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BI6727, a polo-like kinase 1 inhibitor, synergizes with gefitinib to suppress hepatocellular carcinoma cells via a G2/M arrest mechanism

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Hepatocellular carcinoma (HCC) is the second leading cause of cancer death, which indicates that efficient intervention agents or strategies against HCC are urgently needed. In the present study, we firstly found that a combination of gefitinib (an epidermal growth factor receptor (EGFR) inhibitor) and BI6727 (a polo-like kinase 1 (PLK1) inhibitor) could significantly inhibit cell proliferation of HCC cells, which attenuated acquired resistance of gefitinib in HCC cells. Interestingly, our results showed that these anti-tumor effects of gefitinib in combination with BI6727 were associated with G2/M arrest. Moreover, further study revealed that BI6727 could downregulate the protein levels of cell division cycle 25C (Cdc25C) via ubiquitination-dependent pathway, which subsequently induced G2/M arrest. Furthermore, two critical checkpoints proteins ataxia telangiectasia-mutated (p-ATM)/ATM and Rad-3 related(p-ATR) and another hallmark phosphorylated H2AX (γ-H2AX) of DNA damage were positively regulated in HCC cells exposed to gefitinib in combination with BI6727. These results indicated that co-treatment induced G2/M arrest was closely related to DNA damage. In summary, the present study discovered that gefitinib synergizing with BI6727 could significantly facilitate DNA damage and overcome acquired resistance of HCC cells to gefitinib. Our study provides a promising approach for the combination of EGFR inhibitors and PLK1 inhibitors in the clinical treatment for HCC.

1. Introduction

Hepatocellular carcinoma is the second leading cause of cancer death, which is one of the most common cancer all over the world (Torre et al. 2015). In the United States, the incidence of hepatocellular carcinoma has tripled while the 5-year survival rate has remained below 12% during the past two decades (El-Serag 2011). Due to the poor prognosis and insignificant symptoms, neither surgical resection, liver transplantation, nor regional therapy are applicable to patients with advanced HCC while systemic chemotherapy remains the only effective treatment. Currently, sorafenib is the first-line drug for HCC. However, its clinical efficiency is severely limited due to the development of drug resistance. Therefore, exploring potential therapeutic targets or novel combination strategies is urgently needed.

The epidermal growth factor receptor tyrosine kinase (EGFR-TK) plays an essential role in cell proliferation, survival and migration whose altered activity has been implicated in the development and growth of many tumors including HCC. Gefitinib (also called Iressa or ZD1839), a small molecular inhibitor of EGFR-TK, demonstrates anti-tumor ability in HCC as well. The preclinical study indicated that gefitinib potently inhibited growth and induced cell cycle arrest and apoptosis in human HCC cells due to the suppression of EGFR-TK (Hopfner et al. 2004). The blockade of EGFR activity by gefitinib had an anti-tumor effect on the malignant development of HCC (Schiffer et al. 2005). These results indicate that EGFR-TK appears to be a novel therapeutic target against HCC. However, gefitinib monotherapy followed by acquired resistance has limited therapeutic efficacy in HCC. For example, the co-treatment of gefitinib and silibinin had synergistic effects in SNU761 cell line while it was only additive anti-tumor effect in the Huh-BAT cell line (Gu et al. 2015). Furthermore, gefitinib facilitated nuclear translocation of IGF-1R, which might lead to drug resistance in HCC (Bodzin et al. 2012). These facts indicate that it is extremely urgent to create novel combination strategies in order to overcome gefitinib-acquired resistance of HCC cells.

In addition to EGFR-TK, polo-like kinase proteins, a serine/threonine-protein kinase of the cell cycle, might be other potential therapeutic targets against HCC. PLK proteins play central roles in apoptosis, DNA damage response as well as the control of cell cycle progression where suppression of PLK1 results in a block in the G2/M phase (Pellegrino et al. 2010). PLKs is a family of four members including PLK1, PLK2, PLK3 and PLK4. Notably, these PLK proteins possess different functions in HCC. PLK1, which is overexpressed in HCC (He et al. 2009), acting as an oncogene. In sharp contrast, PLK2/3/4 are considered as tumor suppressor genes. Thus, innovative therapeutic approaches which activate PLK1 and/or reactivate PLK2/3/4 might be greatly useful in the treatment of human HCC (Pellegrino et al. 2010). A previous study denoted that PLK1 could dictate sensitivity of tumor cells to irinotecan (SN38) which is a well-known anti-tumor agent (Zuco et al. 2015). Furthermore, overexpression of PLK1 has been reported to be involved in chemoresistance, including HCC (Sero et al. 2014; Gutteridge et al. 2016; Shin et al. 2019). Shin et al. (2021) revealed that dual targeting of gefitinib and PLK1 inhibitors could overcome the chemoresistance in lung adenocarcinoma. Expression of ABC transporters has been reported to become a main resistant factor of chemotherapy, including gefitinib (Beretta et al. 2017; Zhao et al. 2018; Wang et al. 2019). Some studies showed that chemo-resistant cancer cells upregulate the levels of PLK1 with ABC transporters, and inhibition of PLK1 could downregulate the levels of ABC transporters (Shin et al. 2021). Therefore, inhibition of the activity of PLK1 may be a strategy to overcome chemoresistance. Herein, we hypothesize that inhibitors of PLK1 in combination with common anti-tumor agents like gefitinib may exert synergistic effects in the treatment of HCC.

