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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

temperature for 1 h and followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h, and developed with ECL reagents.

2.5. Flow cytometry

NCI-H69 and NCI-H69AR were separately collected and centrifuged at 1000*g* for 10 min in an Eppendorf 5810R centrifuge (Hamburg, Germany) at 4 °C. The supernatant was discarded and the pellet was then fixed with a concentration of 0.5% formaldehyde (Sigma–Aldrich, USA) in PBS overnight. After fixation, cells were washed twice with PBS and incubated with a mouse anti-human *Etk* (BD Biosciences, USA), *bcl-2* (100/D5, Dako, Glostrup, Denmark), *p53* (DO-7, Dako, Glostrup, Denmark) and *bcl-*X_L (Novagen, Darmstadt, Germany) at 1:1000 dilution for 30 min on ice. After PBS washing, the cells were then incubated with a FITC-conjugated goat anti-mouse/rabbit IgG antibody (Jackson Immunoresearch Laboratories) on ice in the dark for 30 min. The level of fluorescence was detected using a Coulter EPICS XL flow cytometer (Beckton Coulter).

2.6. Real-time quantitative reverse transcriptase-PCR (RT-PCR)

Total RNA was prepared by using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. The RNA was assessed by denaturing agarose gel electrophoresis (visual presence of sharp 28S and 18S bands) and was quantitated by spectrophotometry. One microgram of total RNA from each sample was reverse-transcribed using AMV Reverse Transcriptase and Oligo(dT)12–18 primer (Invitrogen). Primers for real-time PCR were designed using Primer Express software version 2.0 (Applied Biosystems) and synthesised by Invitrogen. The sequences of primers for *Etk* were 5′-CATAGTGGGTTCT TCGTGGAC-3′ and 5′-TGCCCGAGGTATCTTCAGC-3′. Real-time PCR was carried out in an ABI Prism 7000 sequence detection system (Applied Biosystems) using SYBR Green according to the manufacturer's instructions. The reaction was performed in a 20-μl final volume with 0.150 μg of purified total RNA, 4 μl of PCR mix provided by the manufacturer, 4 μl of $MgCl_2$ (25 mM), 2.5 μl of each primer (2 μM), and 0.4 μl of enzyme mix and diethyl pyrocarbonate-H_2O. Amplification of the cDNA was achieved in 40 cycles of 95 °C, 10 s; 60 °C, 20 s; and 68 °C, 30 s. Fluorescence was recorded during the elongation phase at 72 °C.

The threshold cycle (Ct) was defined as the PCR cycle number at which the reporter fluorescence crosses the threshold reflecting a statistically significant point above the calculated baseline. The Ct of each target product was determined and normalised against that of the housekeeping gene *GAPDH* (forward, 5′-AAGAAGGTGGTGAAGCAGGC-3′; reverse, 5′-TCCACCACCCTGTTGCTGTA -3′). All samples were amplified at least in triplicate on different days.

2.7. Statistical analysis

$p < 0.05$ was regarded as a significant difference. The flow cytometry data was analysed by Student's *t*-test.

3. Results

3.1. Drug resistance profile of H69AR cells

As shown in Table 1, increased resistance in H69AR cells was observed for several anticancer agents as compared to parent H69 cells. To determine the drug resistance profile of H69AR cells to chemotherapeutic agents, we evaluated the IC_{50} of the existing clinical chemotherapeutic drugs paclitaxel, cisplatin, and so on.

3.2. Comparison of the levels of Etk and bcl-2, bcl-X_L and p53 in parental and drug-resistant H69 cells

To evaluate *Etk* expression at the protein level in drug-resistant H69AR cells, as compared to parental H69 cells, Western blot and flow cytometry analyses were performed. The results revealed a significantly elevated protein level of *Etk* in H69AR cells compared to H69 cells.

Because over-expression of *bcl-2* family members has been associated with drug resistance, we examined if the level of *bcl-2, bcl-*X_L and *p53* was elevated in H69AR cells that acquired resistance to chemotherapeutic drugs. As shown in Fig. 1, the level of *bcl-*X_L was significantly higher in H69AR cells than that in parental H69 cells. However, the level of the anti-apop-

Table 1 – Drug sensitivity of H69AR and H69 cell lines.

