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CGK733 enhances multinucleated cell formation and cytotoxicity induced by taxol in Chk1-deficient HBV-positive hepatocellular carcinoma cells

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most deadly human cancers. Chronic hepatitis B virus (HBV) infection is one of the predominant risk factors associated with the development of HCC and complicates the treatment of HCC. In this study, we demonstrate that a HBV-positive HCC cell line HepG2.2.15, was more resistant to chemotherapy agents than its parental HBV-negative cell line HepG2. HBV-positive HCC cells exhibited defective Chk1 phosphorylation and increased chromosomal instability. CGK733, a small molecule inhibitor reportedly targeting the kinase activities of ATM and ATR, significantly enhanced taxol-induced cytotoxicity in HBV-positive HepG2.2.15 cells. The mechanism lies in CGK733 triggers the formation of multinucleated cells thus promotes the premature mitotic exit of taxol-induced mitotic-damaged cells through multinucleation and mitotic catastrophe in HBV-positive HepG2.2.15 cells. These results suggest that CGK733 could potentially reverse the taxol resistance in HBV-positive HCC cells and may suggest a novel strategy to treat HBV-infected HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant diseases in the world and ranks the second among the most deadly malignant cancers in China and East Asian. Epidemiologically, HCC is associated with chronic infections of hepatitis B virus (HBV) or hepatitis C virus (HCV) [1]. In China, more than 90% HCC patients are found to be positive with HBV surface antigen (HBsAg) [2]. HCC is resistant to most of the conventional radio- and chemo-therapeutic schemes [3], which together with malicious metastasis accounts for the high mortality. Therefore it will greatly benefit the treatment of HCC if the cancer cells could be modulated to become sensitive to radio-/chemo-therapeutic reagents.

Taxol, a microtubule stabilizing agents that arrests cells in mitosis leading to cell death [4], has been widely used as a first-line drug in the chemo-therapy of lung, ovarian and breast cancers [5–7]. However, the application of this chemical in treating HCC is limited, possibly due to drug resistance related to *P*-glycoprotein, Bcl-2, Bcl-xL, HURP, JNK and others mechanisms [8–10].

ATM (Ataxia-telangiectasia mutated) and ATR (ATM- and Rad3-related) are members of phosphatidylinositol-3-kinase-related protein kinase (PIKK) family. Both of these kinases are master regulators that are activated almost immediately after the generation of DNA lesions and coordinate multiple pathways of post-damage responses till damages are eliminated [11]. Previous studies have shown that inhibition of ATM and ATR by caffeine could significantly enhance cellular sensitivity to ionizing radiation [12]. Blocking ATM/ATR signaling with pharmacologic inhibitors, including novel ATM inhibitors KU55933 and CGK733, induced senescent breast, lung, and colon carcinoma cells to undergo cell death [13].

In this study, we have demonstrated that HBV-positive HCC HepG2.2.15 cells were significantly more resistant to taxol and other chemotherapy agents than the parental HBV-negative HepG2 cells. HepG2.2.15 exhibited defective phosphorylation of Chk1 and increased chromosomal instability. Treatment with CGK733, a small ATM/ATR kinase inhibitor, significantly enhanced the cytotoxicity of taxol to HBV-positive HCC cells. The mechanism of this chemo-sensitizing effect is likely because GCK733 accelerates the pre-mature exit of taxol-induced mitosis through formation of multinucleated cells.

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2. Materials and methods

2.1. Cell lines and culture conditions

Human hepatoblastoma cells HepG2 was obtained from Shanghai Biochemistry Institute. A subline HepG2.2.15 (2215) which contains a complete hepatitis B virus (HBV) genome [14] was kindly provided by Prof. Yanmeng Zhou (Zunyi Medical College, China). Both cell lines were maintained in Dulbecco modified Eagle medium (GIBCO) supplemented with 10% fetal bovine serum (Invitrogen) and penicillin–streptomycin (Hyclone). All cells were incubated at 37 °C with 5% CO_2.