Based on these background, we examined the anti-tumor effects of the combination of BI6727, a potent PLK1 inhibitor, and gefitinib in HCC cell lines. We found that the combination of BI6727 and gefitinib exhibited synergistic anti-tumor effects through DNA damage as well as subsequent cell cycle arrest, which conse-

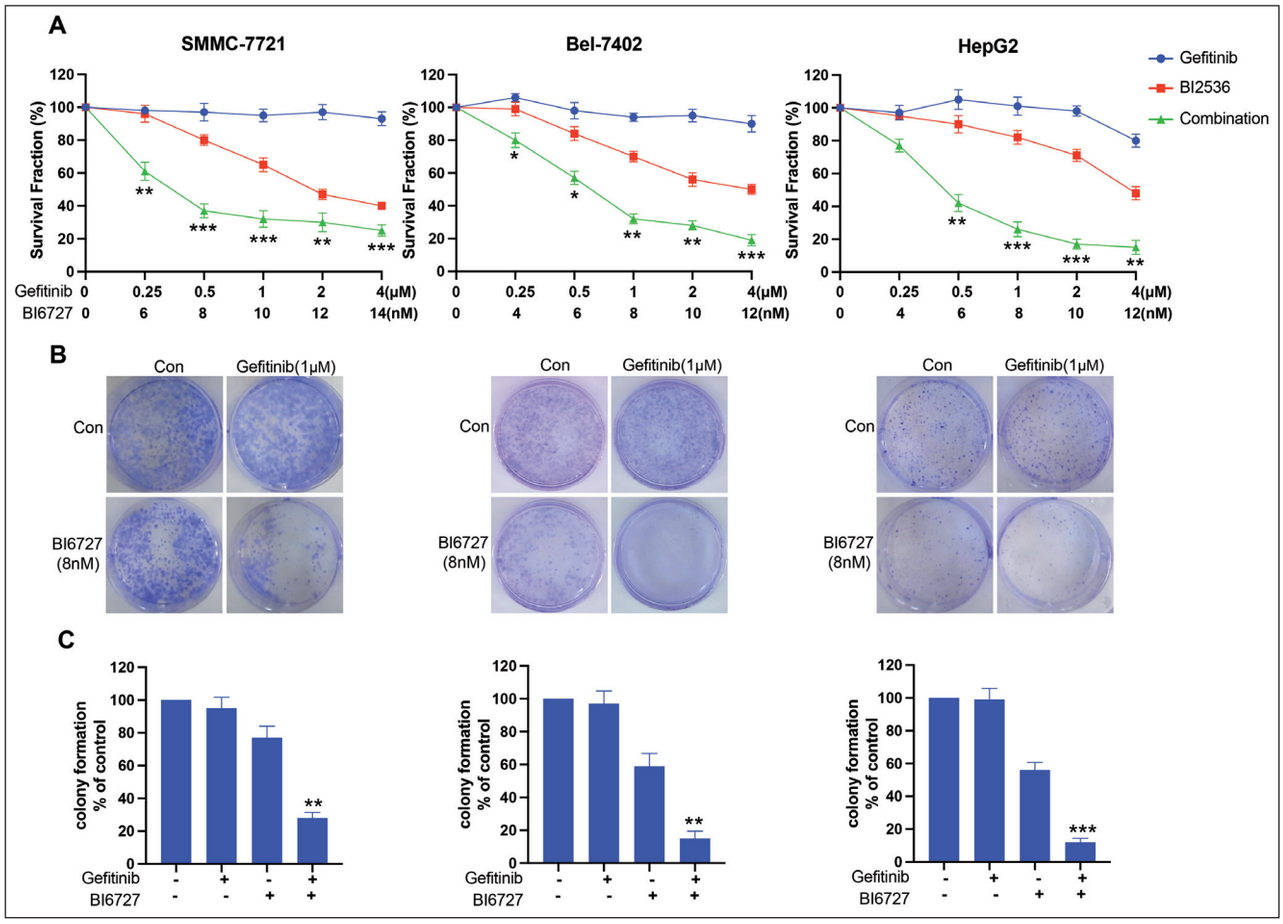


Fig. 1: Combinational cytotoxicity of gefitinib and BI6727 in SMMC-7721, Bel-7402 and HepG2 cell lines. (A) SMMC-7721, Bel-7402 and HepG2 cells were treated with serial concentrations of gefitinib, BI6727 or the combination for 72 h, and subsequently survival fractions of each group were calculated by a SRB staining assay.Data are representative of three independent experiments and are expressed as the mean ± SD. *p<0.01, **p<0.01, ***p<0.001(compare with gefitinib group); Student's t test. (B) Cells treated with gefitinib (0.5 μM) and/or BI6727 (8 nM) for 24 h were cultured at the same count for 9 days. The colonies of each group were photographed after stained by a 0.1% crystal violet solution. A representative picture of each group is shown as well as the colony formation rates in bar chart compared to control (C). Data are representative of three independent experiments and are expressed as the mean ± SD. **p<0.01, ***p<0.001(compare with gefitinib group); Student's t test.

quently resulted in cytotoxicity to HCC cell lines. Thus, combined treatment of gefitinib and BI6727 may constitute a potent clinical therapy approach for the treatment of HCC.