Anticancer agents	IC_{50} (μg/ml $\overline{X} \pm s$)		Resistance index
	H69	H69AR	
ADM	4.90 ± 0.84	135.30 ± 5.32	27.61
DNR	1.53 ± 0.65	30.45 ± 2.63	19.9
VDS	4.26 ± 1.96	63.06 ± 2.55	14.8
TAX	3.12 ± 1.02	30.55 ± 2.36	9.79
DDP	14.52 ± 2.13	136.19 ± 3. 56	9.38
5-FU	4.66 ± 1.35	26.23 ± 2.21	5.63
VP-16	40.13 ± 3.25	208.06 ± 6.26	5.18
MIT	2.87 ± 0.52	13.49 ± 1. 23	4.70

ADM, adriamycin; DNR, daunorubicin; VDS, vincristine sulphate; TAX, paclitaxel; DDP, cisplatin; 5-FU, pyrimidinedione; VP-16, etoposide; MIT, mitoxantrone.

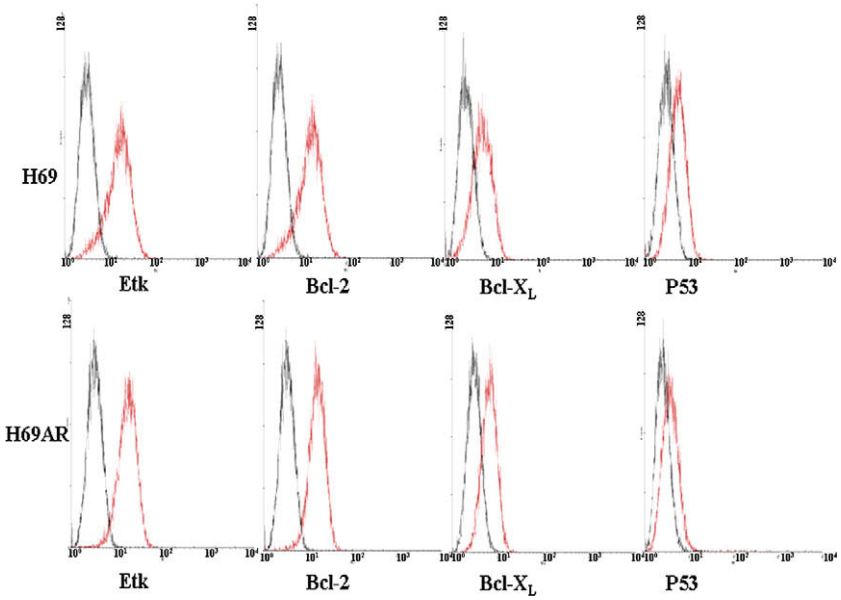

Fig. 1 – Immunoblotting showing *Etk*, *bcl-X$_L$*, *bcl-2* and *p53* expression in drug-resistant H69AR cells and parental H69 cells.

totic protein *bcl-2* and *p53* was slightly increased in H69 cells as compared to H69AR cells (Figs. 1 and 2; Table 2).

3.3. *Inhibition of Etk mRNA and protein expression by Etk siRNA*

Etk siRNA was transfected into H69AR cells with Lipofectamine 2000 *in vitro*. Real-time PCR demonstrated that 48 h after treatment, siRNA substantially reduced *Etk* mRNA levels by 72% ($2^{-\Delta\Delta CT}$: 8.42E–06 versus 2.36E–06) in H69AR as compared to H69AR-siRNA.

To examine whether the *Etk* protein was also silenced, its expression was measured by Western blotting. The negative vector had no significant effect on *Etk* expression. *Etk* protein was expressed at a higher level in normal cells and in cells transfected with negative vector than in those cells transfected with *Etk* siRNA vector (Fig. 3).

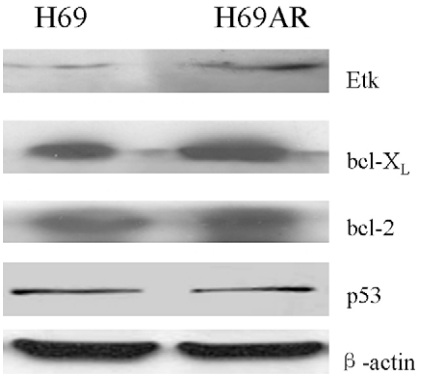

Fig. 2 – Flow cytometry showing *Etk*, *bcl-X$_L$*, *bcl-2* and *p53* expression in drug-resistant H69AR cells and parental H69 cells.

