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Isfahan University of Medical Sciences

Original Article

Apoptotic effect of linifanib on human ovarian cancer OVCAR3 cell line

Han Ki Lee[1], Jongsung Lee[2], Myeong Jin Nam[1], Hyun Gi Koh[3], Kyungmoon Park[3], Kyu Yun Jang[4,5,6,*], and See-Hyoung Park[3,*]

[1]*Department of Biological Science, Gachon University, Seongnam 13120, Republic of Korea.*
[2]*Department of Integrative Biotechnology, Sungkyunkwan University, Suwon 16419, Republic of Korea.*
[3]*Department of Biological and Chemical Engineering, Hongik University, Sejong, 30016, Republic of Korea.*
[4]*Department of Pathology, Jeonbuk National University Medical School, Jeonju 54896, Republic of Korea.*
[5]*Research Institute of Clinical Medicine, Jeonbuk National University, Jeonju 54896, Republic of Korea.*
[6]*Biomedical Research Institute, Jeonbuk National University Hospital, Jeonju 54907, Republic of Korea.*

Abstract

Background and purpose: Linifanib is a multi-targeted receptor tyrosine kinase inhibitor. Although it is widely recognized for its efficacy in inhibiting receptor tyrosine kinases, its anticancer effects in ovarian cancer have not been extensively studied. In this study, we investigated the anticancer effects of linifanib on human ovarian cancer OVCAR3 cells.
Experimental approach: To evaluate cell proliferation capacity, we performed MTT assays, cell counting, morphological analysis, and colony formation assays. Flow cytometry was used to assess the induction of apoptotic cells by linifanib, and DNA fragmentation was evaluated using TUNEL assays. Western blotting was performed to determine the protein expression levels related to apoptosis.
Findings/Results: Our results demonstrated that linifanib significantly inhibited the proliferation of OVCAR3 cells and induced apoptosis. Notably, treatment with linifanib led to the inhibition of phosphorylated Akt at Ser473, accompanied by the activation of FOXO3.
Conclusion and implications: Taken together, these findings indicate that linifanib suppresses the proliferation of human ovarian cancer OVCAR3 cells, highlighting its potential as a therapeutic candidate for ovarian cancer treatment.

Keywords: Apoptosis; FOXO3; Linifanib; Ovarian cancer; OVCAR3.

INTRODUCTION

Ovarian cancer refers to cancer that occurs in the ovaries, which play a crucial role in female reproduction and hormone secretion (1,2). It is also known as a malignant tumor that occurs in the ovaries and most often affects individuals between the ages of 50 and 70 (3). The causes of ovarian cancer include ovulation, genetic factors, mutations, dysfunctional changes in BRCA1 and BRCA2 genes, and environmental factors (4,5). Treatment methods for ovarian cancer, caused by various factors, include surgery, chemotherapy, and radiation therapy (6). Nevertheless, these treatments have some drawbacks, such as the detrimental effects of radiation therapy on normal ovarian tissue (7).

The progression of ovarian cancer cells is closely related to the cell cycle, which regulates cellular division and growth (8). Dysregulation of the cell cycle, particularly through mutations in genes that control checkpoints, can lead to uncontrolled cell proliferation and tumor formation (9).

*Corresponding authors:
K.Y. Jang, Tel: +82-632703136, Fax: +82-632703135
Email: kyjang@jbnu.ac.kr
S.H. Park, Tel: +82-448602126, Fax: +82-448628831
Email: shpark74@hongik.ac.kr

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Therefore, understanding the cell cycle in ovarian cancer cells is essential for developing targeted therapies. Current treatments aim to disrupt this cell cycle, but they often have unintended effects on both cancerous and normal cells (10).

Therefore, researchers and doctors must enhance alternative therapeutic methods to treat ovarian cancer. For example, cisplatin is a representative medication widely used in the treatment of many cancers, especially ovarian cancer (11). While it suppresses ovarian tumors to some extent, it inevitably involves various adverse effects, such as tumor recurrence and drug resistance (12). Therefore, in the treatment of ovarian cancer, it is essential to develop an effective treatment that minimizes side effects.

Standard chemotherapy for ovarian cancer is a vital component in the management of this aggressive disease, particularly in advanced stages. The standard treatment regimens often involve a combination of carboplatin, a kind of platinum-based agent, and paclitaxel, a kind of taxane, which have been shown to improve survival and quality of life outcomes for patients (13). Chemotherapy is typically administered after surgical tumor removal, functioning as adjuvant therapy to eradicate residual cancer cells. In cases where surgery is not feasible, chemotherapy can serve as neoadjuvant treatment to shrink tumors before surgery (14). Despite the effectiveness of these regimens, around 85% of patients experience recurrence, leading to a significant challenge in long-term management due to the development of resistance to standard treatments (15). This has encouraged ongoing research into novel therapies and maintenance strategies, including the use of poly (ADP-ribose) polymerase (PARP) inhibitors and other targeted treatments that aim to enhance patient outcomes (16). As chemotherapy remains a basis of ovarian cancer treatment, developing an alternative option and understanding its working mechanism are essential for optimizing patient outcomes and quality of life.

