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YYB-101, a Humanized Antihepatocyte Growth Factor Monoclonal Antibody, Inhibits Ovarian Cancer Cell Motility and Proliferation

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Abstract. *Background/Aim: Hepatocyte growth factor (HGF) acts as a key regulator in promoting ovarian cancer metastasis. Previously, we observed that YYB-101, a humanized anti-HGF antibody, effectively inhibits ovarian cancer cell migration, invasion, and progression. Here, we evaluated the signaling mechanisms affected by YYB-101 that are important in ovarian cancer cell progression. Materials and Methods: Using cell migration, invasion and proliferation assays, we evaluated the effects of YYB-101 on A2780/luc and SKOV3 cells. The effects of YYB-101 on signaling molecules were determined by immunocytochemistry and immunoblot analysis. Results: YYB-101 inhibited HGF-induced ovarian cancer cell motility by down-regulating paxillin phosphorylation and actin-cytoskeleton rearrangement. Also, YYB-101 inhibited ovarian cancer cell proliferation by reducing c-MET phosphorylation and activating apoptosis in vitro and in vivo. These effects were significantly enhanced by combining YYB-101 treatment with paclitaxel, a standard chemotherapy drug. Conclusion: YYB-101 can be examined as a new therapeutic agent for the treatment of patients with ovarian cancer.*

Ovarian cancer (OC) is one of the top three life-threatening cancers in women, in addition to cervical cancer and breast cancer. Also, OC is the fifth leading cause of women cancer-related deaths in the United States and has the highest mortality rate among gynecological diseases. OC statistics for the year 2020 predict that 13,940 women will die from OC and 21,750 will be newly diagnosed (1). The OC five-year survival rate varies by stage. The survival rate for stage I or II is more than 60%, but the survival rate for advanced stage (stage III or IV) is less than 29% (2). In addition, the OC recurrence rate is more than 80%, and recurrent OC is difficult to treat because they often exhibit chemotherapy resistance. Since there is no effective early screening test for OC and there are no symptoms, most patients are initially diagnosed with advanced OC that spread to the peritoneal cavity and to other organs. Cancer cell peritoneal dissemination leads to an increase in ascites, resulting in high-grade carcinoma progression that is associated with poor prognosis (3-5).

Hepatocyte growth factor (HGF), also called scatter factor, is a multifunctional heterodimer polypeptide produced by mesenchymal stem cells (6, 7). HGF binds its receptor, the MET transmembrane tyrosine kinase (c-MET), and attenuates cell growth, motility, and survival. In particular, HGF is over-expressed in various cancer tissues and is related to tumor development and prognosis (8, 9). The HGF/c-MET signaling pathway can influence epithelial-mesenchymal transition (EMT) by stimulating cancer cell migration from the primary tumor site to metastatic sites. HGF is also secreted by cancer-associated fibroblasts located in the tumor microenvironment and stimulates various biological effects in cancer cells through paracrine signaling (10, 11). In addition, HGF levels are increased in the patients with OC ascites, causing enhanced cancer cell motility and peritoneal dissemination (12, 13). Therefore, HGF-mediated signaling inhibitors are expected to be effective anticancer agents. Foretinib, DCC-2701, and PF-2341066, are c-MET inhibitors that have been shown to exhibit anticancer activity in an OC xenograft model (14-16). Additionally, we have observed that YYB-101, a humanized neutralizing antibody that targets HGF, has anticancer activity against colorectal cancer and glioblastoma (17, 18). We also demonstrated YYB-101 effectively inhibits OC cell migration and invasion. Moreover, combination treatment with paclitaxel and YYB-101 has been shown to significantly reduce tumor growth and increase survival rate in an OC xenograft mouse model (19).

In the present study, we evaluated the YYB-101 effect on HGF-mediated signaling and biological processes. We validated that HGF-induced motility and proliferation were inhibited by

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YYB-101 through actin-cytoskeleton rearrangement, c-MET inactivation, and apoptosis activation *in vitro* and *in vivo*. These results suggest that YYB-101 inhibits OC progression by blocking c-MET signaling and inducing apoptosis.