2.2. Drug treatment and viability measurement

Cells (5×10^3) in logarithmic phase were seeded into 96-well plates in five parallel wells per group and allowed to adhere overnight. Supernatant was then decanted and the medium containing drugs at indicated concentrations was added. In the CGK733 (#C9867, Sigma) and taxol combination groups, CGK733 was added to the cells 1 h prior to taxol treatment [15]. After 72 h, 10 μl of freshly prepared MTT solution (5 mg/ml) was added into each well and the cells were incubated for another 4 h. Supernatants were removed and 150 μl of dimethyl sulfoxide (DMSO) was added. Absorption value of each well at a wavelength of 490 nm (A_{490}) was detected after shaking for 10 min. The relative viability of cells was determined by the average absorbance divided by that of the no-treatment cells. Each experiment was repeated three times.

2.3. Indirect immunofluorescence (IF) assay

Cells were grown on cover slips in 24-well plates and fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature (RT). Cells were then permeabilized with PBS containing 0.1% TritonX-100 and sequentially labeled with primary antibodies (α-tubulin mouse monoclonal, Sigma) and secondary antibodies (anti-mouse-FITC, DAKO) in a humidified chamber. Cover slips were sealed with mounting medium containing DAPI (Vector laboratories) and visualized by Olympus fluorescence microscope.

2.4. Western blot

For whole-cell extracts, cells were lysed directly in SDS sample buffer (50 nM Tris–HCl pH 6.8, 1% SDS, 10% glycerol, 5% β-ME, 0.1% bromphenol blue). Total proteins were separated by 6%–15% polyacrylamide gels and transferred to NC membrane (GE healthcare). Specific proteins were probed with primary antibodies (phospho-S345Chk1 rabbit polyclonal, CellSignaling; Aurora A mouse monoclonal, Sigma; Cyclin B1 rabbit polyclonal, CellSignaling; phospho-Histone H3 mouse monoclonal, CellSignaling) in TBST (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20) supplemented with 5% non-fat milk. Chemoluminescent visualization was performed with horseradish peroxidase-conjugated secondary antibodies (DAKO) and detected by ChemiDocXRS system (Bio-Rad).

2.5. Statistical analysis

Statistical significance of differences between groups was determined by Student's *t*-test. A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. HBV-positive HCC cells exhibit increased resistance to chemotherapy agents

To investigate the impact of HBV on the sensitivity of HCC cells to chemotherapy agents, we examined cell viability after treatment with various drugs commonly used to treat cancers using HCC HepG2 cells and HBV-positive 2215 cells. HepG2 cells were sensitive to the treatment of taxol with only 1/3 of cell viability at the drug concentration of 7 nM as compared with no-treatment control (Fig. 1A). In contrast, 2215 cells displayed significantly increase resistance to taxol treatment with minimal cell viability loss at the drug concentration of 7 nM and around 40% of cell viability remaining at the highest concentration tested (117 nM) (Fig. 1A). Moreover, 2215 displayed similarly increased resistance to Cisplatin and Adriamycin as compared with HepG2 cells (Fig. 1B). These results demonstrated that HBV-positive 2215 cells are more resistant to chemotherapy agents than the parental HCC cells HepG2, presumably due to the impact of the integrated HBV genome.

3.2. HBV-positive HCC cells exhibit defective Chk1 phosphorylation and increased chromosomal instability

Previous studies have suggested that Chk1-deficient cells exhibit increased resistance to taxol [16]. To examine if Chk1 is defective in HBV-positive 2215 cells, we detected the amount of total Chk1 and phosphorylation of Chk1 at Ser345 in HepG2 and 2215 cells after Camptothecine (CPT) treatment by Western blot. HepG2 and 2215 cells were harvested and lysed after CPT (2 μM) treatment at different time points. The results showed that the total as well as phosphorylated (Ser345) Chk1 of 2215 cells following CPT treatment were significantly decreased compared with HepG2 cells (Fig. 2A). This indicate that Chk1 and its phosphorylation on Ser345 were defective in HBV-positive 2215 cells and Chk1 deficiency could be one of the reasons that rendered 2215 cells to be resistant to taxol treatment.

Chk1 deficiency has been found to be associated with chromosomal instability [17]. We therefore assessed the amount of abnormal chromosomes in HBV-positive HCC cells 2215 and the parental HepG2 cells. HBV-positive cells 2215 displayed significantly higher amount of abnormal chromosomes (Fig. 2B) ($P < 0.01$), potentially as a result of Chk1 deficiency.

