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Lenvatinib Inhibits AKT/NF-κB Signaling and Induces Apoptosis Through Extrinsic/Intrinsic Pathways in Non-small Cell Lung Cancer

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Abstract. *Background/Aim: Non-small cell lung cancer (NSCLC) is a serious disease and the leading cause of death globally. Overexpression of protein kinase B/nuclear factor-kappa B (NF-κB) signaling transduction of NSCLC cells was recognized as a potential therapeutic target. Lenvatinib is a multiple kinase inhibitor against vascular endothelial growth factor receptor family. However, whether lenvatinib may affect AKT/NF-κB in NSCLC remains unknown. Materials and Methods: MTT assay, NF-κB reporter gene assay, flow cytometry, tranwell migration/invasion analysis and western blotting were used to identify the alteration of cell viability, NF-κB activation, apoptosis effect, migration/invasion potential and AKT/NF-κB related protein expression, respectively, in CL-1-5-F4 cells after lenvatinib treatment. Results: The cell viability and NF-κB activity were suppressed by lenvatinib. Extrinsic and intrinsic apoptosis were activated by lenvatinib. Additionally, the metastatic potential of CL-1-5-F4 cells was also suppressed by lenvatinib. Conclusion: Altogether, lenvatinib induced extrinsic/intrinsic apoptosis and suppressed migration/invasion ability of NSCLC cells that was associated with AKT/NF-κB signaling inactivation.*

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The treatment results of non-small cell lung cancer (NSCLC) are far beyond satisfied and have room for improvement (1). Oral tyrosine kinase inhibitors (TKI) targeting mutated epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) and immune checkpoint inhibitors have been shown to improve overall survival of patients with NSCLC (2-4). The studies evaluating efficacy and safety of novel TKI in patients with NSCLC are ongoing (5).

Nuclear factor kappa B (NF-κB) is a major transcription factor mediating NSCLC progression through regulating expression of NF-κB target genes involved in tumor cell proliferation, survival, and invasion. Furthermore, NF-κB has been indicated to mediate tumor resistance to chemotherapy and radiotherapy (6-8). Induction of apoptosis through extrinsic and intrinsic pathways are associated with tumor regression in NSCLC elicited by therapeutic agents (9). Cell and animal models presented that both inhibitors of NF-κB signaling and apoptosis modulators not only attenuate NSCLC progression but also enhance anti-NSCLC efficacy of therapeutic agents. Inhibitors of NF-κB signaling and apoptosis modulators have been recognized as promising approaches for treating NSCLC (10, 11).

Lenvatinib, a multi-targeted TKI, has been shown to possess activity against platelet-derived growth factor receptor alpha (PDGFRα), vascular endothelial growth factor

receptors (VEGFR) 1-3, fibroblast growth factor receptors (FGFR) 1-4, and rearranged during transfection (RET) (12). It is used for the treatment of thyroid cancer, hepatocellular carcinoma (HCC), and renal cell carcinoma (RCC) (13-15). Lenvatinib has antitumor efficacy in patients with RET fusion-positive lung adenocarcinoma (16). However, the anti-NSCLC mechanism of lenvatinib has not been yet fully elucidated. Therefore, the main goal of the study was to verify the effect of lenvatinib on NF-κB and apoptotic signaling in NSCLC *in vitro*.

Materials and Methods

Chemical and reagents. Lenvatinib, dimethyl sulfoxide (DMSO) and MTT (3-(4,5-Dimethylthiazol-2-yl-2,5- Diphenyltetrazolium Bromide)) were purchased from SigmaAldrich Corp. (St. Louis, MO, USA). Primary antibodies including AKT (ser473), AKT, β-actin, MCL-1, XIAP, C-FLIP, VEGF, MMP-9, CyclinD1, NF-κB (ser536), and NF-κB p65 were all purchased from Elabscience (Houston, TX, USA). CaspGLOW™ Fluorescence active caspase-3, -8 and -9 staining kit were purchased from BioVision (Milpitas, CA, USA). FITC anti-human CD95 Fas and PE anti-human CD178 FasL antibodies were purchased from BioLegend CNS, Inc. (San Diego, CA, USA).

