Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 25 (2014) 241–249

Journal of Nutritional Biochemistry

Down-regulation of epidermal growth factor receptor by curcumin-induced UBE1L in human bronchial epithelial cells☆

A-Pei Jiang[a], Dong-Hu Zhou[a], Xu-Lian Meng[a], Ai-Ping Zhang[b], Cui Zhang[b], Xiao-Ting Li[a], Qing Feng[a,*]

[a]Department of Nutrition and Food Safety, Key Laboratory of Toxicology, School of Public Health, Nanjing Medical University, Nanjing, Jiangsu 211166, China
[b]Department of Cardiothoracic Surgery, Nanjing First Hospital, Nanjing Medical University Affiliated Hospital, Nanjing, Jiangsu 210001, China

Received 6 March 2013; received in revised form 17 July 2013; accepted 1 November 2013

Abstract

UBE1L, ubiquitin-activating enzyme E1-like, is the activating enzyme of ISG15ylation (ISG15, interferon stimulated gene 15). Loss of UBE1L and activation of epidermal growth factor receptor (EGFR) signaling are common events in lung carcinogenesis. Curcumin, a well-studied chemopreventive agent, is known to down-regulate EGFR. The present study demonstrated that curcumin decreased EGFR expression in human bronchial epithelial (HBE) Beas-2B cells and lung cancer A549 cells. For the first time, UBE1L was found to be induced by curcumin in HBE cells. Interestingly, overexpression of UBE1L reduced EGFR at posttranslational level in HBE cells. UBE1L triggered EGFR membrane internalization and promoted complex formation between ISG15 and EGFR. Curcumin decreased EGFR downstream signaling pAKT and nuclear factor κB (NF-κB). Overexpression or knockdown of UBE1L also resulted in down-regulation or up-regulation of phosphoinositide 3-kinase/AKT/NF-κB correspondently. In human samples, there was an inverse relationship between UBE1L and EGFR/AKT/NF-κB in non-small cell lung cancer tissues and adjacent tissues. These results uncover a novel chemopreventive mechanism of curcumin in inducing UBE1L and down-regulating EGFR signaling in HBE cells.
Crown Copyright © 2014 Published by Elsevier Inc. All rights reserved.

Keywords: Curcumin; UBE1L; EGFR; Down-regulation

1. Introduction

Lung cancer is a highly aggressive malignant disease and is a leading cause of cancer death worldwide. Recent advances and insights into molecular pathogenesis of lung cancer have provided novel molecular targets, offering newer strategies and agents that are tumor specific. Epidermal growth factor receptor (EGFR) is one such target [1].

EGFR is a member of the ErbB family of receptor tyrosine kinases [2]. On binding of ligands such as epidermal growth factor (EGF), the receptors form homodimers or heterodimers with other members of the ErbB family of receptor tyrosine kinases such as ErbB2, ErbB3 and ErbB4 [3], resulting in autophosphorylation and in further activation of downstream signaling events, including phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR), Ras/Raf/Mitogen-activated protein kinase/ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) and Janus kinase/signal transducer and activator of transcription (JAKs/STATs), which trigger antiapoptosis, cell proliferation, angiogenesis and tumor invasion and metastasis [4]. Akt signaling, mainly associated with cell survival [5], is triggered by binding of the Src homology 2 (SH2) domain of PI3K to phosphorylated tyrosine of Her3, which is heterodimerized with EGFR [6]. PI3K/Akt activation is known to induce nuclear factor κB (NF-κB) activation [7]. The PI3K/AKT/NF-κB pathway plays an important role in lung cancer progression [8,9]. Given its roles in signal transduction and development of the malignant phenotype, the EGFR signaling pathway presents feasible targets for pharmacological intervention in lung cancer. Currently, monoclonal antibodies against EGFR (cetuximab, panitumumab and zalutumumab) and small molecule tyrosine kinase inhibitors (gefitinib and erlotinib) are being used for lung cancer treatment in patients [1].

The degradation of EGFR involves the binding of ligand to the EGFR, leading EGFR phosphorylation, and initiates EGFR internalization/degradation along the endolysosomal pathway [10,11]. Ubiquitination plays an important role in this process. Monoubiquitinin targets EGFR for endosomal degradation, whereas polyubiquitination targets EGFR for proteasomal degradation [12,13]. The process of EGFR down-regulation and degradation is the major negative feedback regulatory mechanism that controls the intensity and duration of receptor signaling [13].

UBE1L is E1-like ubiquitin-activating enzyme, which catalyzes ISG15ylation [14]. ISG15 [interferon (IFN)-stimulated gene 15 kDa] is the first reported ubiquitin-like protein [15]. UBE1L is located near a

☆ This work was supported by Science Foundation of Jiangsu Province (No. BK2009421), National Natural Science Foundation of China (No. 30972479), Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry (HG11-4302), Foundation from Graduate Student Scientific Research Innovation Project of Jiangsu Province Ordinary Universities and Foundation from Priority Academic Program Development of Jiangsu Higher Education Institutions.

* Corresponding author. Tel.: +86 25 86868455; fax: +86 25 86868499.
E-mail address: qingfeng@njmu.edu.cn (Q. Feng).

0955-2863/$ - see front matter. Crown Copyright © 2014 Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jnutbio.2013.11.001

chromosome 3 region, which is deleted in lung cancers [16]. Also, UBE1L was not detectable in several human lung cancer cell lines [17]. Overexpression of UBE1L in human bronchial epithelial (HBE) cells and lung cancer cells was showed to promote ISG15ylation of cyclin D1 and to decrease cyclin D1 overall protein levels, which was associated with suppression of cell growth [18]. Furthermore, retinoic acid was able to induce UBE1L and consequently led ISGylation of promyelocytic leukemia (PML)/retinoic acid (RA) receptor α (RARα) for degradation [19]. These findings indicated that UBE1L could be a tumor suppressor gene.

Curcumin, an orange-yellow component of the food flavor turmeric, is a plant polyphenol that has been known as a chemopreventive agent [20–22]. Recent studies indicated that curcumin inhibits cancer initiation and progression through regulation of multiple cellular pathways including EGFR [23], PI3K/AKT [24], NF-κB [25], mitogen activated protein kinase [26], and signal transducer and activator of transcription (STAT) pathways [27]. As research continues, more other molecular mechanisms of curcumin will be explored.

In the present study, we found that curcumin could down-regulate EGFR and its downstream signaling in HBE cells. Interestingly, curcumin was able to induce UBE1L expression in HBE cells. Whether curcumin-induced UBE1L participated in EGFR down-regulation was investigated. The relationship between EGFR and UBE1L was also uncovered.

2. Materials and methods

2.1. Cell culture and reagents

HBE cells (Beas-2B) and human lung carcinoma A549 cells were purchased from the Cell Centre of the Chinese Academy of Medical Sciences (Beijing, China). Beas-2B cells were cultured in LHC-9 media (Biofluids, Rockville, MD, USA). A549 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere. Curcumin and EGF were purchased from Sigma-Aldrich Ltd. (Poole, Dorset, UK).

2.2. Tissue specimens

Institutional approval was acquired from the ethical review board of Nanjing Medical University prior to this study. Non-small cell lung cancer (NSCLC) tissues at TNM stages of I to IIIa, along with matched adjacent tissues, were obtained from patients who received curative resection in the Nanjing First Hospital, a Nanjing Medical University-affiliated hospital. Tissues were grinded in liquid nitrogen and then added lysis buffer to extract protein for Western blot analysis.

