Interleukin 6 augments lung cancer chemotherapeutic resistance via ataxia-telangiectasia mutated/NF-kappaB pathway activation

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Chemotherapeutic resistance is closely associated with multidrug resistance (MDR). Although a relatively good response can be achieved in the initial stages of lung cancer chemotherapy, chemoresistance can develop quickly after initial chemotherapy, which clearly affects patients’ survival.1–3 Hence, chemotherapeutic resistance, especially MDR, is an important issue for chemotherapeutic failure and remains a challenge in the clinical treatment of lung cancer. Proinflammatory cytokines, such as interleukin 6 (IL-6), which are usually secreted by immune cells, have also been documented to be expressed by osteosarcoma, ovarian cancer cells and head and neck squamous cell carcinoma cells.4–6 Further studies reveal that an elevated IL-6 level has a close relationship with poor clinical outcome of advanced lung cancer patients, indicating that IL-6 contributes to chemotherapeutic resistance in lung cancer.7–9 Meanwhile, treatment with IL-6 reveals anti-apoptotic effects and promotes MDR.10–13 However, until now, few studies have explored the role of IL-6 in chemotherapeutic agents-induced MDR.

The activation of protein kinase ataxia-telangiectasia mutated (ATM) is reported to be involved in DNA damage response and cell cycle checkpoints,14 to increase MDR-associated protein expression, and to contribute to chemoresistance.15–17 Treatment with chemotherapeutic agents triggers the phosphorylation of ATM and the export of IKK-gamma, which initiates the activation of TAK1-IKK-NF-kappaB.18 The activation of ATM induces Ubc13-mediated TRAF6 polyubiquitin, promotes TAB 2-dependent TAK1 phosphorylation and increases the nucleus translocation of p65, indicating that ATM is the upstream kinase of the NF-kappaB pathway.19–21 While ATM could be activated by chemotherapeutic agents-induced DNA double strand breaks (DSB),12–14 the phosphorylation of ATM is clearly increased by treatment with hypoxia without apparent DNA damage.18–20 Cisplatin treatment has been demonstrated to increase proinflammatory cytokines release21 and NF-kappaB activation initiates anti-apoptotic protein expression, augments proinflammatory cytokine secretion and contributes to MDR.22 Chemotherapeutic agent-induced MDR raises the question of whether IL-6 secretion could be increased by treatment with chemotherapeutic agents, which, in turn, activate the ATM/NF-kappaB pathway, augment MDR-associated protein expression and contribute to MDR in lung cancer. However, little is known about the effect of ATM/NF-kappaB activation on IL-6-associated lung cancer chemotherapeutic resistance, which is clearly an important issue for treating lung cancer chemotherapeutic resistance.

The aim of the present study was to identify the functions of the ATM/NF-kappaB activation in IL-6-induced chemotherapeutic
resistance by studying changes in MDR-associated protein expression, and to examine their relationships with the ATM/NF-kappaB pathway activation. We found that IL-6 increased lung cancer cell chemotherapeutic resistance and enhanced the expressions of Bcl-2, Mcl-1, Bcl-xl and ABCG2 by activating the ATM/NF-kappaB pathway. The high level of IL-6 reveals not only higher activity of ATM/NF-kappaB but also increases the expression of ABCG2, Bcl-2, Mcl-1 and Bcl-xl. Importantly, treatment with cisplatin and camptothecin could increase IL-6 secretion in lung cancer cell lines by activating the p38/NF-kappaB pathway. These results indicate that both IL-6 and ATM are potential targets for the treatment of chemotherapeutic resistance in lung cancer.

Materials and Methods

Reagents. Recombinant Human IL-6 and Matched IL-6 Antibody Pairs were obtained from eBioscience (San Diego, Table 1. Primer sequence