Cell culture of CL1-5-F4 cells. CL1-5-F4 cells were kindly provided from by Dr. Chia-Lin Hsieh (Taipei Medical University, Taiwan, ROC). Cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were incubated in incubator with 5% CO_2 and under 37˚C humidified atmosphere. Mediums and reagents were all purchased from Thermo Fisher Scientific Inc., MA, USA.

Plasmid transfection, stable clone selection and reporter gene assay. As described in previous study, CL1-5-F4 cells were transfected with NF-κB-luciferase 2 vector (pNF-κB/*luc2*) using jetPEI transfection reagent (Polyplus Transfection, Strasbourg, Alsace, France) (17, 18). Cells with stable NF-κB expression were selected by Hygromycin B (200 μg/ml) and named as CL1-5-F4/NF-κB/*luc2* cells. For reporter gene assay, 5×10^5 CL1-5-F4/NF-κB/*luc2* cells were seeded in 96 wells overnight and treated with 0-70 μM lenvatinib for 24 and 48 h. The activation of NF-κB in CL1-5-F4/NF-κB/*luc2* cells were detected by IVIS 200 Imaging System and normalized by cell viability results as previous described.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) cell viability assay. The viability of CL1-5-F4 cells after lenvatinib (0-70 μM) treatment for 24 and 48 h were performed by MTT assay. The detail procedure was described in previous study (18). In brief, the DMSO dissolves MTT reagents were finally detected by SpectraMax iD3 microplate reader at 570 nm (Molecular Devices, San Jose, CA, USA).

Apoptosis analyses. CL1-5-F4 cells were seeded in 6 wells plate with 5×10^5 cells per well overnight. Then, CL1-5-F4 cells were treated with 0, 30, 50 μM of lenvatinib for 48 h. For caspase related marker detection, cells were stained as manufacture protocol by caspGLOW™ Fluorescence active caspase-3, -8 and -9 staining kit, respectively. For cell cycle analysis, CL1-5-F4 cells were fixed with 75% ethanol at –20˚C overnight and stained by 40 μg/ml Propidium iodide (PI) and RNase mixture dye (with 100 μg/ml RNase and 1% Triton X-100) in phosphate-buffered saline buffer (PBS) for 30 min in the dark at 37˚C. For Annexin-V/PI staining, the procedure was followed as protocol provided from commercial Annexin V-FITC staining kit (Vazyme Biotech Co. Lt, Nanjing City, PR China) (19). For FAS and FAS-L analysis, CL1-5-F4 cells were stained with FAS-FITC and FASL-PE antibodies for 15 min (20). For mitochondria membrane potential ($\Delta\Psi_m$) analysis, CL1-5-F4 cells were stained by 4 μM $DiOC_6$ in 0.5 ml of PBS for 30 min at 37˚C. After different staining procedures, the signal of florescence was finally investigated by NovoCyte flow cytometry and quantified by NovoExpress® software (Agilent Technologies Inc., Santa Clara, CA, USA). The detail procedure was mentioned in previous study (21, 22).

Transwells invasion and migration assay. CL1-5-F4 cells were seeded in 10 cm plate with 2×10^6 cells per plate overnight. Then, CL1-5-F4 cells were treated with 0, 30, 50 μM of lenvatinib for 48 h. After treatment, 3×10^5 cells were seeded in serum-free medium and placed into transwell insert (with matrigel or without). The transwell insert were then placed into 24-wells for 24 h with serum medium to let cells to migrate and invade. Further fixed and staining procedure was described in previous studies (23).

