TLE1 corepressor promotes gefitinib resistance in lung cancer A549 cells via E-cadherin silencing

XIN YAO[1], NASIR ROBERTS[1], PRINCE IHEUKWUMERE[1], ALANA CARMOUCHE[1], RENWEI CHEN[2], MA. CARMELA DELA CRUZ[3] and HECTOR BILIRAN[1]

[1]Department of Biology, Xavier University of Louisiana, New Orleans, LA 70125, USA; [2]Marine Science Institute, University of California, Santa Barbara, CA 93106, USA; [3]College of Medicine, University of the Philippines Manila, Ermita, Manila 1000, Philippines

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Abstract. As a putative lung specific oncogene, the transducin-like enhancer of split 1 (TLE1) corepressor drives an anti-apoptotic and pro-epithelial-mesenchymal transition (EMT) gene transcriptional programs in human lung adenocarcinoma (LUAD) cells, thereby promoting anoikis resistance and tumor aggressiveness. Through its survival- and EMT-promoting gene regulatory programs, TLE1 may impact drug sensitivity and resistance in lung cancer cells. In the present study, a novel function of TLE1 was uncovered as an inhibitor of the antitumor effects of the epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) gefitinib in the human LUAD cell line A549, which exhibits moderate sensitivity to EGFR-TKI. While upregulation of TLE1 expression potently inhibited the proliferation inhibitory and apoptotic effects of gefitinib in A549 cells, downregulation of endogenous TLE1 in these cells enhanced their sensitivity to gefitinib. In experimentally derived gefitinib-resistant A549 cells (A549GR) that have acquired EMT, TLE1 expression is upregulated as compared with parental A549 cells, and acute ablation of TLE1 expression is sufficient to partially restore gefitinib sensitivity and attenuate EMT phenotype. Mechanistic studies showed that TLE1 confers gefitinib resistance in A549 cells in part via downregulation of E-cadherin, a known potentiator of EGFR-TKI sensitivity and apoptosis induction. Importantly, the TLE1/E-cadherin transcriptional axis is negatively regulated by gefitinib to trigger apoptosis via the Bcl-2-inhibitor of transcription 1 cell death pathway. In conclusion, these results indicate a novel role of TLE1 in modulating EGFR-TKI sensitivity in lung cancer cells via regulation of E-cadherin expression, and its upregulation may potentiate EGFR-TKI resistance in LUAD.

Correspondence to: Dr Hector Biliran, Department of Biology, Xavier University of Louisiana, 1 Drexel Drive, New Orleans, LA 70125, USA
E-mail: hbiliran@xula.edu

Abbreviations: TLE1, transducin-like enhancer of split 1; EGFR, epidermal growth factor receptor; EGFR-TKI, EGFR tyrosine kinase inhibitor; EMT, epithelial-to-mesenchymal transition; Bit1, Bcl-2 inhibitor of transcription

Key words: TLE1, EGFR, EGFR-TKI, gefitinib, EMT, E-cadherin

Introduction

Non-small cell lung cancer (NSCLC) is a highly aggressive disease with lung adenocarcinoma (LUAD), the most diagnosed histological subtype of NSCLC, having a 5-year patient survival rate of only 15%. Recent molecular advances in tumor biology have identified epidermal growth factor receptor (EGFR) to be highly expressed and/or mutated in NSCLC, and EGFR inhibition [for example, tyrosine kinase inhibitors (TKIs)] has shown promise in the treatment of patients with LUAD (1). Gefitinib, a first-generation EGFR-TKI, shows effective antitumor activity in patients with EGFR-mutant LUAD as compared with chemotherapy. Despite their initial response, numerous EGFR-TKI-treated patients eventually acquire resistance. EGFR-TKI resistance mechanisms include secondary EGFR mutations (for example, T790M), bypass signaling activations (for example, MET amplification) and phenotypic transformation [for example, epithelial-mesenchymal transition (EMT)] (2). In addition, some patients with EGFR-TK mutations do not respond to EGFR-TKIs (intrinsic resistance) while others with wild type EGFR respond to this drug (3). Hence, it is likely that other molecular factors beyond EGFR mutations determine the sensitivity of lung cancer cells to EGFR-TKIs.

A major antitumor effect of EGFR targeted therapy is through induction of apoptosis (4). To date, the molecular mechanisms underlying EGFR-TKI-induced apoptosis have not been fully elucidated. In addition to induction and/or activation of the intrinsic mitochondrial pro-apoptotic Bcl-2 family members such as BIM, downregulation of anti-apoptosis effectors (for example, survivin) has been shown to promote gefitinib sensitivity (5,6). While key survival signaling pathways impacted by EGFR-TKIs are yet to be identified, very little information is currently available on the effects of EGFR-TKIs on gene transcription and epigenetic machineries. Undoubtedly, EGFR-TKIs may alter transcriptional programs and induce reprogramming to exert antitumor activity. Molecular characterization of the transcriptional

and epigenetic machineries regulated by EGFR-TKIs will yield novel targets to potentiate the antitumor effect of EGFR inhibition therapy.

In addition to genetic mutations, aberrant function of epigenetic regulators contributes to EGFR-TKI resistance. Dysfunction of epigenetic regulators in the form of transcriptional coactivators and corepressors may result in altered DNA and histone proteins through recruitment of chromatin remodelling enzymes to target gene promoter and in transcriptional profiles that render cancer cells resistant to targeted therapy. Importantly, with the ability to impact multiple gene regulatory and cell signaling pathways due to their interaction with diverse transcription factors (TFs), transcription coregulators represent viable molecular targets to circumvent drug resistance.

Transducin-like enhancer of split 1 (TLE1) is a transcriptional corepressor that exerts a lung specific oncogenic function. In a transgenic mouse model, overexpression of Grg1 gene (the mouse homologue of TLE1) resulted in lung tumors that resemble human lung adenocarcinoma (7). Aligning with this *in vivo* data, our laboratory has previously shown that the transcriptional corepressor TLE1 regulates a survival- and an EMT-promoting gene expression programs in LUAD cells to promote anoikis resistance and anchorage-independent growth *in vitro* and tumorigenicity *in vivo* (8,9). The anti-apoptosis and pro-EMT function of TLE1 in part involves inhibiting the tumor suppressive Bcl-2-inhibitor of transcription 1 (Bit1) cell death pathway. Despite its known function in orchestrating anti-apoptotic and EMT transcriptional programs which are key determinants of drug resistance, TLE1 effects on anticancer drug resistance particularly in LUAD remain unknown. As a 'master' regulator of lung cancer cell apoptosis resistance and EMT phenotype, it is hypothesized that inhibiting the TLE1 nuclear function can block its oncogenic function and its potential antagonistic effects on molecular targeted therapy including EGFR-TKI. In the present study, it was identified that TLE1 reduces gefitinib-induced growth inhibition and apoptosis in LUAD A549 cells in part via downregulation of E-cadherin, and TLE1 expression is upregulated in the experimentally generated gefitinib-resistant A549 (A549GR) cell line to promote EMT and gefitinib insensitivity.

Materials and methods

Cell culture and transduction assays. The human LUAD cell line A549 (cat. no. CCL-185) and HCC827 (cat. no. CRL 2868) were obtained from the American Type Culture Collection. A549 was cultured in Dulbecco's modified Eagle's medium (DMEM) with glutamine containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. HCC827 was cultured in RPMI-1640 with 10% FBS and penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.). To express exogenous TLE1 in A549 cell line, parental cells were transduced with lentiviral GFP-TLE1 or the empty control GFP construct (Horizon Discovery) (8,9). Briefly, the lentiviral products were produced using the second-generation system by transfecting the 293T cell line with 1 μg of lentiviral plasmid, 1.2 μg of packaging plasmid, and 6.6 μl of Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in 250 μl OPTI-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated at 37˚C humidified incubator with 5% CO_2. Following a 16-h incubation, culture medium was harvested and clarified via centrifugation at 500 x g at 5 min followed by 0.45-μm filtration. Lentivirus-containing culture medium was used immediately, or stored at 4˚C. Lentiviral transduction of the parental A549 cell line was performed in a 24-well plate using a 0.5 multiplicity of infection followed by incubation at 37˚C in a humidified incubator with 5% CO_2 for 48 h prior to selection and subsequent experiments. Two control GFP clones and three distinct exogenous TLE1-expressing GFP-TLE1 clones were pooled together to generate the control GFP and GFP-TLE1 pools, respectively. Meanwhile, to generate the stable A549 TLE1 shRNA and control shRNA cells, the parental A549 cell line was transfected with 0.5 μg of the control short hairpin RNA (shRNA) or TLE1-specific shRNA construct (OriGene Technologies, Inc.) cells in OPTI-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and cultured at 37˚C humidified incubator with 5% CO_2. A total of 24 h post-transfection, transfected cells were treated with 1 μg/ml puromycin (Invitrogen; Thermo Fisher Scientific, Inc.) to select for stable clones. The TLE1 shRNA is sense, 5'-GGAATGTGAGAAACTGGCAAG TGAA-3' and antisense, 5'-UUCACUUGCCAGUUUCUC ACAUUCC-3'; and the control shRNA with sense, 5'-UUC UCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGA CACGUUCGGAGAATT-3' does not target any annotated human genes. To restore TLE1 expression, TLE1 shRNA cells were transfected with a TLE1 plasmid containing silent mutations in the region targeted by the TLE1 shRNA. Lastly, the gefitinib-resistant A549 (A549GR) cell line was established using a previous protocol (10), wherein the parental A549 cells were continuously exposed to a concentration of 20 μM gefitinib.