2.3. Plasmids and transient transfection

The pSG5-UBE1L, hemagglutinin (HA)-EGFR, pcDNA3-histidine (His)-UBP43, His_6-tagged pcDNA3-ISG15 were kindly provided by Dr. Ethan Dmitrovsky (Dartmouth Medical School, Hanover, NH, USA). The EGFP-ubiquitin was provided by Dr. Jianwei Zhou (Nanjing Medical University, Nanjing, China). Transient transfection was carried out with Effectene transfection reagent (Qiagen, Hilden, Germany).

2.4. Knockdown of UBE1L

Cells were cultured in 35-mm culture plates with 2×10^5 cells per well and transfected with UBE1L interfering RNA containing three target-specific small interfering RNAs (siRNAs) (sc-106657; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or control siRNA (sc-37007) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.5. Immunoblot analysis

Cells were lysed with ice-cold lysis buffer (KeyGEN, Nanjing, China). Sixty micrograms of lysates were loaded on polyacrylamide gel before transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). A polyclonal antibody recognizing UBE1L was kindly provided by Dr. Ethan Dmitrovsky (Dartmouth Medical School). Other primary antibodies for immunoblot assays included anti-HA, anti-His, and anti-ISG15 (Abmart, Shanghai, China); anti-β-actin (BOSTER, Wuhan, China); and antiubiquitin, anti-EGFR (D38B1), anti-AKT, anti-p-AKT and anti-NF-κB (Cell Signaling Technology, Wilmington, DE, USA). Horseradish peroxidase-conjugated antirabbit or antimouse immunoglobulin G was used as the secondary antibody (BOSTER). The protein bands were detected using the Chemiluminesence Detection System (Cell Signaling Technology, Danvers, MA, USA).

2.6. Immunoprecipitation and pull-down analysis

After the cells were lysed, 1 mg of total protein was incubated with indicated antibody at 4°C for 2 h. Then 40 μl protein A/G plus-agarose beads (Santa Cruz

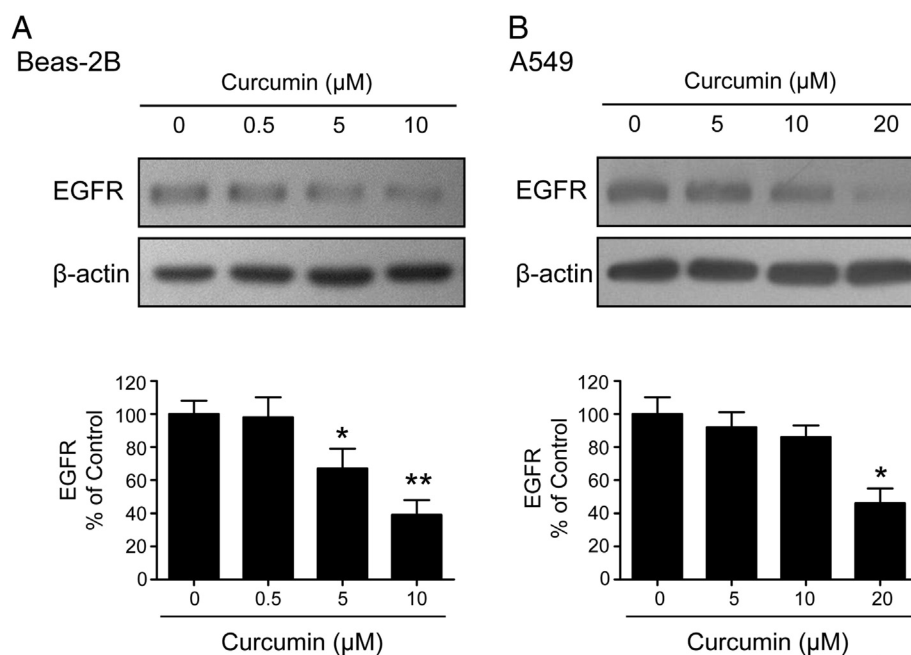


Fig. 1. Effect of curcumin on EGFR protein expression in HBE Beas-2B cells and NSCLC A549 cells. (A) Curcumin decreased EGFR expression in Beas-2B cells in a dose-dependent manner. Beas-2B cells were incubated in the absence or presence of curcumin (0.5–10 μM) for 24 h. EGFR protein expression was assessed by Western blot analysis. β-Actin expression served as a loading control. (B) Curcumin decreased EGFR expression in A549 cells in a dose-dependent manner. A549 cells were incubated in the absence or presence of curcumin (5–20 μM) for 24 h. Then, the cells were harvested and lysed for the detection expression of EGFR by Western blot analysis. β-Actin expression served as a loading control. Densitometry quantification of protein expression levels was shown as fold changes. Data were expressed as means±S.D. *P<.05, **P<.01 vs. control group.

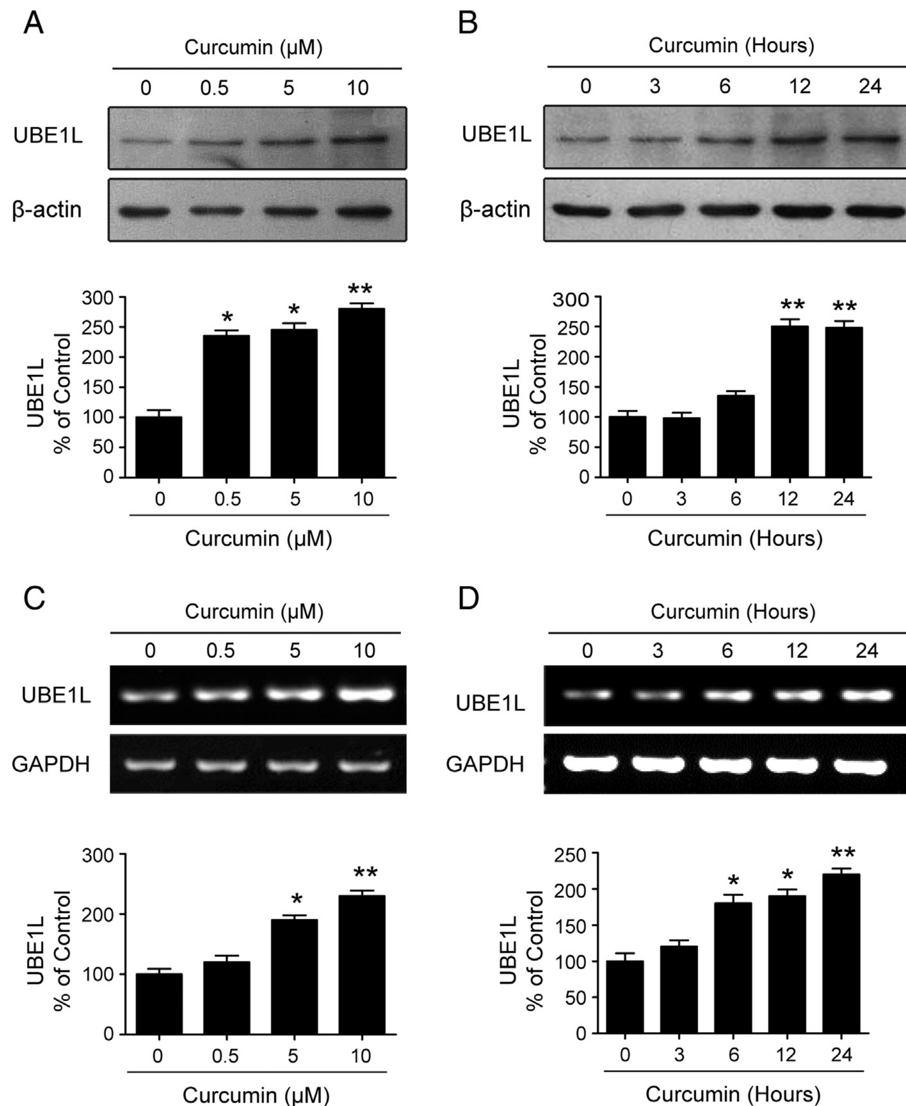


Fig. 2. Effect of curcumin on UBE1L protein expression in HBE Beas-2B cells. (A) Curcumin induced UBE1L protein expression in a dose-dependent manner. HBE cells were incubated in the absence or presence of curcumin (0.5–10 μM) for 24 h. β-Actin expression served as a loading control. (B) Curcumin induced UBE1L protein expression in a time-dependent manner. HBE cells were incubated with curcumin (10 μM) for 3, 6, 12 and 24 h. β-Actin expression served as a loading control. (C) Curcumin induced UBE1L mRNA expression in a dose-dependent manner. HBE cells were incubated in the absence or presence of curcumin (0.5–10 μM) for 24 h. GAPDH used as an internal control. (D) Curcumin induced UBE1L mRNA expression in a time-dependent manner. HBE cells were incubated with curcumin (10 μM) for 3, 6, 12 and 24 h. GAPDH used as an internal control. Densitometry quantification of protein expression levels was shown as fold changes. Data were expressed as means±S.D. *P<.05, **P<.01 vs. control group.