Linifanib is a small molecule that acts as a multi-targeted receptor tyrosine kinase inhibitor (17,18). Especially, it is a vascular endothelial growth factor receptor (VEGFR) kinase inhibitor and platelet-derived growth factor receptor (PDGFR) inhibitor designed to suppress tumor progression by preventing the formation of new blood vessels that supply the tumor with oxygen and nutrients and by inhibiting major angiogenic signaling pathways (19). Linifanib is developed to treat hematologic malignancies and solid tumors (20,21). Linifanib has the potential to be an efficient reagent for cancer treatment; however, the effects of linifanib on cancer development have not been sufficiently investigated (22). Since the effect of linifanib on human ovarian cancer has not been studied, we investigated the anti-carcinogenic effect of linifanib on human ovarian cancer OVCAR3 cells in this study.

This study aimed to elucidate the mechanisms by which linifanib induces cell death in ovarian cancer cells. To achieve this, we conducted a series of experiments to investigate the signaling pathways involved in cancer cell apoptosis. Our findings demonstrated that linifanib induces apoptotic cell death, causes cell cycle arrest, and activates the tumor suppressor protein forkhead box O3 (FOXO3) in human ovarian cancer OVCAR3 cells. Based on these results, we propose that linifanib may promote apoptotic responses by downregulating specific genes that promote cell survival and upregulating pro-apoptotic genes. The biological and pathological significance of this novel mechanism of linifanib in the treatment of ovarian cancer is presented in the following sections.

MATERIALS AND METHODS

Chemical reagents and antibodies

Linifanib was purchased from Selleckchem (Houston, TX, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). A concentration of 40 mM stock solution of linifanib was stored at -80 ℃. Mouse anti-ß-actin (1:5000 dilution) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-Akt (1:1000 dilution), rabbit anti-pAkt (S473) (1:1000 dilution), rabbit anti-FOXO3 (1:1000 dilution), rabbit anti-cleaved PARP (1:1000 dilution), rabbit anti-PARP (1:1000 dilution), rabbit anti-cleaved caspase-3 (1:1000 dilution), rabbit anti-caspase-3 (1:1000 dilution), rabbit

anti-Bax (1:1000 dilution), and rabbit anti-Bcl2 (1:1000 dilution) were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

OVCAR3 cells were obtained from the Korean cell line bank (Seoul, Korea) and maintained under standard culture conditions in a humidified incubator at 37 ℃ with 5% CO_2. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone, Logan, UT, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS; GW Vitek, Seoul, Korea) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Grand Island, NY, USA). The culture medium was refreshed one day before subculturing to ensure optimal cellular physiology. Subculturing was performed every three days or when cells reached 80-90% confluency, using 0.25% trypsin-EDTA solution (Gibco, USA). For experimental consistency, all assays were conducted using cells between passages 5 and 10.

MTT assay

OVCAR3 cells were seeded in 96-well plates at a density of 3×10^3 cells per well and incubated at 37 ℃ in a humidified incubator containing 5% CO_2 in air for 24 h. Subsequently, the cells were treated with various concentrations of linifanib (0, 10, 20, and 40 μM). After 24, 48, and 72 h of treatment, 20 μL of MTT dye (5 mg/mL) was added to each well, and the cells were incubated for 3 h at 37 ℃. The supernatants were removed, and the cells were treated with DMSO to form formazan. The plates were incubated in a shaker at room temperature (25 ℃) for 30 min. After that, the absorbance was measured at 570 nm using a microreader (Molecular Devices, Mountain View, CA, USA).

Cell counting assay

OVCAR3 cells were seeded in 6-well plates at a density of 5×10^4 cells per well and incubated at 37 ℃ in a humidified incubator containing 5% CO_2 in air for 24 h. Subsequently, the cells were treated with various concentrations of linifanib (0, 10, 20, and 40 μM). After 24, 48, and 72 h of treatment, the cells were stained using 0.4% trypan blue solution (Sigma-Aldrich). Cell numbers were counted using a hemocytometer.

Morphological analysis

To assess the morphological characteristics associated with linifanib-induced cellular stress and apoptosis, OVCAR3 cells were seeded in 12-well plates at a density of 2×10^4 cells per well and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were subsequently treated with linifanib at concentrations of 0, 10, 20, and 40 μM for 24, 48, and 72 h. Cell morphology was observed using a phase-contrast microscope (CKX53, Olympus, Tokyo, Japan) at a magnification of 40×. Representative images were captured to document morphological changes such as cell shrinkage, membrane blebbing, and formation of apoptotic bodies (hallmarks indicative of apoptotic cell death). This qualitative analysis provided preliminary visual evidence of cytotoxic effects and apoptosis progression in response to linifanib treatment.

Colony formation assay

OVCAR3 cells were seeded in 6-well plates at a density of 5×10^2 cells per well and incubated at 37 ℃ in a humidified incubator containing 5% CO_2 in air for 24 h. Subsequently, the cells were treated to various concentrations of linifanib (0, 10, 20, and 40 μM) for 24 h, and then the media was replaced with fresh media. After 14 days of treatment, the cells were fixed with 4% formaldehyde (Sigma-Aldrich) for 30 min at 4 ℃ and stained with 1% crystal violet (Sigma-Aldrich) solution for 30 min. Colony numbers were counted.