2. Investigations and results

2.1. Synergistic anti-tumor effects of gefitinib and BI6727 in HCC cell lines

To evaluate the anti-proliferative effects of different doses of BI6727, alone or in combination with gefitinib, we did a SRB assay on a panel of HCC cell lines. Human HCC cell lines SMMC-7721, Bel-7402 and HepG2 were treated with a series concentration of gefitinib (0-4 μM), BI6727 (0-14 nm) or the combination for 72 h. As shown in Fig. 1A, single treatment of gefitinib imposed moderate anti-proliferative effects on human HCC cell lines, which indicated that those human HCC cell lines were insensitive toward gefitinib. In contrast, a 72h exposure to BI6727 and gefitinib could greatly extend the anti-cancer activities as indicated by the significantly reduced survival fractions in all the three tested HCC cell lines. These results suggested that a combination of gefitinib and BI6727 had synergistic effects in growth arrest, which might overcome the gefitinib resistance of HCC cell lines.

Next, we observed the morphology of cells exposed to gefitinib and/or BI6727. Human HCC cells lines treated with gefitinib (1 μM), BI6727 (8 nM) or the combination were imaged by optical microscopy. As shown in Fig. 1B, cell viability in the combination group significantly decreased compared to treatment with either single agent.

We then tested the long-term synergistic effects of BI6727 (8 nM) alone and in combination with gefitinib (0.5 μM) on anchorage-independent colony formation of HCC cell lines *in vitro*. As shown in Fig. 1C, HCC cells showed little sensitivity to single-agent gefitinib treatment. Whereas, the sensitivity of HCC to gefitinib was largely enhanced when treated with BI6727 in combination and the formation of colony was deducted to less than 20% of untreated cells compared to untreated cells.

2.2. Gefitinib in combination with BI6727 induced G2/M arrest in human HCC cell lines

We further asked whether the increased anti-proliferative effect induced by BI6727 *plus* gefitinib would be the result of an increased cell cycle arrest. Therefore, FACS analysis was further utilized to investigate the cell cycle distribution in human HCC cell lines. After exposure to gefitinib (1 μM), BI6727 (6 nM) and the combination for 24 h, cell cycle distribution of SMMC-7721, Bel-7402 and HepG2 cell lines were analyzed respectively. As shown in Fig. 2, flow cytometry analysis of SMMC-7721, Bel-7402 and HepG2 revealed that minimal changes in cell cycle distribution were detected in all the three tested HCC cell lines exposed to gefitinib alone. Notably, in consistent with the observation achieved on cell proliferation assay, cells treated with gefitinib in combination with BI6727 were significantly arrested in G2/M-phase (71.2% in HepG2, 69.9% in Bel-7402, 70.1% in SMMC-7721), concomitant with a dramatic decrease in G1-phase population. These findings further validated that BI6727 in combination with gefitinib exerted

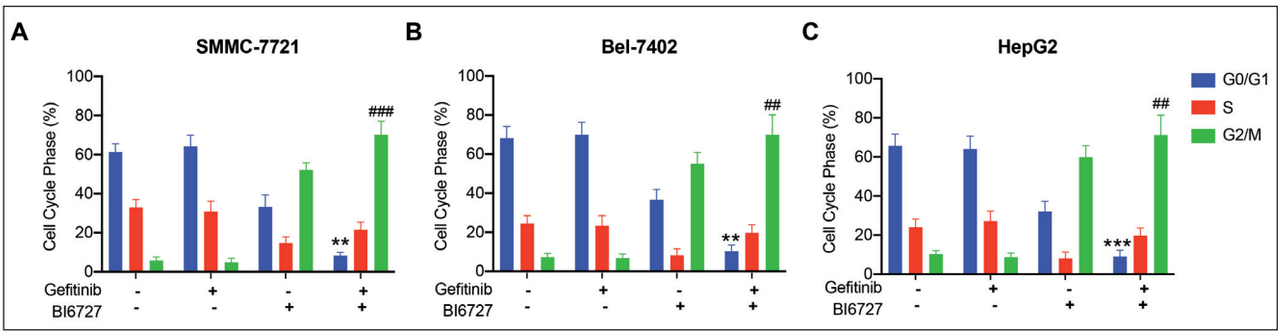


Fig. 2: Cell cycle distribution of SMMC-7721, Bel-7402 and HepG2 incubated with gefitinib (1 μM) and/or BI6727 (6 nM) for 24 h. A-C, Representative histograms of cell cycle analyses were shown, which reflect the percent of cells in each phase. Data are representative of three independent experiments and are expressed as the mean ± SD. **p<0.01, ***p<0.001(compare with control group); ##p<0.01, ###p<0.001(compare with control group); Student's t test.

enhanced anti-proliferative effects by inducing G2/M phase cell cycle arrest, which subsequently overcome gefitinib resistance of human HCC cells.

