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Non-receptor tyrosine kinase *Etk* regulation of drug resistance in small-cell lung cancer

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ABSTRACT

Epithelial and endothelial tyrosine kinase (*Etk*), also known as *Bmx* (bone marrow X kinase), plays an important role in the apoptosis of epithelial cells. The aim of this study was to investigate whether *Etk* is involved in the chemoresistance of small cell lung cancer (SCLC) and to correlate the drug resistance associated proteins such as *bcl-2*, *bcl-X_L* and *p53*. Drug-resistant small lung cancer cells (H69AR) which were originally developed by ADM and which demonstrated multi-drug resistance to chemotherapeutic agents were used in the study. Western blot analysis revealed that H69AR cells over-expressed the proteins *Etk* and *bcl-X_L*, but not *bcl-2* and *p53* when compared to parent H69 cells. Knockdown of *Etk* expression by *Etk*-specific small interfering RNA sensitised H69AR cells to chemotherapeutic drugs and inhibited *bcl-X_L* expression but not *bcl-2* and *p53*. Co-immunoprecipitation was performed to further evaluate the relationship between *Etk* and *bcl-X_L* with anti-*Etk* and anti-phospho-*Etk* antibodies. The *bcl-X_L* was accompanied with a robust increase of *Etk* and tyrosine phosphorylated Etk at Tyr-40 in H69AR cells. In conclusion, our results suggest that non-receptor tyrosine kinase *Etk* is involved in drug resistance to SCLC by mediating *bcl-X_L* via Tyr(P)-40. The potential approach for downregulation of *Etk* activity on expression would be a novel, potentially clinically practical strategy for interfering with chemoresistance in SCLC.

1. Introduction

Lung cancer is one of the leading malignant tumours in the world at present. Small cell lung cancer (SCLC) represents around 15% of all lung cancers.[1,2] The mainstay of treatment for SCLC remains chemotherapy including cisplatin, etoposide and paclitaxel. Despite its marked sensitivity to induction chemotherapeutic drugs, SCLC is characterised by high relapse rates and a subsequent poor prognosis because of drug resistance. For patients with limited-stage disease (LD), 5-year survival rate is 12–26%, but is less than 5% in patients with extensive-stage disease (ED).[3–5] Accordingly, chemoresistance has become one of the major obstacles in the treatment of SCLC and is clinically a very important issue for improving the poor prognosis of SCLC.

Epithelial and endothelial tyrosine kinase (*Etk*), also known as bone marrow X kinase (*Bmx*), is one member of the Tec family of non-receptor tyrosine kinases (*BTK/AKT, ITK/EMT/TSK, ETK/BMX, TEC*). It shares a homologous structure with the other members of the Btk family, consisting of the conserved structural domains-a PH (pleckstrin homology), SH3 (SRC homology 3), SH2 (SRC homology 2)-from the amino terminus, and the kinase domain in the carboxyl terminus.[6–9] *Etk* is expressed in epithelial cells as well as distributed in lympho-haematopoietic cells.[7,10,11] It has been investigated that *Etk* could be both anti- and pro-apoptotic.

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Etk can protect prostate carcinoma cell line LNCap from apoptosis induced by thapsigargin (TG) and photodynamic therapy (PDT), whereas it sensitises mast cell line 32D toward apoptosis upon treatment with G-CSF. The bidirectional role of *Etk* in apoptosis was explained through *Etk* being a direct substrate for caspase. The caspase cleavage contains an intact C-terminal SH2 domain and kinase domain. This molecule has an enhanced kinase activity and, while not apoptotic on its own, possesses an ability to enhance apoptosis induced by other agents.[12–14] Therefore, we propose that *Etk* may confer apoptosis and resistance to chemotherapeutic drugs in SCLC.

To better understand the molecular mechanisms of multidrug resistance in SCLC, we investigate whether or not *Etk* is involved in the chemoresistance of SCLC in this study. We first demonstrated that Etk mRNA and protein expression increased in drug-resistant small lung cancer cells (H69AR), and that down-regulation of *Etk* markedly sensitised SCLC cells to chemotherapeutic drugs. We then used *Etk* and Tyr(P) co-immunoprecipitation to identify candidate *Etk* downstream substrates in SCLC cells, which revealed an interaction between *Etk* and *bcl-X_L*. These studies showed that *bcl-X_L* is a downstream target of *Etk* pathway in the drug resistance of SCLC cells.

2. Materials and methods

2.1. Cell culture

The human small lung cancer cell line NCI-H69 and the drug-resistant subline H69AR were purchased from the American Type Culture Collection (ATCC, USA) and maintained in RPMI 1640 medium containing L-glutamine with 10% foetal calf serum in an incubator at 37 °C with 5% CO_2. H69AR was alternately fed with drug-free medium and medium containing 0.8 μM of Adriamycin (ADM), and overexpresses *MRP1/ABCC1*.[15–17] The resistant cell line was tested regularly for maintained resistance to the selected drugs. Growth and morphology of all cell lines were monitored on a weekly basis.