Cell cycle analysis

OVCAR3 cells were seeded in 6-well plates at a density of 2×10^5 cells per well and incubated at 37 ℃ in a humidified incubator containing 5% CO_2 in air for 24 h. Subsequently, the cells were treated with various concentrations of linifanib (0, 10, 20, and 40 μM). After 24 and 48 h of treatment, the cells were harvested with trypsinization and fixed with ice-cold 70% ethanol overnight. Subsequently, the cells were centrifuged at 1350 rpm for 5 min and stained with 50 μg/mL

of propidium iodide (PI) and 200 µg/mL of RNase in PBS at 37 ℃ for 30 min. The cell cycle was analyzed using a flow cytometer (Beckman Coulter, Brea, CA, USA).

Annexin V/PI staining assay

The percentage of apoptotic cells was measured using the fluorescein isothiocyanate (FITC) annexin V apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA). OVCAR3 cells were seeded in 6-well plates at a density of 2×10^5 cells per well and incubated at 37 ℃ in a humidified incubator containing 5% CO_2 in air for 24 h. Subsequently, the cells were treated with various concentrations of linifanib (0, 10, 20, and 40 µM). After 24 and 48 h of treatment, the cells were harvested with trypsinization and suspended in 1× binding buffer. Then, the cells were stained with 5 µL of FITC-labeled annexin V and 50 µg/mL of PI in binding buffer at room temperature (25 ℃) in the dark for 10 min. The cell population was analyzed using a flow cytometer (Beckman Coulter, Brea, CA, USA).

TUNEL assay

OVCAR3 cells were seeded in 6-well plates at a density of 2×10^5 cells per well and incubated at 37 ℃ in a humidified incubator containing 5% CO_2 in air for 24 h. Subsequently, the cells were treated with various concentrations of linifanib (0, 10, 20, and 40 µM). After 24 and 48 h of treatment, the cells were fixed with 4% formaldehyde solution at 4 ℃ for 30 min and permeabilized with 0.2% Triton X-100. The cells were treated with 50 µL terminal deoxynucleotidyl transferase (TdT) enzyme mixture (equilibration buffer 45 µL, nucleotide mix 5 µL, and TdT enzyme 1 µL). The nuclei of cells were stained with Hoechst 33342 stain solution (Sigma-Aldrich). DNA fragmentations were detected by fluorescence microscope (Nikon Eclipse TE 2000-U, Tokyo, Japan).

Western blot analysis

Western blotting was performed to evaluate the expression of apoptosis-related proteins following linifanib treatment. OVCAR3 cells were seeded in 6-well plates at a density of 3×10^5 cells/well and incubated at 37 ℃ in a humidified incubator containing 5% CO_2 in air for 24 h. Subsequently, the cells were treated with various concentrations of linifanib (0, 10, 20, and 40 µM). After 24 h of treatment, the whole cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Danvers, MA, USA) containing protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined using a Bradford protein assay. Equal amounts of protein (10 µg) were loaded per lane. Then, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 2 h and transferred to methanol-activated polyvinylidene fluoride (PVDF; Sigma-Aldrich) membrane at 100 V (4 ℃) for 1 h. After that, membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich) at room temperature for 1 h and incubated with the specific primary antibodies at room temperature for 1 h. The membranes were incubated with an anti-rabbit or mouse secondary antibody (1:10000 dilution, Sigma-Aldrich). Chemiluminescence was detected using enhanced chemiluminescence solution (GE Healthcare, Chicago, IL, United States) and a Chemi-doc detection system (Bio-Rad, Hercules, CA, USA). Band intensities were quantified using ImageJ software (NIH, USA) and normalized to ß-actin to obtain relative protein expression levels.

Statistical analysis

The results are expressed as mean ± SEM. To compare the statistical significance between the groups, Student's T-test was used for statistical analysis, and a P-value < 0.05 was considered statistically significant. Experiments were repeated three times.

RESULTS

The effect of linifanib on the proliferation of OVCAR3 cells

The MTT and cell counting assays were carried out to identify the anti-proliferative effect of linifanib in OVCAR3. As shown in Fig. 1A, the viability of OVCAR3 cells decreased after treatment with linifanib in a concentration and time-dependent manner. The viability of

cells treated with 0, 10, 20, and 40 μM of linifanib was 100, 87.3, 77.4, and 69.1% for 24 h, 100, 78.9, 68.3, and 62.9% for 48 h, and 100, 64.2, 52.6, and 48.3% for 72 h. The cell counting assay results confirmed the anti-proliferation ability of linifanib (0, 10, 20, and 40 μM) in OVCAR3 (Fig. 1B). Especially, the cell number in OVCAR3 cells treated with linifanib (40 μM) was significantly lower than that of the control at 72-h incubation time. We also observed the more visible anti-proliferative effect of linifanib through cell morphological alteration and colony formation assay. As shown in Fig. 1C, the cell density decreased as linifanib concentration increased. Colony formation assay was also performed using the same treatment condition of linifanib against OVCAR3 cells. The number of colonies increased to 140 over 2 weeks in the control group (Fig. 1D and E). On the other hand, it was observed that colonies were barely visible with 10, 20, and 40 μM of linifanib. Taken together, these results suggested that linifanib inhibited cell proliferation against OVCAR3 cells in a concentration and time-dependent manner.