2.3. Cdc25C downregulation by BI6727 contributed to enhanced anti-cancer effects by the combination

The above-mentioned data suggested that the G2/M arrest of HCC might play critical roles in the combination therapy. To elucidate the mechanism causing G2/M arrest in gefitinib *plus* BI6727-treated cells, we determined its effects on the expression of proteins important in the G2/M transition. Mounting evidence has shown that Cdc25C, a cell cycle regulator, plays a central role in M-phase entry (Perdiguero and Nebreda 2004; Boutros et al. 2007; Giono et al. 2017). Cdc25C is inactive during most of cell cycle. Once activated at the onset of M-phase, Cdc25C dephosphorylates cell division cycle protein 2 homolog (Cdc2)/Cyclin B complex and suppresses G2/M transition. In the present study, we explored whether combination therapy-induced G2/M arrest was associated with Cdc25C. Therefore, Cdc25C, which triggers entry into mitosis, was chosen to further validate findings mentioned above. Our first test was to determine whether the protein level of Cdc25C altered in response to co-treatment of gefitinib and BI6727.

As shown in Fig. 3A, we firstly found that gefitinib (1 μM) alone imposed minimal effect on Cdc25C protein levels in SMMC-7721 cells. In contrast, BI6727 (6 nM) could significantly downregu-