Western blotting. CL1-5-F4 cells were seeded in 10 cm plate with 2×10^6 cells per plate overnight. Then, CL1-5-F4 cells were treated with 0, 30, 50 μM of lenvatinib for 48 h. After lenvatinib treatment, CL1-5-F4 cells were lysed by lysis buffer and protein concentration of each group was analyzed by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc.). The protein expression of MCL-1, XIAP, C-FLIP, VEGF, MMP-2, MMP-9, CyclinD1, NF-κB p65 (ser536), NF-κB p65, AKT (ser473), AKT, β-actin after lenvatinib treatment were assayed by Western blotting. In brief, proteins were separated by 8-10 % SDS-PAGE and transferred onto polyvinylidene difluoride membrane (PVDF). The membrane with protein was further conjugated with primary and secondary antibodies. The membrane was then added with Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, Burlington, MA, USA) for visualization and detected by a chemiluminescent image system (ChemiDoc-It 515, UVP). The procedure details were described in previous study (21).

Statistical analysis. For statistical analysis, one-way ANOVA was performed between control and lenvatinib treated groups. When *p*-value smaller than 0.05, it may be recognized as significant difference. Results were all displayed as mean±standard deviation.

Results

Lenvatinib induced the cytotoxicity of NSCLC cells and is associated with AKT/NF-κB signaling inactivation. To identify whether lenvatinib may decrease the viability of NSCLC and inactivate AKT/NF-κB relative signal transduction, we performed MTT assay, NF-κB reporter gene assay and western blotting. As illustrated in Figure 1A, the viability of CL1-5-F4 cells was decreased in a dose- and time-dependent manner. The IC_{50} of lenvatinib treatment for 48 h in CL1-5-F4 cells was around 50 μM. In Figure 1B-C, the activation of NF-κB of