The effect of linifanib on the OVCAR3 cell cycle

The sub-G1 cell population refers to cells that have undergone significant DNA fragmentation and loss, a hallmark of apoptosis. These cells appear in the sub-G1 region of a DNA content histogram, which is to the left of the G0/G1 peak, indicating they have a reduced DNA content due to the extraction of fragmented DNA oligomers. To examine whether treatment with linifanib affects cell cycle distribution in OVCAR3 cells, we performed cell cycle analysis using flow cytometry. OVCAR3 cells were treated with various concentrations of linifanib (0, 10, 20, and 40 μM) for 24 and 48 h, and the cell population was analyzed. We could find the increased sub-G1 population after treating cells with linifanib in a concentration- and time-dependent manner. As shown in Fig. 2, the sub-G1 population of OVCAR3 cells was increased, followed by treatment with various concentrations of linifanib. Thus, our results suggest that linifanib induced sub-G1 cell cycle arrest in OVCAR3 cells.

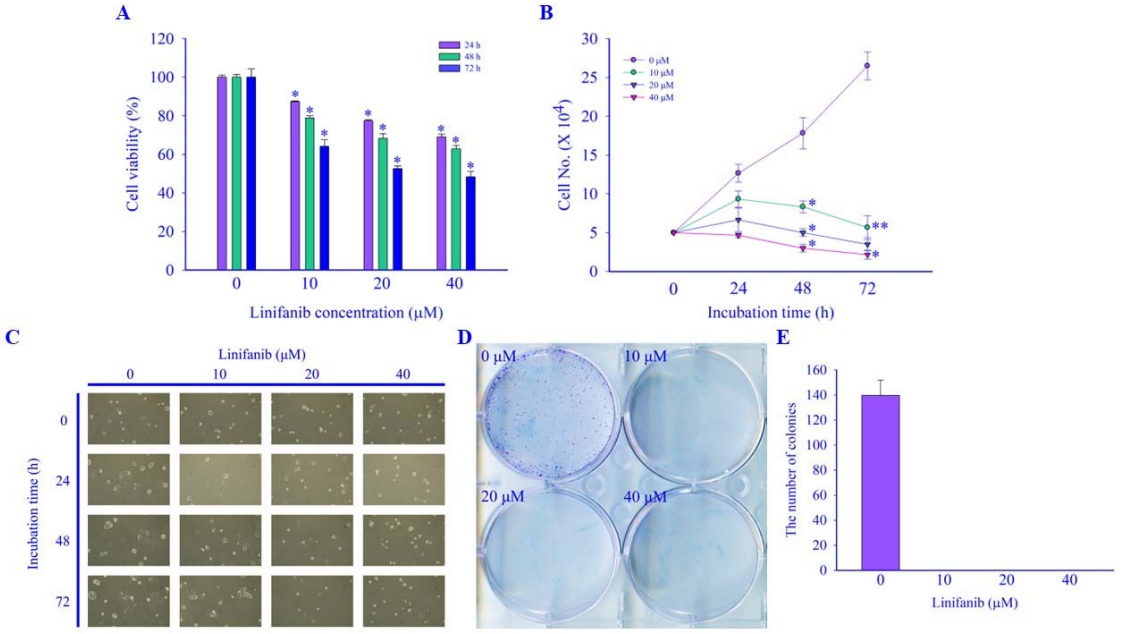

Fig. 1. Anti-proliferative effects of linifanib in OVCAR3 cells. Concentration- and time-dependent effects of linifanib (0, 10, 20, and 40 μM) in OVCAR3 cells after linifanib treatment for 24, 48, and 72 h. (A) The cell viability was examined using the MTT assay. (B) Cell counting assay after treatment with linifanib. (C) Morphological alteration pictures of OVCAR3 cells treated with linifanib. (D) Colony formation assay after treatment with linifanib. (E) Quantification of colony inhibition is shown in the bar graph. Data are expressed as mean ± SEM. *P < 0.05 and **P < 0.01 indicate significant differences compared to the control group (0 concentration of linifanib).