3.3. CGK733 enhances taxol-induced cytotoxicity in HBV- positive HCC cells

In order to explore strategies to enhance the chemo-sensitivity of HBV-positive HCC cell, we investigated the effect of an ATM/ATR inhibitor CGK733 on the efficacy of taxol in 2215 cells. CGK733 inhibited the growth of 2215 cells in a dose-dependent manner starting from 4.2 ng/ul ($P < 0.01$) (Fig. 3A). While treatment of 4.2 ng/μl CGK733 and 35 nM taxol individually for 72 h only caused less than 50% of cell viability loss, the combinational treatment exhibited significantly improved cytotoxicity on 2215 cells which resulted residual cell viability (Fig. 3B). Thus CGK733 significantly enhanced taxol-induced cytotoxicity in HBV-positive HCC cells.

3.4. CGK733 accelerates the formation of multinucleated cells and promotes the exit of mitosis in taxol-treated HBV-positive HCC cells

Taxol can activate the spindle assembly checkpoint (SAC), thereby blocking cells in mitosis [18]. To explore the mechanisms

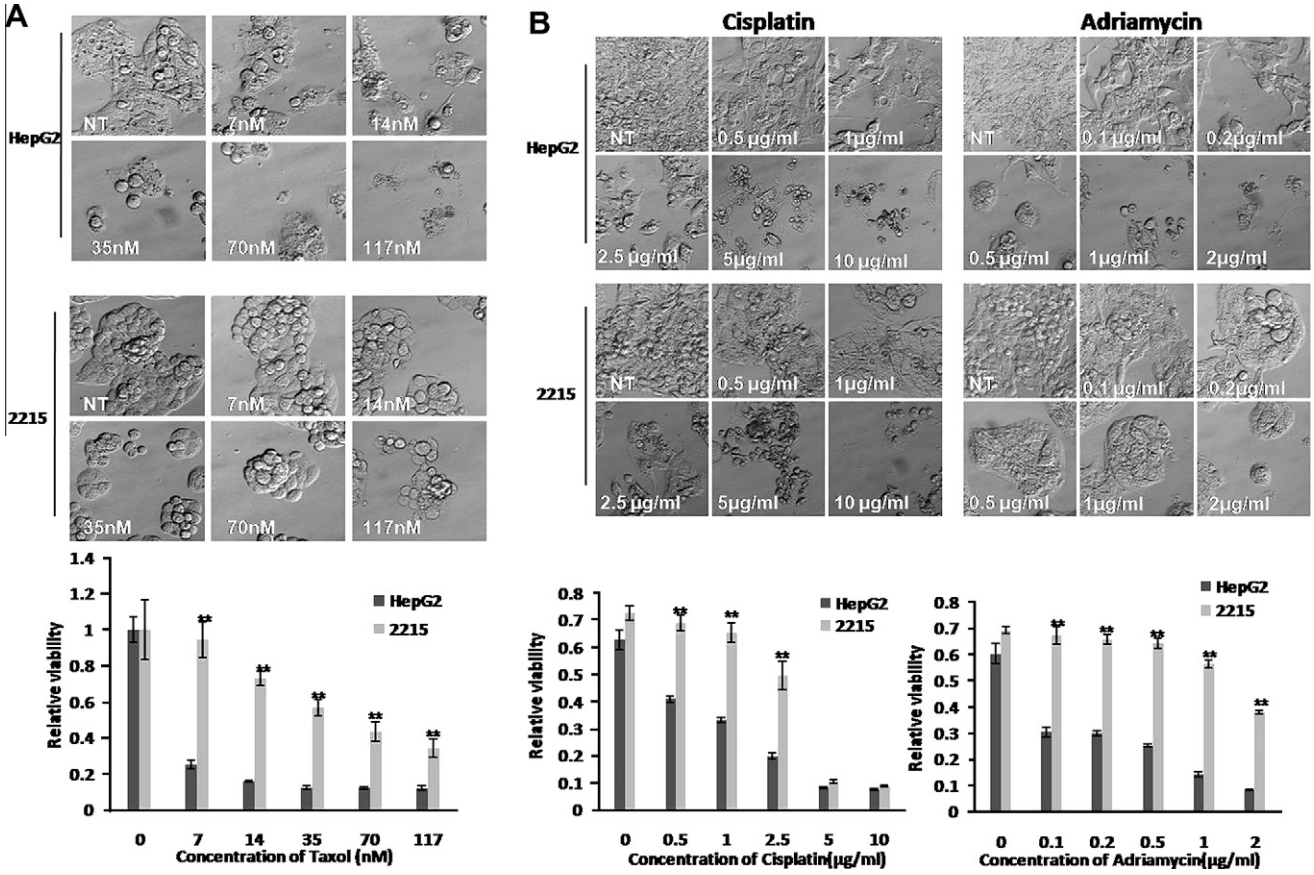

Fig. 1. HBV-positive HCC cells are resistant to chemotherapy agents treatment. (A) HCC HepG2 cells and HBV-positive subline 2215 cells were treated with indicated doses of taxol for 72 h and cell viability was determined by light microscope (upper panel) and