Chemical reagents, antibodies and plasmids. The Cell Fractionation Kit (cat. no. ab109719) was purchased from Abcam. The anti-COX IV (1:1,000; cat. no. 4850T) was purchased from Cell Signaling Technology, Inc. The mouse monoclonal anti-myc (1:1,000; cat. no. MAI-980) was from Invitrogen; Thermo Fisher Scientific, Inc. The anti-E-cadherin (1:1,000; cat. no. 610181) and anti-vimentin (1:1,000; cat. no. 550513) were acquired from BD Biosciences while anti-GFP (1:500; cat. no. sc-53882), anti-β-actin (1:1,000; cat. no. sc-81178), anti-GAPDH (1:1,000; cat. no. sc-47724) and anti-TLE1(1:200; cat. no. sc-137098) antibodies were obtained from Santa Cruz Biotechnology, Inc. The anti-AES antibody (1:1,000; cat. no. PA5-121149) was purchased from Thermo Fisher Scientific, Inc. The EGFR-TKI gefitinib and z-VAD-fmk were purchased from Selleck Chemicals. The Bit1-myc tagged construct which encodes for the mitochondrial localized Bit1 protein was generated as previously described (8,9). The GFP-TLE1 and the full-length E-cadherin encoding plasmids were obtained from Origene Technologies, Inc.

Small interfering RNA (siRNA) and plasmid transfection. For acute knockdown studies, control non-targeting siRNA or pool of siRNAs specifically targeting TLE1 (Santa Cruz Biotechnology, Inc.) or AES (Invitrogen; Thermo Fisher Scientific, Inc.) were transfected into A549 cells ($2x10^5$)

using the Lipofectamine RNAiMAX transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated at 37˚C in a humidified incubator with 5% CO_2 for 24 h followed by subsequent experimentation (8,9). The TLE1 siRNA pool consisted of 3 different siRNA duplexes: TLE1 siRNA1 sense, 5'-GGACCGGAUUAAAGAGGAATT-3' and antisense, 5'-UUCCUCUUUAAUCCGGUCCTT-3'; TLE1 siRNA2 sense, 5'-GGCACUAUGUGAUGUAUUATT-3' and antisense, 5'-UAAUACAUCACAUAGUGCCTT-3'; and TLE1 siRNA3 sense, 5'-GAAGGCUACAGUCUAUGAATT-3' and antisense, 5'-UUCAUAGACUGUAGCCUUCTT-3'. The AES siRNA pool consisted of two different siRNA duplexes: AES siRNA1 sense, 5'-CAAAGACGAAUUUCAGCUATT-3' and antisense, 5'-GAACAUCGAGAUGCACAAATT-3'; and AES siRNA2 sense, 5'-GAACAUCGAGAUGCACAAATT-3' and antisense, 5'-UUUGUGCAUCUCGAUGUUCAA-3'. For siRNA experiments, the negative control siRNA with no homology to any known human genes is sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. For plasmid DNA constructs, transient transfection assays were conducted using lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for A549 cells in OPTI-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) as prescribed by the manufacturer with the total amount of plasmid used normalized with the corresponding empty vector construct.

Cell viability, apoptosis and migration assays. Cells were treated with various concentrations of gefitinib (0-5 μM) or osimertinib (0-1 μm) for 48 h and subjected to the metabolic activity-based Alamar Blue assay to assess cell viability as previously described (8,9). Briefly, the number of metabolically active cells was measured using the PrestoBlue Cell Viability Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) with fluorescence reading at 485 nm excitation wavelength and 520 nm emission wavelength in a microplate reader. In parallel, cells treated with various concentrations of gefitinib (0-5 μM) or osimertinib (0-1 μm) for 48 h were subjected to Cell Death Apoptosis ELISA (cat. no. 11774425001; Roche Molecular Diagnostics) to quantify the amount of DNA histone fragments (8,9). The migratory ability of cells was quantified with the use of a Boyden chamber cell migration assay as previously described (8). Briefly, cells ($3x10^4$) were added to the upper chamber of 24-well plates (BD Falcon) that contained cell culture inserts (8.0-μm pores); and 10% FBS was added to the lower chamber to serve as a chemoattractant. After 18 h, cells that migrated through the membrane and attached on the underside of the membrane were stained with 0.1% crystal violet at room temperature for 1 h and counted using a light microscope.

Protein preparation, western blotting and subcellular fractionation assays. Protein preparation and western blotting were performed as previously described (8,9). Protein lysate was prepared using the Mem-PER Plus eukaryotic membrane protein extraction reagent kit (Thermo Fisher Scientific, Inc.). For western blot analysis, equal amounts of proteins (35 μg) were resolved on 4-20% gradient Tris-glycine gels (Invitrogen; Thermo Fisher Scientific, Inc.) and electrophoretically transferred to nitrocellulose membrane. The membranes were then incubated with primary antibodies overnight at 4˚C, followed by incubation with appropriate secondary antibodies [Amersham ECL Rabbit IgG, HRP-linked whole Ab (1:20,000; cat. no. NA934V; Cytiva); Amersham ECL Mouse IgG, HRP-linked whole Ab (1:25,000; cat. no. NA931V; Cytiva)]. Visualization of protein bands on the membranes was performed using the ECL detection system (cat. no. RPN2232; Cytiva), and band intensities were quantified by densitometric analysis using ImageJ software (National Institutes of Health). Preparation of the mitochondrial, cytoplasmic and nuclear containing fractions was conducted using the Cell Fractionation Kit (Abcam). The protein concentration in different fractions was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc.) with BSA (cat. no. 23208; Thermo Fisher Scientific, Inc.) as the standard.

Total RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from $5x10^6$ cultured cells using the Qiagen RNeasy miniprep kit (Qiagen Sciences, Inc.) as prescribed by the manufacturer and the quantified by spectrophotometry (NanoDrop 8000; Thermo Fisher Scientific, Inc.). Total RNA was subjected to a one-step real-time RT-qPCR using the iTaq Universal SYBR (Bio-Rad Laboratories, Inc.) by RT-qPCR on the BIO-RAD CFX96 Touch Real-Time PCR Detection System utilizing the following primers: human E-cadherin forward, 5'-AGGCTAGAGGGTCACCGCGTC-3' and reverse, 5'-GCTTTGCAGTTCCGACGCCAC-3'; and TLE1 forward, 5'-CCTCCTACACAGCAGCAGTT-3' and reverse, 5'-TCTGCATCGTGGTGCTTCTT-3'. In parallel, human GAPDH forward, 5'-CCCACTCCTCCACCTTTGAC-3' and reverse, 5'-TTGCTGTAGCCAAATTCGTTGT-3' were used as control. The thermocycling conditions were as follows: reverse transcription reaction was 10 min at 50˚C, polymerase activation and DNA denaturation was 1 min at 95˚C, and then amplification; denaturation for 10 sec at 95˚C, annealing/extension for 30 sec at 60˚C, and run for 40 cycles. The melt-curve analysis was following 65-95˚C (0.5˚C increment 5 sec/step). The relative levels of mRNAs were analyzed using the ΔΔCq method (11).