Materials and Methods

Cell culture. The human epithelial ovarian adenocarcinoma cell lines SKOV3 and A2780 cells were obtained from KBCC (Korea Biotechnology Commercialization Center; Incheon, Republic of Korea) and Sigma-Aldrich (St. Louis, MO, USA), respectively. A2780 cells over-expressing firefly luciferase (A2780/luc cells) were generated as previously described (20). The cells were maintained in Roswell Park Memorial Institute (RPMI) medium (Thermo Fisher Scientific; Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific).

Cell migration, invasion, and wound-healing assay. Cell migration and invasion assays were performed as previously described (19). Briefly, to evaluate YYB-101 (CellabMED Inc.; Seoul, Republic of Korea) efficacy, 0.3 or 3 μM YYB-101 were added to 2% FBS culture medium in the presence or absence of 80 ng/ml rhHGF (recombinant human Hepatocyte Growth Factor; R&D Systems; Minneapolis, MN, USA) and loaded into the bottom chamber. For the migration assay, A2780/luc cells (3×10^5 cells per well) or SKOV3 cells (1×10^5 cells per well) were seeded into the upper chamber and maintained at 37˚C for 48 h (A2780/luc cells) or 8 h (SKOV3 cells). For invasion assays, each Transwell plate was coated with 1 mg/ml Matrigel matrix (BD Biosciences; San Jose, CA, USA). After incubating overnight, A2780/luc cells (2×10^5 cells per well) or SKOV3 cells (0.5×10^5 cells per well) were seeded into each upper chamber and maintained at 37˚C for 72 h. After incubation, medium containing cells was removed from the upper chamber. Chambers were stained using a Diff-Quick staining kit (Sysmex; Kobe, Japan) and migrated cells were observed using light microscopy. For the wound-healing assay, SKOV3 cells (1×10^4 cells per well) were seeded into 96-well culture plates. After incubation overnight, a scratch wound was created using an automated 96-pin wound maker (Essen Bioscience; Hertfordshire, UK). After wounding, cell debris was aspirated, cells were washed once with culture medium, and refed with 2% FBS medium containing 0.3 μM YYB-101, 1 pM paclitaxel, or 0.3 μM YYB-101 plus 1 pM paclitaxel. Triplicate wells were used for both conditions. The plate was incubated in an IncuCyte ZOOM incubator (Essen Bioscience) for 24 h and images were taken automatically every two h in the same field of each well. The relative wound density was calculated using IncuCyte Chemotaxis Software (Essen Bioscience).

Immunocytochemistry. A2780/luc cells (1×10^3 cells per well) were plated on Nunc® Lab-Tek® II chamber slides (Thermo Fisher Scientific). After 24 h, the culture medium was replaced using 2% FBS medium containing 0.3 μM YYB-101 or 1 pM paclitaxel for 24 h. Cells were then fixed with 4% formaldehyde for 10 min and washed twice with PBS. Cells were blocked using PBS containing 1% BSA and incubated with anti-phospho-paxillin antibody (Cell Signaling Technology; Danvers, MA, USA) overnight at 4˚C. After washing three times with PBS, cells were subsequently incubated with rabbit Alexa Fluor 488-labeled secondary antibody (Invitrogen; Waltham, MA, USA) and Rhodamine-phalloidin at room temperature for 1 h. The nuclei were stained using Hoechst 33342 for 5 min and cells were mounted with mounting solution. Stained cells were imaged with confocal microscope (LSM510, Carl Zeiss; Oberkochen, Germany).

Cell proliferation assay. A2780/luc cells (3×10^3 cells per well) or SKOV3 cells (1.5×10^3 cells per well) were seeded into a 96-well culture plate and incubated overnight. The medium was replaced with serum-free medium containing 0.3 μM YYB-101, 3 μM YYB-101, 1 pM PTX, or 0.3 μM YYB-101 plus 1 pM PTX. After 72 h, the medium was changed with serum-free medium, and 10 μl WST-1 solution (TAKARA; Kusatsu, Japan) was added to the culture medium and incubated for 1 h. The absorbance of each well was detected using a VersaMax microplate reader (Molecular Devices; San Jose, CA, USA) at 450 nm.

