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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