MTT assays (lower panel). NT, no-treatment. (B) Viability of HepG2 and 2215 cells following treatment of Cisplatin and Adriamycin for 72 h. Results were represented by mean ± S.E. from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ as compared with HepG2 cells with the same treatment.

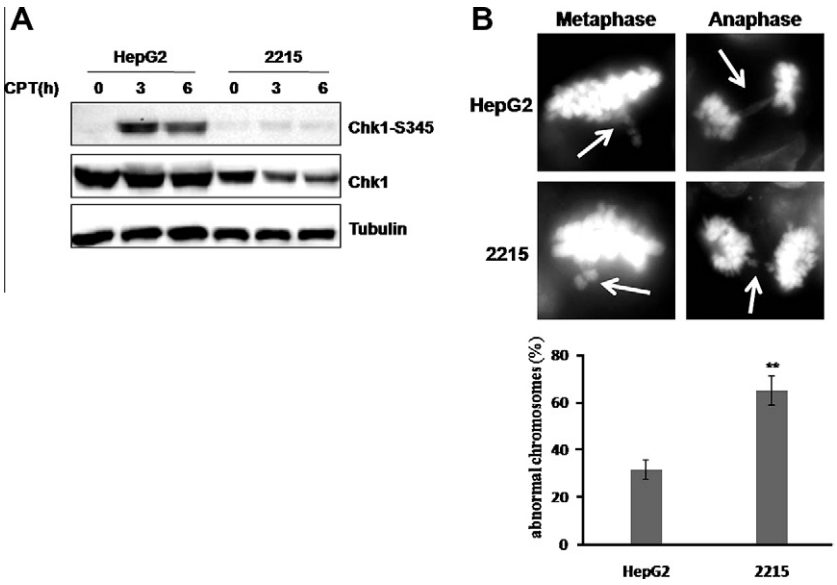

Fig. 2. HBV-positive HCC exhibits defective phosphorylation of Chk1and increased chromosomal instability. (A) HepG2 and 2215 cells were lysed and analyzed for total Chk1 and phosphorylation of Chk1 Ser345 after CPT treatment at the indicated time points by Western blot. (B) Mitotic chromosomes of HepG2 and 2215 cells were visualized with mounting medium with DAPI (upper panel). Arrows indicate the lagging chromosome lagging behind most of the segregating chromosomes. The histogram (lower panel) is the quantitative results with means and standard deviations from three independent experiments, counting 100 cells in each experiment. $^{**}P < 0.01$ as compared with HepG2 cells.

that CGK733 enhances taxol-induced cytotoxity on HBV-positive HCC, we examined the effect of CGK733 on the formation of mitosis in taxol-treated cells. As shown in Fig. 4A, taxol-induced mitotic cells exhibited similar kinetics in both HepG2 and 2215 cells with a