Immunoblot analysis. All antibodies used in immunoblot assays were purchased from Cell Signaling Technology, Inc.. A2780/luc cells (1×10^6 cells per well) were seeded into six-well plates and cultured in 2% FBS RPMI medium for 24 h. The medium was replaced with serum-free medium containing 3 μM hIgG, 1 pM PTX, 3 μM YYB-101, or 1 pM PTX plus 3 μM YYB-101. After 24 h, cells were lysed with SDS-sample buffer and subjected to western blot analysis using antibodies against c-MET, phospho-c-MET (Tyr1234/1235), GAB1, phospho-GAB1 (Tyr627), Src, phospho-Src (Tyr416), ERK 1/2, phospho-ERK 1/2 (Thr 202/Tyr 204), Bax, Caspase-3, Cleaved Caspase-3, or PARP or α-tubulin.

Orthotopic mouse model of ovarian cancer. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC, NCC-16-342) of the National Cancer Center, Republic of Korea (NCCRI). NCCRI is an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) accredited facility that abides to the Institute of Laboratory Animal Resources (ILAR) guide. Female BALB/C-nude mice (Orient Bio; Sungnam, Korea) were anesthetized using isoflurane, and the right lateral sides of their abdomens were incised. A2780/luc cells (1×10^5 cells in 10 μl) were implanted into the ovarian bursa, and the wound was sealed. One week after cell implantation, 10 mg/kg paclitaxel were administered once a week by intraperitoneal injection, or 40 mg/kg YYB-101 were administered by intravenous injection. 30 d later, the ovary tumor tissue was isolated and frozen with liquid nitrogen. The frozen tissue was ground with a mortar and pestle in the presence of liquid nitrogen, and the resulting tissue powder was subjected to protein extraction by RIPA buffer (Biosesang; Seongnam, Korea) containing a protease inhibitor cocktail (Sigma-Aldrich). After BCA protein assay, equal amounts of total protein (20 μg) were separated by SDS-PAGE and western blot analysis was performed using specific antibodies.

Results

YYB-101 inhibits HGF-induced motility in OC cells through cytoskeleton rearrangement. We first evaluated YYB-101 efficacy on HGF-induced A2780/luc and SKOV3 cell using the migration and invasion Transwell system. Both cells were treated with 80 ng/ml of HGF with or without 0.3 μM or 3 μM YYB-101. The migration of A2780/luc and SKOV3 cells was decreased following treatment with YYB-101