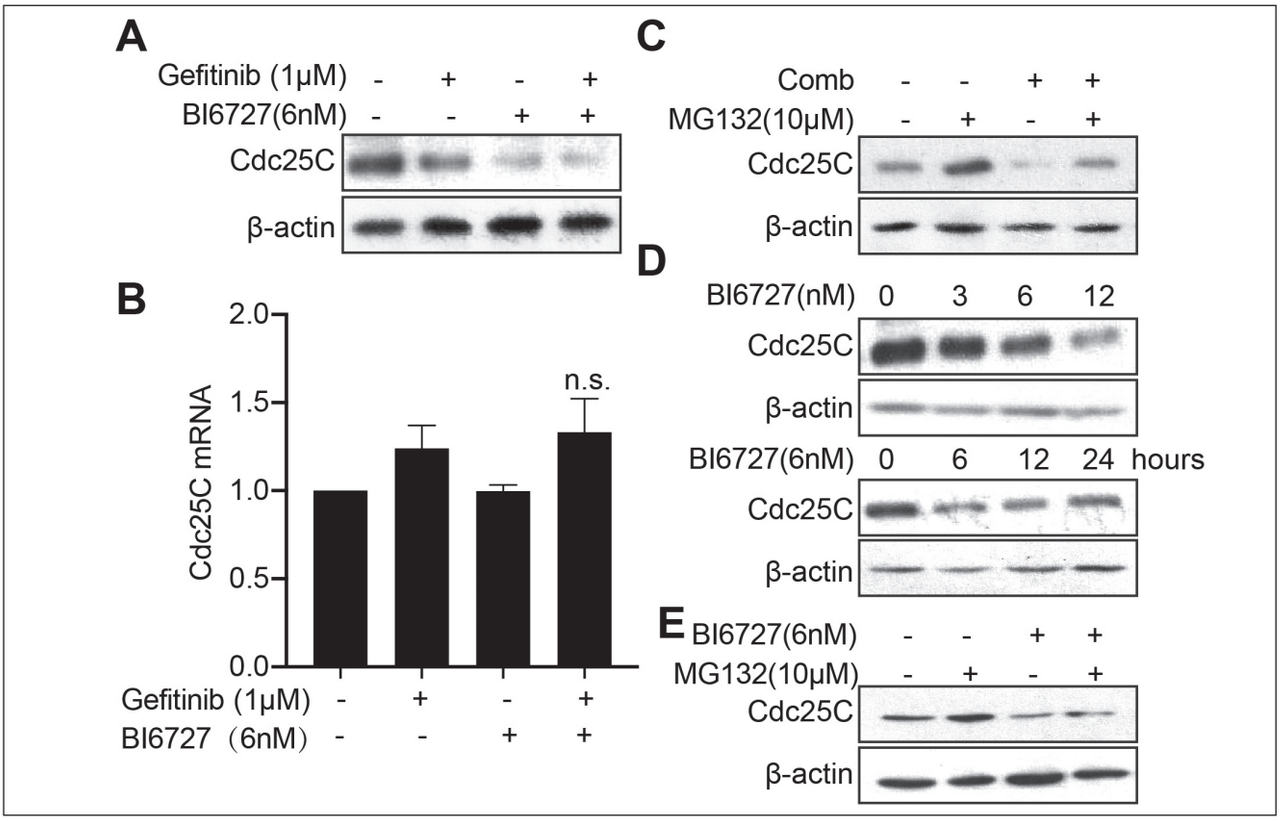
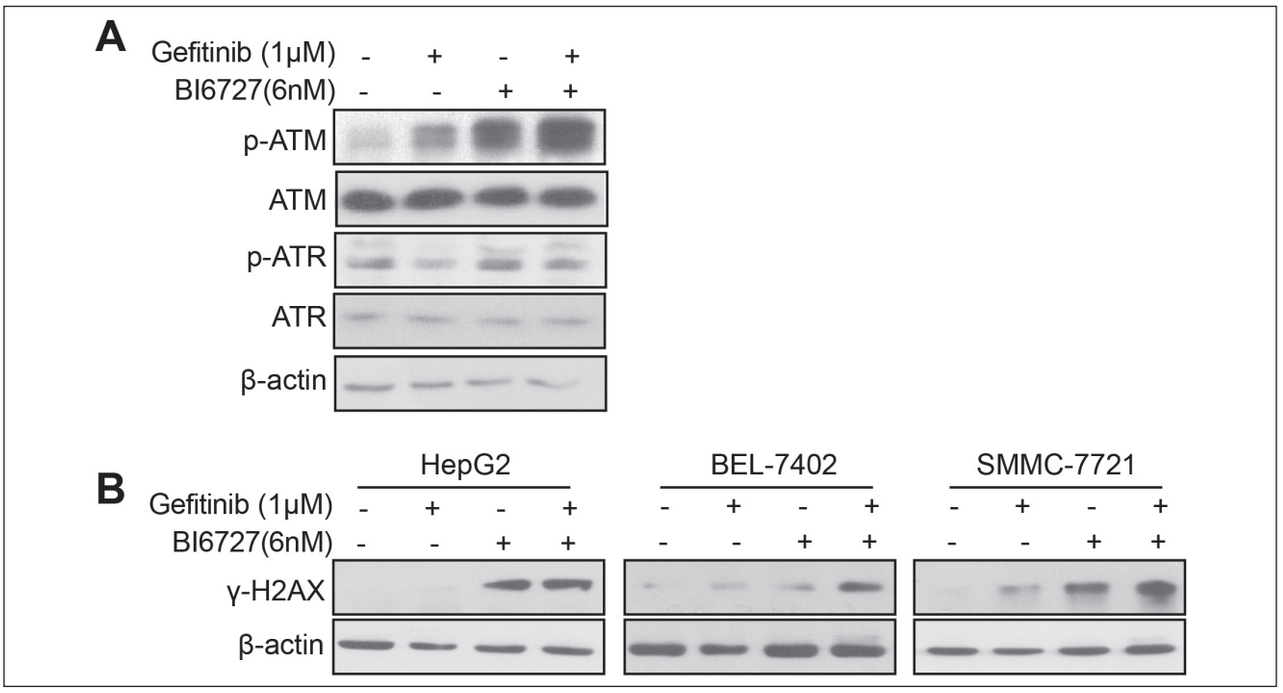


Fig. 3: Combination therapy aggravated downregulation of Cdc25C in SMMC-7721 cell lines. (A) SMMC-7721 cells were treated with gefitinib (1 μM) and/or BI6727 (6 nM) for 24 h. Then effects on protein expression of Cdc25C were analyzed through western blot. Meanwhile, we probed for β-actin as the loading control. (B) SMMC-7721 cells were treated with gefitinib (1 μM) and/or BI6727 (6 nM) for 24 h. Then relative mRNA levels of Cdc25C were analyzed through qRT-PCR. Data are representative of three independent experiments and are expressed as the mean ± SD. n.s. represent no siginificance. (C) SMMC-7721 cells were treated with gefitinib (1 μM) plus BI6727 (6 nM) for 24 h. Eight hours before test, cells were treated with MG132 (10 μM) according to the purpose of experiments, then the protein levels of Cdc25C was detected by western blot. Meanwhile, we probed for β-actin as the loading control. (D) SMMC-7721 cell lines were treated with BI6727 alone in a serial concentration (3, 6, 12 nM) for 24 h or incubated with BI6727 (6 nM) for different times (0, 6, 12, 24 h). western blot was utilized to measure the protein level of Cdc25C. Meanwhile, we probed for β-actin as the loading control. (E) SMMC-7721 cells exposed to BI6727 (6 nM) or not for 24 h. Eight hours before test, cells were treated with MG132 (10 μM) according to the purpose of experiments. Then western blot was performed to analyze protein level of Cdc25C. Meanwhile, we probed for β-actin as the loading control.