Biotechnology) were added overnight at 4°C. The immunoprecipitates were washed three times with the lysis buffer without phenylmethanesulfonyl fluoride (PMSF) and eluted with sodium dodecyl sulfate (SDS) loading buffer by boiling for 5 min. The immunoprecipitates were analyzed by Western blotting. For pull-down analysis, the cells were lysed by sonication and centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was added to 10 ml of nickel nitrilotriacetic acid resin (Ni-NTA; Invitrogen, Carlsbad, CA, USA). Proteins were separated by SDS/polyacrylamide gel electrophoresis using 10% gel.

2.7. Reverse transcription polymerase chain reaction

Total RNAs were isolated from cells using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Primers were synthesized by Invitrogen (Shanghai, China). The UBE1L forward primer was 5′-AGGTGGCCAAGAACTTGGTT-3′, and reverse primer was 5′-CACCACCTGGAAGTCCAACA-3′. The EGFR forward primer was 5′-GTGACCGTTTGG-GAGTTGATGA-3′, and reverse primer was 5′-GGCTGAGGGAGGCGTTCTC-3′. The GAPDH forward primer was 5′-CAAGGTCATCCATGACAACTTTG-3′, and reverse primer was 5′-GTCCACCACCCTGTTGCTGTAG-3′. Reverse transcription reaction was carried out by RevertAid Fist Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada). The polymerase was RevertAid Reverse Transcriptase. Polymerase chain reaction (PCR) was carried out at 95°C for 3 min, followed by 35 cycles for UBE1L (94°C, 1 min; 65°C, 1 min; 72°C, 3 min), 40 cycles for EGFR (95°C, 20 s; 54°C, 30 s; 72°C, 30 s), 20 cycles for GAPDH (95°C, 30 s; 56°C, 30 s; 72°C, 40 s) and a final elongation at 72°C for 10 min. PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining.

2.8. EGFR internalization analysis

Beas-2B cells were transfected with pSG5-UBE1L. The amount of EGFR presented at the cell surface was determined by labeling of the unpermeabilized and fixed cells with an anti-EGFR antibody directly conjugated to Alexa 488 (Beyotime, Haimen, China). EGFR surface labeling was analyzed by the flow cytometer (BD, San Jose, CA, USA).

2.9. Immunofluorescence staining

Beas-2B cells seeded in chamber slides were transfected with pSG5-UBE1L. The cells were rinsed in cold phosphate-buffered saline and fixed with 4% paraformaldehyde. After fixation, cells were blocked in phosphate buffered saline with tween-20 (PBST) containing 10% normal goat serum. Immunofluorescence labeling was performed using the combinations of anti-EGFR antibody and specific secondary antibody Alexa 488 (Beyotime). Microscopy was carried out using confocal microscope (Zeiss, Jena, Germany).