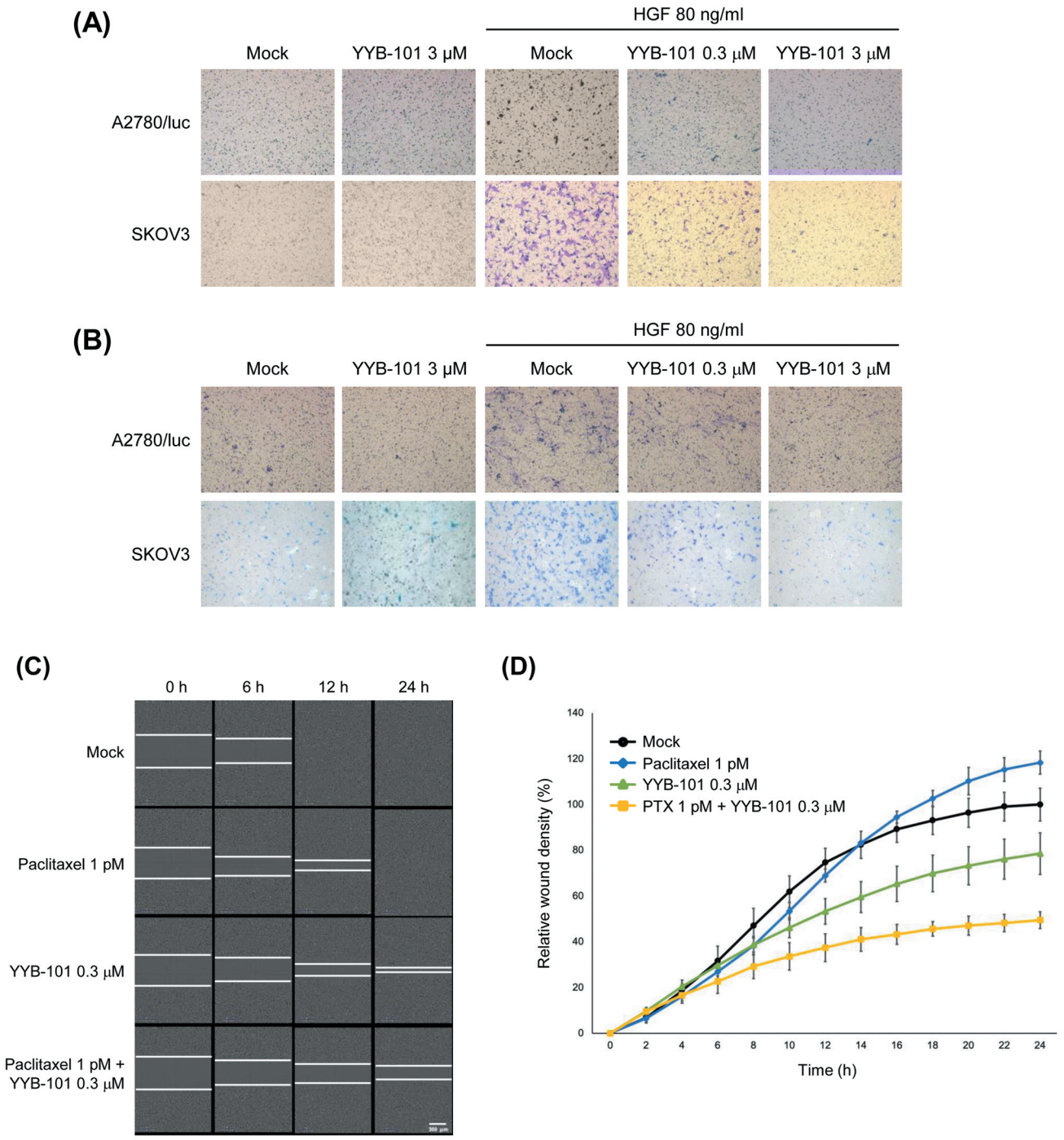

Figure 1. *The YYB-101 effect on HGF-induced motility and the synergistic effect of paclitaxel and YYB-101 on ovarian cancer cell migration. (A) A2780/luc cells (3×10^5) and SKOV3 cells (1×10^5) were loaded into the upper Transwell compartments, while the lower chambers contained 0.3 μM YYB-101, 80 ng/ml HGF, or 80 ng/ml HGF with 0.3 μM or 3 μM YYB-101. After incubation for 48 h (A2780/luc cells) or 8 h (SKOV3 cells), migrated cells were fixed, stained, and observed using light microscope (10×). (B) For invasion assay, A2780/luc cells (2×10^5) and SKOV3 cells (0.5×10^5) were loaded onto Matrigel-coated upper Transwell compartments. Lower chambers contained 0.3 μM YYB-101, 80 ng/mL HGF, or 80 ng/ml HGF with 0.3 μM or 3 μM YYB-101. After culturing for 72 h (A2780/luc cells) or 48 h (SKOV3 cells), invaded cells were fixed, stained, and observed using light microscope (10×). (C) SKOV3 (1×10^4) cells were seeded into 96-well culture plates. After incubation 24 h, the scratch wound was formed by a wound maker, and the cells were treated with 1 pM paclitaxel, 3 μM YYB-101, or 1 pM paclitaxel with 3 μM YYB-101, and images were taken at the indicated times in the same field of each well. (D) The relative wound density was recorded over time using the IncuCyte ZOOM live-cell imaging program.*

(Figure 1A). Similar to its effect on migration, YYB-101 inhibited cell invasiveness in both treatment conditions (Figure 1B).

