Camptothecin and cisplatin upregulate ABCG2 and MRP2 expression by activating the ATM/NF-κB pathway in lung cancer cells

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Abstract. Multidrug resistance (MDR) formation is an important problem in lung cancer chemotherapy. Our study showed that both camptothecin and cisplatin could not only induce ATM and NF-κB activation but also upregulate expression of the MDR-related genes ABCG2, MRP2 in NCI-H446 cells. Moreover, camptothecin and cisplatin-induced ABCG2 and MRP2 upregulation could be impaired by ATM and NF-κB inhibitors, indicating a relationship between ATM, NF-κB activation and MDR formation in lung cancer chemotherapy. Our study indicates that ATM may serve as a potential molecular target for MDR formation in lung cancer chemotherapy.

Introduction

Small cell lung cancer (SCLC), which accounts for ~15% of all lung cancer cases, is the most aggressive metastatic form of lung cancer and does not respond well to surgery or radiotherapy (1). Although up to 90% of small cell lung cancer (SCLC) initially responds to chemotherapy, patients with SCLC often relapse with multidrug resistant formation (MDR), leading to 5-year survival <5% (2,3) indicating that MDR is a major obstacle for successful small cell lung cancer chemotherapy. ATP-binding cassette transporter proteins such as ATP-binding cassette (ABC) transporters P-glycoprotein (P-gp, MDR1), multidrug resistance-associated protein (MRP) and breast cancer resistance protein (BCRP/ABCG2) (4,5), transporting a wide variety of chemical compounds in ATP-dependent manner, have been found to contribute to MDR formation in a variety of tumors arising from gastric, renal, endometrium, melanoma and soft tissue (6-12). However, the role of ABCG2 and MRP2 in lung cancer associated MDR formation is still uncertain and this is important for dealing with lung cancer associated multidrug resistance.

Various cellular pathways might be simultaneously involved in the clinical drug resistance of cancer patients (13-22). Ataxia telangiectasia mutated (ATM), a nuclear serine-threonine kinase involving in DNA double strand break (DSB) repair, was reported to upregulate MDR associated genes expression and contribute to multidrug resistance (14-16). NF-κB, which is involved in regulating apoptosis, inflammatory response, cell survival and immune response, was also reported to play roles in tumor growth, invasion and metastasis (18-22). Although the phosphorylation of NEMO Ser85 by ATM was shown in HEK293 cells (23), the role of ATM-NF-κB pathway in lung cancer associated MDR formation is still uncertain and needs further exploration.

In the present study, human small cell lung cancer NCI-H446 cells were used as lung cancer model and the roles of ATM/NF-κB activation induced by chemotherapeutic drugs in MDR formation were explored. The results showed that: firstly, the expressions of ABCG2, MRP2 and Bcl-2 were upregulated in response to camptothecin and cisplatin treatment; secondly, ATM and NF-κB pathways were activated by camptothecin or cisplatin treatment; moreover, NF-κB activation was dependent on ATM phosphorylation in camptothecin or cisplatin treatment conditions; most importantly, both ABCG2 and MRP2 upregulation induced by camptothecin could be impaired by NF-κB and ATM inhibitors. These findings indicated that chemotherapeutic drugs such as camptothecin and cisplatin could upregulate ABCG2 and MRP2 expression by activating ATM/NF-κB pathway in small cell lung cancer chemotherapy, providing ATM as potential target molecular for overcoming lung cancer chemotherapy-associated multidrug resistance.

Materials and methods

Reagents. Reagents were purchased from the following companies: Rhodamine 123 (rho123; Molecular Probes, Eugene, OR, USA) was the dye used to detect early stage of cell apoptosis.
Calcein-AM (Molecular Probes) is a cell permeate dye that can be used to determine cell viability. Camptothecin (CPT) and cisplatin (DDP) were purchased from Calbiochem (San Diego, CA, USA). Anti-phospho-IκBα, anti-p65, anti-phospho-p65, anti-ATM, anti-phospho-ATM, anti-ABCG2, anti-MRP2 and anti-Bcl-2 were from Cell Signaling Technology (Beverly, MA, USA). Anti-histone H3, anti-β-actin and anti-tubulin were from Santa Cruz Bioethenology (Santa Cruz, CA, USA).