Bioinformatics analysis. The Cancer Genome Atlas TCGA database (https://portal.gdc.cancer.gov/) was analysed to compare TLE1 mRNA level in LUAD and lung squamous cell carcinoma (LUSC) vs. normal lung tissues. Kaplan-Meier survival plots were generated using the R Package survival (https://cran.r-project.org/web/packages/survival/) to assess the prognostic significance of TLE1 expression. To examine TLE1 expression in patients with EGFR-TKI resistant and sensitive LUAD, the Gene Expression Omnibus (GEO) Datasets (http://www.ncbi.nlm.nih.gov/geo/gds), hosted by the National Center for Biotechnology Information (NCBI), was employed as the database for dataset retrieval. The search query used was '[(LUAD) AND (EGFR-TKI resistance) OR (gefitinib resistance) OR (osimertinib resistance)]', with additional filters set for the organism as 'Homo sapiens' and entry type as either 'DataSets' or 'Series'. The resulting datasets were then screened to include only samples derived directly from patients, excluding those from cell lines. Ultimately, dataset GSE231938 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE231938) was selected, comprising samples from one EGFR-TKI-sensitive patient and two EGFR-TKI-resistant

patients (12). The levels of TLE1 mRNA in these samples were subsequently analyzed using Geo2R, with expression levels reported in Transcripts Per Million. To assess possible enrichment of TF binding motif in the TLE1 promoter region, the EPD Eukaryotic promoter database was utilized as a source of the human TLE1 promoter sequence (13). The TLE1 promoter region (-1,000 to 100 base pair (bp) relative to transcription start site (TSS) was scanned with a cut-off P-value of 0.001.

Statistical analysis. Data are presented as the mean ± standard deviation (SD) of at least three independent experiments. All calculations were performed using the NCSS statistical software (NCSS, LLC). Statistical analyses were performed using two-tailed Student's t test for experiments with two groups and one-way ANOVA with Tukey's post hoc test for comparisons among multiple groups. $^{*}P<0.05$ was considered to indicate a statistically significant difference.

Results

TLE1 expression is upregulated and functions as a poor prognosis factor in patients with LUAD. To determine clinical significance of TLE1 in NSCLC, TLE1 mRNA level in LUAD and LUSC (two major NSCLC subtypes) was examined using the TCGA database. As shown in Fig. 1A, TLE1 expression was elevated in both LUAD and LUSC as compared with normal counterparts. Importantly, high TLE1 expression strongly associated with shorter overall survival (OS) and disease-free survival in patients with LUAD (Fig. 1B). As shown in Fig. 1C, high TLE1 expression failed to correlate with LUSC patient survival rates. Due to crossing over of the OS curves in Fig. 1C, the period of analysis was restricted to exclude this late-stage crossover event. Reanalysis failed to show statistically significant difference in the survival rate between the high TLE1 expressing and low TLE1 expressing LUSC groups (Fig. S1B). These data are consistent with TLE1 corepressor functioning as a molecular determinant of LUAD aggressiveness and further indicate that TLE1 expression may serve as a poor prognosis factor in patients with LUAD.

To assess the potential role of TLE1 in EGFR-TKI resistance, one available GEO dataset GSE231938 was also examined and the level of TLE1 mRNA was compared between EGFR-TKI sensitive and resistant LUAD tumor samples. The two patients exhibiting resistance to EGFR-TKI therapy demonstrated elevated levels of TLE1 compared with the patient who is sensitive to EGFR-TKI treatment (Fig. S1A). However, due to the limited sample size, statistical significance could not be determined.

TLE1 modulates the sensitivity of A549 cells to EGFR-TKI gefitinib. In addition to tumor invasiveness, chemoresistance is another hallmark of cancer aggressiveness. To address the possibility that TLE1 may regulate drug resistance, the impact of TLE1 expression on molecular targeting therapy against EGFR was examined in LUAD. Since EMT is a known mechanism for EGFR-TKI resistance in the human LUAD A459 cells (14) which were previously shown to develop EMT phenotype upon exogenous TLE1 expression (8,9), stable exogenous GFP-TLE1-expressing and control GFP A549 clonal pool of cells (Fig. 2A) were treated with the EGFR-TKI gefitinib and their proliferation (Fig. 2B) and basal apoptosis (Fig. 2C) were assessed. Treatment of control GFP A549 cells with gefitinib resulted in proliferation inhibition and apoptosis induction, indicating sensitivity of A549 cells to gefitinib as previously reported (10,15). As compared with control GFP cells, the GFP-TLE1 A549 cells showed enhanced proliferation and reduced apoptosis following gefitinib treatment, signifying that exogenous TLE1 expression conferred gefitinib resistance in A549 cells.

To confirm the specificity of ectopic TLE1 effects on the antitumor activity of gefitinib, endogenous TLE1 expression in A549 cells, which exhibit moderate levels of TLE1, was downregulated via the shRNA technology (Fig. 2D). Following gefitinib treatment, TLE1 shRNA cells exhibited greater proliferation inhibition and apoptosis as compared with control shRNA cells (Fig. 2E and F). The enhanced gefitinib sensitivity of TLE1 shRNA cells was lost upon restoration of TLE1 expression with a TLE1 plasmid containing silent mutations in the shRNA target sequence (TLE1m) (Fig. 2D-F). Collectively, these findings indicated that TLE1 expression attenuates the proliferation-inhibitory and apoptosis-inducing effects of gefitinib in the A549 cell line, and its upregulation may contribute to gefitinib resistance in these cells. Consistent with these results, TLE1 expression in A549 cells also conferred protection against the proliferation-inhibitory and apoptotic effects of the third generation EGFR-TKI osimertinib (Fig. S2A-D) and attenuated the antitumor effect of gefitinib in the EGFR-TKI sensitive, EGFR-mutant LUAD HCC827 cell line (Fig. S3A-C).

TLE1 enhances gefitinib resistance in A549 cells through E-cadherin repression. As a transcriptional corepressor, TLE1 promotes EMT in LUAD cells via epigenetic silencing of E-cadherin expression (8,9) (Fig. S3A). Since E-cadherin expression correlates with gefitinib sensitivity in lung cancer (16), it was investigated if TLE1 confers resistance to gefitinib through transcriptional silencing of E-cadherin. At first, the levels of E-cadherin expression in A549 cells in the presence or absence of gefitinib were examined. As demonstrated in Fig. 3A, gefitinib treatment upregulated E-cadherin (a) protein and (b) mRNA expression levels. The E-cadherin upregulation by gefitinib treatment coincided with a significant re-localization of the nuclear TLE1 protein to the cytoplasm as evidenced by subcellular fractionation and western blotting assays (Fig. 3B), consistent with the notion that the observed gefitinib-induced E-cadherin expression is associated with inhibition of TLE1 nuclear function. Importantly, exogenous TLE1 expression significantly inhibited the upregulation of E-cadherin by gefitinib (Fig. 3Ca and Cb). These findings indicated that TLE1 serves as a downstream target of gefitinib, and its upregulation antagonizes the gefitinib-induced E-cadherin expression in A549 cells.

The aforementioned findings raise the possibility that TLE1 may confer EGFR-TKI resistance through silencing of E-cadherin. To test directly whether the TLE1-mediated gefitinib resistance can be attributed to regulation of E-cadherin, forced expression of E-cadherin in TLE1 expressing A549 cells was performed followed by gefitinib treatment. The upregulation of E-cadherin expression in GFP-TLE1 A549 cells was confirmed by immunoblotting (Fig. 3Da) and