2.2. In vitro drug-resistance assay

In vitro drug cytotoxicity was measured by Cell Counting Kit-8 (CCK-8) assays. The cells incubated without drugs (i.e. control wells) were set at 100% survival and were used to calculate the concentration of each cytostatic drug lethal to 50% of the cells (LC_{50}). The ranges of drug concentrations were based on earlier studies and aimed at obtaining an LC_{50} value both for highly sensitive and resistant cases. A total of eight anticancer drugs [Daunorubicin (DNR; Pharmacia & Upjohn, Italy), Vincristine Sulfate (VDS; MingXin, Guangzhou, China), Cisplatin (DDP; Shangdong, China), Mitoxantrone (MIT; Sichuang, China), Etoposide (VP-16; Jiangshu, China), Paclitaxel (TAX; Hunan, China), Pyrimidinedione (5-FU; Sichuang, China) and Adriamycin (ADM; Jiangshu, China)] were obtained from commercial sources and were dissolved according to the manufacturer's instructions and tested in five concentrations. Anticancer drugs-induced cell death was quantified using the CCK-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H- tetrazolium, monosodium salt] assay. Cells were seeded into 96-well plates (4000 cells/well) and then treated for 24 h in 200 μl of medium with anticancer drugs. CCK-8 reagent (Dojindo, Kumamoto, Japan) was then added and the cells were incubated at 37 °C for 4 h before reading the absorbency using a micro-plate reader (μQuant, Bio-Tek Instruments Inc.) at 450 nm. The assay was conducted in six replicate wells for each sample and three parallel experiments were performed.

2.3. Transfection of small interfering (si) RNA

The oligonucleotides encoding *Etk* siRNA were 5′-TGG AGCTGGGAAGTGGCCAGTTCAAGAGACTGGCCACTTCCCAGC-TCCTTTTTTC-3′ and 5′-TCGAGAAAAAAGGAGCTGGGAAGT GGCCAGTCTCTTGAACTGGCCACTTCCCAGCTCCA-3′. siRNA transfection was done according to the protocol supplied by Invitrogen (Shanghai, China). Briefly, 1×10^5 cells were seeded into six-well plates containing antibiotic-free medium and incubated overnight. For each well, 1 μl each of XL1 and XL2 was mixed together with 183 μl of OPTI-MEM I. The mixture was then combined with a solution prepared with 3 μl of Oligofectamine and 15 μl of OPTI-MEM I. After 15 min of incubation at room temperature, the final mixture was added to each well, which had been washed and contained FCS-free medium. The final concentration of siRNA was 200 nmol/L. We used the same concentration of luciferase-specific siRNA as a control oligonucleotide.

2.4. Western blot and immunoprecipitation

Cells were lysed and sonicated in a solution containing 0.5% sodium deoxycholate (w/v), 0.2% SDS (w/v), 1% Triton X-100 (v/v), 5 mM EDTA, 10 mg/ml leupeptin, 10 mg/ml aprotinin and 1 mM phenylmethyl sulfonyl-uoride supplemented with 1:1000 dilution of protease inhibitor cocktail. The homogenates were then centrifuged at 4 °C for 10 min to remove cell debris. Supernatants were harvested and concentrations were determined by the *DC* protein assay (Bio-Rad Laboratories, Hercules, CA). Equivalent amounts of protein were electrophoresed on an 8% polyacrylamide gel and then transferred to a PVDF membrane. For Western immunoblotting, the membrane was blocked with 5% milk (w/v) in TBS containing 0.1% Tween 20 (TBS/T) at room temperature for 2 h and incubated with primary antibodies including *Etk* (BD Biosciences, USA), *bcl-2* (100/D5, Dako, Glostrup, Denmark), *p53* (DO-7, Dako, Glostrup, Denmark) and *bcl-X_L* (Novagen, Darmstadt, Germany) overnight at 4 °C, washed three times with PBST, followed by incubation with appropriate secondary antibodies at room temperature for 1 h. The immune complexes were detected by an enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL, USA). For co-immunoprecipitation, protein extracts (0.2 mg) were incubated with anti-*bcl-X_L* antibody overnight at 4 °C. Subsequently, antibodies were collected with protein A-/protein G-Sepharose beads, and protein complexes were washed three times at 4 °C with the lysis buffer, electrophoresed on an SDS–polyacrylamide gel under reducing conditions, and transferred onto PVDF membrane. Immunoblotting was performed as described above. Blots were incubated with the indicated primary antibodies, either an anti-Etk antibody or an anti-phosphotyrosine(Tyr40) antibody (Cell Signalling, USA) at room