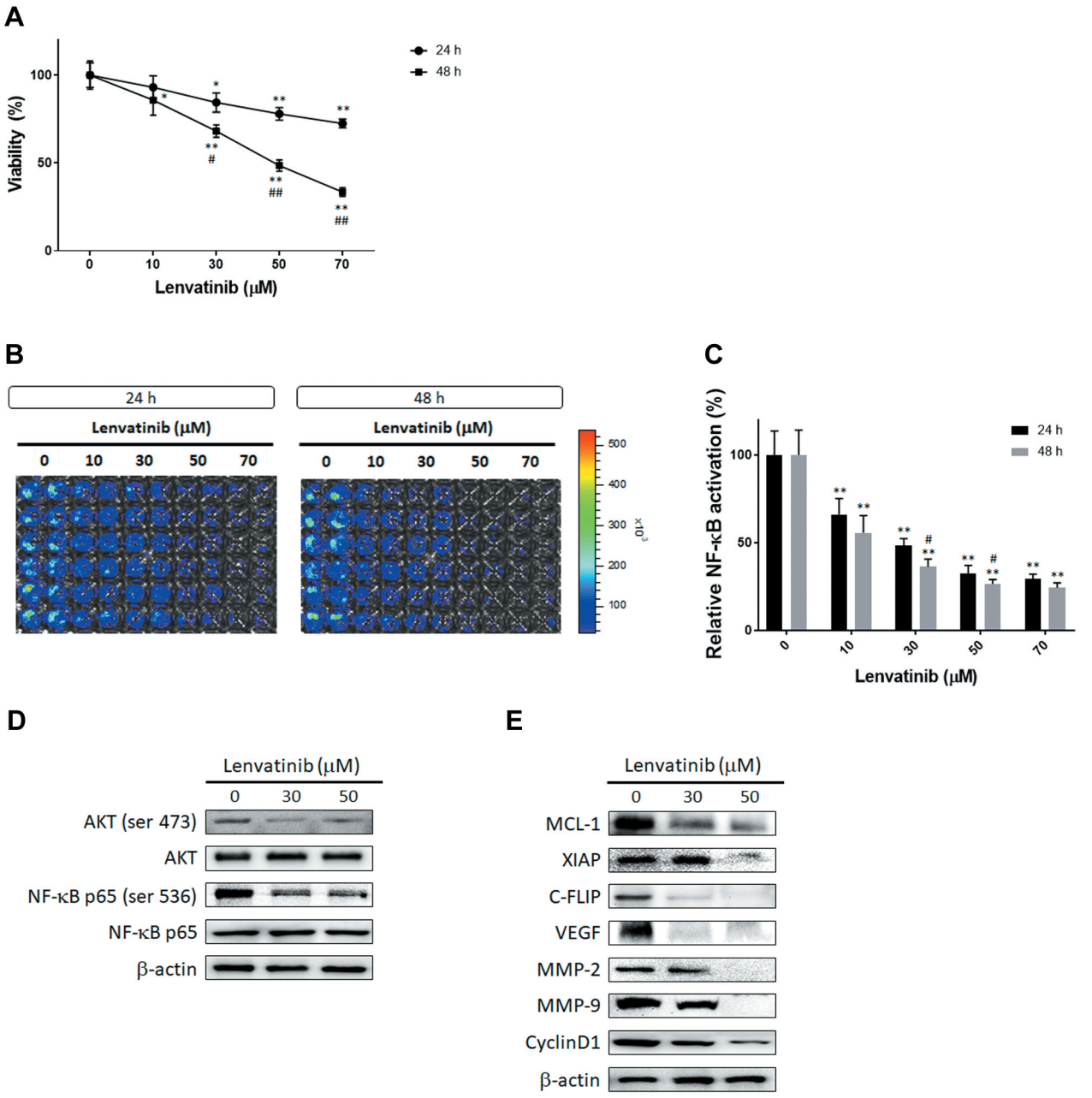

Figure 1. *Cell viability of CL1-5-F4 was decreased by lenvatinib and was associated with AKT/NF-κB inactivation. CL1-5-F4 and CL1-5-F4/NF-κB/luc2 cells were treated with 0-70 μM lenvatinib for 24 and 48 h. We then performed MTT assays for cell viability and NF-κB reporter gene assays for NF-κB activation detection. Results of (A) cell viability, (B-C) NF-κB reporter gene assay and (D-E) western blotting are displayed. (*p<0.05, **p<0.01 vs. 0 μM lenvatinib; #p<0.05, ##p<0.01 vs. 24 h or 30 μM lenvatinib).*

CL1-5-F4/NF-κB/*luc2* cells was also suppressed by lenvatinib in a dose- and time-dependent manner. The inhibition efficacy of NF-κB activity was around 50-70% at 30-50 μM lenvatinib treatment for 48 h. In addition, we also investigated whether the phosphorylation of AKT and NF-κB were decreased by lenvatinib. As shown in Figure 1D, lenvatinib effectively reduced the phosphorylation of AKT and NF-κB. The AKT/NF-κB relative proteins, such as MCL-1, XIAP, C-FLIP, VEGF, MMP-2, MMP-9, CyclinD1 were also inhibited by lenvatinib treatment (Figure 1E). Taken together, the cytotoxicity induction of lenvatinib in NSCLC cells may associate with AKT/NF-κB inactivation.