| Genes     | F/R | Sequence                           |
|-----------|-----|------------------------------------|
| Beta-actin| F   | 5'-TCAAGATCATCTGCTCCTCCTG-3'       |
| Beta-actin| R   | 5'-CTGCTTGCTGATCCATCCTG-3'         |
| IL-6      | F   | 5'-CCACACAGACAGCCACTCACC-3'        |
| IL-6      | R   | 5'-CTGATTTGCGAGAGCCCTC-3'          |
| ABCG2     | F   | 5'-ACTGCTTGAACACTGACA-3'           |
| ABCG2     | R   | 5'-ATAGGCTTCGAGGATGAA-3'           |
| Bcl-2     | F   | 5'-GGAGGTGATGCTGGCTCTT-3'          |
| Bcl-2     | R   | 5'-TCACTTGCTGAGTCTAGAGGC-3'        |
| Mcl-1     | F   | 5'-TGCAAGGTGTGCTGAGTACAG-3'        |
| Mcl-1     | R   | 5'-CTCCTTGCCACTCTTCTTC-3'          |
| Bcl-xl    | F   | 5'-GAGCTTGTTGTCTCTCCCTTC-3'        |
| Bcl-xl    | R   | 5'-TCCATCTCCAGATTCCAGCCT-3'        |

Fig. 1. Interleukin 6 (IL-6) treatment contributes to chemotherapeutic resistance in lung cancer cells. (a–f) The whole cellular RNA, protein and supernatant were prepared from cultured NCI-H446/A549 (a–d), NCI-H209/LTEP-a-2 (e, f) cells and the expression of IL-6 was determined by RT-PCR (a), real-time PCR (b,e), western blot (c) and ELISA (d, f) analyses. Beta-actin was used as an internal control. NCI-H446/A549 (g–i) and NCI-H209 cells (j) were pretreated with (2 ng/mL) IL-6 (h–j) or PBS (g) prior to cisplatin (DDP) (4 μg/mL) or camptothecin (CPT) (0.5 μg/mL) treatment and cell viabilities were determined by microscope (g, h), flow cytometry (i), and Trypan Blue cell count (j), analyses. The data are presented as the mean ± SEM. ***P < 0.001, **P < 0.01, Student's t-test or one-way ANOVA with the Newman-Keuls post test. One representative from three experiments is shown.
CA, USA). Camptothecin and cisplatin were purchased from Calbiochem (San Diego, CA, USA). Anti-phospho and total kinase antibodies were acquired from Cell Signaling Technology (Beverly, MA, USA). An Annexin V/PI Detection Kit was obtained from KeyGEN Biotech (Nanjing, China). DAPI was acquired from Vector Laboratories (Burlingame, CA, USA). The siRNA of IL-6, ATM, p65 and controls was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). RPMI-1640, DMEM and FBS were acquired from Hyclone (Logan, UT, USA). Lipofectamine 2000 was purchased from Invitrogen (Grand Island, NY, USA). SYBR Premix Ex Taq, Trizol and Prime-Script Reverse Transcriptase were obtained from TaKaRa Biotechnology (Dalian, China).

Cell lines. NCI-H446, NCI-H209, NCI-H1299, LTEP-a-2 cells, a kind gift from Professor GH Jin (Xiamen University), were grown in RPMI-1640 or DMEM media containing 10% FBS at 37°C under 5% CO2. Cells were synchronized by serum starvation for at least 12 h before treatment.

Flow cytometric measurements. Cell apoptosis assay was assayed as described previously. Briefly, cells were pretreated with IL-6 (2 ng/mL) for 5 h prior to cisplatin (4 μg/mL) treatment. Then, the cells were stained with Annexin V-FITC and propidium iodide for 20 min at room temperature. Flow cytometry was performed using a FACSCalibur Flow Cytometer, and the data were analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA).

ELISA. To determine the effect and mechanism of chemotherapeutic agent treatment on IL-6 release, 1 × 10⁵ cells were treated with cisplatin or camptothecin for indicated periods, and the supernatant was collected. IL-6 concentration was determined via ELISA.

Confocal immunofluorescence assays. The effects of IL-6 on ATM and p65 phosphorylation were investigated using immunofluorescence assays. Briefly, cells were treated with IL-6 (2 ng/mL), fixed and permeabilized in 100% methanol. Then, the cells were blocked with 10% non-fat milk and incubated with primary antibodies overnight at 4°C, followed by staining with fluorescence-conjugated IgG (1:100 dilution). Images were captured using a confocal fluorescence microscope at 488 or 546 nm.

siRNA transfection. Cells plated at a density of 3 × 10⁴ cells/well were transfected with relative siRNA using Lipofectamine 2000 (Invitrogen). The cells were collected for further experiments 48 h after transfection. The final concentration for each siRNA is 100 nM.