Fig; 4: Enhanced effects of gefitinib plus BI6727 on upregulation of p-ATM, p-ATR and γH2AX. (A) HepG2 cells were exposed to gefitinib (1 μM) and/or BI6727 (6 nM) for 24 h respectively. Protein levels of p-ATM/p-ATR were tested. (B), HepG2, Bel-7402 and SMMC-7721 cells were exposed to gefitinib (1 μM) and/or BI6727 (6 nM) for 24 h respectively.were detected by western blot analysis. Meanwhile, we probed for β-actin as the loading control.

late Cdc25C protein levels. Notably, expression of Cdc25C could be completely abolished when exposed to gefitinib and BI6727 in combination. To explore the mechanism of downregulation of Cdc25C protein level, qRT-PCR analyses were performed to explain whether combination-induced downregulation of Cdc25C protein level was dependent on transcription inhibition. The results of qRT-PCR showed that mRNA level of Cdc25C was not distinctly changed when treated with gefitinib (1 μM) and BI6727 (6 nM) (Fig. 3B), suggesting that the downregulation of Cdc25C protein level was owning to the post-translation modifications rather than the transcription inhibition.

The ubiquitin-proteasome pathway is a fundamental mechanism that mediates post-translational modulation of intracellular proteins. Some recent evidence revealed that ubiquitination-dependent degradation pathways were involved in downregulation of Cdc25C protein level (Chen et al. 2002; Eymin et al. 2006; Chou et al. 2013; Giono et al. 2017). Based on these studies, MG132, a proteasome inhibitor, was utilized to probe the specific protein degradation mechanisms. Initially, we incubated SMMC-7721 cells with proteasome inhibitor MG132 (10 μM) before combination treatment of gefitinib (1 μM) and/or BI6727 (6 nM). The western blot results in Fig. 3C showed that combination treatment dramatically decreased Cdc25C level, meanwhile, MG132 was able to rescue co-treatment induced Cdc25C degradation. Analysis mentioned above suggested that gefitinib in combination with BI6727 arrested the cell cycle at G2/M-phase through induction of Cdc25C degradation via ubiquitin–proteasome pathway.

Since the inhibition of PLK1 consequently resulted in a block in the G2/M phase (Pellegrino et al. 2010). BI6727, a PLK1 inhibitor, was considered to play a fundamental role in the synergistic effects. Therefore, we examined the effects of BI6727-monotreatment in facilitating degradation of Cdc25C. As shown in Fig. 3D, BI6727-monotreatment was able to downregulate Cdc25C levels in a time- and dose-dependent manner. Strikingly, we found that treatment of BI6727 alone was able to induce degradation of Cdc25C (Fig. 3E). Moreover, MG132, a proteasome inhibitor was able to reverse the protein level of Cdc25C to the baseline. These data denoted that gefitinib *plus* BI6727 could promote degradation of Cdc25C through ubiquitination-dependent pathways and BI6727 dominantly contributed to the enhanced anti-cancer effects.

2.4. DNA damage was induced by combination therapy of gefitinib and BI6727

We further attempted to identify the upstream regulators that can induce gefitinib *plus* BI6727 co-treatment-mediated degradation of Cdc25C. Cdc25C is a well-known downstream effector substrate of ATM/ATR-checkpoint kinase (Chk) 1/Chk2-Cdc25A/Cdc25B/Cdc25C signaling pathway. Besides, the phosphorylation of ATM/ATR plays an important role in DNA damage, and cell cycle arrest (Boutros et al. 2007; Kinner et al. 2008). Therefore, p-ATM and p-ATR, two major members of the phosphatidylinositol 3-kinase related kinase (PIKK) family, were chosen as key checkpoint proteins to explore the further mechanism. Meanwhile, the study by Rothkamm et al. (2015) elucidated that γ-H2AX is also one of essential hallmarks to reflect the presence of double-strand breaks during DNA damage. We also included the level of γ-H2AX in our study to support our conclusions. To this end, SMMC-7721, Bel-7402 and HepG2 cells exposed to gefitinib (1 μM) and/or BI6727 (6 nM) for 24 h were utilized to detect intracellular levels of p-ATM, p-ATR and γ-H2AX. As shown in Figs. 4A and 4B, minimal changes in protein levels were detected in all the three tested HCC cell lines exposed to gefitinib alone. Whereas, the monotreatment of BI6727 upregulated levels of p-ATM, p-ATR and γ-H2AX. Notably, combination therapy remarkably increased levels of p-ATM, p-ATR and γ-H2AX, suggesting that combination of gefitinib and BI6727 significantly induced DNA damage, which may contribute to the cell cycle arrest and the subsequent apoptosis elicited by the combination.

3. Discussion

Advanced HCC remains virtually incurable due to the complicated pathogenesis so that tumor response to chemotherapy is quite low. The lack of efficient intervention agents or strategies against HCC is the main reason of this detrimental situation. In the present study, we have shown that combination treatment of gefitinib and BI6727 produced significantly enhanced anti-tumor effects on HCC cell lines by arresting cells at G2/M-phase, which was dictated by Cdc25C.