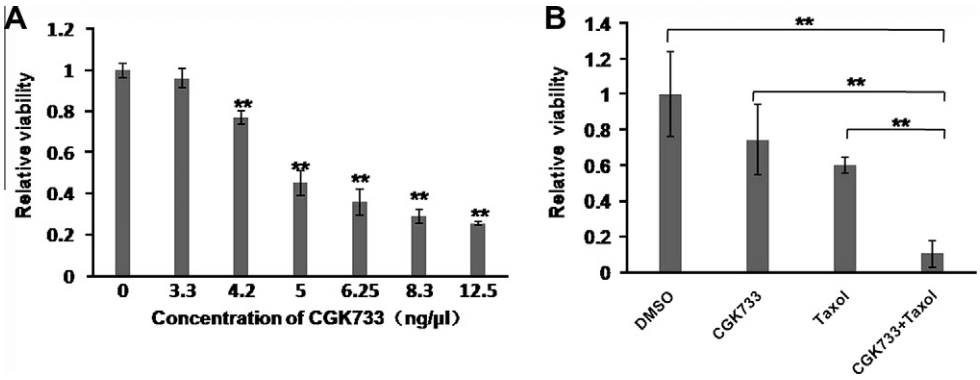

Fig. 3. CGK733 enhances taxol-induced cytotoxicity in HBV- positive HCC cells. (A) Viability of 2215 with treatment of CGK733 (0–12.5 ng/μl) for 72 h. $^{**}P < 0.01$ as compared with no-treatment. (B) Viability of 2215 cells following treatment of CGK733 (4.2 ng/μl), taxol (35 nM), or in combination. DMSO was used as a control. Results represent the mean ± S.E. from three independent experiments. $^{**}P < 0.01$.

peak at 24 h following treatment and then declined rapidly. Moreover, the amount of mitotic cells induced by taxol treatment in 2215 was comparable to that in HepG2 cells, suggesting HBV did not interfere with taxol-treated HCC cells entering mitosis. CGK733 treatment slightly enhanced the amount of HepG2 mitotic cells but not the amount of 2215 mitotic cells induced by taxol. The kinetics of mitosis in 2215 was confirmed by phosphorylation of Histone 3 (H3-S10) which increased at 24 h in cells treated with taxol alone and taxol in combination with CGK733 and then decrease afterwards (Fig 4B). Aurora-A is an important protein involved in several mitotic phases and cyclin B is a mitotic cyclin. The amount of protein level of Aurora A and cyclin B did change after treatment, suggesting that 2215 cells were not arrested at mitosis (Fig 4B).

Taxol is an inducer of mitotic catastrophe, a way of cell death. With treatment of taxol, cells exit mitosis in the presence of misaligned or mis-segregated chromosomes, known as a process progressing from mitotic slippage to multinucleated status, eventually, cells died. We next examined the effect of CGK733 on cell nucleus in taxol-treated cells using antibodies against α-tubulin and DAPI. HepG2 cells, but not the HBV-positive 2215 cells, exhibited multinucleated cells formation following taxol-treatment. While addition of CGK733 did not significantly influence the multinucleated cell formation on HepG2 cells, 2215 cells treated with CGK733 in combination with taxol displayed significantly increased multinucleated cells. Notably, the amount of multinucleated cells induced by CGK733 in combination with taxol in 2115 (Fig. 4C) reached a level comparable to that observed in parental HepG2 cells (Fig. 4D). These results suggest that CGK733 promotes the exit of taxol-induced mitosis in 2215 cell by enhancing the multinucleated cell formation.

4. Discussion

HCC is one of the most prevalent and deadly cancers in the world. For the patients with HCC, effective chemotherapeutic drugs are greatly desired in addition to surgical resection and radiofrequency ablation. Chronic HBV infection is a major risk for the development of HCC. In China, more than 90% patients of HCC are associated with HBV infection [2]. Therefore research for reagents to attenuate the resistance of HBV-positive HCC cell to chemotherapeutic drugs is highly demanded.

In this study, we confirmed that 2215, a HBV-positive HCC cell line, exhibits increased resistance to taxol and other chemotherapeutic agents than the HBV-negative parental cell line HepG2. HBV-positive 2215 cells also exhibit defective Chk1 phosphorylation and increased chromosomal instability. A small molecule, CGK733, significantly enhanced the taxol-induced cytotoxity in HBV-positive HCC cells. The mechanism probably lies in CGK733 triggers the multinucleated cell formation in taxol-treated cells thus promotes premature exit of mitosis.