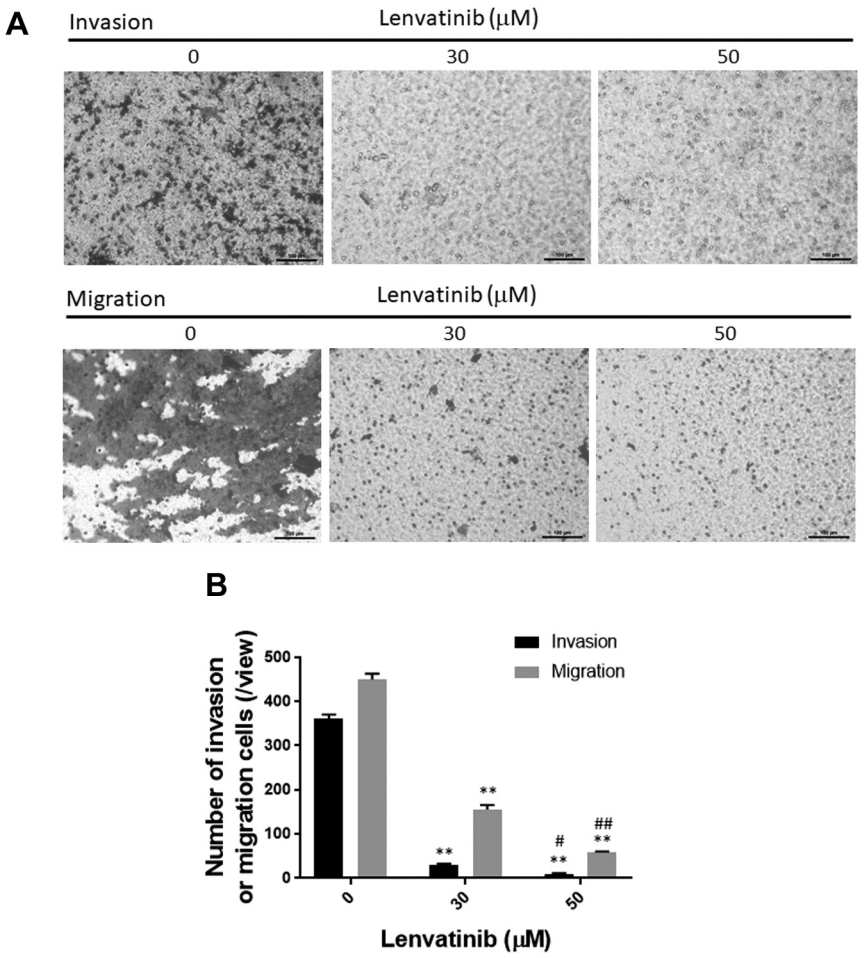

Figure 2. *The migration and invasion ability of CL1-5-F4 cells were suppressed by lenvatinib. CL1-5-F4 cells are treated with 30, 50 μM lenvatinib for 48 h. (A) Invasion/migration trend and (B) quantification results of CL1-5-F4 cells after lenvatinib treatment are displayed. (**p<0.01 vs. 0 μM lenvatinib; #p<0.05, ##p<0.01 vs. 30 μM lenvatinib).*

Lenvatinib suppressed the migration and invasion ability of NSCLC cells. We then performed tranwell migration and invasion assays to identify whether lenvatinib may regulate the migration and invasion potential of CL1-5-F4 cells. As displayed in Figure 2A, the number of invasion and migration cells was markedly decreased by lenvatinib treatment. The number of invasion and migration cells was also quantified and displayed as bar chart in Figure 2B. A significant suppression of CL1-5-F4 cells was found in the lenvatinib-treated group in a dose-dependent manner. In summary, the migration and invasion function in NSCLC cells may be inhibited by lenvatinib.

Lenvatinib triggered the apoptosis effect of NSCLC cells. The activation of cleaved caspase-3, Annexin-V and the accumulation of subG_1 were used as the marker to validate whether the apoptosis signaling may be triggered by lenvatinib. In Figure 3A, the activation of cleaved caspase-3 in the lenvatinib treatment group was increased to 10-20% as compared to the non-treatment group. In annexin-V/PI staining results, the PI and annexin-V double positive stained groups (Q2-2) was recognized as late apoptosis population. As illustrated in Figure 3B, the late apoptosis population was markedly increased by lenvatinib. Whereas, the percentages of sub-G_1 phase (apoptotic cells, Figure 3C) was significantly increased after cells treated with 30 and 50 μM lenvatinib for 48 h. In conclusion, the apoptosis effect can be triggered by lenvatinib in NSCLC cells.

Lenvatinib activated both extrinsic and intrinsic apoptosis signaling. To further identify the effect of lenvatinib on apoptosis mechanism, we investigated whether lenvatinib may trigger the activation of extrinsic apoptosis and intrinsic apoptosis-related markers. The activation of Fas was increased