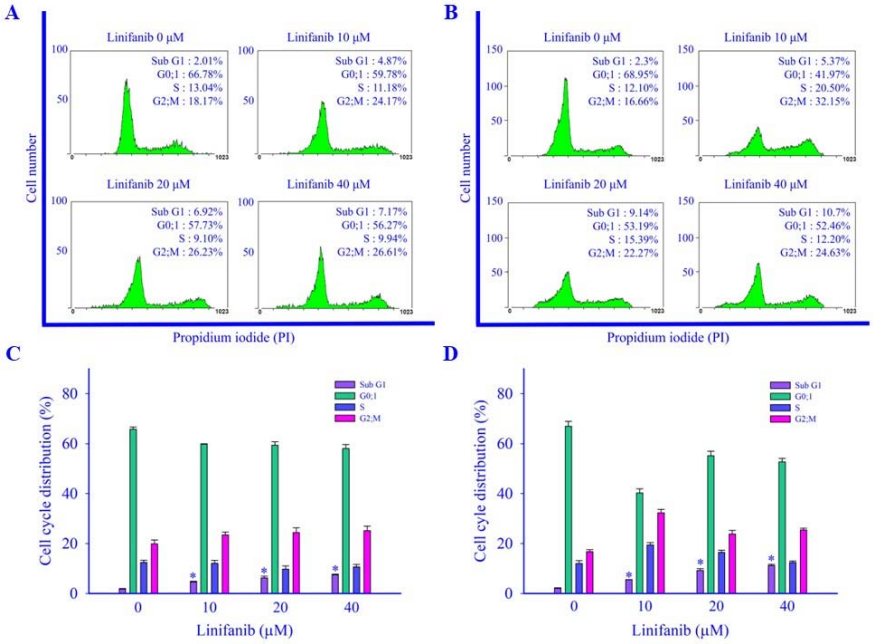

Fig. 2. Cell cycle distribution of OVCAR3 cells after linifanib treatment. Flow cytometry analysis showing changes in cell cycle phases, including increased Sub-G1 population, after treatment with various concentrations of linifanib for 24 h and 48 h. (A and B) Representative cytograms; (C and D) quantification of cell cycle distribution. Data are expressed as mean ± SEM. **P* < 0.05 indicates significant differences compared to the respective control group (0 concentration of linifanib).

The effect of linifanib on OVCAR3 cell apoptosis

It was investigated whether treatment with linifanib induces apoptotic cell death in OVCAR3 cells. The annexin V staining method for detecting apoptosis works by exploiting the early event of phosphatidylserine externalization from the inner to the outer leaflet of the plasma membrane, a hallmark of apoptotic cells. Annexin V, which has a strong Ca^{2+}-dependent affinity for phosphatidylserine, binds to these exposed phosphatidylserine residues, allowing for the detection of apoptotic cells through flow cytometry or fluorescence microscopy. Annexin V/PI assay results showed that apoptotic cell death rates were increased after treatment with linifanib in a concentration- and time-dependent manner (Fig. 3). The total apoptotic cell rate of OVCAR3 cells treated with 0, 10, 20, and 40 μM of linifanib was 2.6, 6.3, 6.5, and 7.2% for 24 h and 1.6, 8.7, 11.2, and 13.4% for 48 h, respectively. These results elucidate that linifanib induced apoptosis in OVCAR3 cells.

The effect of linifanib on DNA fragmentation in OVCAR3 cells

The TUNEL assay is a widely used method for detecting DNA fragmentation, a hallmark of apoptotic cell death, by labeling the 3'-hydroxyl termini of DNA breaks, and it is used as a reliable marker to identify and quantify apoptotic cells. To investigate DNA fragmentation in OVCAR3 cells after treatment with linifanib, the TUNEL assay was adopted. As shown in Fig. 4A-D, OVCAR3 cells were treated with various concentrations of linifanib (0, 10, 20, and 40 μM) for 24 h and 48 h. TUNEL-positive cells increased in a concentration- and time-dependent manner. After treatment with 0, 10, 20, and 40 μM of linifanib, the percentage of TUNEL-positive cells of OVCAR3 was 0, 0, 1, and 4% for 24 h and 0, 1.5, 3, and 11.7% for 48 h. These overall results suggest that linifanib promotes DNA fragmentation in OVCAR3 cells, which is a downstream phenomenon of apoptosis.

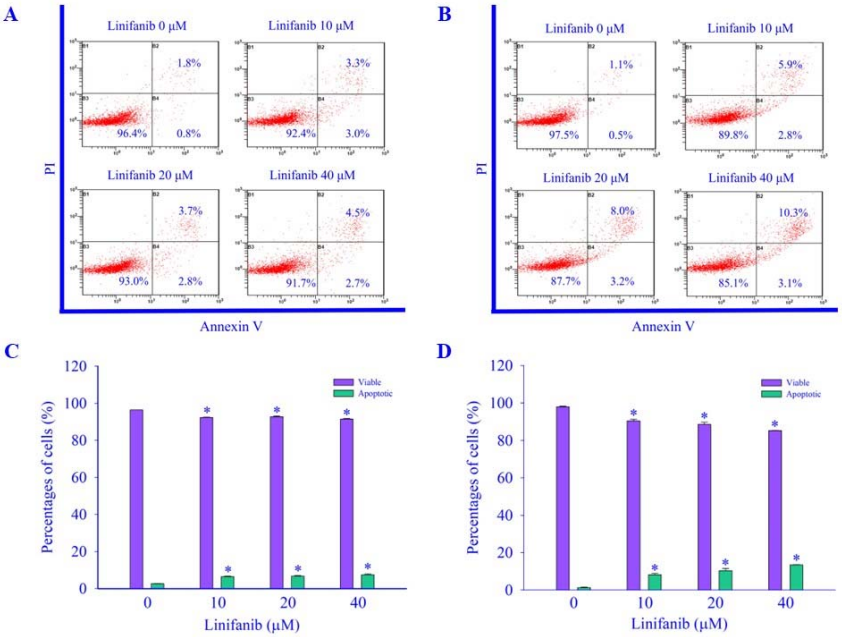

Fig. 3. Annexin V/ propidium iodide staining results following 24 h and 48 h of treatment with linifanib (0, 10, 20, and 40 μM) in OVCAR3 cells. The percentages of apoptotic cells against (A and B) OVCAR3 cells are shown in the cytogram. (C and D) Percentages of viable and apoptotic cells were indicated in the bar graphs. Data are expressed as mean ± SEM. **P* < 0.05 indicates significant differences compared to the respective control group (0 concentration of linifanib).