Since paclitaxel is used as a first-generation chemotherapeutic drug for patients with advanced OC, we hypothesized that treatment with the combination of paclitaxel and YYB-101 could have a synergistic effect on inhibiting ovarian cancer cell motility. We evaluated the synergistic effect on the motility of OC cells using a wound-healing assay. Treatment with paclitaxel alone did not affect wound-healing rate, and treatment with YYB-101 showed a moderate effect. As predicted, treatment with the combination of paclitaxel and YYB-101 showed a synergistic effect in reducing SKOV3 cell migration (Figure 1C and D).

Next, we tested the YYB-101 effect on the structure of the cytoskeleton of OC cells by immunocytochemistry using anti-phospho-paxillin and F-actin staining. YYB-101 treatment inhibited paxillin phosphorylation and destabilization of F-actin in A2780/luc cells, whereas paclitaxel treatment did not (Figure 2). These results showed that YYB-101 inhibits OC cell motility by inhibiting cytoskeleton rearrangement.

YYB-101 inhibits OC cell proliferation in vitro. Next, we investigated the YYB-101 effect on the viability of OC cells using the WST-1 assay. Several studies have suggested that HGF is not associated with an increase in OC cell viability. As in previous studies, a single YYB-101 treatment did not affect A2780/luc or SKOV3 cell viability. However, the cell viability was significantly reduced following treatment with the combination of YYB-101 and paclitaxel (Figure 3A and B).

We observed changes in the c-MET signaling pathway in A2780/luc cells by immunoblot analysis. c-MET Tyr1234/1235 phosphorylation was decreased by YYB-101 treatment. In addition, the levels of phosphorylated GRB2-associated-binding protein 1 (GAB1), proto-oncogene tyrosine-protein kinase (Src), and extracellular signal-regulated kinase (ERK 1/2), which are downstream signaling molecules of c-MET, were decreased by YYB-101 treatment. Furthermore, treatment with the combination of paclitaxel and YYB-101 further reduced the phosphorylation of these signaling molecules. To verify whether YYB-101 treatment induces apoptosis, we assayed for the levels of Bax (bcl-2-like protein 4) as well as cleaved caspase-3 and poly (ADP-ribose) polymerase (PARP). Bax, a proapoptotic molecule, was induced when cells were treated with paclitaxel and YYB-101 alone or in combination. In addition, higher levels of cleaved caspase-3 were found after YYB-101 treatment than after paclitaxel, and cleaved PARP levels were increased after the combined treatment.

YYB-101 affects c-MET signaling and apoptosis in vivo. Previously, we have reported that YYB-101 inhibits OC growth in intraperitoneal and orthotopic mouse xenograft models. Here, we validated the signaling changes upon YYB-101 treatment in tumor tissue. We generated orthotopic xenograft mice and treated them with PBS, paclitaxel, YYB-101, or YYB-101 and paclitaxel. Thirty days after treatment, the ovary tumor tissues were extracted and the signaling molecules were analyzed by western blot. Similar to the *in vitro* experiment results, of c-MET, GAB1, Src, and ERK 1/2 phosphorylation were reduced and Bax, cleaved caspase-3, and cleaved PARP were increased by paclitaxel or YYB-101 treatment. These changes in the signaling molecules were even more increased by the paclitaxel and YYB-101 combination treatment (Figure 4).