**Cell line and Treatment condition.** Small cell lung cancer (NCI-H446) cells (American Type Culture Collection, HTB-171, Bethesda, MD, USA) were grown in RPMI-1640 mediacontaining penicillin/streptomycin (Gibco, Gaithersburg, MD) and 10% fetal bovine serum (Hyclone, Logan, UT) at 37˚C in 5% CO2. NCI-H446 cells were passaged every 2 days. Cells were synchronized by serum starvation (in RPMI-1640 without serum) for ≥12 h before treated with dose-escalated camptothecin or cisplatin for 18 h.

**Cell apoptosis assay.** Cell apoptosis assay was determined by flow cytometry according to the method described previously (24). Briefly, NCI-H446 cells were treated with camptothecin or cisplatin for 18 h at indicated final concentration. Then, cells were removed by trypsinization, rinsed with PBS and re-suspended in binding buffer containing Annexin V-FITC and propidium iodide (PI) for 20 min at room temperature. The samples were analyzed on FACSCalibur and data were analyzed with CellQuest software.

**Measurement of mitochondrial membrane potential by flow cytometry.** Mitochondrial membrane was monitored using the fluorescent dye Rhodamine 123, which detected the early stage of cell apoptosis (25). Briefly, NCI-H446 cells were treated with camptothecin or cisplatin for 18 h at indicated final concentration. Then, cells were rinsed with PBS and Rhodamine 123 was added at a final concentration of 1 µg/ml. After 15 min co-incubation at 37˚C, the cells were collected, washed twice with PBS and then analyzed by flow cytometry.

Calcein-AM has been used as an excellent tool for the studies of cell membrane integrity and is a true end-point assay for cell viability. Calcein-AM (final concentration of 0.5 µM) was added to cells after camptothecin or cisplatin treatment as described above. After 15-min co-incubation at 37˚C, the cells were collected, washed twice and analyzed by flow cytometry.

**Confocal immunofluorescence assay.** The effect of ATM phosphorylation and p65 nuclear translocation were investigated by immunofluorescence assay as described previously (26). Briefly, NCI-H446 cells were serum-starved for ≥12 h followed by 0.5 µg/ml camptothecin stimulation for indicated periods. Then, cells were fixed and permeabilized in 100% methanol for 15 min, washed with PBS and blocked with 10% non-fat milk in PBS for 3 h. Primary antibodies (phospho-p65 or phospho-ATM) were incubated in a humid chamber overnight at 4˚C. Finally, FITC-conjugated secondary antibodies were incubated for 1 h at 37˚C and DAPI counterstaining was performed to visualize cell nuclei. The cells were observed and images were recorded by a confocal fluorescence microscope at the wavelength of 488 nm. ATM phosphorylation inhibitor CGK (20 µM) was pre-treated for 1 h before camptothecin treatment to observe the effect of ATM activation on p65 phosphorylation and translocation. Cells were washed three times in each step to remove non-binding substance.

**Cytoplasmic and nuclear extracts isolation.** Cytoplasmic and nuclear extracts from whole cell extracts were prepared as described previously (27). Briefly, NCI-H446 cells were treated with camptothecin or cisplatin for 18 h at indicated final concentration. Then, cells were suspended in ice-cold CER buffer (cytoplasmic extraction reagent), vortexed for 10 min and ice-cold CER was added. The cytosolic fraction (supernatant) was separated by centrifugation (16000 x g, 5 min, 4˚C) and the nuclear protein was separated by incubating insoluble fraction with ice-cold NER (nuclear extraction reagent) for 40 min and centrifuged at 16000 x g for 10 min, 4˚C. Protein concentration was estimated using the Bio-Rad protein assay reagent and an equal amount of proteins per sample of nuclear extract was further analyzed by Western blotting.

**Western blot analysis.** Proteins were obtained in lysis buffer as previously described (28). To investigate the effects of camptothecin or cisplatin on MDR-related genes expression, NCI-H446 cells were treated with camptothecin or cisplatin at indicated concentration. Protein lysates were electrophoresed on 8-10% SDS-PAGE gels, then transferred to PVDF membranes and blotted with primary antibodies. Followed by appropriate peroxidase-conjugated secondary antibodies and detected by chemiluminescence ECL. β-actin, tubulin or histone were used as loading control.

**Statistical analysis.** Each experiment was repeated at least 3 times and confirmed that similar data were obtained. All data were expressed as mean and standard error means. Statistical significance was tested using one-way ANOVA with post Newman-Keuls test. Statistical differences were considered to be significant at p<0.05.