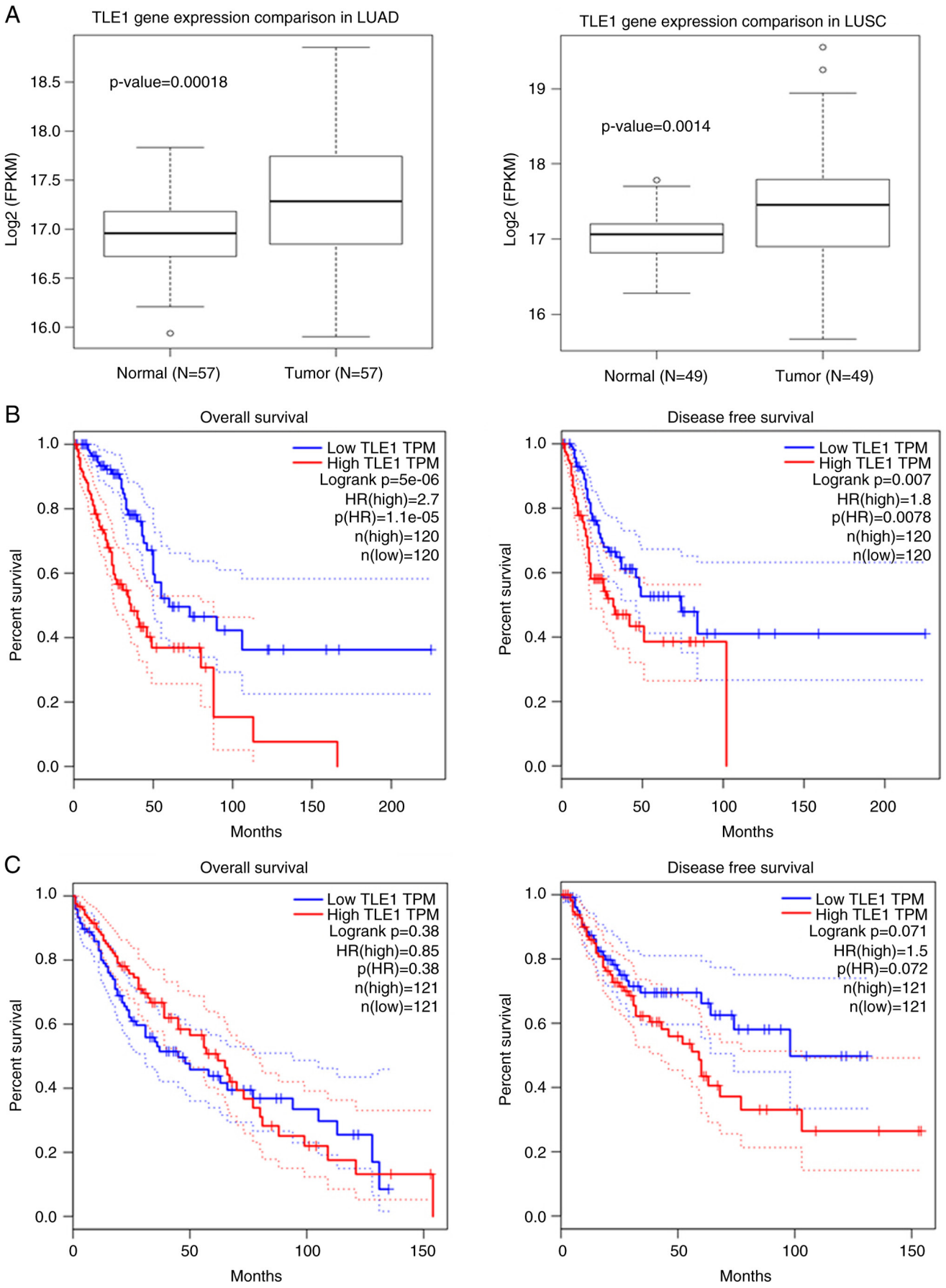

Figure 1. TLE1 expression correlates with poor prognosis in patients with LUAD. (A) Comparison of TLE1 mRNA expression in LUAD and LUSC vs. normal lung tissue (TCGA data, P-values obtained using Student's t-test). (B and C) Kaplan-Meier survival analysis of overall survival and disease-free survival for high and low expression levels of TLE1 in patients with (B) LUAD and (C) LUSC. TLE1, transducin-like enhancer of split 1; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma

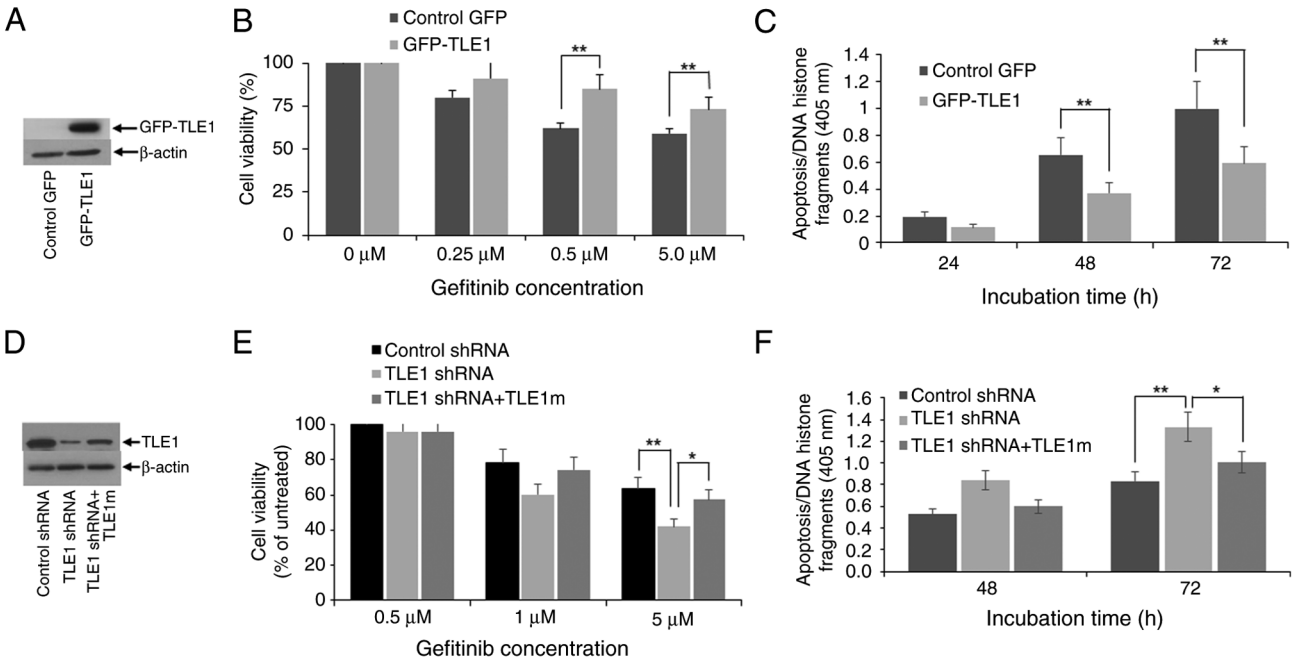

Figure 2. TLE1 regulates the sensitivity of A549 cells to gefitinib. (A and B) Control GFP and GFP-TLE1 pool of A549 cells were subjected to (A) western blotting against specific antibodies to GFP and B-actin and treated with the indicated concentration of gefitinib (mmol/l) for 48 h followed by (B) Alamar blue assay. Data are expressed as a percentage of the value for untreated cells. (C) The control GFP and GFP-TLE1 cells were treated with 10 mmol/l gefitinib at the indicated times followed cell death ELISA apoptosis assay. (D-F) The A549 derived control shRNA, TLE1 shRNA, and TLE1 shRNA cells transfected with a TLE1 plasmid containing silent mutations in the shRNA target sequence were subjected to (D) western blotting with the indicated antibodies, (E) Alamar blue assay 48 h post-treatment with indicated concentration of gefitinib, and (F) cell death ELISA apoptosis assay at the indicated times following treatment with 10 mmol/l gefitinib. The results are representative of three independent experiments. *P<0.05 and **P<0.01 [(B and C) Student's t-test and (E and F) one-way ANOVA with post hoc Tukey's test]. Error bars indicate SD. TLE1, transducin-like enhancer of split 1; shRNA, short hairpin RNA.

RT-qPCR (Fig. 3Db) assays. As shown in Fig. 3Dc, ectopic E-cadherin expression significantly increased the level of gefitinib-induced apoptosis in exogenous TLE1 expressing A549 cells, indicating partial restoration of their gefitinib sensitivity. Consistent with this finding, forced upregulation of E-cadherin in exogenous TLE1 expressing HCC827 cell line which exhibited decreased E-cadherin expression similarly attenuated the TLE1-mediated gefitinib resistance (Fig. S4A and B). These data indicated that E-cadherin repression in part underlies the TLE1-mediated resistance to gefitinib in lung cancer cells.

Increased TLE1 expression in gefitinib-resistant A549 (A549GR) cells confers EMT features and gefitinib resistance. To explore the role of TLE1 in acquired EGFR-TKI resistance in lung cancer cells, gefitinib-resistant A549 cells (A549GR) were established from the parental A549 cell line through a continuous low dose exposure to gefitinib. In line with a previous study (10), the A549GR cells exhibited EMT features such as acquisition of increased cell size, flattened phenotype (Fig. 4Aa), and enhanced cell migration capacity (Fig. 4Ab and Ac) as compared with parental A549 cells. Molecular changes associated with EMT were also observed in A549GR cells including decreased E-cadherin and increased vimentin expression (Fig. 4Ad). Importantly, the A549GR cells displayed reduced sensitivity to gefitinib-induced apoptosis relative to parental A549 cells (Fig. 4B).