RT-PCR and quantitative PCR. The effects of IL-6 on MDR-related protein expression were investigated via RT-PCR and real-time PCR analyses, as described previously. Briefly, whole cellular RNA was extracted, and reverse transcription was performed using PrimeScript Reverse Transcriptase (TaKaRa Biotechnology, Dalian, China). PCR amplification was conducted using the following conditions: 95°C for 3 min, followed by 30 cycles of 95°C for 5 s, 60°C for 30 s and 72°C for 60 s, and a final extension at 72°C for 10 min. Beta-actin was used as an internal control. To quantify gene amplification, real-time PCR analysis was performed using an ABI 7000 Sequence Detection System in the presence of SYBR Green (TaKaRa Biotechnology, Dalian, China). The cycling parameters were 95°C for 5 min, followed by 32 cycles of 95°C for 5 s, 55°C for 30 s and 72°C for 60 s, with a final extension at 72°C for 10 min; a melting curve analysis was subsequently conducted. The relative expression levels (defined as fold changes) of the target genes were normalized to the folds of the corresponding control cells. The primer sequences outlined in Table 1 were used in these assays.

Western blot analysis. The cells were treated with IL-6 (2 ng/mL) and the expression of related proteins was determined via western blot analysis.

Statistical analysis. All experiments were repeated at least three times to confirm the results. The data are presented as the mean ± SEM. Student’s t-test and one-way ANOVA with the Newman–Keuls post test were applied. Differences were considered significant at P < 0.05.

Results

Interleukin 6 treatment contributes to chemotherapeutic resistance in lung cancer cells. To explore the role of IL-6 in MDR
in lung cancer, the relationship between IL-6 and lung cancer chemotherapeutic resistance was investigated. Compared with NCI-H446 and NCI-H209 cells, A549 (Fig. 1a-d) and LTED-a-2 (Fig. 1e-f) cells clearly have higher IL-6 expression. Meanwhile, A549 (Fig. 1g) and LTED-a-2 cells (Fig. S1a) have higher chemotherapeutic resistance to treatment with camptothecin and cisplatin. When IL-6 replenishment was performed, increased survival rates were achieved in NCI-H446 (Fig. 1h), NCI-H209 (Fig. 1j) and A549 cells (Fig. S1b). In addition, microscope observation confirmed that IL-6 replenishment increases cells’ resistance to camptothecin or cisplatin treatment in both NCI-H446 (Fig. 1h) and NCI-H1299 cells (Fig. S1c-e). Taken together, these findings indicate that proinflammatory cytokines, such as IL-6, play an important role in lung cancer chemotherapeutic resistance.

Higher level of interleukin 6 promotes ABCG2, Mcl-1, Bcl-xl and Bcl-2 expression in lung cancer cells. To understand the role of IL-6 in lung cancer MDR, the relationships between IL-6 level and the expressions of Bcl-xl, Mcl-1, Bcl-2 and ABCG2 in lung cancer cells were investigated. Compared with NCI-H446 cells, A549 cells, which have higher IL-6 expression (Fig. 1a-d), clearly have higher expressions of ABCG2, Bcl-xl, Mcl-1 and Bcl-2 at both translational (Fig. 2a) and transcriptional (Fig. 2b) levels. Transcriptional determination of ABCG2, Bcl-xl, Mcl-1 and Bcl-2 in NCI-H209/LETP-a-2 (Fig. 2d) or NCI-H1299/LETP-a-2 cells (Fig. S2a) leads to similar conclusions. The IL-6 replenishment efficiently increased the expression of ABCG2, Bcl-xl, Mcl-1 and Bcl-2 in NCI-H446 cells at both the protein (Fig. 2c) and RNA (Fig. 2d) levels in both NCI-H446 (Fig. 2c-e) and NCI-H209 cells (Fig. 2f). In addition, when IL-6 expression was knocked down by IL-6 siRNA transfection, the expressions of ABCG2, Bcl-xl, Mcl-1 and Bcl-2 were decreased in A549 (Fig. 2g), LTED-a-2 cells (Fig. 2h) and NCI-H446 cells (Fig. S2b). Similar results were observed in the lung cancer cell line NCI-H1299 and (Fig. S2c). IL-6 administration obviously increased the Bcl-2 expression of adoptive transferred NCI-H446 cells and normal tissues (Fig. S2d). Taken together, these findings indicate that the level of IL-6 directly regulates ABCG2 and anti-apoptotic protein expression in lung cancer cells.