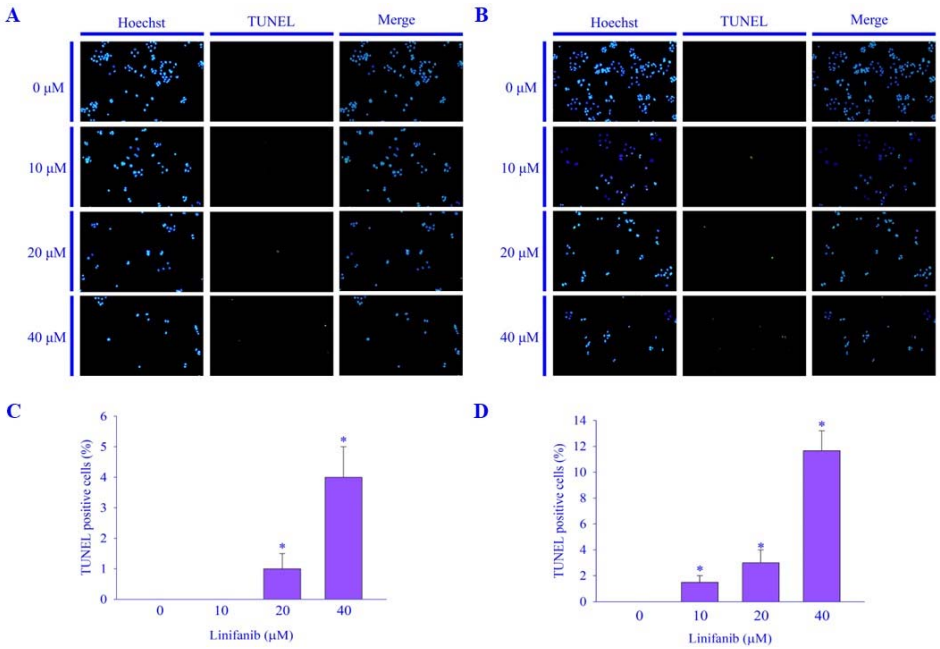

Fig. 4. TUNEL assay results of OVCAR3 cells treated with linifanib (0, 10, 20, and 40 μM) for 24 h and 48 h. DNA fragmentation was shown by fluorescence microscopy. (A and B) Blue fluorescence represents the nuclei stained using Hoechst 33342, and green fluorescence indicates the TUNEL-positive cells. (C and D) The bar graph showed the percentages of TUNEL-positive cells after linifanib treatment. Data are expressed as mean ± SEM. **P* < 0.05 indicates significant differences compared to the respective control group (0 concentration of linifanib).

The effect of linifanib on apoptotic signaling pathways in OVCAR3 cells

To elucidate the apoptotic effect of linifanib in OVCAR3 cells, Western blot analysis was performed and revealed the expression levels of proteins related to cancer cell apoptosis after treatment with 0, 10, 20, and 40 μM of linifanib. Cleaved caspase-3 is a key indicator of apoptosis, as it is the active form of Caspase-3, which is the primary executioner caspase responsible for the proteolytic cleavage of various cellular substrates. PARP, when cleaved by caspase-3, is another marker of apoptosis. PARP is involved in DNA repair, but during apoptosis, its cleavage by caspase-3 prevents it from performing its repair functions, contributing to the irreversible cell death process. As shown in Fig. 5, cleaved caspase-3 and cleaved PARP expression levels increased after treatment with linifanib. However, the expression levels of the pro-form (caspase-3 and PARP) decreased after linifanib treatment. The expression of Bax and Bcl-2 in mitochondria plays a crucial role in regulating apoptosis, which is the process of programmed cell death. The expression level of pro-apoptotic protein, such as Bax, increased, but anti-apoptotic protein, such as Bcl-2, decreased after linifanib treatment. Additionally, the expression level of FOXO3 protein, which is known as a tumor suppressive transcriptional factor, increased after treatment with linifanib. Then, we demonstrated that the expression level of phosphorylated Akt (pAkt (Ser473)) was downregulated after treatment with linifanib. Total Akt expression level was constant regardless of linifanib treatment. Phosphorylated (activated) Akt phosphorylates FOXO3, leading to its cytoplasmic retention and inactivation *via* proteasome-mediated degradation, thereby preventing FOXO3 from entering the nucleus and performing its tumor-suppressive functions, such as inducing apoptosis and cell cycle arrest. Therefore, these results elucidate linifanib-stimulated apoptotic signaling pathways such as Akt/FOXO3 in OVCAR3 cells.