3.4. *Effect of Etk siRNA on cell sensitivity to chemotherapeutic drugs*

To further evaluate the role of *Etk* in chemoresistance, we analysed the viability of H69AR cells when exposed to chemotherapeutic drugs after treatment with *Etk* siRNA. The growth inhibitory IC_{50} values to chemotherapeutic drugs for siRNA-treated H69AR cells was 2.64–9.36-fold greater in the siRNA-treated cells (Table 3) than that in the control cells. These data indicated that downregulation of *Etk* sensitised SCLC cells to chemotherapeutic drugs.

3.5. *Effect of Etk siRNA on bcl-2 family members and p53 expression*

The above reported results showed that both *Etk* and *bcl-X$_L$* expression increased in drug-resistant H69AR cells as compared to parental H69 cells. To further evaluate the relation-

Table 2 – Expression of *Etk*, *bcl-2*, *bcl-X$_L$* and *p53* in H69 and H69AR cells by flow cytometry (% $\bar{X} \pm s$).

Cell lines	*Etk*	*bcl-2*	*bcl-X$_L$*	*p53*
H69	46.0 ± 1.5	85.2 ± 3.07	28.1 ± 1.8	11.3 ± 1.4
H69AR	92.8 ± 2.8	94.6 ± 1.0	45.9 ± 2.4	12.4 ± 0.9

H69 versus H69AR, $^*p < 0.05$.

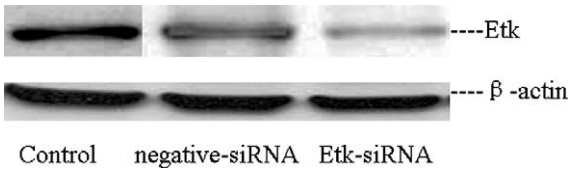

Fig. 3 – Immunoblotting showing downregulation of *Etk* expression after treatment with *Etk* siRNA in H69AR cells.

Table 3 – Drug sensitivity of H69AR after treatment with *Etk* siRNA.

Anticancer agents	IC_{50} (μg/ml $\overline{X} \pm s$)			Resistance index
	Control	Negative-siRNA	Etk-siRNA	
ADM	132.95 ± 5.02	103.54 ± 4.87	14.20 ± 1.68	9.36
DNR	35.14 ± 2.23	32.63 ± 2.11	4.72 ± 1.30	7.45
VDS	59.86 ± 2.21	54.79 ± 2.18	9.04 ± 1.92	6.62
TAX	31.34 ± 2.36	28.91 ± 2.40	6.48 ± 2.02	4.84
DDP	132.41 ± 3.60	116.56 ± 2.98	28.08 ± 3.13	4.72
5-FU	22.37 ± 2.21	19.96 ± 1.85	6.11 ± 1.45	3.66
VP-16	211.31 ± 5.66	192.72 ± 5.23	58.26 ± 3.25	3.63
MIT	14.43 ± 1.17	12.66 ± 1.03	5.47 ± 1.02	2.64

ADM, adriamycin; DNR, daunorubicin; VDS, vincristine sulphate; TAX, paclitaxel; DDP, cisplatin; 5-FU, pyrimidinedione; VP-16, etoposide; MIT, mitoxantrone.

ship between *Etk* and *bcl-2*, *bcl-X_L* and *p53* in H69AR, we detected these proteins at 48 h after treatment with *Etk* siRNA. The protein level of *bcl-X_L* was greatly reduced, while *bcl-2* and *p53* presented no visible change with *Etk* siRNA treatment (Fig. 4).

3.6. *Correlation between Etk and bcl-X_L protein expression*

The above data provided evidence that *Etk* may be closely associated with *bcl-X_L*. We performed co-immunoprecipitation to further evaluate whether *bcl-X_L* expression was directly regulated by *Etk* in H69AR cells. As shown in Fig. 5, *bcl-X_L* was co-immunoprecipitated with anti-*Etk* and anti-phospho-*Etk* (Tyr-40) antibodies. The *bcl-X_L* was accompanied with a robust increase of *Etk* and tyrosine phosphorylated *Etk* at Tyr-40 in drug-resistant H69AR cells (Fig. 5).

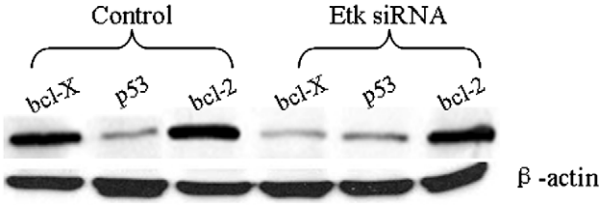

Fig. 4 – Immunoblotting showing downregulation of *bcl-X_L* expression but not *bcl-2* and *p53* after treatment with *Etk* siRNA in H69AR cells.