IL-6 treatment induces ATM and NF-kappaB pathways in lung cancer cells. Although ATM, which could be activated by DNA DSB, contributes to chemoresistance, the phosphorylation of ATM can also be increased without apparent DNA damage. Compared with NCI-H446 cells, A549 cells, which have a higher level of IL-6 (Fig. 1a-d), have higher levels of the phosphorylated ATM and IxBz (Fig. 3a). The finding that IL-6 contributes to lung cancer MDR (Figs 1, 2) therefore raises the question of whether the ATM/NF-kappaB pathway could be activated by IL-6 treatment. Our results revealed that the phosphorylation of ATM at Ser1981 was clearly increased following IL-6 treatment (Fig. 3b), as were the levels of IkappaBalpha and p65 phosphorylation (Fig. 3b). The confocal microscopy results led to a similar conclusion that IL-6 treatment induces ATM and NF-kappaB activation in both NCI-H446 (Fig. 3c) and NCI-H1299 cells (Fig. 3d) Taken together, these findings indicate that IL-6 activates the ATM and NF-kappaB pathways in lung cancer cells.

Inhibition of ataxia-telangiectasia mutated and NF-kappaB activation abrogates the effects of interleukin 6 on ABCG2 and anti-apoptotic protein upregulation. To understand the role of ATM and NF-kappaB activation in augmented MDR through increased IL-6, inhibitors were used to inhibit related kinase activities. These inhibitors were invariably able to eliminate ATM or NF-kappaB kinase activity (Fig 4a e, Fig. S3a). The inhibition of ATM or NF-kappaB activity clearly abrogated the effect of IL-6 on ABCG2 and anti-apoptotic protein expression.

![Interleukin 6 (IL-6) activates the ataxia-telangiectasia mutated (ATM) and NF-kappaB pathways in lung cancer cells.](image-url)
expression at both the translational (Fig. 4c) and transcriptional (Fig. 4d) levels in NCI-H446 cells. Meanwhile, the ATM and p65 knockdown results of NCI-H446 cells led to a similar conclusion (Fig. 4e). Similar results were observed in the lung cancer cell line NCI-H209 (Fig. 4f), NCI-H1299 (Fig. S3b) and LTEP-a-2 cells (Fig. S3c). Taken together, the observation that inhibition of ATM or NF-kappaB activity eliminated the effect of IL-6 on ABCG2 and anti-apoptotic protein expression indicates that the ATM and NF-kappaB pathways are involved in IL-6-mediated MDR in lung cancer cells.

Inhibition of p38 and NF-kappaB activation abrogates the effects of chemotherapeutic agents on interleukin 6 upregulation in lung cancer cells. It has been documented that treatment with chemotherapy increases IL-6 levels in advanced cancer patients.\(^{7,8}\) To elucidate the mechanism of chemotherapy upregulating IL-6 expression, lung cancer NCI-H446 (Fig. 5a–d), NCI-H209 (Fig. 5e,f) and NCI-H1299 (Fig. S4a,b) cells were treated with camptothecin or cisplatin, and the expression of IL-6 was analyzed. The treatment with cisplatin (Fig. 5a,c,e) and camptothecin (Fig. 5b,d,f) increased IL-6 expression. The inhibition of p38 or NF-kappaB activity clearly abrogated the effects of cisplatin and camptothecin on IL-6 release at the translational levels (Fig. 5g,h) in NCI-H446 cells. Meanwhile, the p38 and p65 knockdown results of NCI-H446 cells led to a

