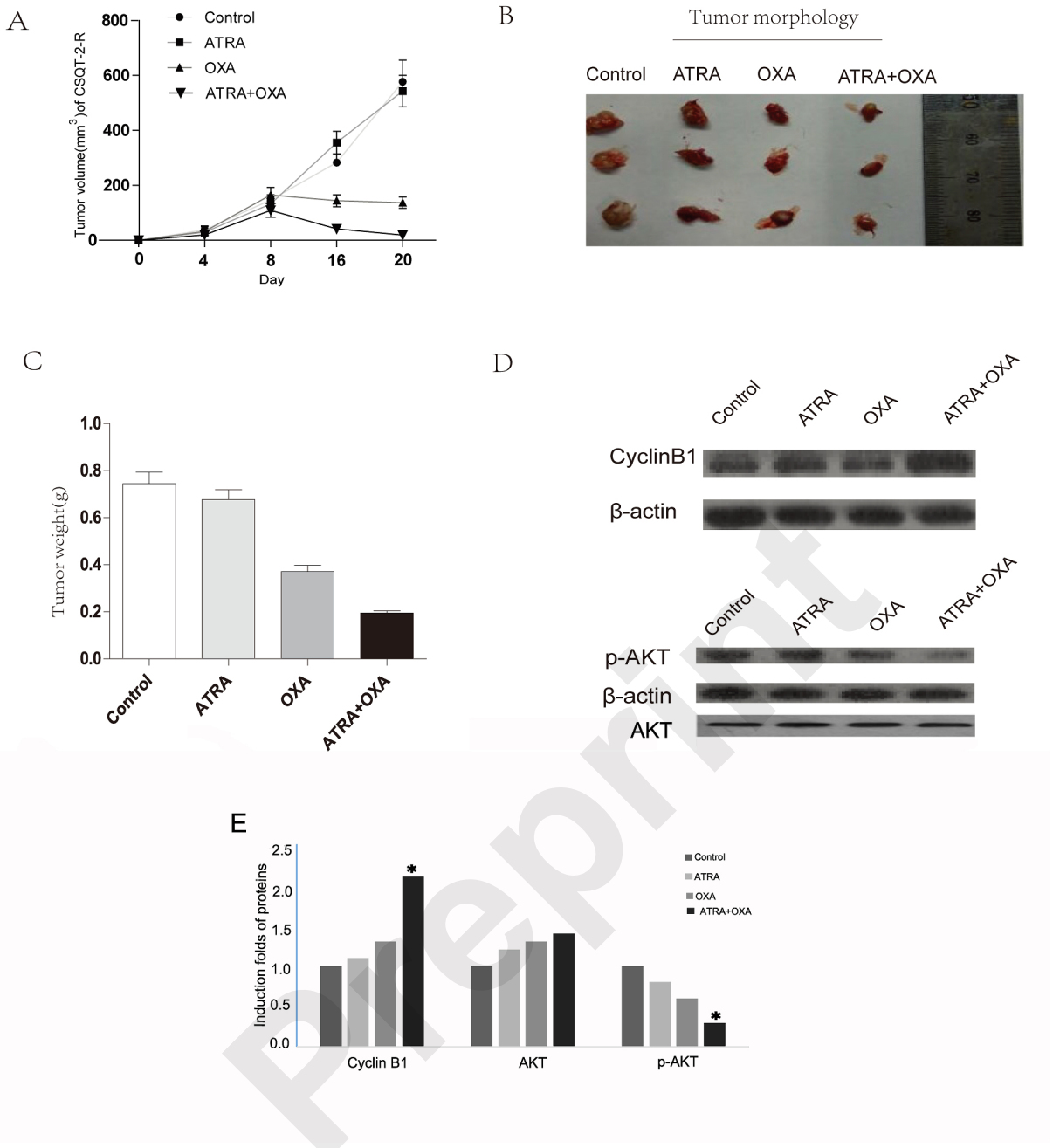

Figure 4 OXA and ATRA combined treatment suppressed tumor growth of nude mice in vivo. (A) Compared with single drug treatment, co-treatment with OXA and ATRA significantly reduced the tumor volume; (B) Xenograft images; (C) Co-treatment with OXA and ATRA significantly reduced the tumor weight; (D) An increased expression of CyclinB1 and a decreased expression of p-AKT were observed in combined treatment group. ATRA, all-trans retinoic acid; OXA, oxaliplatin.