**Results**

**Both cisplatin and camptothecin independently change mitochondrial membrane potential and induce cell apoptosis.** To investigate the pro-apoptosis effect of camptothecin and cisplatin, NCI-H446 cells were exposed to different concentrations of camptothecin or cisplatin for 18 h and analyzed by flow cytometry. The results showed that: both camptothecin and cisplatin could effectively induce apoptosis in a concentration-dependent manner, as both of 0.5 µg/ml camptothecin and 1 µg/ml cisplatin could induce ~10% apoptosis (Fig. 1A-C). Analysis of early stage cell apoptosis found that camptothecin or cisplatin efficiently changed the mitochondrial membrane potential, which was demonstrated by enhanced release of Rhodamine 123 from the mitochondria and mean of fluorescence index increase in intracellular fluorescence (Fig. 1D and E). Cell viability determination of Calcein-AM analysis showed that 0.1 µg/ml camptothecin or 0.5 µg/ml cisplatin treatment affected cell viabilities of NCI-H446 cells (Fig. 1F and G). These data indicated that >1 µg/ml camptothecin or cisplatin efficiently induced cell apoptosis, while lower
The expression of ABCG2, MRP2 and Bcl-2 was upregulated by camptothecin or cisplatin stimulation in NCI-H446 cells.

To investigate the effects of camptothecin and cisplatin on expression of ABCG2, MRP2 and Bcl-2, NCI-H446 cells were treated with camptothecin or cisplatin and the expressions of ABCG2, MRP2 and Bcl-2 was determined by Western blotting. The results showed that cisplatin stimulation obviously increased ABCG2 and MRP2 expressions in a concentration- (0.1-5 µg/ml) (Fig. 2A) and time-dependent manner (3-24 h) (Fig. 2C), which reach the maximum at 1 µg/ml and 24 h respectively. Similar to cisplatin, camptothecin upregulated ABCG2, and MRP2 expression in a concentration- (0.01-1 µg/ml) (Fig. 2B) and time-dependent manner (1-24 h) (Fig. 2D). Interestingly, not only ABCG2 and MRP2 but also Bcl-2 was upregulated by cisplatin or camptothecin stimulation from 3 to 24 h (Fig. 2C and D). Collectively, these data indicated that camptothecin or cisplatin treatment induced the expression of multidrug resistance protein and anti-apoptosis protein, contributing to MDR formation in NCI-H446 cells.
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Figure 2. Treatment with cisplatin or camptothecin upregulates ABCG2, MRP2 and Bcl-2 expression in NCI-H446 cells. NCI-H446 cells were treated with cisplatin (A and B) or camptothecin (C and D) in indicated concentration for 18 h for indicated periods. Then, the cellular protein was extracted and ABCG2, MRP2, Bcl-2 expression was determined by Western blotting. β-actin was used as loading control. Data shown are representative of three independent experiments (n=3). DDP, cisplatin; CPT, camptothecin.

Figure 3. Treatment with cisplatin or camptothecin induces ATM phosphorylation in NCI-H446 cells. NCI-H446 cells were treated with cisplatin (A) or camptothecin (B and C) at a final concentration of 0.5 µg/ml for indicated periods. ATM phosphorylation was determined by Western blotting and confocal immunofluorescence assay, respectively, with anti-phospho-ATM antibodies. Both cisplatin (A) and camptothecin (B and C) treatments enhanced ATM phosphorylation. Data shown are representative of three independent experiments (n=3). β-actin was used as loading control. DDP, cisplatin; CPT, camptothecin.
Camptothecin or cisplatin activates ATM in NCI-H446 cells. ATM, a serine/threonine protein kinase, could be activated by ionizing radiation or other agents such as etoposide which induces DNA double strand breaks (29). To determine the effect of camptothecin and cisplatin on ATM activation in lung cancer cells, NCI-H446 cells were treated with camptothecin or cisplatin and ATM phosphorylation was observed by Western blotting and laser confocal microscope respectively. The results showed that 0.5 µg/ml camptothecin or cisplatin and ATM phosphorylation was observed by Western blotting and laser confocal microscope respectively. The results showed that 0.5 µg/ml camptothecin or cisplatin treatments obviously increased ATM phosphorylation in NCI-H446 cells in time-dependent manner which reach the maximum at 1 h after the exposure to camptothecin or cisplatin (Fig. 3A and B). The immunofluorescence observation found that camptothecin increased the phosphorylation of ATM from 0.5 to 3 h, which reach the maximum at 1 h after camptothecin stimulation (Fig. 3C).