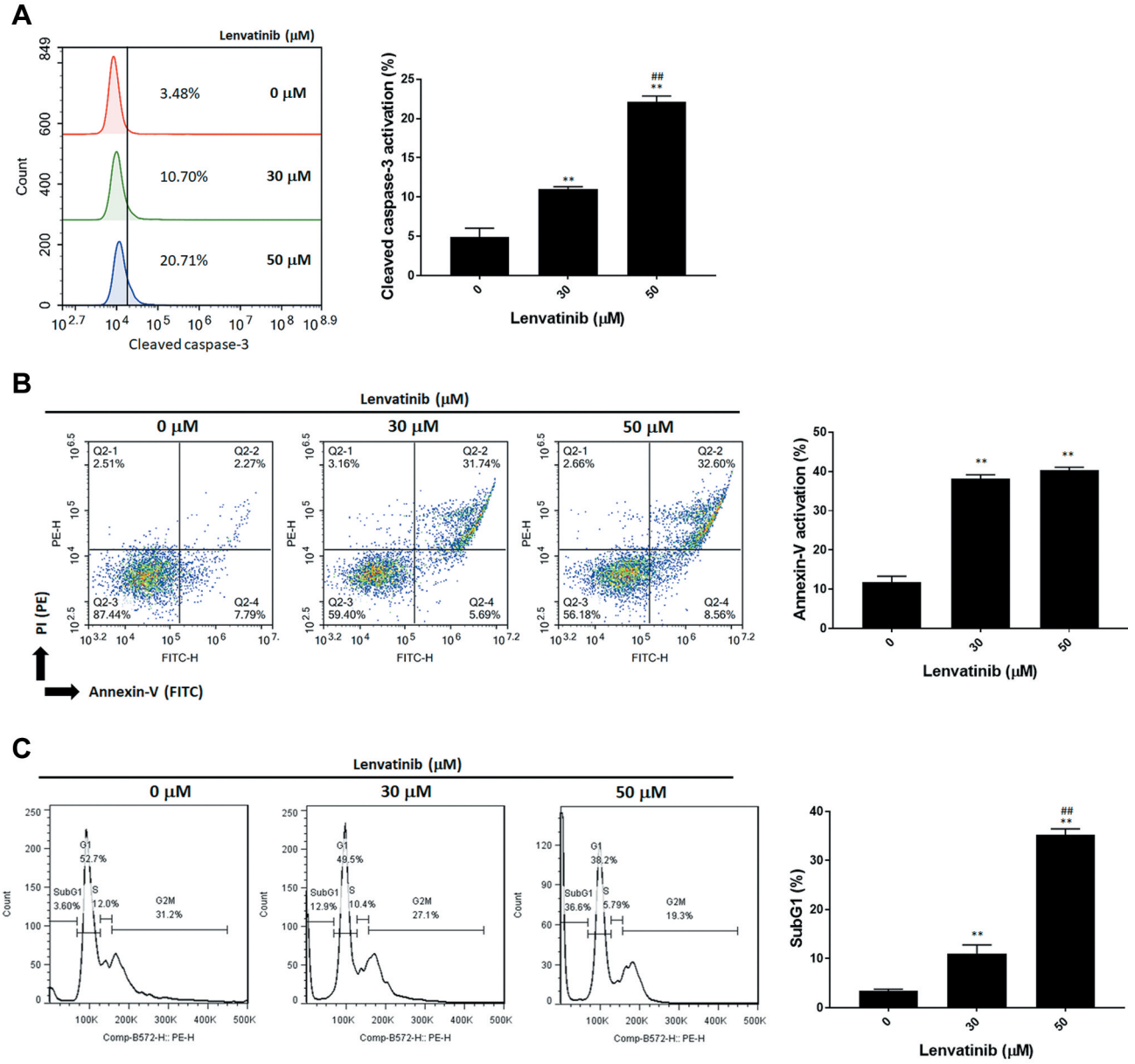

Figure 3. *Apoptosis effect of CL1-5-F4 cells were promoted by lenvatinib. CL1-5-F4 cells are treated with 30, 50 μM lenvatinib for 48 h. Activation of (A) cleaved caspase-3, (B) annexin-V and (C) accumulation of subG$_1$ population of CL1-5-F4 cells after lenvatinib treatment are displayed. (**p<0.01 vs. 0 μM lenvatinib; ##p<0.01 vs. 30 μM lenvatinib).*