Based on the present study, there is no doubt that combination treatment of molecular targeted drugs is expected to elevate treatment effects. Sorafenib, a multi-kinase inhibitor, was known as a first-line drug for the clinical treatment of advanced HCC. Tumor response rates are usually quite low in mono-treatment of sorafenib while some studies have proved that sorafenib possesses synergistic anti-HCC effects in combination with other molecular targeted drugs such as mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor (CI-1040) (Ou et al. 2009), Akt Kinase (AKT) inhibitor (GDC0068) (Zhai et al. 2014), dual phosphoinositide 3-kinase(PI3K)/mTOR inhibitor (PI-103) (Gedaly et al. 2010), hypoxia-inducible factor 1-alpha(HIF1-α) inhibitor (EF24) (Liang et al. 2013) etc. The results mentioned above suggest that anti-HCC agents combined with other molecular targeted drugs might exert synergistic effects against HCC. When it comes to gefitinib, which was initially used for clinical studies of lung cancer, it could also synergize with other anti-tumor agents such as silibinin so that the growth-inhibiting effects were enhanced in HCC (Gu et al. 2015). Meanwhile, two clinical trials (NCT00071994, NCT00282100) of gefitinib in patients with hepatocellular carcinoma have been carried out. These clinical and pre-clinical studies indicated that gefitinib might potentially play a key role in treatment of hepatocellular carcinoma. Similar to the present study, a previous study has confirmed that gefitinib in combination with SN-38 significantly facilitated the ubiquitin-proteasome-dependent degradation of Rad51 protein, subsequently gave rise to more DNA damages, and ultimately resulted in the synergistic anti-tumor activity (Shao et al. 2016), which suggested that synergism of gefitinib and other anti-tumor agents was closely associated with DNA damage and subsequent cell cycle arrest.

Majorities of anti-tumor drugs possess abilities to induce DNA damage and subsequently mediated cell cycle arrest, which triggers apoptosis. Therefore, combination of molecular targeted drugs with agents that induce DNA damage or cell cycle arrest is expected to exert an augmented anti-tumor efficacy. Similar to our study, erlotinib was known as another EGFR inhibitor and erlotinib synergizing with arsenic trioxide (ATO), which was a traditional drug inducing DNA damage, dramatically enhanced anti-tumor efficacy (Kryeziu et al. 2013). However, most drugs with ability to induce DNA damage show excessive cytotoxicity which might be considered as side effects. Notably, BI6727 acting as a PLK1 inhibitor was superior to traditional DNA damage inducers, which suggested that molecular targeted drugs with low side effects were promising therapeutic agents against HCC.

During the study of combination mechanism, we discovered that Cdc25C was such a key pivot that linked DNA damage to G2/M arrest. Cdc25C is a dual-specificity protein phosphatase that controls entry into mitosis by dephosphorylating the protein kinase Cdc2 (Peng et al. 1997), which suppresses the activity of Cdc2/Cyclin B complex and results in G2/M arrest. In hepatocellular carcinoma, mounting studies have proved that a great number of agents exert anti-tumor effects through Cdc25C-associated mechanisms. Huang et al. (2013) confirmed that dihydromyricetin inhibited the viability of HCC cells by inducing G2/M arrest via the Chk1/Chk2/Cdc25C pathway. Sun et al. (2017) verified that romidepsin reduced activity of Cdc25C through the ERK/cdc25C/cdc2/Cyclin B pathway and induced G2/M arrest followed by apoptosis in HCC cells. Additionally, UCN-01 induced S and G2/M cell cycle arrest and then suppressed invasion in HCC cells (Wu et al. 2013). All these results suggested that Cdc25C might dictate G2/M arrest and was closely related to proliferation as well as invasion in HCC. Consistent with other studies, we further found that the PLK1 inhibitor BI6727 significantly negatively regulated the total protein level of Cdc25C via ubiquitin-proteasome dependent pathway, which resulted in G2/M arrest. Notably, this effect could be dramatically enhanced when combined with gefitinib. Our results indicated that EGFR inhibitors might facilitate the ability of PLK1 inhibitors to reduce activity of Cdc25C.

In summary, the present study revealed that BI6727 downregulated the protein level of Cdc25C, which triggered G2/M arrest and exerted the anti-proliferative efficacy in HCC cells. Notably, the EGFR inhibitor gefitinib dramatically enhanced these anti-tumor effects when synergizing with BI6727. All the results suggested that combination of EGFR inhibitors and PLK1 inhibitors was expected to be a promising therapy strategy against HCC. Thus, there is an urgent need to validate our conclusions in vivo, and further explore synergistic effects of other EGFR inhibitors plus different PLK1 inhibitors.

4. Experimental

4.1. Cell lines and cell culture

Tree human hepatocellular carcinoma cell lines (SMMC-7721, Bel-7402, HepG2) were utilized for all the *in vitro* studies, all of which were purchased from Cell Bank of China Science (Shanghai, China). SMMC-7721 and Bel-7402 cells were routinely maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). HepG2 cells were cultured in dimethyl sulfoxide (DMEM; Gibco, Grand Island, NY, USA) containing 10% FBS (Gibco, Grand Island, NY, USA). All three cell lines were incubated at 37 °C in a 5% CO_2humidifed atmosphere.