Camptothecin or cisplatin can activate the NF-κB pathway in NCI-H446 cells. Phosphorylation and degradation of IκBα, the natural blocker of NF-κB and a direct downstream protein activated by phosphorylated IKK, is well known as an important prerequisite for the activation of NF-κB (16,26). To investigate the effects of camptothecin and cisplatin on NF-κB pathway activation, NCI-H446 cells were treated with camptothecin or cisplatin and phosphorylation of IκBα, p65 and p65 translocation were determined by Western blotting. The results showed that both IκBα and p65 phosphorylation in cytoplasm was increased at 3 h and remained elevated at 8 h after camptothecin or cisplatin treatment. In contrast, total IκBα and total p65 in cytoplasm decreased accordingly in a time-dependent manner (Fig. 4A and B). Consistent with phosphorylation of IκBα and p65 in cytoplasm, the translocation of p65 from cytoplasm to nuclei was observed at 3 h and reached the maximum at 12 h, which remained elevated at 24 h after camptothecin or cisplatin treatment (Fig. 4C and D), indicating that camptothecin or cisplatin stimulation induced NF-κB pathway activation in NCI-H446 cells.

ATM phosphorylation is involved in camptothecin or cisplatin-induced NF-κB activation in NCI-H446 cells. Two independent studies have reported an essential role of ATM in DSB-induced NF-κB activation (14,30). To explore the role of ATM phosphorylation in camptothecin and cisplatin-induced NF-κB activation, ATM phosphorylation inhibitor CGK was used prior to camptothecin or cisplatin treatment and NF-κB activation, the translocation was determined by Western blotting and confocal microscope observation, respectively. The results showed that camptothecin stimulation effectively induced both p65 and IκBα phosphorylation from 3 to 12 h (Fig. 5A) and enhanced p65 translocation from cytoplasm to nucleus (Fig. 5B). As BAY, an NF-κB activation inhibitor, could effectively inhibit p65 phosphorylation (Fig. 5A) and translocation (Fig. 5B), the reduction of NF-κB activation derived by the usage of CGK indicated that camptothecin induced NF-κB activation was ATM phosphorylation dependent in NCI-H446 cells.

Camptothecin upregulates the expressions of ABCG2, MRP2 and Bcl-2 by activating the ATM/NF-κB pathway. To explore the role of ATM/NF-κB activation in camptothecin-induced upregulation of ABCG2, MRP2 and Bcl-2 in NCI-H446 cells, ATM phosphorylation inhibitor CGK and NF-κB inhibitor BAY were used prior to camptothecin stimulation and the expressions of ABCG2, MRP2 and Bcl-2 were determined by Western blotting. The results showed that camptothecin stimulation obviously enhanced MRP2, Bcl-2 (Fig. 6A) and
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ABCG2 (Fig. 6B) expression in a CGK concentration-dependent manner. NF-κB activation inhibitor BAY was also found to block MRP2, Bcl-2 and ABCG2 upregulation induced by camptothecin. However, the inhibitory effect of PDTC on MRP2, Bcl-2 and ABCG2 upregulation was not clear, which might be due to PDTC that did not inhibit camptothecin-induced NF-κB activation, as Togashi et al showed that PDTC did not inhibit tumor necrosis factor-α-induced NF-κB activation in astrocytes (31). As CGK (20 μM) and BAY (20 μM) were capable of sufficiently inhibiting NF-κB activation, the inhibition effect of CGK and BAY on ABCG2, MRP2, Bcl-2 expressions indicated that camptothecin upregulated ABCG2, MRP2 and Bcl-2 expression, respectively, by activating ATM/NF-κB pathway in NCI-H446 cells.

Discussion

Although doxorubicin has been reported to induce MRP1 expression by activating the c-jun kinase pathway in human small cell lung cancer cell lines (1), a definitive conclusion with regard to the impact of drug resistance factors can not be derived due to the heterogeneity of the present study. Thus, MDR associated genes and mechanism need further exploration for dealing with chemotherapeutic multidrug resistance. In the present study, ABCG2 and MRP2 were used as representative multidrug resistance proteins and the roles of ATM-NF-κB activation in lung cancer-associated MDR formation were investigated in camptothecin or cisplatin treatment conditions. Our results showed that: ATM and NF-κB activation, resulting in cell survival, play an essential role in MDR development in camptothecin or cisplatin treated NCI-H446 cells; importantly, camptothecin or cisplatin was able to upregulate ABCG2, MRP2 expression by activating the ATM-NF-κB pathway, which was demonstrated by ATM and NF-κB inhibitors abrogating camptothecin-increased ABCG2 and MRP2 expression, indicating that ATM inhibitor might be useful for overcoming multidrug resistance in lung cancer chemotherapy.