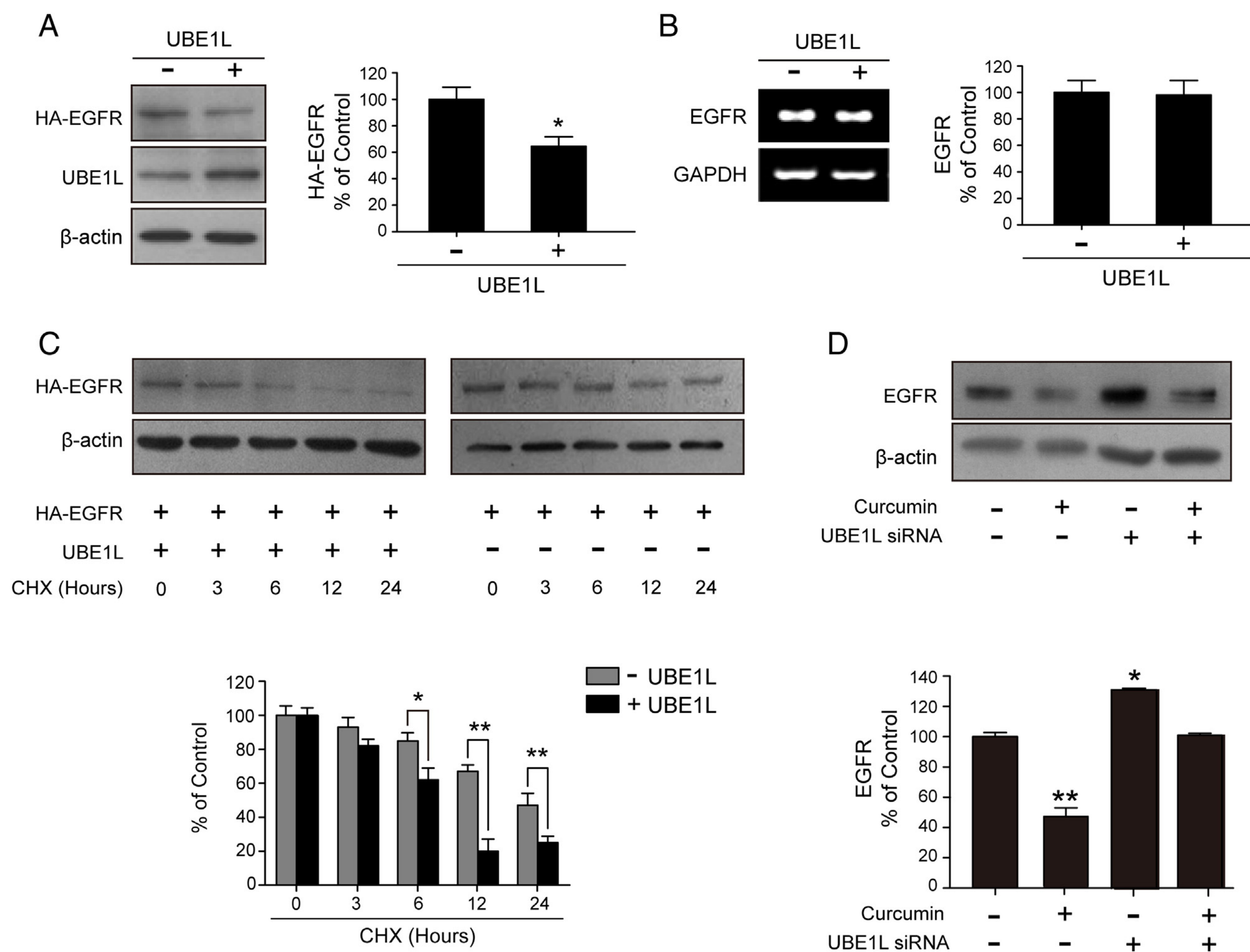


Fig. 3. Effects of UBE1L on EGFR in HBE cells. (A) UBE1L transfection decreased EGFR protein expression. After Beas-2B cells were transfected with UBE1L and HA-tagged EGFR for 24 h, the cells were harvested and lysed for the detection expression of HA by Western blot analysis. β-Actin expression served as a loading control. (B) UBE1L did not change EGFR mRNA expression. After Beas-2B cells were transfected with UBE1L for 24 h, total RNAs were prepared and subjected to reverse transcription PCR using primer for EGFR. GAPDH was used as an internal control. (C) UBE1L destabilized exogenous EGFR protein turnover. HBE cells were transfected with HA-tagged EGFR and with or without UBE1L in the presence or absence of CHX (40 μg/ml) for 24 h. EGFR protein was detected by an anti-HA antibody. β-Actin expression served as a loading control. (D) Curcumin reduced the increased EGFR level as knockdown of UBE1L. HBE cells were transfected with UBE1L siRNA, in the presence or absence of curcumin (5 μM) for 24 h. The cells were harvested and lysed for the detection expression of EGFR by Western blot analysis. β-Actin expression served as a loading control. Densitometry quantification of protein expression levels was shown as fold changes. Data were expressed as means±S.D. *P<.05 vs. control group.

2.10. Statistical analysis

Data were derived from three independent experiments. The parameters were expressed as mean±S.D. Statistical analysis was performed using analysis of variance with Newman–Keuls test. P values of <.05 and <.01 were considered significant. Immunoblots were quantified using ImageJ software [28] (National Institutes of Health).

3. Results

3.1. Curcumin inhibits expression of EGFR in HBE cells and NSCLC cells

Curcumin is known to down-regulate EGFR in several cancer cell lines such as human colon cancer cells, prostate cancer cells, NSCLC cells and bladder cancer cells [23,29–31]. In this study, the inhibitory effect of curcumin on EGFR expression was confirmed in HBE Beas-2B cells and NSCLC A549 cells. Cells were treated with curcumin at indicated concentrations for 24 h. Whole-cell extracts were prepared. Western blotting analyses demonstrated that curcumin significantly inhibited protein expression of EGFR in HBE Beas-2B and A549 cells, as shown in Fig. 1A and B.

3.2. Curcumin induces UBE1L expression in HBE cells

Previously, retinoic acid and type I IFN were found to induce UBE1L expression [19,32]. In the current study, we investigated whether cucumin could induce UBE1L in the cells. Beas-2B cells were treated with curcumin at doses from 0.5 to 10 μM for 24 h, and then the cell lysates were subjected to Western blot analysis to determine UBE1L protein level. As shown in Fig. 2A, curcumin induced UBE1L expression in a dose-dependent manner. When the cells were treated with curcumin at a dose of 10 μM for the indicated time, curcumin time dependently induced UBE1L protein expression (Fig. 2B). We further examined the induction of UBE1L messenger RNA (mRNA) expression by curcumin treatment. Total RNAs were isolated from the cells incubated with curcumin with the indicated doses for 24 h and subjected to reverse transcription PCR. UBE1L mRNA level was increased when the cells were treated with 0.5–10 μM curcumin, as shown in Fig. 2C. Also, curcumin induced UBE1L mRNA level in a time-dependent manner (Fig. 2D). This indicates that curcumin up-regulated UBE1L expression at the transcriptional level. However, A549 cells did not express UBE1L and curcumin failed to reactivation UBE1L (data not shown).

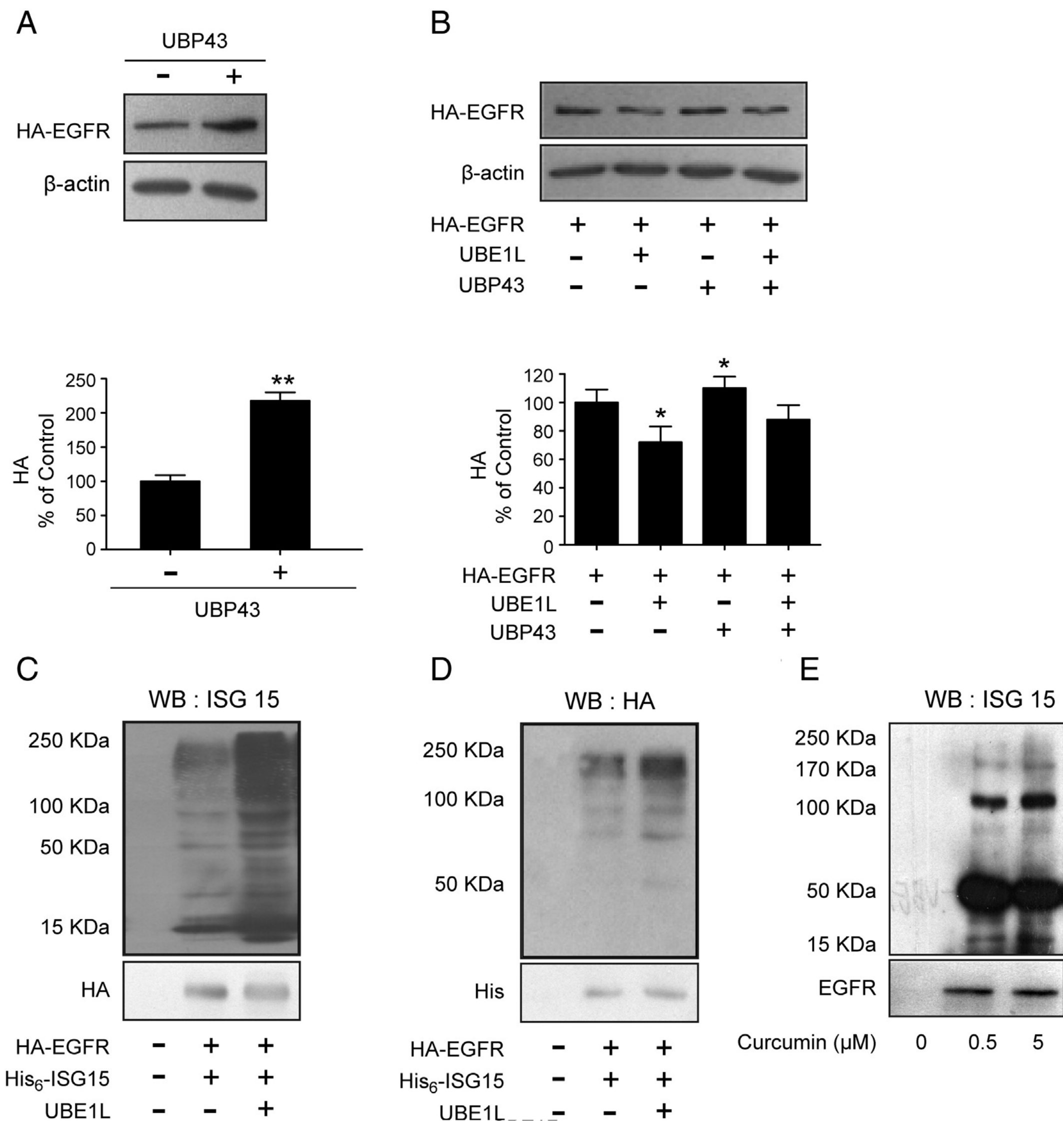


Fig. 4. Effects of UBP43 on EGFR and the complex between ISG15 and EGFR in HBE Beas-2B cells. (A) UBP43 increased EGFR expression. Beas-2B cells were cotransfected with UBP43 and HA-tagged EGFR for 24 h. EGFR protein was detected by an anti-HA antibody. β-Actin expression served as a loading control. (B) UBP43 antagonized UBE1L-mediated inhibition of EGFR. HBE cells were cotransfected with HA-tagged EGFR with or without UBE1L and UBP43 for 24 h. The cells were harvested and lysed for the detection expression of HA by Western blot analysis. β-Actin expression served as a loading control. (C) Complex between ISG15 and EGFR. Beas-2B cells were cotransfected with HA-tagged EGFR and His-tagged ISG15 with or without UBE1L for 24 h. Immunoprecipitation was done with an anti-HA antibody followed by immunoblot with anti-ISG15 antibody. HA expression served as input protein. (D) Results in panel C were independently confirmed in Beas-2B cells by Ni-NTA pull-down of these tagged ISG15 species. EGFR-HA conjugates were identified with an anti-HA antibody. His expression served as input protein. (E) Curcumin increased ISG15 modification of EGFR. Beas-2B cells were treated with curcumin (0.5 μM, 5 μM) for 24 h. Immunoprecipitation was done with EGFR antibody followed by immunoblot with anti-ISG15 antibody. EGFR expression served as input protein. Densitometry quantification of protein expression levels was shown as fold changes. Data were expressed as means±S.D. *P<.05, **P<.01 vs. control group.