Fig. 5 – The association between *Etk* and *bcl-X_L* in H69AR cells was determined by co-immunoprecipitation with *bcl-X_L* antibody and followed by immunoblotting with anti-Etk or anti-phospho-Etk (Tyr-40) antibody.

4. Discussion

Etk, a member of the Tec family of tyrosine kinases, is shown to be involved in various cellular processes including proliferation, differentiation and apoptosis.[5,7,11–13,17] In this study, we first showed that *Etk* mRNA and protein expression increased in drug-resistant small lung cancer cells (H69AR). H69AR was originally developed by ADM and presented multi-drug resistance to chemotherapeutic drugs including DNR, VDS, DDP, MIT, VP-16, TAX, 5-FU. Over-expression of *Etk* in H69AR suggested that *Etk* may have a relationship with chemoresistance in SCLC. To further investigate whether *Etk* is involved in drug resistance, we examined the effect of siRNA-mediated *Etk* down-regulation on cell sensitivity to chemotherapeutic drugs in H69AR cells. The IC_{50} of H69AR cells to chemotherapeutic drugs was significantly decreased by *Etk*-specific siRNA. H69AR was much more sensitive to chemotherapeutic agents compared to that in negative or normal groups with a greater decrease in the mRNA and protein expression of *Etk*. Our findings indicate that *Etk* may be closely associated with chemoresistance and may be a novel, potentially clinically practical strategy for interfering with chemoresistance in SCLC.

Etk has been reported to have both antiapoptotic and proapoptotic potential depending on the cell contexts and the *Etk* exhibits increased kinase activity. Over-expression of *Etk* rendered prostate cancer cell line LNCaP more resistant to photodynamic therapy or thapsigargin-induced apoptosis.[12] Down-regulation of *Etk* expression by a specific siRNA sensitised prostate cancer cells to doxorubicin-induced apoptosis. However, *Etk* overexpression promoted the MDA-MB-468 breast cancer cell line to EGF-induced apoptosis and mast cell line 32D toward apoptosis upon treatment with G-CSF.[13,14,18] At the present time, the mechanism of this putative switch remains unknown. In addition to the potential therapeutic impact of *Etk*, our studies also shed important light on the mechanisms by which *Etk* mediates multi-drug resistance in SCLC. It has been reported previously that *bcl-2*, *bcl- X_L* and *p53* may be closely related to chemoresistance in SCLC.[19–21] To gain insight into the molecular basis of *Etk* responsible for sensitivity to chemotherapeutic agents, we carried out an analysis on the relationship between *Etk* expression and *bcl-2*, *bcl- X_L* and *p53* in H69AR. In the present study, we showed that the level of *bcl-2*, *bcl-X_L* and *p53* expression in-

creased in H69AR cells compared to H69 corresponding to *Etk*. We also found that the protein level of *bcl-X_L* was greatly reduced after treatment with *Etk* siRNA. *Etk* tended to be associated with *bcl-X_L* but not *bcl-2* and *p53*. To further confirm their relationship, we also performed co-immunoprecipitation and demonstrated that *bcl-X_L* expression directly interacts with *Etk* in H69AR cells. Our data suggested that *Etk* and its effector *bcl-X_L* function to render H69AR cells more resistant to chemotherapeutic drugs.

The mechanism for *Etk* activation by various stimuli is not fully understood. Tyrosine phosphorylation of *Etk* is one of the critical steps. Phosphorylation of *Etk* at Tyr-40, a conserved tyrosine residue in the PH domain, might be a phosphorylation site and its phosphorylation could be required for *Etk* activation in endothelial and epithelial cells.[22] In this study, we demonstrated that tyrosine phosphorylated *Etk* protein increased in drug-resistant H69AR cells compared to parental H69 cells. *Bcl-X_L* expression was also associated with phosphorylated *Etk* in H69AR cells. The data suggested that the potential phosphorylation site Tyr(P)-40 located in the PH domain will become accessible to *bcl-X_L*-associated drug resistance.

In summary, our data have provided a novel mechanism by which 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 or expression would be expected to sensitise SCLC to chemotherapeutic drugs. Therefore, our findings indicate that *Etk* should be a valuable therapeutic target for interfering with chemoresistance in SCLC.

Conflict of interest statement

None declared.

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