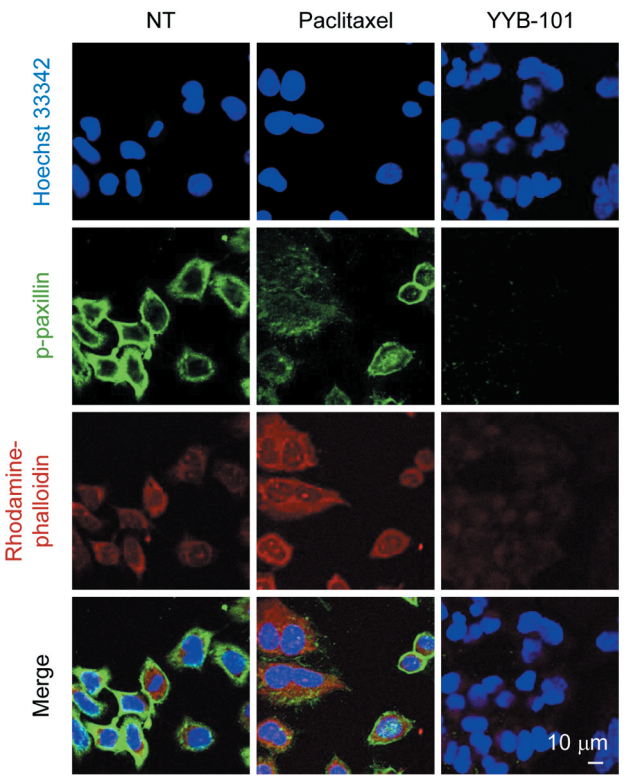

Figure 2. *The YYB-101 effect on cytoskeleton rearrangement in A2780/luc cells. A2780/luc cells (1×10^3) were loaded into chamber slides and treated with 1 pM paclitaxel or 0.3 μM YYB-101. 24 h later, the cells were fixed, stained with anti-phospho-paxillin antibody or rhodamine-phalloidin, and visualized by confocal microscopy. The nuclei were stained using Hoechst 33342. Scaled bar: 10 μm.*

Discussion

OC is primarily treated by cytoreductive surgery, and all patients except those with early-stage disease are treated with chemotherapy following surgery. The standard OC chemotherapy treatment includes platinum drugs (*e.g.*,