3.3. UBE1L represses EGFR protein expression

Since EGFR is overexpressed in 50%–70% of human lung cancers [33] and UBE1L is not detected in several human lung cancer cell lines [17], we hypothesized that UBE1L can suppress EGFR expression. To investigate this hypothesis, HBE Beas-2B cells were cotransfected with UBE1L and HA-tagged EGFR plasmids. Fig. 3A shows that overexpression of UBE1L suppressed EGFR expression in the HBE cells. However, UBE1L transfection did not influence EGFR mRNA expression (Fig. 3B). To determine whether UBE1L affected EGFR protein stability, UBE1L was cotransfected with HA-tagged EGFR into the HBE cells in the presence or absence of cycloheximide (CHX; 40 μg/ml). Fig. 3C shows that UBE1L destabilized exogenous EGFR protein. These results indicated that down-regulation of EGFR by UBE1L may be occurred at the posttranslational level. To confirm whether curcumin inhibited EGFR via increasing UBE1L, Beas-2B cells were transfected with UBE1L siRNAs, in the presence or absence of curcumin treatment. The Western blot analysis showed that knockdown of UBE1L increased EGFR level. When the cells were transfected with UBE1L siRNA in the presence of curcumin, the increased EGFR level was down (Fig. 3D). This result indicated that UBE1L induction by curcumin was involved in down-regulation of EGFR.

3.4. UBE1L promotes the formation of the complex between ISG15 and EGFR

UBP43 is the deconjugating enzyme of ISG15, removing ISG15 from conjugated proteins [34]. We further tested whether UBP43 reversed the effect of UBE1L on EGFR. Fig. 4A shows that EGFR expression increased with UBP43 transfection. This is consistent with

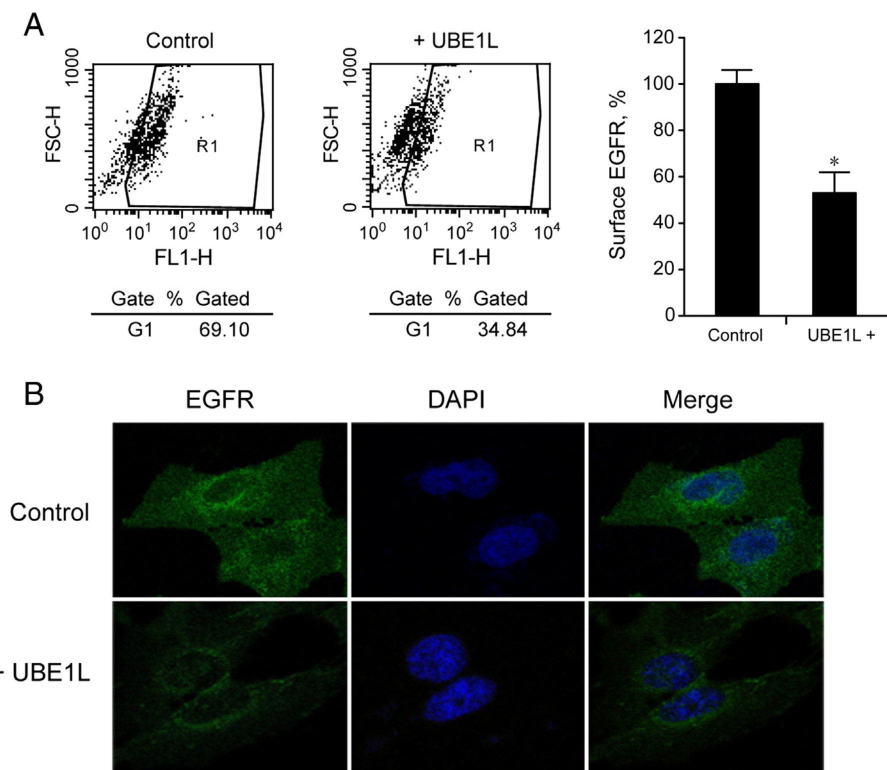


Fig. 5. UBE1L induces EGFR internalization. (A) FACS analyzed EGFR internalization. Beas-2B cells were transfected with UBE1L for 24 h. The amount of EGFR present at the cell surface was determined by FACS analysis and normalized to control cells. (B) Immunocytochemistry analyzed EGFR internalization. Beas-2B cells were transfected with UBE1L for 24 h. Localization of EGFR (green) and nuclei (blue) were visualized by confocal microscopy. Densitometry quantification of fluorescence levels was shown as fold changes. Data were expressed as means±S.D. **P*<.05 vs. control group.

the results that UBP43 overexpression led to EGFR up-regulation [35]. Transfection of UBE1L inhibited EGFR expression in Beas-2B cells, but UBP43 cotransfection antagonized this effect as shown in Fig. 4B. UBE1L triggers substrate conjugate to ISG15, while UBP43 makes substrate de-conjugate from ISG15 [34]. Therefore, it was hypothesized that ISG15 could form complex with EGFR. HBE cells were transiently transfected with or without UBE1L and with EGFR and ISG15 expression vectors. Lysates were subjected to protein A/G beads or Ni-NTA agarose for immunoprecipitation or pull-down assay before immunoblot analyses. Fig. 4C and D reveals conjugates of EGFR following cells cotransfection of ISG15 and EGFR. When the cells cotransfected with HA-tagged EGFR, His-tagged ISG15 and UBE1L, ISG15ylated species increased (Fig. 4C and D). As curcumin can induce UBE1L, whether curcumin itself can promote the formation of ISG15ylation of EGFR was investigated. As shown in Fig. 4E, curcumin itself increased ISG15 modification of EGFR.