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** Inhibition of ataxia-telangiectasia mutated (ATM) and NF-kappaB activation abrogates the effects of interleukin 6 (IL-6) on ABCG2 and anti-apoptotic protein upregulation. (a, b) NCI-H446 cells were pretreated with 20 \(\mu\)M CGK733 prior to IL-6 (2 ng/mL) treatment. The role of ATM phosphorylation in IL-6 induced NF-kappaB activation was determined by western blot (a) and confocal microscopy assays (b). Bay11-7082 was used as a negative control. (c–f) NCI-H446 (c–e) and NCI-H209 (f) cells were pretreated with 20 \(\mu\)M CGK733/Bay11-7082 (c, d) or ATM/p65 siRNA transfection (e, f) prior to IL-6 (2 ng/mL) treatment. The roles of ATM and NF-kappaB activation in IL-6 increased the expressions of ABCG2 and anti-apoptotic protein (Mcl-1, Bcl-xl and Bcl-2), which were determined via western blot (c) and real-time PCR assays (d–f). The data are presented as the mean ± SEM. **P < 0.001, one-way ANOVA with the Newman–Keuls post test. One representative from three experiments is shown. Beta-actin was used as the loading control.
similar conclusion (Fig. S1j). Similar results were observed in the lung cancer cell line NCI-H209 (Fig. 5k,l) and NCI-H1299 cells (Fig. S4c,d). Taken together, the observation that inhibition of p38 or NF-kappaB activity eliminated the effects of cisplatin and camptothecin on IL-6 expression indicates that the p38 and NF-kappaB pathways are involved in chemotherapeutics agents-induced IL-6 expression, which, in turn, contributes to MDR in lung cancer.

Discussion
Chemotherapeutic agents can not only induce tumor cell apoptosis but also trigger inflammation in the tumor microenvironment. Proinflammatory factors, such as IL-2, IL-7 and IL-6, are documented to be involved in MDR in head and neck squamous cells, glioma cells, prostate cancer, lung cancer and breast cancer. Meanwhile, a higher level of circulating IL-6, increased by chemotherapeutic agent treatment, is associated with patients’ survival in gastric carcinoma, prostate cancer, breast cancer and lung cancer. Inhibition of p38 and NF-kappaB pathways are involved in chemotherapeutic resistance in lung cancer cells (Figs 1, 2). Moreover, inhibition of ATM-NF-kappaB activities abrogates the effect of IL-6 on ABCG2 and anti-apoptotic protein expression (Fig. 4), indicating that IL-6 and the ATM-NF-kappaB pathway are potential therapeutic targets in inflammation-associated lung cancer chemotherapeutic resistance. As tumor tissues are composed of tumor cells, fibroblasts and immune cells, the finding that fibroblasts and immune cells synthesize and secrete IL-6 cannot exclude the possibility that other components of lung cancer tissues might contribute to the elevation of serum IL-6. In the present study, cancer cells were shown to secrete IL-6 under chemotherapeutic agent treatment conditions, indicating that IL-6 promotes MDR in both a paracrine and autocrine manner in lung cancer.

Ataxia-telangiectasia mutated, a nuclear serine-threonine kinase involved in DNA DSB repair and MDR, can be activated by chemotherapeutic drugs. The phosphorylation of ATM can also be increased by hypoxia treatment without apparent DNA damage. The present study demonstrated that IL-6 treatment clearly increases ATM phosphorylation (Fig. 3) without cell apoptosis (Fig. 1g), indicating that IL-6 activates ATM kinase in DNA damage in an independent manner. In the present study, both inhibition of ATM-NF-kappaB activities and gene silence eliminate the effect of IL-6 on ABCG2 and anti-apoptotic protein expression (Fig. 4), indicating that IL-6 and the ATM-NF-kappaB pathway are potential therapeutic targets for lung cancer chemotherapy. We also notice that constitutive activation of signal transducer and activator of transcription 3 (STAT3) upregulate P-gp expression and augment chemotherapeutic resistance. Kim et al. also found that afatinib activates IL-6R/JAK1