To investigate the role of TLE1 in acquired gefitinib resistance of A549 cells, it was first examined if TLE1 expression is altered between A549 and A549 GR cells. Both RT-qPCR (Fig. 4Ca) and western blotting (Fig. 4Ac) assays demonstrated induction of TLE1 expression at both the mRNA and protein levels, respectively. To gain mechanistic insights on the observed upregulation of TLE1 in gefitinib resistant cells, the TLE1 promoter region (-1,000 to 100 base pair (bp) relative to TSS, cut-off P=0.001) was examined for TF binding motifs using the EPD eukaryotic promoter database (13), with emphasis on TFs that are associated with EGFR-TKI resistance in human lung cancer cells. It was found that the TLE1 promoter region is enriched for binding motif for transcription factors STAT3 and ZNF263. The transcriptional activator STAT3 has been shown to be activated upon acquisition of EGFR-TKI resistance (17,18), while loss of expression of the transcriptional repressor ZNF263 has been observed in EGFR-TKI resistant LUAD cells (19). Thus, the activation of STAT3 and/or downregulation of ZNF263 may underlie the observed transcriptional upregulation of TLE1 expression in gefitinib-resistant A549GR cells.

TLE1 expression was then downregulated in these cells via a previously validated pool of TLE1-specific or control siRNAs (8,9) (Fig. S5), followed by gefitinib treatment and apoptosis assay. As demonstrated in Fig. 4Cb, acute ablation of TLE1 expression in A549GR cells partially restored their sensitivity to gefitinib-induced apoptosis. Concurrent with the attenuation of gefitinib resistance, loss of TLE1 expression in A549GR cells also resulted in induced expression of the epithelial marker E-cadherin (Fig. 4Cc) with concomitant inhibition of cell migration (Fig. 4Cd and Ce), indicating that increased TLE1 promotes EMT in A549GR cells.

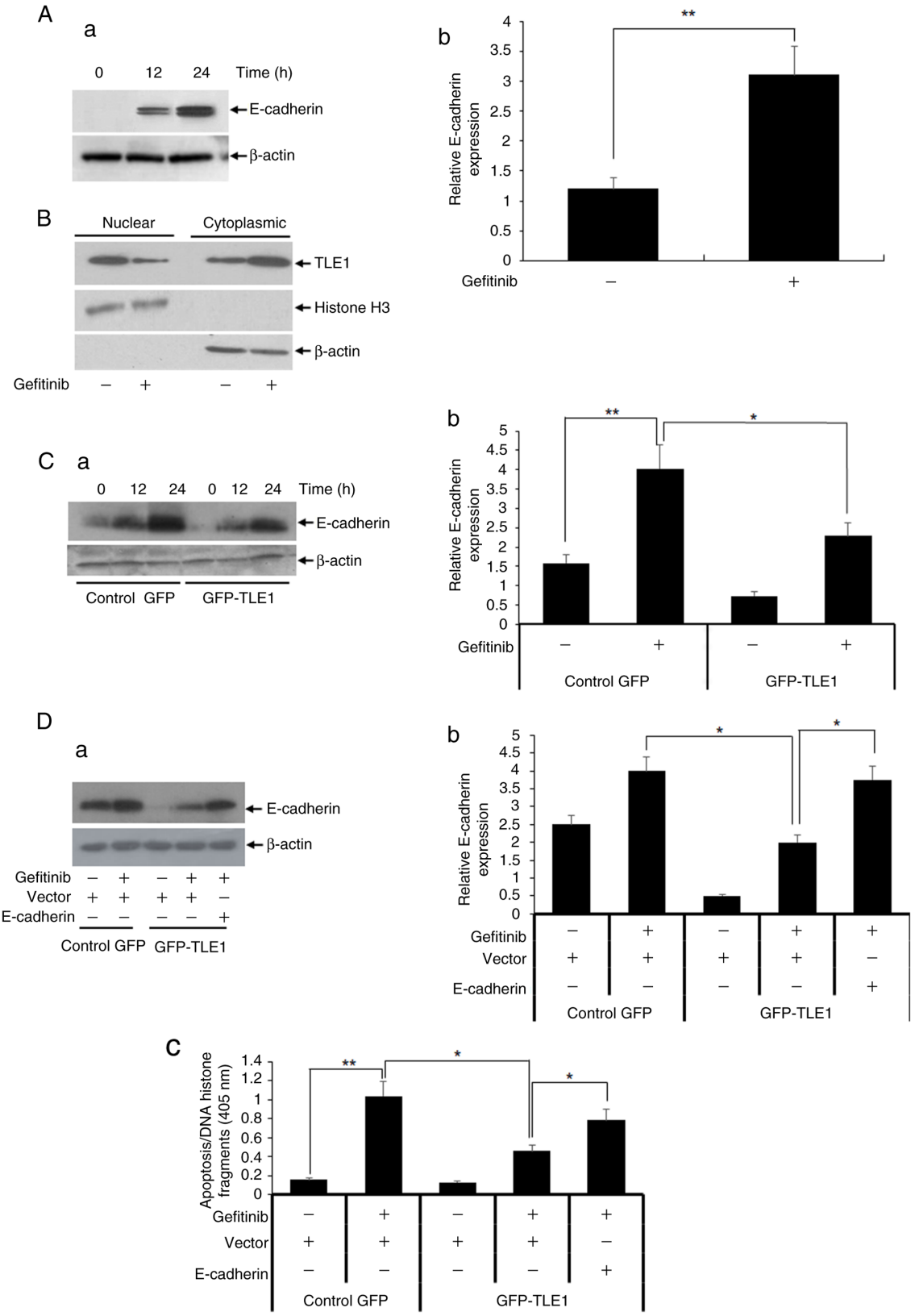

Figure 3. TLE1 promotes gefitinib resistance via silencing of E-cadherin expression. (A) A549 cells were treated with 10 mmol/l gefitinib at the indicated times, and then cells were harvested and subjected to (Aa) western blotting with the indicated antibodies and (Ab) RT-qPCR analysis using specific E-cadherin primers. (B) A549 cells were cultured in the presence or absence of 10 mmol/l gefitinib for 24 h and then subjected to cell fractionation assay. The resulting nuclear and cytoplasmic fractions were analysed by western blotting with the indicated antibodies. (C) Stable control GFP and GFP-TLE1 A549 cells treated with 10 mmol/l gefitinib at the indicated times were subjected to (Ca) western blotting and (Cb) RT-qPCR analysis to assess for E-cadherin protein and mRNA levels, respectively. (D) GFP-TLE1 A549 cells transfected with the vector or E-cadherin expressing construct were subjected to (Da) western blotting with the indicated antibodies and (Db) RT-qPCR analysis using specific E-cadherin primers or further cultured in the presence or absence of 10 mmol/l gefitinib for 48 h followed (Dc) cell death ELISA apoptosis assay. The results are representative of three independent experiments. *P<0.05 and **P<0.01 [Student's t test (Ab) and one-way ANOVA with post hoc Tukey's test (Cb, Db and Dc)]. Error bars indicate SD. TLE1, transducin-like enhancer of split 1; RT-qPCR, reverse transcription-quantitative PCR.