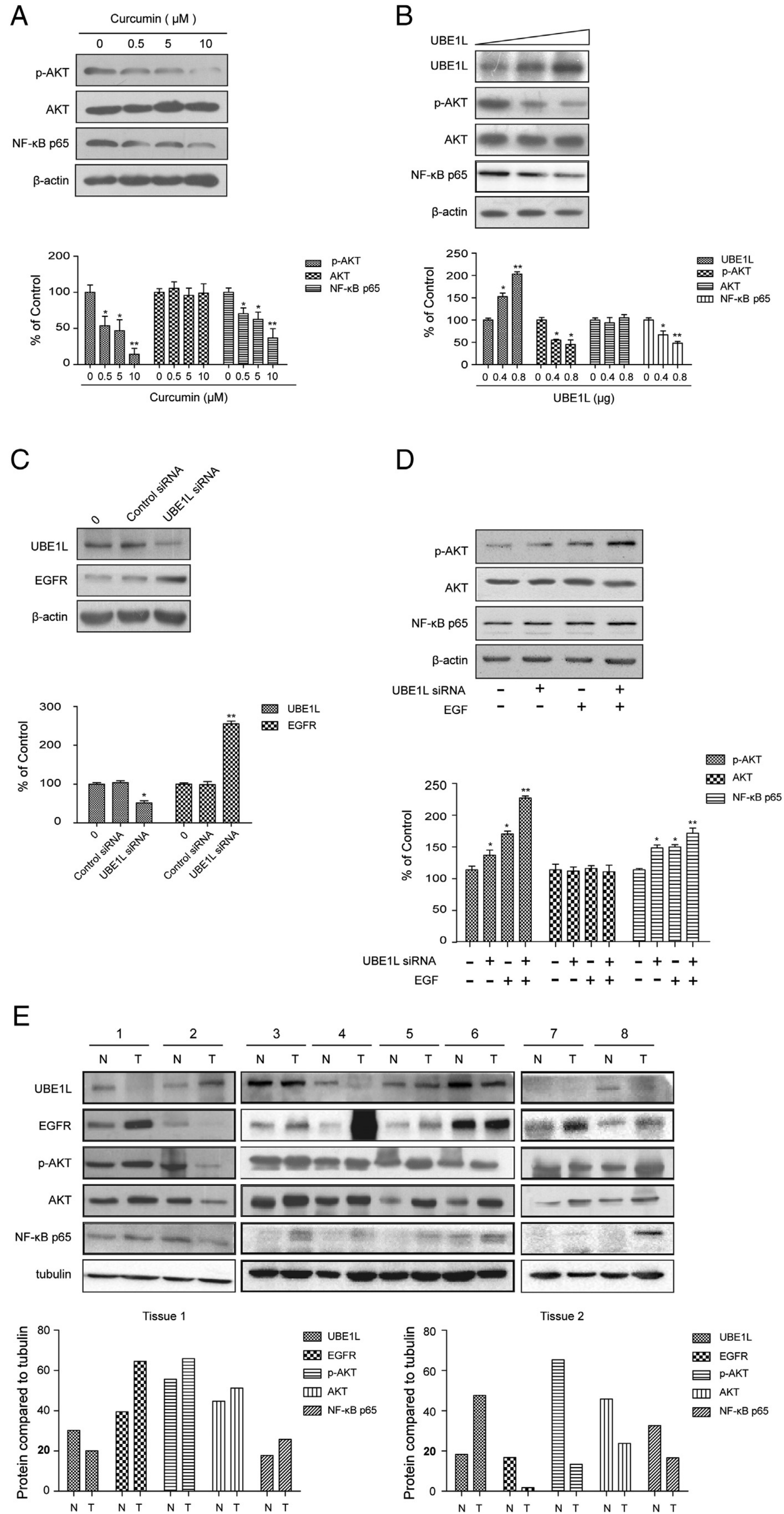
3.5. UBE1L enhances EGFR membrane internalization

Endocytic down-regulation is a pivotal mechanism turning off signaling from the EGFR [36]. Thus, to investigate whether UBE1L affect the internalization of EGFR, HBE cells were transfected with UBE1L or empty vector (as control) for 24 h. Then, the amount of EGFR presenting at the cell surface was determined by Fluorescence Activated Cell Sorter (FACS) analysis. As seen in Fig. 5A, compared to control, EGFR at the surface was substantially decreased after transfected with UBE1L. To further test the internalization, Beas-2B cells were transfected with UBE1L or empty vector (as control) for 24 h. They were subsequently fixed and labeled for EGFR (green) and nuclei (blue). Fig. 5B shows that EGFR was primarily located at the cell surface in the control cells; UBE1L transfection led to trafficking of EGFR from the plasma membrane into the cells. This indicated that UBE1L promoted internalization of the EGFR.

3.6. Curcumin and UBE1L down-regulate EGFR downstream signaling

Curcumin has been reported to suppress EGFR downstream signaling such as AKT and NF-κB [37]. In the present study, we confirmed this effect in HBE Beas-2B cells. Fig. 6A shows that curcumin decreased pAKT and NF-κB in a dose-dependent manner, but had no influence on total AKT level. We next investigated the direct effect of UBE1L on EGFR downstream signaling protein. As

Fig. 6. Effect of curcumin and UBE1Lon EGFR downstream signaling. (A) Curcumin decreased pAKT and NF-κB p65 proteins in the HBE cells in a dose-dependent manner. Beas-2B cells were incubated in the absence or presence of curcumin (0.5–10 μM) for 24 h. Then, the cells were harvested and lysed for the detecting expressions of pAKT, AKT and NF-κB p65 proteins by Western blot analysis. β-Actin expression served as a loading control. (B) UBE1L decreased pAKT and NF-κB p65 proteins in the HBE cells. Beas-2B cells were transiently transfected with UBE1L (0.4 μg, 0.8 μg) for 24 h. The cells were harvested and lysed for the detecting expressions of pAKT, AKT and NF-κB p65 proteins by Western blot analysis. UBE1L expression was detected for the transfection efficiency. β-Actin expression served as a loading control. (C) Knockdown of UBE1L increased EGFR proteins in the HBE cells. Beas-2B cells were transfected with siRNAs targeting UBE1L or nontargeting control siRNAs for 24 h. Cells were harvested and lysed for the detecting expressions of EGFR proteins by Western blot analysis. UBE1L expression was detected to confirm the efficiency of siRNA knockdown. β-Actin expression served as a loading control. (D) Knockdown of UBE1L increased pAKT and NF-κB p65 proteins in the HBE cells. Beas-2B cells were transfected with siRNAs targeting UBE1L for 24 h, in the presence or absence of EGF (20 ng/ml) for 2 h. Cells were harvested and lysed for the detecting expressions of pAKT, AKT and NF-κB p65 proteins by Western blot analysis. β-Actin expression served as a loading control. (E) Proteins levels in the NSCLC cancer tissues (T) and the adjacent tissues (N). The NSCLC cancer tissues and the adjacent tissues were lysed for the detection expressions of UBE1L, EGFR, pAKT, AKT and NF-κB p65 proteins by Western blot analysis. Tubulin expression served as a loading control. Densitometry quantification of protein expression levels was shown as fold changes. Data were expressed as means±S.D. **P*<.05, ***P*<.01 vs. control group.


A
Curcumin (μM)
0 0.5 5 10
p-AKT
AKT
NF-κB p65
β-actin
% of Control
p-AKT
AKT
NF-κB p65
Curcumin (μM)
B
UBE1L
UBE1L
p-AKT
AKT
NF-κB p65
β-actin
% of Control
UBE1L
p-AKT
AKT
NF-κB p65
UBE1L (μg)
C
0
Control siRNA
UBE1L siRNA
UBE1L
EGFR
β-actin
% of Control
UBE1L
EGFR
D
p-AKT
AKT
NF-κB p65
β-actin
UBE1L siRNA
EGF
p-AKT
AKT
NF-κB p65
% of Control
UBE1L siRNA
EGF
E
1 2 3 4 5 6 7 8
N T N T N T N T N T N T N T N T
UBE1L
EGFR
p-AKT
AKT
NF-κB p65
tubulin
Tissue 1
Protein compared to tubulin
UBE1L
EGFR
p-AKT
AKT
NF-κB p65
Tissue 2
Protein compared to tubulin
UBE1L
EGFR
p-AKT
AKT
NF-κB p65

shown in Fig. 6B, after transfection with UBE1L, the phosphorylation levels of AKT in the cells were decreased dramatically, whereas the total AKT level remained unchanged. After transfected with UBE1L, NF-κB (the downstream molecule of AKT) in the cells decreased. When the cells were transfected with UBE1L siRNAs, the expression of EGFR increased (Fig. 6C). To confirm if EGFR downstream signaling was influenced by the knockdown of UBE1L, Beas-2B cells were transfected with UBE1L siRNA targeting UBE1L in the presence or absence of EGF. The expression of pAKT, AKT and NF-κB were detected by Western blot analysis. The result showed that knockdown of UBE1L-enhanced EGF mediated activation of pAKT and NF-κB expression, whereas the total AKT level remained unchanged (Fig. 6D). These results indicate that curcumin-induced down-regulation of EGFR signaling is mediated through induction of UBE1L, which down-regulates the EGFR/AKT/NF-κB signal pathway.