to 8-20% after lenvatinib treatment in CL1-5-F4 cells (Figure 4A). Not only Fas activation pattern of CL1-5-F4 cells was found in lenvatinib treatment, Fas-L was also found to be effectively increased (Figure 4B). In the meantime, caspase-8, a downstream factor of Fas/Fas-L was also activated by lenvatinib. The cleavage form of caspase-8 was increased by 11-25% after lenvatinib treatment (Figure 4C). Moreover, lenvatinib may induce the loss of $\Delta\Psi m$ (Figure 4D) and the activation of cleaved caspase-9 (Figure 4E). Altogether, lenvatinib may not only induce Fas/Fas-L activation, a death receptor dependent apoptotic pathway, but also trigger intrinsic mitochondria-dependent apoptotic pathways.

Discussion

Lenvatinib has been demonstrated to effectively reduce cell growth and induce apoptosis in HCC and thyroid cancer (24, 25). We found that lenvatinib also significantly inhibited cell

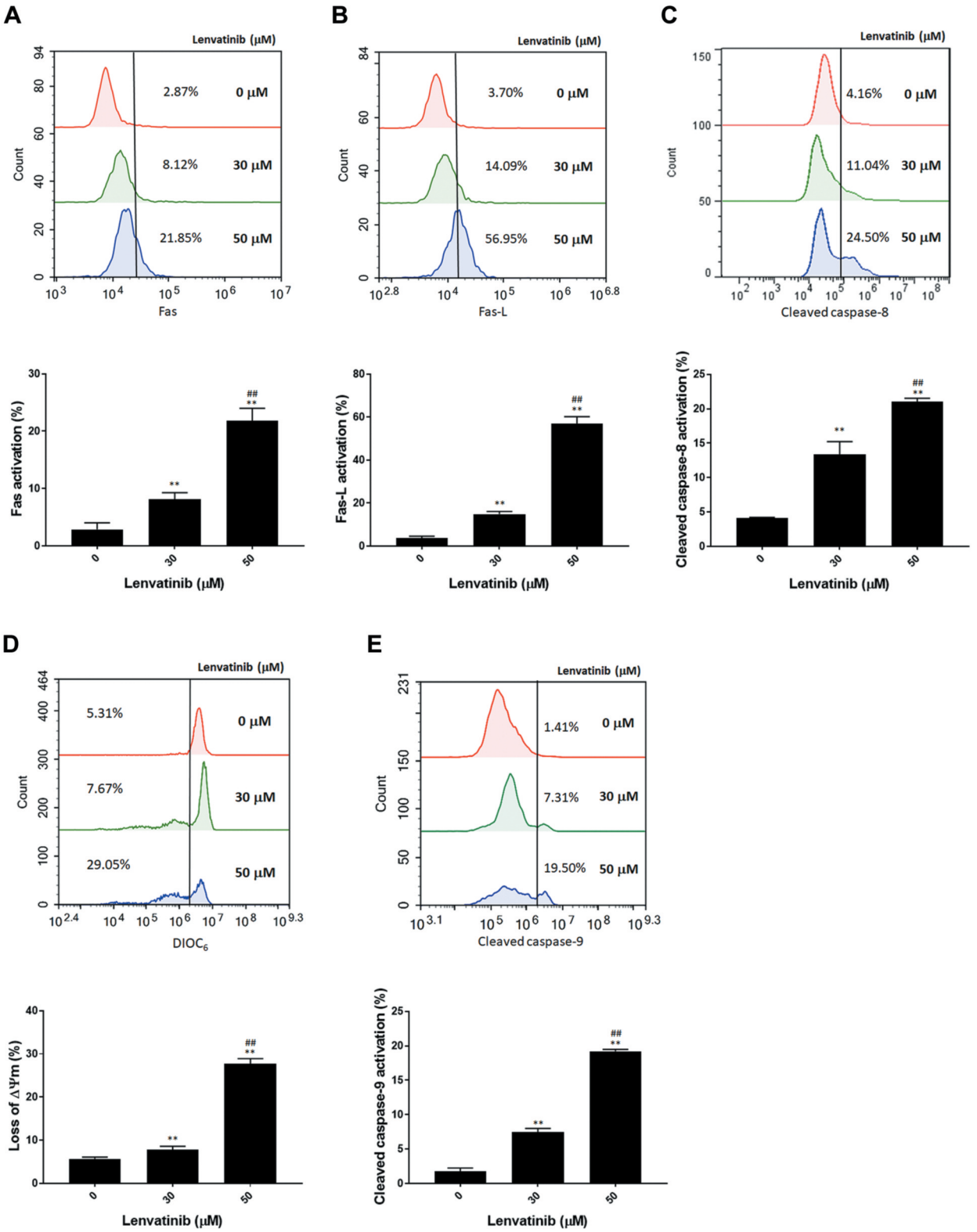

Figure 4. *Death receptor dependent and mitochondria dependent apoptotic pathway of CL1-5-F4 cells were activated by lenvatinib. CL1-5-F4 cells are treated with 30 and 50 μM lenvatinib for 48 h. Activation of (A) Fas, (B) Fas-L and (C) cleaved-caspase 8 of CL1-5-F4 cells after lenvatinib treatment are displayed. (D) The loss of $\Delta\Psi_m$ and (E) the activation of cleaved-caspase 9 are presented. (**p<0.01 vs. 0 μM lenvatinib; ##p<0.01 vs. 30 μM lenvatinib).*

proliferation and triggered apoptosis in NSCLC CL1-5-F4 cells (Figures 1, 3 and 4). Cleavage of caspase-3 is required for the formation of apoptotic deoxyribonucleic acid (DNA) fragmentation and is increased by death receptor (extrinsic) and mitochondrial (intrinsic) apoptotic signaling (26, 27). Our results showed that lenvatinib significantly augmented cleavage of caspase-3 and promoted extrinsic/intrinsic apoptotic signaling [increased death receptor Fas expression, Fas ligand (FasL) activation, cleaved caspase-8 expression, and loss of $\Delta\Psi_m$] (Figures 3A-D and 4). Cleaved caspase-9 is an apoptotic protein that regulates the intrinsic apoptotic pathway (28). We found that protein expression of cleaved caspase-9 was increased by lenvatinib treatments (Figure 4E).