Mitochondrial membrane potential is a key indicator of cellular viability, which reflects the pumping of hydrogen ions across the inner membrane during the process of electron transport and oxidative phosphorylation. In the present study, mitochondrial membrane was monitored by Rhodamine 123 and Calcein-AM flow cytometry. As Rhodamine 123 preferentially partition into active mitochondria based on depolarization of mitochondrial membrane potential when early stage cell apoptosis results in the release of Rhodamine 123 from the mitochondria and an increase in intracellular fluorescence (25). Calcein-AM is a widely used green fluorescent cell marker which is membrane permeant and can be introduced into cells via incubation. Once inside the cells, calcein-AM, a non-fluorescent molecule itself, is hydrolyzed by endogenous esterase into the highly negatively charged green fluorescent calcein and retained in the cytoplasm in live cells (32).

Function-deficient mutations of ATM has been reported in ataxia telangiectasia, which account for autosomal recessive disorder of cerebella ataxia, ocularcutaneous telangiectasia, immunodeficiency, radiation sensitivity, growth retardation, premature aging and cancer predisposition (33).
ATM was also implicated recently in metabolic pathways seemingly unrelated to DNA damage (34). However, as DNA damage sensor (14), ATM is a nuclear serine-threonine kinase involved in DNA double strand break (DSB) repair and plays an important role in chemotherapeutic drug induced MDR formation. Although the present study showed that ATM could be activated by camptothecin and cisplatin and ATM phosphorylation was crucial for the upregulation of ABCG2 and MRP2, the mutation of ATM induced by chemotherapeutic drug and the relationship of ATM mutation and MDR formation could be very important for lung cancer chemotherapy and needs further clarification.

Korita et al (35) reported that MRP2 expression determines the efficacy of cisplatin-based chemotherapy in patients with hepatocellular carcinoma. The MRP1 upregulation in tumors after chemotherapy attributed to selection of pre-existing MDR cells was also reported (12,36-38). Several studies have been performed to investigate potential correlation between ABCG2 expression and clinical outcomes. Given the specific tissue localizations, the role of ABCG2 in healthy tissue may be to protect an organism or tissue from potentially harmful toxins. Here we showed that significant increases of ABCG2, MRP2 and Bcl-2 were observed within 12 h of exposure of NCI-H446 cells to camptothecin or cisplatin (Fig. 2).

IKK or NF-κB activation is ATM phosphorylation-dependent in transformed cells (39-41). In the present study, CGK, the ATM inhibitor, not only inhibited the phosphorylation of IκBα and p65 but also blocked p65 translocation from cytoplasm to nucleus (Fig. 5), indicating that ATM phosphorylation is necessary for IκBα phosphorylation in camptothecin or cisplatin-induced NF-κB activation. As IKKγ/NEMO was reported as NF-κB essential modulator (39) and PI3K/AKT activation in response to IL-1 stimulation leads to NF-κB activation (42), both IKKγ/NEMO and PI3K/AKT might induce NF-κB activation. Although ATM phosphorylation was necessary for camptothecin-induced NF-κB activation and MDR formation, the mechanisms of phosphorylated ATM inducing IκBα phosphorylation by interacting with NEMO or AKT are still unknown and need further investigation.

NF-κB is involved in apoptotic response of cells exposed to chemotherapeutic agents (20-23). Activated NF-κB binds to specific DNA sequences of target genes and regulates gene transcription involved in chemoresistance and radioresistance, including COX-2, cyclin D1, Bcl-2, Bcl-xl, survivin and XIAP (43). In the present study, we have characterized the role of ATM and p65 activation in camptothecin or cisplatin induced ABCG2, MRP2 and Bcl-2 upregulation in NCI-H446 cells (Fig. 5A). Whether other NF-κB components also regulate ABCG2, MRP2 and Bcl-2 expression is still uncertain.

Collectively, our study demonstrated that camptothecin or cisplatin treatment increased expression of ABCG2, MRP2 and Bcl-2 by activating the ATM/NF-κB pathway in human NCI-H446 cells. This study may explain one of the key mechanisms of MDR development following lung cancer chemotherapy. Furthermore, the present research suggests that combined treatment of ATM and NF-κB inhibitors might prevent the development of MDR under clinical conditions.

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References