Taxol causes cell death mainly through microtubule stabilization and the cell cycle arrest in mitosis [4]. The mitotic arrest caused by taxol is dependent on the spindle checkpoint. The spindle checkpoint signaling involves the function of several highly conserved proteins and including Mad1, Mad2, Mad3/BubR1, Bub1, Bub3 and Mps1 [19]. With prolonged treatment, cells exit mitosis in the presence of misaligned or mis-segregated chromosomes, known as a process progressing from mitotic slippage to multinucleated status [20]. Multinucleation is a morphological marker for the detection of mitotic catastrophe (MC), which normally leads to cell death [21]. MC continues to be an attractive endpoint for the development of novel anticancer agents [22].

Taxol is widely used in the treatment of solid tumors including breast cancer, ovarian cancer and lung cancer [23]. However, the application of taxol in treating HCC is limited, possibly due to drug resistance from some uncharacterized mechanisms. There are multiple mechanisms that cancer cells may develop resistance against taxol. Bcl-2 and Bcl-x(L) play an important role in mediating resistance to taxol in HCC cells [8]. In addition, HURP acts as a novel survival protein that protects HCC cells against taxol-induced cell death [9]. And resistance to taxol in hepatoma cells is related to static JNK activation [10].

It has been reported that Chk1-deficient cells exhibit increased resistance to taxol. Chk1, a component of the DNA damage and replication checkpoints, protects cells from spontaneous chromosome mis-segregation and chromosomal instability. In addition, Chk1 functions in spindle checkpoint because a fraction of this protein localizes to spindle and Chk1-deficient cells fail to sustain mitotic arrest in the presence of taxol [16]. Intriguingly, we found that 2215 cells was phenotypically similar to Chk1-deficient cells with total Chk1 amount as well as its phosphorylation on ser345 being impaired after CPT treatments. Moreover, 2215 cells exhibited more chromosomal instability than HepG2 cells. The upstream Chk1 kinase ATR phosphorylates Chk1 on both S317 and S345 [24]. We speculate the ATR pathway was impaired by some molecules encoded by HBV, which may have caused the defection and inactivation of Chk1and resulted in chromosomal instability and resistance to taxol.

Nowadays, the clinical use of taxol is limited mainly by drug resistance and toxicity [23,25]. Therefore agents that can increase the efficacy of taxol thus patients can be treated with a lower dose of the chemotherapy agents will be beneficial. At present, application of small molecular inhibitor-based therapeutics has gained