Expression of anti-apoptosis, invasion, and proliferation-associated proteins was correlated with constitutive NF-κB activation in NSCLC (29). Protein kinase B (PKB or AKT), a key component of phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway, has been shown as an upstream modulator of NF-κB signaling to up-regulate NF-κB activity and expression of NF-κB-mediated proteins (30). Increased expression of NF-κB or AKT activation was associated with shorter overall survival of patients with NSCLC (31-33). The attenuation of NF-κB activation effectively diminished the invasion ability, protein levels of MCL-1, XIAP, C-FLIP, VEGF, MMP-9, MMP-2, and cyclin-D1 (19, 30). In addition, AKT inactivation reduced NF-κB activity in CL1-5-F4 cells (30). Our results demonstrated that lenvatinib significantly suppressed NF-κB activity, cell migration, and invasion (Figures 1B-D and 2). The expression of above-mentioned anti-apoptosis, invasion, and proliferation-associated proteins was also inhibited by lenvatinib treatment (Figure 1E).

In conclusion, the present study indicated that lenvatinib induced apoptosis through extrinsic/intrinsic pathways and inhibited AKT/NF-κB signaling-mediated anti-apoptotic and invasion potential in CL1-5-F4 cells. We suggest that both induction of apoptosis and suppression of AKT/NF-κB signaling are associated with anti-NSCLC capability of lenvatinib. The strength of this work is that we identified that the anti-NSCLC potential was associated with AKT/NF-κB inhibition. The major limitation of this project was that the effect of lenvatinib had only been performed on *in vitro* NSCLC cells model; thus, further *in vivo* studies need to be performed in the future.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this article.

Authors' Contributions

Data curation, YC Liu, BH Huang, and FT Hsu; funding acquisition, WL Liu and FT Hsu; writing—original draft, YC Liu; writing—review, WL Liu, FT Hsu and SS Lin. All Authors have read and agreed to the published version of the manuscript.

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