4.2. Cell proliferation assay

Human HCC cells (SMMC-7721, Bel-7402 and HepG2) were plated in 96-well plates for 24 h growth in 5% CO_2 incubator at 37 °C. Then cells were treated with the compounds at various doses for 72 h, subsequently a sulforhodamine B (SRB) staining assay (Sigma-Aldrich, St Louis, MO, USA) was employed to measure cell proliferation. Cells (4000/well) were incubated with 10% trichloro acetic acid (TCA) for 1 h (4 °C) followed by the staining with SRB for 30 min. The SRB was then washed away with 1% glacial acetic acid, after which 100 μL of 1% Tris-base was added to each well. The optical density (OD) was determined at 510 nm by a Multiskan Spectrum plate reader (Termo Electron Corporation, Marietta, OH, USA).

4.3. Clonogenicity assay

During a soft agar cloning assay, cells seeded into 6-well plates were treated with gefitinib (0.5 μM), BI6727 (8 nM) or the combination for 24 h, and then cells in each group were sparsely plated into 60 mm dishes respectively (approximately 1,000 cells per dish). The compound-containing medium was replaced every 3 days and the drugs were re-added. After a routinely culture for 9 days, the colonies were stained by a 0.1% crystal violet solution and subsequently dishes in each group were photographed. Finally, a 10% acetic acid was utilized to extract the stained cells and the absorbance was determined at 595 nm by a Multiskan Spectrum plate reader (Thermo Electron Corporation, Marietta, OH, USA). The colony formation ability was calculated as ((the absorbance of treated cells - the absorbance of blank) / (the absorbance of control cells - the absorbance of blank)) ×100%.

4.4. PI staining for cell cycle analysis

The cell cycle analysis was performed after PI staining. Firstly, SMMC-7721, Bel-7402 and HepG2 cells were incubated with gefitinib (1 μM) and/or BI6727 (6 nM) for 24 h and subsequently harvested and fixed with 75% ethanol at -20 °C. Secondly, cells in each group were incubated with RNaseA and subsequently with PI (Sigma-Aldrich, St Louis, MO, USA) in the dark for 30 min. Finally, at least 5×10^4 cells for each group were analyzed by an FACS-Calibur cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

4.5. Western Blot

Firstly, cells were incubated in six-well plates (approximately 2×10^5 cells per well) and then treated with gefitinib and/or BI6727 in specified concentration for 24 h. Eight hours before test, cells were treated with MG132 (10 μM) according to the purpose of experiments. Secondly, cell lysis buffer (50 mmol/L Tris-HCl, 25 mmol/L NaF, 150 mmol/L NaCl, 25 mmol/L b-sodium glycerophosphate, 2 mmol/L EDTA, 2 mmol/L EGTA, 0.3% Triton X-100, 0.3% NP-40, 0.1% PMSF, 0.1% $NaVO_3$,0.25% Leupeptin) were utilized to resuspend and lysate cells. Then all these samples were sonicated for 6 time (6 s at a time), after which the samples were subsequently centrifuged at 12×10^3r.p.m. for 30 min at 4 °C. Thirdly, during the process of western blot, protein samples were separated on Tris-glycine gels transferred to nitrocellulose membrane (0.45 μm) and subsequently incubated with corresponding HRP-linked antibodies. Finally, protein bands were detected using the Amersham Imager 600 (General Electric Company, MA, USA).

4.6. Real-time reverse transcription-PCR (qRT-PCR)

Firstly, SMMC-7721 cells were incubated with gefitinib (1 μM) and/or BI6727 (6 nM) for 24 h (approximately 2×10^5 cells per well) in a six-well plate. Secondly, TRIZOL (Invitrogen, Boston, MA, USA) was utilized to extract total RNA from these cells and then total RNA was dissolved in DEPC-treated water. Thirdly, cDNA was synthesized through reverse transcription of 2 mg samples of total RNA using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (Beijing Transgenbiotech, Beijing, China). Fourthly, specific primers for Cdc25C (See below) and Actin (See below) were utilized to perform qRT-PCR. PCR was run on Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Woburn, MA, USA).

Cdc25C Forward: 5'-CTGCCACTCAGCTTACCACT-3'
Reverse: 5'-TCTATGGCCACGGTCCAAAC-3'
Actin Forward: 5'-CACCATTGGCAATGAGCGGTTC-3'
Reverse: 5'-AGGTCTTTGCGGATGTCCACGT-3'

4.7. Statistical analyses

All data are presented as mean±SD. One-way ANOVA analysis or student's t-test were performed to analyze statistical significance using SPSS 19.0 (IBM, New York, NY, USA). Differences with $p<0.05$ were considered statistically significant

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Conflicts of interest: No potential conflicts of interest were disclosed.

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