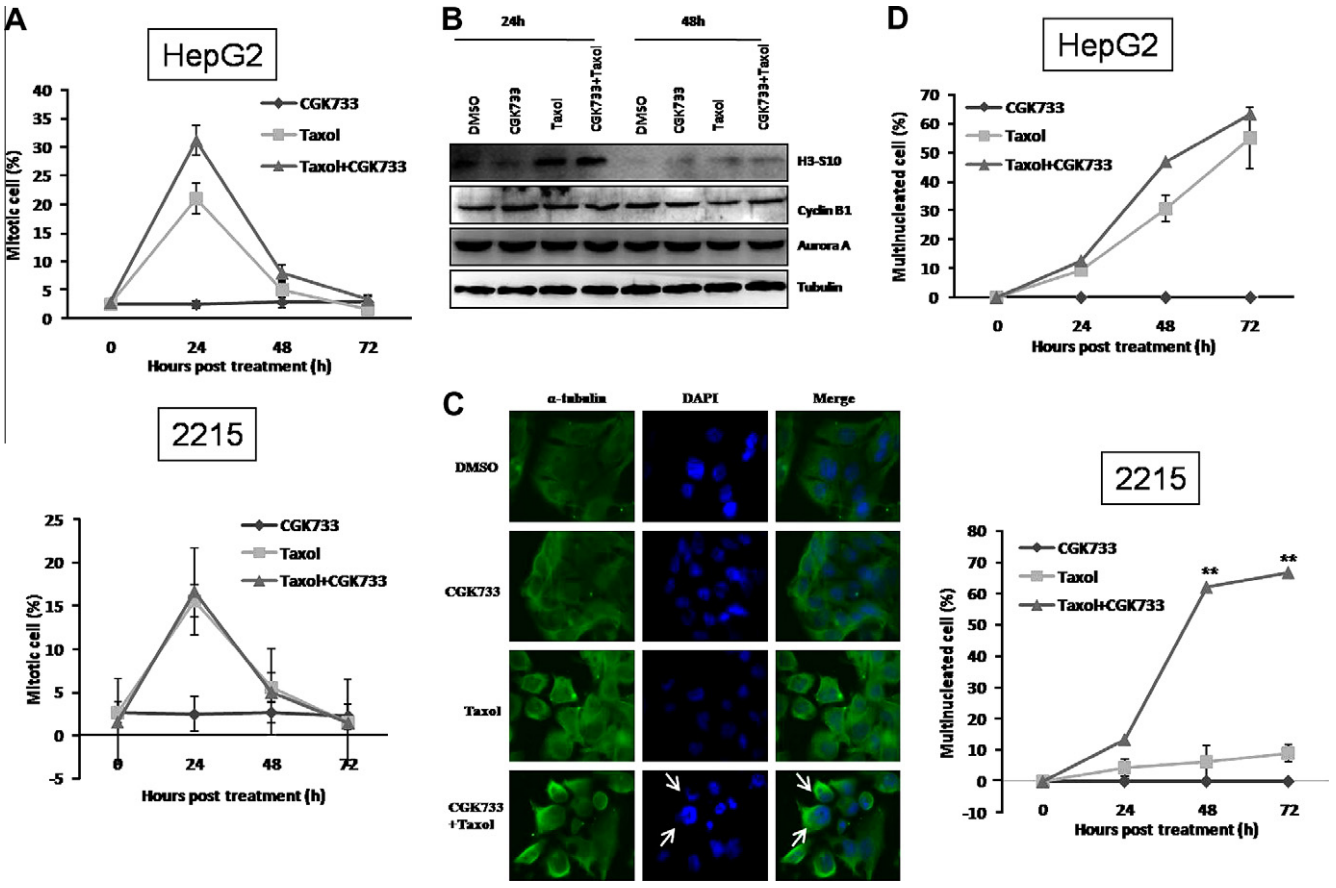

Fig. 4. CGK733 induces the increase of multinucleated cells and promotes the exit of mitosis caused by taxol. HepG2 and 2215 cells were treated DMSO, CGK733, taxol or CGK733 in combination with taxol. Mitosis and multinucleated cell formation were evaluated under fluorescence microscope. (A) Three hundred cells per group were counted to calculate the mitotic cells at different time points. (B) 2215 cells were harvested after treatment with taxol (35 nM) and CGK733 (4.2 ng/μl) at indicated time points, and cell lysates were subject to immunoblotting with indicated antibodies. (C) HBV-positive HCC 2215 cells were treated with GCK733 and taxol individually or in combination for 48 h. Cells were then stained with α-tubulin antibody (green) for detection of microtubules and with DAPI (blue) for detection of nuclei. Representative fluorescence microscopy images (1000×) were shown with arrow indicating multinucleated cell. (D) Three hundred cells per group were counted to calculate the multinucleated cells at different time points, $^{*}P < 0.05$; $^{**}P < 0.01$ as compared with taxol alone.

considerable attention due to their enormous potential to cause cell death in cancer cells [26–28].We found that CGK733, a specific inhibitor targeting the kinase activities of ATM and ATR without affecting any other domains required for cell proliferation [29], may also play a role in mitotic checkpoint. Our study suggests that CGK733 can enhance the sensitivity of 2215 cells to taxol by accelerating the process in cells from misaligned or mis-segregated chromosomes slippage to multinucleated status, resulting in mitotic catastrophe and cell death. More importantly, taxol showed fairly good lethal effect with lower concentration (35 nM) when combined with CGK733, while it exhibited the cytotoxicity to other cancer cells at much higher concentrations (micromolar level) in the previous studies [30,31]. These results suggest that the combination treatment of CGK733 with lower concentration of taxol not only increased the sensitivity of 2215 cells to taxol but also can potentially reduce toxicity caused by taxol in chemotherapy.

In conclusion, we have demonstrated the combination of CGK733 and taxol was effective in the reversal of the taxol-resistance of Chk1-deficient, HBV- positive HCC 2215 cells. It can be explained that the combination of CGK733 and taxol provoked premature mitotic exit of mitotic-damaged cells followed by multinucleation and mitotic catastrophe caused by taxol. Our findings have indicated a novel strategy in the therapy of HBV-related HCC that have been resistant to most radio/chemo-therapies.

Acknowledgments

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