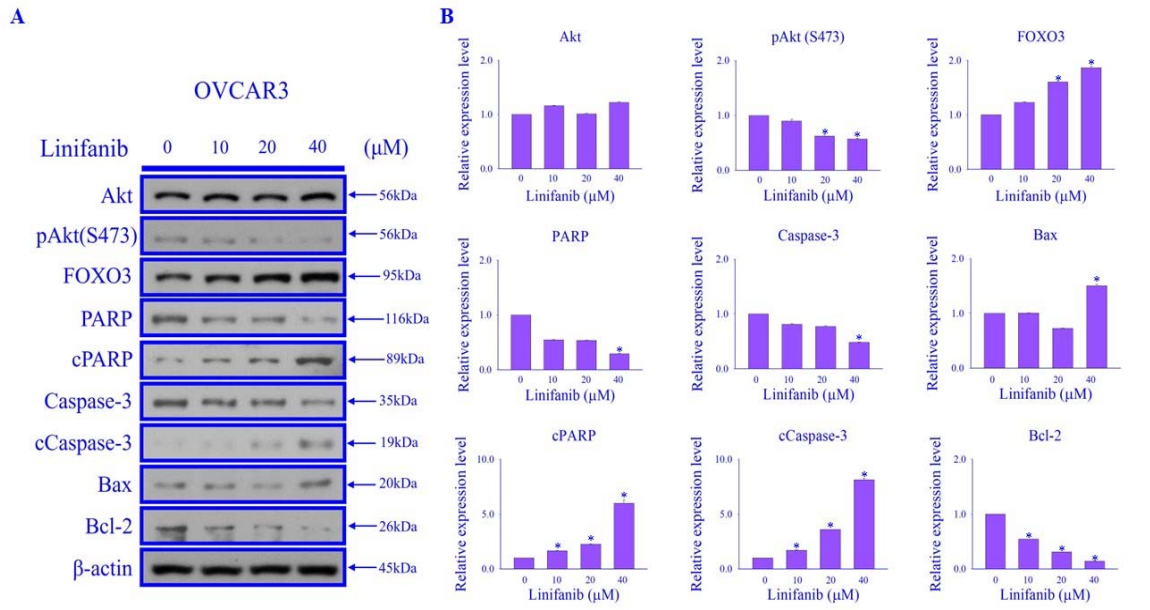

Fig. 5. (A) Western blot assay of OVCAR3 cells after linifanib treatment (0, 10, 20, and 40 μM) for 24 h. (B) The relative expression levels of specific proteins associated with apoptosis were analyzed by Western blot assay. Data are expressed as mean ± SEM. **P* < 0.05 indicates significant differences compared to the respective control group (0 concentration of linifanib). ß-actin was used as a loading control protein. cCaspase-3, cleaved capsase-3; cPARP, cleaved poly (ADP-ribose) polymerase, FOXO3, Forkhead box O3.

DISCUSSION

In this study, we investigated linifanib, also known as ABT-869, which displays anticancer effects in ovarian cancer cells. Linifanib (ABT-869) is a structurally novel and potent inhibitor of receptor tyrosine kinases, specifically targeting VEGF and PDGF receptors. It is designed to suppress tumor growth by preventing the formation of new blood vessels that supply the tumor with oxygen and nutrients, and it also inhibits key angiogenic signaling pathways such as the VEGFR and PDGFR pathways (23). Recently, many reports have been published on the effectiveness of VEGFR/PDGFR inhibitors in treating cancers and other diseases with poor prognosis. Clinically, a VEGFR inhibitor, bevacizumab, enhanced the duration of progression-free survival in patients with ovarian cancer (24). Meanwhile, in addition to clinical data, numerous reports have been published on nonclinical study results of the VEGFR inhibitor linifanib. Linifanib is reported to be used as one of the chemical cocktails to reverse cellular aging. In particular, the drug restores the separation function between the nucleus and cytoplasm of the cells and plays a role in reversing the expression of aged genes in the cell to a young state (25). It is reported that linifanib induces apoptosis in acute monocytic leukemia MV4-11 cells. Linifanib, when administered orally, inhibits VEGF-induced edema and angiogenesis and demonstrates potent anti-tumor activity in various cancer models, including breast, fibrosarcoma, colon, and small cell lung carcinoma, leading to tumor size reduction and regression (26). Therefore, we tried to demonstrate how linifanib would be more effective in treating ovarian cancer.

Linifanib also showed therapeutic and apoptotic effects in cancers of various human tissues. It was reported that linifanib, when combined with veliparib, has anticancer effects by reducing cell proliferation in head and neck squamous cell carcinoma, regulating the apoptosis marker cleaved PARP, and enhancing DNA double-strand breaks to increase γ-H2AX (27). It was reported that the combination treatment of linifanib and irinotecan, an anticancer drug that is a topoisomerase I inhibitor, significantly improved survival in mice with anaplastic thyroid cancer (28). Furthermore, it was confirmed that linifanib is efficacious in the xenograft model of human non-small cell lung cancer, and F-fluorodeoxyglucose-positron emission tomography is a potential pharmacodynamic biomarker strategy for linifanib therapy (29). Particularly, linifanib induces apoptosis in human ovarian cancer SKOV3 cells *via* activation of FOXO3 and reactive oxygen species (30). Thus, numerous studies have shown that linifanib triggers apoptosis in various cancers by engaging different cell signaling pathways and mechanisms. Building on these findings, we conducted research demonstrating that linifanib also exhibits anticancer effects in ovarian cancer OVCAR3 cells. Linifanib is a multi-targeted tyrosine kinase inhibitor with anti-angiogenic activity *via* VEGFR and PDGFR inhibition. However, its clinical use has been associated with adverse effects such as hypertension and proteinuria (31,32). To mitigate these toxicities, optimized dosing strategies, such as combination with standard chemotherapeutics or administration at lower concentrations, may be required in future therapeutic applications.