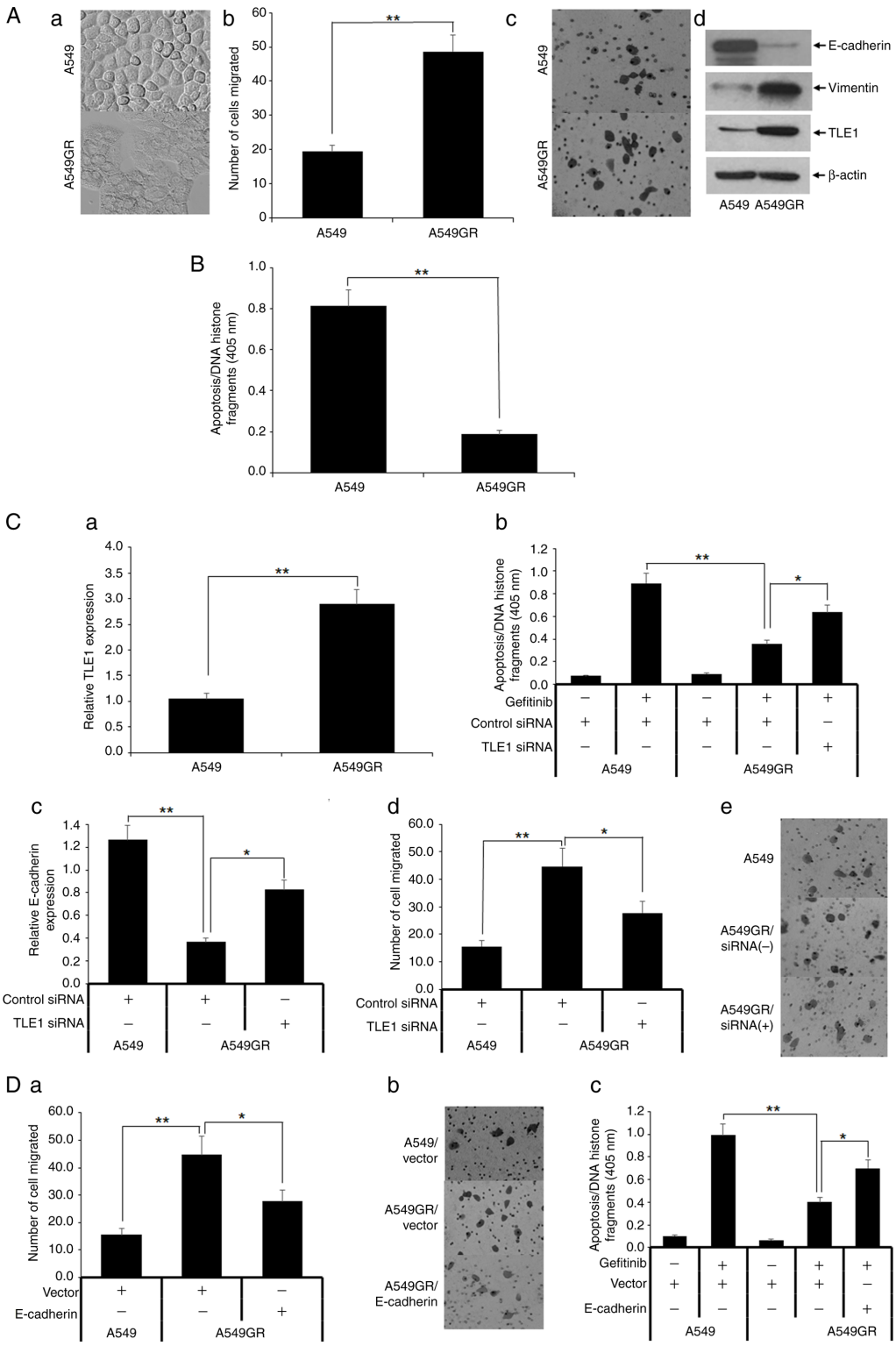

Figure 4. Gefitinib-resistant A549 (A549GR) cells display EMT and increased TLE1 expression, and knockdown of TLE1 attenuates the EMT phenotype and gefitinib resistance of A549GR cells. (A) The parental A549 and gefitinib-resistant A549GR cells were subjected to (Aa) phase contrast light microscopy to assess their morphology, (Ab and Ac) Borden chamber assay to evaluate migration potential, and (Ad) western blotting to measure protein expression of different EMT markers including TLE1 with specific antibodies. (B) A549 and A549GR cells were cultured in the presence or absence of 10 mmol/l gefitinib for 48 h followed by cell death ELISA apoptosis assay. (C) A549 and A549GR cells were subjected to (Ca) RT-qPCR analysis to measure TLE1 mRNA expression level; (Cb) A549GR cells transfected with a pool of TLE1 specific, or control siRNAs were cultured in the presence of absence of 10 mmol/l gefitinib for 48 h followed by cell death ELISA apoptosis assay. The control siRNA or TLE1 siRNA transfected A549GR cells were also subjected to (Cc) RT-qPCR analysis to assess E-cadherin mRNA level and (Cd and Ce) Boyden chamber migration assay. (D) A549GR cells transfected with a E-cadherin expressing or vector construct were subjected to (Da and Db) a Boyden chamber migration or cultured in the presence of absence of 10 mmol/l gefitinib for 48 h followed by (Dc) cell death ELISA apoptosis assay. In Fig. 4Ab, Ac, Cd, Ce, Da and Dc, cells were added to the upper compartment of the Boyden chamber, and after 12 h, cells attached on the underside of the membrane were stained with 0.1% crystal violet, counted (4Ab, Cd and Da), and images were captured (4Ac, Ce, and Db). In the aforementioned experiments, the results are representative of three independent experiments. *P<0.05 and **P<0.01 [Student's t test (Ca) and one-way ANOVA with post hoc Tukey's test (Cb, Cc, Cd, Da and Dc)]. Error bars indicate SD. EMT, epithelial-mesenchymal transition; TLE1, transducin-like enhancer of split 1; RT-qPCR, reverse transcription-quantitative PCR; siRNA, small interfering RNA.