We further investigated UBE1L and EGFR in the NSCLC specimens. We examined expression levels of UBE1L, EGFR and EGFR downstream AKT/NF-κB in the NSCLC tissues and adjacent tissues by Western blot analysis. Fig. 6E show that UBE1L levels in cancer tissues were lower than in adjacent tissues, or undetected, while the levels of EGFR, pAKT, AKT and NF-κB were higher in the malignant tissues. There was only one exception in cancer tissue no. 2, which showed higher UBE1L level and lower levels of EGFR, pAKT, AKT and NF-κB. Therefore, these results demonstrate that there is an inverse relationship between UBE1L and EGFR/AKT/NF-κB expression in NSCLC, which suggests that UBE1L plays an important role as a tumor suppressor in lung carcinogenesis.

4. Discussion

The present study clearly shows that curcumin is able to induce UBE1L expression and subsequently suppress EGFR levels in HBE cells. The significance of this novel discovery is that it provides a new chemopreventive mechanism for curcumin in the induction of UBE1L. We also demonstrate that UBE1L can down-regulate EGFR and its downstream signals both *in vitro* and *in vivo*. These data provide strong support for the proposal that UBE1L is a potential target for lung cancer chemoprevention and therapy.

UBE1L expression is up-regulated by certain stresses such as bacterial and viral infections [38]. These stresses mainly induce type I IFN signaling. Chemotherapy drug doxorubicin and camptothecin dramatically elevated UBE1L protein and mRNA levels, leading to ISG15ylation of several cellular proteins [39]. UBE1L-triggered ISG15ylation has characteristics of its vital role in the innate immune response to viral infections. Several reports have indicated that curcumin had an antiviral activity against the coxsackie virus [40] and the hepatitis B virus [41]. Therefore, we investigated whether the ISG15 system was a candidate target of curcumin. Like other elements of the ISG15ylation system stimulated by IFNs and all-*trans*-retinoic acid, ISG15 protein levels were up-regulated by curcumin in Beas-2B cells (data not shown). UBE1L triggers the first step for conjugation of ISG15 to target proteins. It was assumed that curcumin may enhance conjugation of substrate proteins by ISG15 (ISG15ylation). Curcumin may enhance immune response through induction of cellular ISG15ylation. The precise mechanism of curcumin induction of UBE1L in Beas-2B will be discussed in another article.

UBE1L promotes ISG15ylation of cyclin D1 and PML/RARα and leads to destabilization of cyclin D1 and PML/RARα [18,19]. These results suggest that ISG15ylation of protein may trigger protein down-regulation. In this study, curcumin induced UBE1L and ISG15 expression in Beas-2B cells, and UBE1L promoted a complex between ISG15 and EGFR. These could be a possible mechanism of curcumin-triggered down-regulation of EGFR.

Curcumin is also known to induce EGFR protein degradation through ubiquitination of EGFR [30]. In the current study, UBE1L did not affect the ubiquitination of EGFR (data not shown). ISGylation may potentially interfere with the ubiquitination pathway at the level of E2 and E3 enzymes, as some of the E2 (e.g., UbcH8) and E3 enzymes (e.g., EFP) can transfer both ubiquitin and ISG15 conjugate to target proteins [42].

Curcumin has been extensively studied and found to have diverse pharmacological activities. However, poor aqueous solubility and low bioavailability limit curcumin developing as a useful drug. Nanoparticles, liposomes, micelles and phospholipid complexes are promising novel formulations to enhance the aqueous solubility and bioavailability of curcumin [43]. One phase I clinical trial that applied oral dosing of 4–8 g of curcumin to human showed peak plasma levels of 0.41–1.75 μM of curcumin after 1 h of dosing [44]. The concentration of curcumin used in our experiments was from 0.5 to 10 μM, which could overcome the bioavailability challenges if novel formulations of curcumin are developed in future.

Down-regulation of EGFR-dependent signaling is achieved by internalization of activated EGFR and its degradation in lysosomes [45]. Our results showed that UBE1L induced EGFR membrane internalization, which may regulate trafficking of ligand-activated internalized EGFR and cause EGFR degradation. Thus, destabilization of EGFR by UBE1L is likely to be an important mechanism underlying the anticancer effect of the UBE1L, particularly in tumors driven by EGFR signaling. However, the precise mechanism of how UBE1L triggers EGFR membrane internalization needs to be further investigated.

EGFR and its downstream signaling pathways are involved in the development and progression of several human tumors, including lung cancer. One main intracellular pathway activated by EGFR is the PI3K/AKT pathway [46]. As mentioned above, PI3K/Akt activation is known to induce NF-κB activation. AKT/NF-κB is a major antiapoptotic/prosurvival pathway. We observed that curcumin could down-regulate the AKT/NF-κB pathway. Also, there was an inverse correlation between UBE1L and EGFR downstream pathway AKT and NF-κB expression in human NSCLC. An increased EGFR and enhanced/maintained EGFR downstream signaling were observed in the absence or low expression of UBE1L in human NSCLC specimens. We hypothesize that the low expression of UBE1L in lung cancer may lead to EGFR/AKT/NF-κB activation. More human NSCLC specimens need to be studied to test this hypothesis.

Taken together, our findings provide a novel chemopreventive mechanism of curcumin as well as the antioncogenic activity of UBE1L. These discoveries may lead to new strategies for cancer prevention and therapy in treating EGFR-dependent cancers.

References

[1] Perez-Soler R. HER1/EGFR targeting: refining the strategy. Oncologist 2004;9:58–67.

[2] Suzuki S, Dobashi Y, Sakurai H, Nishikawa K, Hanawa M, Ooi A. Protein overexpression and gene amplification of epidermal growth factor receptor in nonsmall cell lung carcinomas. An immunohistochemical and fluorescence in situ hybridization study. Cancer 2005;103:1265–73.

[3] Riese DN, Stern DF. Specificity within the EGF family/ErbB receptor family signaling network. Bioessays 1998;20:41–8.

[4] Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001;2:127–37.

[5] Goswami A, Ranganathan P, Rangnekar VM. The phosphoinositide 3-kinase/Akt1/Par-4 axis: a cancer-selective therapeutic target. Cancer Res 2006;66:2889–92.

[6] Engelman JA, Janne PA, Mermel C, Pearlberg J, Mukohara T, Fleet C, et al. ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. Proc Natl Acad Sci U S A 2005;102:3788–93.

[7] Kane LP, Shapiro VS, Stokoe D, Weiss A. Induction of NF-kappaB by the Akt/PKB kinase. Curr Biol 1999;9:601–4.

[8] Richmond A. Nf-kappa B, chemokine gene transcription and tumour growth. Nat Rev Immunol 2002;2:664–74.