Fig. 5. Inhibition of p38 and NF-kappaB activation abrogates the effects of chemotherapeutic agents on interleukin 6 (IL-6) upregulation in lung cancer cells. (a-f) NCI-H446 (a–d) and NCI-H209 cells (e, f) were exposed to 0.5 μg/mL cisplatin (DDP) or camptothecin (CPT) and IL-6 expression was determined by flow cytometry (a, b) and ELISA analyses (c–f), respectively. For the flow cytometry analysis, the numbers in the histogram indicate the geometric mean fluorescence (MFI) of the tested samples. *P < 0.05; **P < 0.01; ***P < 0.001. Student’s t-test. (g–l) NCI-H446 (g–j) and NCI-H209 cells (k, l) were pretreated with 20 μM inhibitor of SB203580 (20 μM)/Bay 11-7082 (20 μM) (g, h, k, l) or p38/p65 siRNA transfection (i, j) prior to cisplatin or camptothecin treatment. The roles of p38 and NF-kappaB activation in cisplatin or camptothecin-increased IL-6 expression were determined by ELISA analyses. Data are given as mean ± SEM, n = 3. *P < 0.05; **P < 0.01; ***P < 0.001, one-way ANOVA with post Newman–Keuls test. The data are presented as the mean ± SEM, *P < 0.05, **P < 0.01, one-way ANOVA with the Newman–Keuls post test. A representative of three independent experiments is shown.
/STAT3 signaling via autocrine IL-6 secretion. As IL-6 treatment clearly increases Jak2-Stat3 (Fig. S6) activation and the ATM phosphorylation is involved in Stat3 activation,\textsuperscript{40} the exact interactions of ATM and Stat3 in IL-6 promoted chemotherapeutic resistance requires further exploration.

The treatment with chemotherapeutic agents triggers the export of ATM and sumoylated NEMO (IKK-\gamma), which activates XIAP-containing and ELKS-containing signalosome\textsuperscript{15} in response to LPS or afatinib treated patients,\textsuperscript{39,42,43} the exact effects and mechanism of IL-6 on lung cancer stem cell marker expression requires further investigation. The present study reveals that IL-6 treatment increases ABCG2 expression at both the translational and transcriptional levels (Fig. 4), and contributes to chemotherapeutic resistance (Fig. 1), indicating that IL-6 treatment facilitates lung cancer cells acquiring cancer stem-like phenotypes. The exact effects and mechanism of IL-6 on lung cancer stem cell marker expression requires further investigation.

Taken together, our experiments reveal for the first time that IL-6 increases ABCG2, anti-apoptotic protein expression, and contributes to IL-6 augmented lung cancer chemotherapeutic resistance by activating the ATM and NF-kappaB pathways, indicating that ATMs and IL-6 are potential targets for managing lung cancer chemotherapeutic resistance.

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Disclosure Statement
The authors have no conflict of interest to declare.
IL-6 induces Jak2 and Stat3 phosphorylation.

Fig. S6.

Inhibition of ATM and NF-κB activation abrogates the effects of VEGF on ABCG2 and anti-apoptotic protein up-regulation.

Fig. S5.

H1299 and LTEP-a-2 cells.

Fig. S2.

IL-6 increases ABCG2, Mcl-1, Bcl-xl and Bcl-2 expression in lung cancer NCI-H1299 and NCI-H446 cells.

Fig. S4.

Inhibition of p38 and NF-kappaB activation abrogates the effects of chemotherapeutic agents on IL-6 up-regulation in NCI-H1299 cells.

Fig. S3.

Inhibition of ATM and NF-kappaB activation abrogates the effects of VEGF on ABCG2 and anti-apoptotic protein up-regulation.

Fig. S1.

IL-6 treatment contributes to chemotherapeutics resistance in lung cancer cells.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. IL-6 treatment contributes to chemotherapeutics resistance in lung cancer cells.

Fig. S2. IL-6 increases ABCG2, Mcl-1, Bcl-xl and Bcl-2 expression in lung cancer NCI-H1299 and NCI-H446 cells.

Fig. S3. Inhibition of ATM and NF-kappaB activation abrogates the effects of IL-6 on ABCG2 and anti-apoptotic protein up-regulation in NCI-H1299 and LTEP-a-2 cells.

Fig. S4. Inhibition of p38 and NF-kappaB activation abrogates the effects of chemotherapeutic agents on IL-6 up-regulation in NCI-H1299 cells.

Fig. S5. Inhibition of ATM and NF-kappaB activation abrogates the effects of VEGF on ABCG2 and anti-apoptotic protein up-regulation.

Fig. S6. IL-6 induces Jak2 and Stat3 phosphorylation.