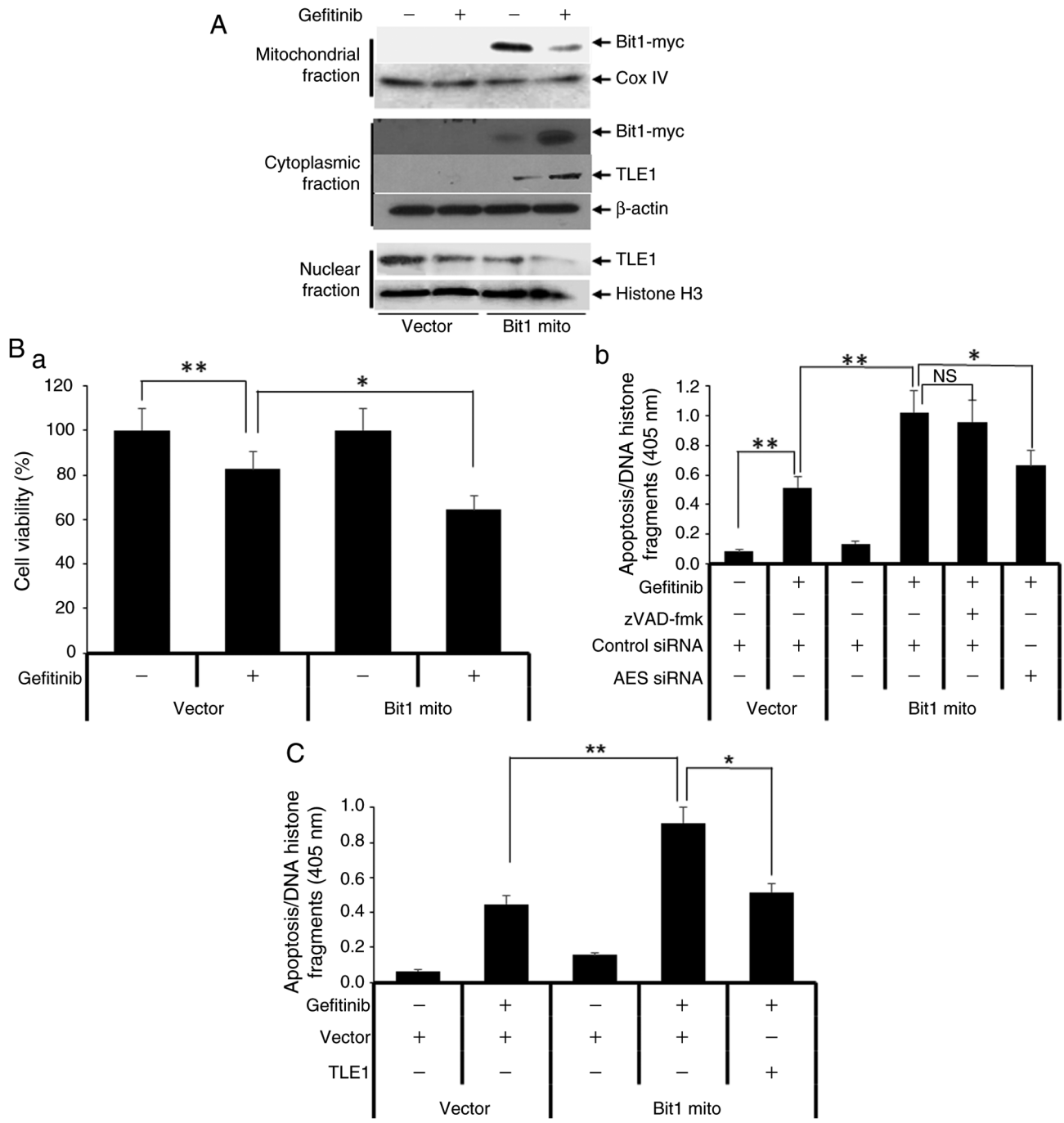

Figure 5. Cell death effector Bit1 enhances gefitinib-induced apoptosis in A549 cells by targeting nuclear TLE1 protein to the cytoplasm. (A) A549 cells transfected with a C-terminally myc-tagged mitochondrial Bit1 expressing or vector construct were cultured in the presence or absence of 10 μmol/l gefitinib for 16 h followed by Cell Fractionation assay. The resulting mitochondrial, nuclear, and cytoplasmic fractions were subjected to western blotting with the indicated antibodies. (B) A549 cells transfected with a mitochondrial Bit1 expressing or vector construct were cultured in the presence of absence of 10 mmol/l gefitinib for 48 h followed by (Ba) Alamar blue or (Bb) cell death ELISA apoptosis assays. In (Bb), mitochondrial Bit1 expressing cells were pretreated with or without 20 mmol/l Z-VAD-fmk or transfected with a pool of AES specific or control siRNAs followed by gefitinib treatment and cell death Elisa apoptosis assay. (C) The mitochondrial Bit1 expressing A549 cells were transfected with TLE1 expressing or vector construct, and 24 h post-transfection cells were cultured in the presence or absence of 10 mmol/l gefitinib for 48 h followed by cell death Elisa apoptosis assay. The results are representative of three independent experiments. *P<0.05 and **P<0.01 [one-way ANOVA with post hoc Tukey's test (Ba, Bb, C)]. Error bars indicate SD. Bit1, Bcl-2-inhibitor of transcription 1; TLE1, transducin-like enhancer of split 1; AES, Amino Enhancer Split; siRNA, small interfering RNA; ns, not significant.

Since EMT is a known determinant of acquired EGFR-TKI resistance in lung cancer cells (14,20) and considering the present findings that E-cadherin repression, a hallmark of EMT, underlies the TLE1-mediated gefitinib resistance in A549 cells, a possibility remains that TLE1 may contribute to gefitinib resistance in A549GR cells via EMT. Hence, it was examined whether reversing induced EMT in A549GR cells by ectopic expression of the TLE1 target E-cadherin gene restores their sensitivity to gefitinib. Forced expression of E-cadherin in A549GR cells attenuated not only their increased motility (Fig. 4Da and Db) but also gefitinib resistance (Fig. 4Dc). Taken together, these results indicated

that the TLE1-E-cadherin transcriptional axis plays a role in acquired gefitinib resistance of A549 cells.

The cell death effector Bit1 potentiates gefitinib-induced apoptosis by inhibiting the TLE1 nuclear function in A549 cells. To induce cell death or apoptosis, the mitochondrial Bit1 protein is released to the cytoplasm and complexes with the transcriptional regulator Amino Enhancer Split (AES) protein to turn off the TLE1-mediated survival gene transcriptional program (21,22). While the mechanistic details underlying the Bit1 apoptosis function remain to be fully delineated, the formation of the pro-apoptotic Bit1-AES complex may channel pre-existing nuclear AES-TLE1 hetero-oligomers to the cytoplasm and lower nuclear TLE1 level, thus turning off the survival promoting TLE1 gene transcriptional program (21,22). To further address the role of TLE1 as an inhibitor of EGFR-TKI-mediated apoptosis and a determinant of EGFR-TKI resistance, it was investigated whether Bit1 can potentiate EGFR-TKI-mediated apoptosis in A549 cells by targeting the TLE1 nuclear function. To test this possibility, Bit1 expression in the mitochondria of A549 cells was targeted via transfection with a C-terminally myc-tagged Bit1 (Bit1 mito) or empty vector plasmid followed by treatment with or without gefitinib. In untreated conditions, the exogenous C-terminally myc tagged Bit1 protein is localized primarily in the mitochondria, and gefitinib treatment resulted in a significant shuttling of the Bit1 mito protein to the cytoplasm (Fig. 5A). The cytoplasmic relocalization of Bit1 following gefitinib treatment is associated with significantly greater gefitinib-mediated proliferation inhibition and apoptosis (Fig. 5B). Consistent with the role of Bit1 as a caspase-independent apoptotic effector (21), the Bit1 induction of gefitinib-mediated apoptosis in A549 cells is unresponsive to pan-caspase inhibitors pretreatment (Fig. 5Bb). Importantly, the observed potentiation of gefitinib-induced apoptosis in Bit1 transfected cells was significantly attenuated by knocking down the expression of AES (the Bit1 pro-apoptotic partner) with the use of a pool of AES-specific siRNA (Figs. 5Bb and S5), indicating specificity of Bit1 effect on gefitinib-induced apoptosis.

To test if TLE1 is a downstream target in the Bit1 regulation of gefitinib-mediated apoptosis, it was examined if Bit1 impinges on the nuclear localization of TLE1. As shown in Fig. 5A, the gefitinib-induced apoptosis in Bit1 expressing cells was associated with cytoplasmic translocation of nuclear TLE1, consistent with our previous findings that Bit1 triggers apoptosis by inhibiting TLE1 nuclear function (21,22). To directly examine the role of TLE1 as a downstream target of Bit1 regulation of gefitinib apoptosis, exogenous TLE1 was expressed in Bit1 overexpressing cells followed by gefitinib treatment and apoptosis assay. As revealed in Fig. 5C, forced expression of nuclear TLE1 significantly inhibited the Bit1 induction of gefitinib-mediated apoptosis in A549 cells. These collective data indicated that Bit1 augments gefitinib-induced apoptosis in part by inhibiting the pro-survival nuclear function of TLE1, thus highlighting TLE1 as a negative regulator of EGFR-TKI sensitivity.

Discussion

Previous studies have provided evidence in support of the TLE1 corepressor as an oncogenic driver of NSCLC through induction of anoikis resistance and EMT *in vitro* and tumorigenicity *in vivo* (7-9). In the present study, it was demonstrated that TLE1 expression is upregulated in both human LUAD and LUSC tumors but exerts poor prognostic function only in LUAD tumors. This finding is consistent with the *in vivo* transgenic mice data demonstrating that overexpression of Grg1 (mouse homologue of TLE1) resulted in lung tumors that resemble human LUAD (7). These findings highlight the lung specific oncogenic function of TLE1 in LUAD and underscore the different biological and genetics signatures of LUAD and LUSC.

In the present study, a novel TLE1 function in inhibiting gefitinib's antiproliferative and apoptotic effects in A549 cells was uncovered. Exhibiting moderate sensitivity to gefitinib, the EGFR wild-type A549 cell line serves as a model system to investigate molecular factors other than EGFR-TK mutations that regulate sensitivity of EGFR-TKIs. Importantly, such cells develop EGFR-TKI resistance via acquisition of EMT phenotype (10). To the best of our knowledge, this is the first study to implicate the TLE1 corepressor in drug sensitivity and resistance in human lung cancer. As illustrated in Fig. 6, a model is proposed by which inhibition of TLE1 nuclear function and upregulation of E-cadherin and other TLE1 target genes' expression contributes to EGFR-TKI-mediated apoptosis, and potentiation of TLE1/E-cadherin transcriptional silencing axis is a determinant of EGFR-TKI resistance.

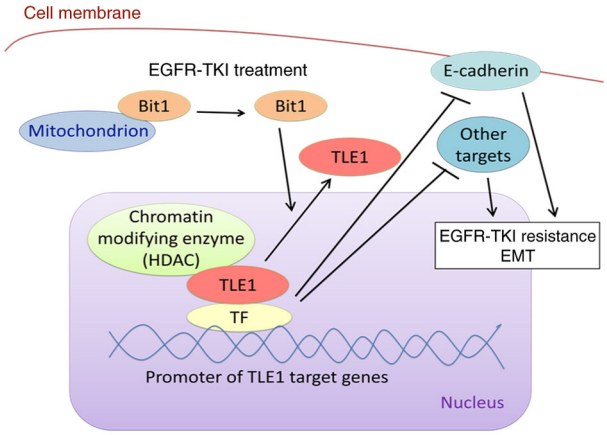

Figure 6. Working model illustrating how TLE1 may drive EGFR-TKI resistance in lung cancer cells via its transcriptional and epigenetic program. As a transcriptional corepressor, TLE1 in conjunction with a chromatin remodelling enzyme (such as HDAC) binds to a TF (such as Zeb1) and is recruited to its target gene promoters, repressing transcription of E-cadherin and other target genes. The silencing of E-cadherin and other target genes functions to block EGFR-TKI-induced apoptosis and drive EGFR-TKI resistance as well as EMT. To initiate apoptosis, EGFR-TKI induces cytoplasmic release of the cell death effector mitochondrial Bit1 protein followed into the cytoplasm to trigger translocation of nuclear TLE1 to the cytoplasm, thus inhibiting TLE1 transcriptional silencing function. The ability of cytoplasmic Bit1 to trigger TLE1 nuclear to cytoplasmic redistribution is in part dependent on the Groucho transcriptional regulator Amino Enhancer Split protein (21,22). The resulting upregulation of E-cadherin and other TLE1 target genes promotes EGFR-mediated cell death. In the development of acquired EGFR-TKI resistance, the observed induction TLE1 expression and activation of the TLE1 transcriptional pathway ensures complete blockade of the cell death and anti-EMT genetic program. TLE1, transducin-like enhancer of split 1; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; HDAC, histone deacetylase; TF, transcription factor; EMT, epithelial-mesenchymal transition; Bit1, Bcl-2-inhibitor of transcription 1.