[9] Akca H, Demiray A, Tokgun O, Yokota J. Invasiveness and anchorage independent growth ability augmented by PTEN inactivation through the PI3K/AKT/NFkB pathway in lung cancer cells. Lung Cancer-J Iaslc 2011;73:302–9.
[10] Waterman H, Katz M, Rubin C, Shtiegman K, Lavi S, Elson A, et al. A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling. EMBO J 2002;21:303–13.
[11] Johannessen LE, Pedersen NM, Pedersen KW, Madshus IH, Stang E. Activation of the epidermal growth factor (EGF) receptor induces formation of EGF receptor- and Grb2-containing clathrin-coated pits. Mol Cell Biol 2006;26:389–401.
[12] Haglund K, Sigismund S, Polo S, Szymkiewicz I, Di Fiore PP, Dikic I. Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat Cell Biol 2003;5:461–6.
[13] Mizuno E, Iura T, Mukai A, Yoshimori T, Kitamura N, Komada M. Regulation of epidermal growth factor receptor down-regulation by UBPY-mediated deubiquitination at endosomes. Mol Biol Cell 2005;16:5163–74.
[14] Yuan W, Krug RM. Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein. EMBO J 2001;20:362–71.
[15] Haas AL, Ahrens P, Bright PM, Ankel H. Interferon induces a 15-kilodalton protein exhibiting marked homology to ubiquitin. J Biol Chem 1987;262:11315–23.
[16] Kok K, Hofstra R, Pilz A, van den Berg A, Terpstra P, Buys CH, et al. A gene in the chromosomal region 3p21 with greatly reduced expression in lung cancer is similar to the gene for ubiquitin-activating enzyme. Proc Natl Acad Sci U S A 1993;90:6071–5.
[17] McLaughlin PM, Helfrich W, Kok K, Mulder M, Hu SW, Brinker MG, et al. The ubiquitin-activating enzyme E1-like protein in lung cancer cell lines. Int J Cancer 2000;85:871–6.
[18] Feng Q, Sekula D, Guo Y, Liu X, Black CC, Galimberti F, et al. UBE1L causes lung cancer growth suppression by targeting cyclin D1. Mol Cancer Ther 2008;7:3780–8.
[19] Shah SJ, Blumen S, Pitha-Rowe I, Kitareewan S, Freemantle SJ, Feng Q, et al. UBE1L represses PML/RAR{alpha} by targeting the PML domain for ISG15ylation. Mol Cancer Ther 2008;7:905–14.
[20] Lin JK. Molecular targets of curcumin. Adv Exp Med Biol 2007;595:227–43.
[21] Nagaraju GP, Aliya S, Zafar SF, Basha R, Diaz R, El-Rayes BF. The impact of curcumin on breast cancer. Integr Biol (Camb) 2012;4:996–1007.
[22] Anand P, Sundaram C, Jhurani S, Kunnumakkara AB, Aggarwal BB. Curcumin and cancer: an "old-age" disease with an "age-old" solution. Cancer Lett 2008;267: 133–64.
[23] Chen A, Xu J, Johnson AC. Curcumin inhibits human colon cancer cell growth by suppressing gene expression of epidermal growth factor receptor through reducing the activity of the transcription factor Egr-1. Oncogene 2006;25:278–87.
[24] Yu S, Shen G, Khor TO, Kim JH, Kong AN. Curcumin inhibits Akt/mammalian target of rapamycin signaling through protein phosphatase-dependent mechanism. Mol Cancer Ther 2008;7:2609–20.
[25] Aggarwal S, Takada Y, Singh S, Myers JN, Aggarwal BB. Inhibition of growth and survival of human head and neck squamous cell carcinoma cells by curcumin via modulation of nuclear factor-kappaB signaling. Int J Cancer 2004;111:679–92.
[26] Squires MS, Hudson EA, Howells L, Sale S, Houghton CE, Jones JL, et al. Relevance of mitogen activated protein kinase (MAPK) and phosphotidylinositol-3-kinase/-protein kinase B (PI3K/PKB) pathways to induction of apoptosis by curcumin in breast cells. Biochem Pharmacol 2003;65:361–76.
[27] Bharti AC, Donato N, Aggarwal BB. Curcumin (diferuloylmethane) inhibits constitutive and IL-6-inducible STAT3 phosphorylation in human multiple myeloma cells. J Immunol 2003;171:3863–71.
[28] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671–5.
[29] Dorai T, Gehani N, Katz A. Therapeutic potential of curcumin in human prostate cancer. II. Curcumin inhibits tyrosine kinase activity of epidermal growth factor receptor and depletes the protein. Mol Urol 2000;4:1–6.
[30] Lee JY, Lee YM, Chang GC, Yu SL, Hsieh WY, Chen JJ, et al. Curcumin induces EGFR degradation in lung adenocarcinoma and modulates p38 activation in intestine: the versatile adjuvant for gefitinib therapy. PLoS One 2011;6:e23756.
[31] Chadalapaka G, Jutooru I, Burghardt R, Safe S. Drugs that target specificity proteins downregulate epidermal growth factor receptor in bladder cancer cells. Mol Cancer Res 2010;8:739–50.
[32] de Veer MJ, Holko M, Frevel M, Walker E, Der S, Paranjape JM, et al. Functional classification of interferon-stimulated genes identified using microarrays. J Leukoc Biol 2001;69:912–20.
[33] Normanno N, Bianco C, De Luca A, Maiello MR, Salomon DS. Target-based agents against ErbB receptors and their ligands: a novel approach to cancer treatment. Endocr Relat Cancer 2003;10:1–21.
[34] Malakhov MP, Malakhova OA, Kim KI, Ritchie KJ, Zhang DE. UBP43 (USP18) specifically removes ISG15 from conjugated proteins. J Biol Chem 2002;277:9976–81.
[35] Duex JE, Sorkin A. RNA interference screen identifies Usp18 as a regulator of epidermal growth factor receptor synthesis. Mol Biol Cell 2009;20:1833–44.
[36] Gui A, Kobayashi A, Motoyama H, Kitazawa M, Takeoka M, Miyagawa S. Impaired degradation followed by enhanced recycling of epidermal growth factor receptor caused by hypo-phosphorylation of tyrosine 1045 in RBE cells. BMC Cancer 2012;12:179.
[37] Binion DG, Heidemann J, Li MS, Nelson VM, Otterson MF, Rafiee P. Vascular cell adhesion molecule-1 expression in human intestinal microvascular endothelial cells is regulated by PI 3-kinase/Akt/MAPK/NF-kappaB: inhibitory role of curcumin. Am J Physiol Gastrointest Liver Physiol 2009;297:G259–68.
[38] Kim KI, Malakhova OA, Hoebe K, Yan M, Beutler B, Zhang DE. Enhanced antibacterial potential in UBP43-deficient mice against *Salmonella typhimurium* infection by up-regulating type I IFN signaling. J Immunol 2005;175:847–54.
[39] Jeon YJ, Jo MG, Yoo HM, Hong SH, Park JM, Ka SH, et al. Chemosensitivity is controlled by p63 modification with ubiquitin-like protein ISG15. J Clin Invest 2012;122:2622–36.
[40] Si X, Wang Y, Wong J, Zhang J, McManus BM, Luo H. Dysregulation of the ubiquitin–proteasome system by curcumin suppresses coxsackievirus B3 replication. J Virol 2007;81:3142–50.
[41] Rechtman MM, Har-Noy O, Bar-Yishay I, Fishman S, Adamovich Y, Shaul Y, et al. Curcumin inhibits hepatitis B virus via down-regulation of the metabolic coactivator PGC-1alpha. FEBS Lett 2010;584:2485–90.
[42] Zhang D, Zhang DE. Interferon-stimulated gene 15 and the protein ISGylation system. J Interferon Cytokine Res 2011;31:119–30.
[43] Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of curcumin: problems and promises. Mol Pharm 2007;4:807–18.
[44] Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, et al. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res 2001;21:2895–900.
[45] Sorkin A, Von Zastrow M. Signal transduction and endocytosis: close encounters of many kinds. Nat Rev Mol Cell Biol 2002;3:600–14.
[46] Berg M, Soreide K. EGFR and downstream genetic alterations in KRAS/BRAF and PI3K/AKT pathways in colorectal cancer: implications for targeted therapy. Discov Med 2012;14:207–14.