The use of OVCAR3 cells provides a valuable platform for the development of therapeutic agents targeting ovarian cancer. OVCAR3 cells are widely recognized as a representative model of high-grade serous ovarian cancer, the most common and aggressive subtype of ovarian cancer. This makes them highly relevant for studying the biology and treatment of this disease (33). OVCAR3 cells have been extensively characterized through various studies, including bioinformatics, cytotoxicity assays, and molecular/functional analyses. This thorough characterization provides a solid foundation for understanding their genetic and molecular profiles, which is crucial for drug development (33). OVCAR3 cells exhibit drug resistance to certain chemotherapeutic agents, such as cyclophosphamide, cisplatin, and doxorubicin, which is typical of many ovarian cancer cases (34). However, they also show sensitivity to other agents such as carboplatin

and PARP inhibitors (*e.g.*, rucaparib), making them useful for studying mechanisms of resistance and sensitivity (35). Studies using OVCAR3 cells have provided insights into apoptosis and cell death mechanisms. For example, these cells can be induced to undergo apoptosis through various treatments, such as combinations of all-trans retinoic acid (ATRA) and zoledronic acid, which helps in understanding the pathways involved in cell death (35). The use of OVCAR3 cells in ovarian cancer drug development offers a well-characterized, relevant, and versatile model that can provide valuable insights into the biology of the disease and the efficacy of potential therapeutic agents.

In this study, we not only confirmed linifanib's anti-proliferative and pro-apoptotic effects but also investigated the molecular mechanisms responsible for these effects, focusing on the Akt/FOXO3 signaling pathway. FOXO3 is a critical transcription factor that regulates genes involved in cell survival, apoptosis, and cell cycle arrest (36). It has been shown to upregulate the expression of pro-apoptotic and cell cycle-inhibitory proteins, such as p21, p27, and Bim, while its activity is negatively regulated by Akt phosphorylation (37). Akt is a serine/threonine kinase that promotes cell survival; when phosphorylated (*e.g.*, at Ser473), it inactivates FOXO3 by sequestering it in the cytoplasm. Our study demonstrated that linifanib treatment led to a dose-dependent decrease in phosphorylated Akt (Ser473) and a corresponding increase in FOXO3 protein levels. These results suggest that linifanib induces apoptosis in OVCAR3 cells through inhibition of the Akt pathway and subsequent activation of FOXO3. Further supporting this mechanism, we observed that linifanib treatment increased the expression of pro-apoptotic proteins such as Bax and decreased anti-apoptotic Bcl-2 levels. These changes indicate a shift in the balance toward apoptosis and further support the involvement of FOXO3-mediated transcriptional regulation. Additionally, several other studies have been done. MicroRNA-148a has also been identified to exert tumor-suppressive effects *via* the regulation of FOXO3 in ovarian cancer cell lines (40). Finally, linifanib has been investigated across various solid tumors, such as non-small cell lung cancer, breast cancer, liver cancer, and colorectal cancer. It suppresses tumor progression by inhibiting cell proliferation, inducing apoptosis, and disrupting intracellular signaling pathways, including AKT/mTOR and MEK/ERK (20,41).

Taken together, our findings provide novel evidence that linifanib inhibited ovarian cancer cell growth through both extracellular (VEGFR/PDGFR) and intracellular (Akt/FOXO3) mechanisms. This dual mode of action enhances its potential as a therapeutic agent in ovarian cancer, particularly in drug-resistant settings. Future studies could investigate its therapeutic potential using *in vivo* models and evaluate its synergistic effects when combined with existing chemotherapeutic or targeted agents. In our future studies, we plan to evaluate the combinatorial efficacy of linifanib with clinically approved PARP inhibitors such as olaparib, niraparib, and rucaparib. Through these investigations, we aim to propose a novel therapeutic strategy that could enhance treatment outcomes and expand the current options available for ovarian cancer management.

CONCLUSION

Overall, we have investigated the effects of linifanib on ovarian cancer cells and found that it inhibits cell proliferation, induces cell cycle arrest and DNA fragmentation, and triggers apoptosis. Through Western blot analysis, we have explored the mechanisms of cell death related to apoptosis. Thus, we have established substantial evidence that linifanib exerts anticancer effects on ovarian cancer *in vitro*. However, further investigations into metastasis and the detailed working mechanisms could provide more robust data. Additionally, it may be beneficial to expand our research by including another ovarian cancer cell line. Conducting xenograft mouse studies could further solidify our understanding of linifanib's therapeutic efficacy. Taken together, these approaches may enhance the evidence supporting linifanib as a potential treatment for ovarian cancer.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

H.K. Lee and J. Lee equally contributed to the conceptualization, investigation, and writing of the original draft. M.J. Nam and H.G. Koh contributed to the conceptualization and investigation. K. Park contributed to the conceptualization, investigation, writing, review, and editing of the article. K.Y. Jang and S.H. Park contributed to the investigation, supervision, writing, review, and editing of the article. The finalized article was read and approved by all authors.

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