A major antitumor effect of EGFR-TKIs is to induce apoptosis. To date, the mechanisms by which EGFR-TKIs trigger apoptosis are yet to be fully delineated. Detailed understanding of apoptotic pathways targeted by EGFR-TKIs is imperative to generate strategies to circumvent EGFR-TKI resistance, which develops in part due to lack of cell death induction following EGFR inhibition. In the last decade, while significant effort has been made to determine the cell death pathways and apoptotic effectors impacted by EGFR-TKIs, knowledge on the transcriptional and epigenetic machineries downstream of EGFR remains limited. In the present study, the findings indicated that the corepressor TLE1 serves a cellular target of gefitinib to trigger apoptosis and whose activation may contribute to EGFR-TKI resistance. First, gefitinib treatment induced cytoplasmic relocalization of nuclear TLE1 in part via activation of the cell death Bit1 pathway resulting in inhibition of its gene transcriptional silencing function. Second, forced expression of nuclear TLE1 was sufficient to attenuate the level of gefitinib-mediated cell death and downregulation of endogenous TLE1 potentiated gefitinib-induced apoptosis. Third, the development of acquired gefitinib resistance in A549 cells resulted in upregulation of TLE1 expression which functions to safeguard cells from EGFR-mediated apoptosis. As described in our model, TLE1 may drive lung cancer resistance to EGFR-TKI via suppression of a cell death and/or anti-EMT genetic program (Fig. 6).

The current data indicates that the transcriptional repression of E-cadherin contributes to TLE1-mediated attenuation of EGFR-TKI apoptosis. In addition to promoting cell-cell adhesion, the tumor suppressor E-cadherin may also induce apoptosis in several cellular models (16,23,24). Consistent with the present results, previous studies demonstrated that restoration of E-cadherin expression is sufficient to induce EGFR-TKI-mediated apoptosis and EGFR-TKI sensitivity in lung cancer cells (16,25). The exact mechanisms underlying the apoptosis function of E-cadherin however remain to be determined but may involve alteration of signaling pathways downstream of and regulated by E-cadherin. To this end, forced E-cadherin expression in breast carcinoma cells promoted etoposide-induced apoptosis via inhibition of the anti-apoptotic Bcl-2 expression (24). The authors are currently exploring which E-cadherin regulated pathways are involved in EGFR-TKI-mediated apoptosis and their potential regulation by TLE1. As a transcriptional corepressor that has ability to interact with several DNA-binding TFs and regulate distinct gene regulatory or signaling pathways, TLE1 corepressor likely regulates a survival promoting transcriptional program and not just a single gene (for example, E-cadherin). The authors' future direction is to identify novel TLE1 target genes by performing an integrated RNA-sequencing and ChIP-sequencing study and molecularly characterize their function in regulating EGFR-TKI apoptosis.

While our current data supports the hypothesis that the TLE1 antagonistic effect on EGFR-TKIs involves turning off the Bit1 apoptosis pathway, TLE1 may inhibit alternative cell death mechanisms such as the BIM pathway, a key effector of EGFR-TKI-mediated apoptosis. It is noteworthy that TLE1 has been shown to transcriptionally upregulate Bcl-2 expression (21). Thus, an exciting possibility exists that the TLE1-mediated induction of Bcl-2 may lead to sequestration and inactivation of BIM through direct Bcl-2-BIM interaction, thereby inhibiting BIM apoptosis function. Whether the activation of Bit1 cell death pathway impacts BIM dependent apoptosis in LUAD cells in the context of EGFR-TKIs remains to be determined.

At this time, the possibility that TLE1-mediated gefitinib resistance maybe related to its ability to promote EMT cannot be excluded. Numerous studies have shown that acquisition of EMT is associated EGFR-TKI resistance in EGFR-mutant and wild-type NSCLC, and restoration of the epithelial marker E-cadherin expression and reversal of EMT potentiate sensitivity of lung cancer cells to EGFR-TKI (16,25). While the exact mechanisms of how EMT contributes to EGFR-TKI resistance remain to be defined, our data raises a possibility that the EMT-mediated EGFR-TKI resistance may channel through TLE1. Indeed, the gefitinib resistant A549 resistant cell line used in the present study exhibits high levels of TLE1 expression and pronounced EMT. While the presence of other gefitinib-resistant promoting mutations cannot be excluded, sole downregulation of TLE1 in these cells is sufficient to reverse EMT and attenuate gefitinib resistance, indicating that TLE1 may drive EGFR-TKI acquired resistance via EMT. Importantly, inhibiting the pro-EMT function of TLE1 via ectopic expression of E-cadherin was associated with increased gefitinib sensitivity. To further examine the role of TLE1 in EMT as a mechanism of EGFR-TKI resistance, it will be interesting to determine if TLE1 regulates acquired resistance to EGFR-TKIs driven by known EMT effectors such as the TF Zeb1 (26) and if TLE1 downregulation and/or inactivation is sufficient to restore EGFR-TKI sensitivity in these contexts. It is noteworthy that it has been previously shown that Zeb1 is a critical TF in mediating TLE1-induced silencing of E-cadherin expression and TLE1-mediated EMT in LUAD cells (9). In addition to Zeb1, the authors are exploring other TFs that are associated with LUAD EGFR-TKI resistance as potential mediators of TLE1 resistance. Via proteomics and genetic approaches, these TLE1-binding TFs will be identified and their role in TLE1 gene regulatory and EGFR-TKI resistance function will be characterized. It is noteworthy that HES1, a known TLE1 interacting TF, is a determinant of EGFR-TKI resistance in LUAD.

To generate mechanistic insights on the upregulation of TLE1 expression during the development of EGFR-TKI resistance, the promoter region of TLE1 was examined for binding sites for TFs that are associated with EGFR-TKI resistance in human lung cancer cells. Strikingly, using the EPD Eukaryotic promoter database, it was found that the TLE1 promoter region is enriched for TFs STAT3 and ZNF263 binding motif. While numerous data in literature demonstrate that the transcriptional activator STAT3 is activated upon acquisition of EGFR-TKI resistance (17,18), there are evidence supporting loss of expression of the transcriptional repressor ZNF263 in EGFR-TKI resistant LUAD cells (19). Thus, the activation of STAT3 and/or downregulation of ZNF263 may result in transcriptional upregulation of TLE1 expression. The authors are currently performing molecular genetic studies to determine which of these TFs contributes to the elevated TLE1 expression in EGFR-TKI resistant cells.

Detailed knowledge and understanding of the molecular components of the TLE1-mediated transcriptional and epigenetic machinery may yield new therapeutic strategies to overcome TLE1-mediated oncogenic and EGFR-TKI resistance. For example, inhibiting TLE1 nuclear function via small molecules and chemicals that target the individual components of the TLE1 transcriptional machinery would be a viable approach. As a transcription corepressor, the authors previously showed that TLE1 recruits histone deacetylase (HDAC) to the E-cadherin gene promoter to promote histone deacetylation and gene silencing. Thus, it will be of interest to investigate if HDAC inhibitors could alleviate the TLE1-mediated EGFR-TKI resistance.

In summary, it was demonstrated that the transcriptional corepressor TLE1 suppresses gefitinib-induced proliferation inhibition and apoptosis in part by silencing the E-cadherin expression. Consistent with its role as a determinant of EGFR-TKI resistance, TLE1 expression is upregulated in the experimentally derived gefitinib-resistant cell line and its downregulation partially restores gefitinib sensitivity. While the detailed mechanism by which TLE1 inhibits gefitinib-induced apoptosis is yet to be elucidated, our collective data indicate that TLE1 may serve as a negative predictive marker of EGFR-TKI sensitivity in LUAD with invasive EMT phenotype.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

XY, NR, PI, AC and MCDC performed molecular and cell-based experiments and analysed the data. RC and HB wrote the manuscript. RC and HB designed the experiments, wrote and edited the manuscript. XY, NR, PI, AC, RC, CDC and HB confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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