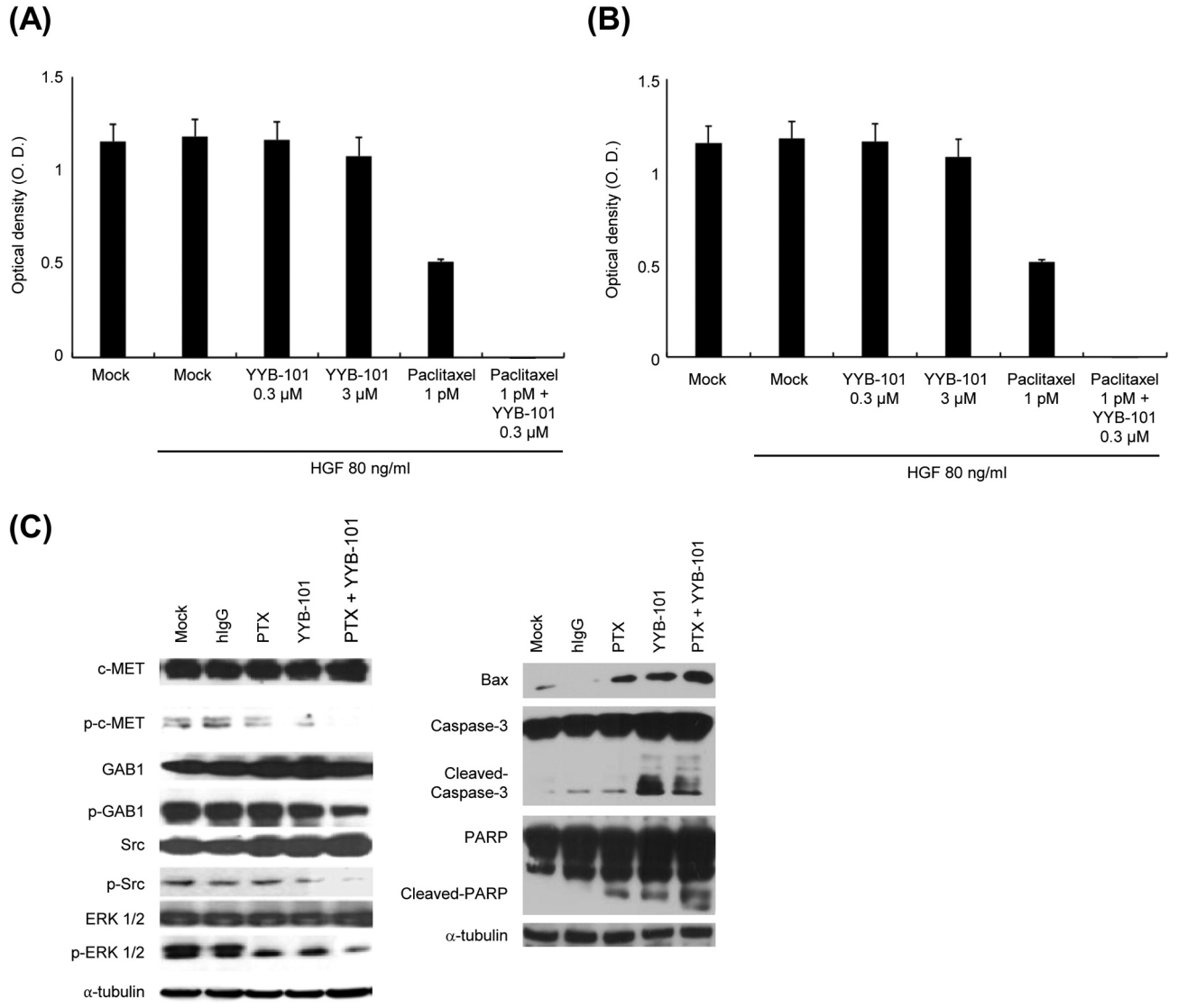

Figure 3. *The YYB-101 effect on ovarian cancer cell proliferation. (A) A2780 cells (3×10^3) and (B) SKOV3 cells (1.5×10^3) were seeded into 96-well plates and incubated overnight. The cells were treated with 80 ng/ml HGF containing 0.3 μM YYB-101, 3 μM YYB-101, 1 pM paclitaxel, or 0.3 μM YYB-101 with 1 pM paclitaxel for 72 h. Cell proliferation was determined using a WST-1 assay. (C) A2780 cells (2×10^6) were plated into 6-well plates and treated with 1 μM hIgG, 3 μM YYB-101, 1 pM paclitaxel, or 3 μM YYB-101 with 1 pM paclitaxel for 24 h. The levels of c-MET signaling molecules (c-MET, p-c-MET, GAB1, p-GAB1, Src, p-Src, ERK 1/2, and p-ERK 1/2) and apoptosis-related molecules (Bax, caspase-3, cleaved caspase-3, PARP, and cleaved PARP) were determined by western blot analysis. α-tubulin was used as a control.*

cisplatin or carboplatin) and a taxane, such as paclitaxel. Previous studies have shown that adjuvant chemotherapy in patients with early-stage (I to IIA) OC improves OS and recurrence rates. However, at the initial diagnosis, 60%-70% of patients have an advanced stage (III or IV) disease, in which cancer cells have seeded in the peritoneum and have metastasized to other organs. These patients have a recurrence rate over 80% after chemotherapy, and their therapeutic options are limited because they exhibit resistance to adjuvant chemotherapeutic agents (21). Therefore, there is a need for new therapeutic agents for advanced stage and recurrent OC. Accumulated reports suggest that HGF is important for OC progression, and anti-HGF antibodies have been developed as a targeted OC therapeutics. Several HGF-targeting antibodies, such as Rilotumumab, Ficlatuzumab, and HuL2G have been tested in clinical trials, but they were discontinued because no therapeutic effects were observed in patients with gastric

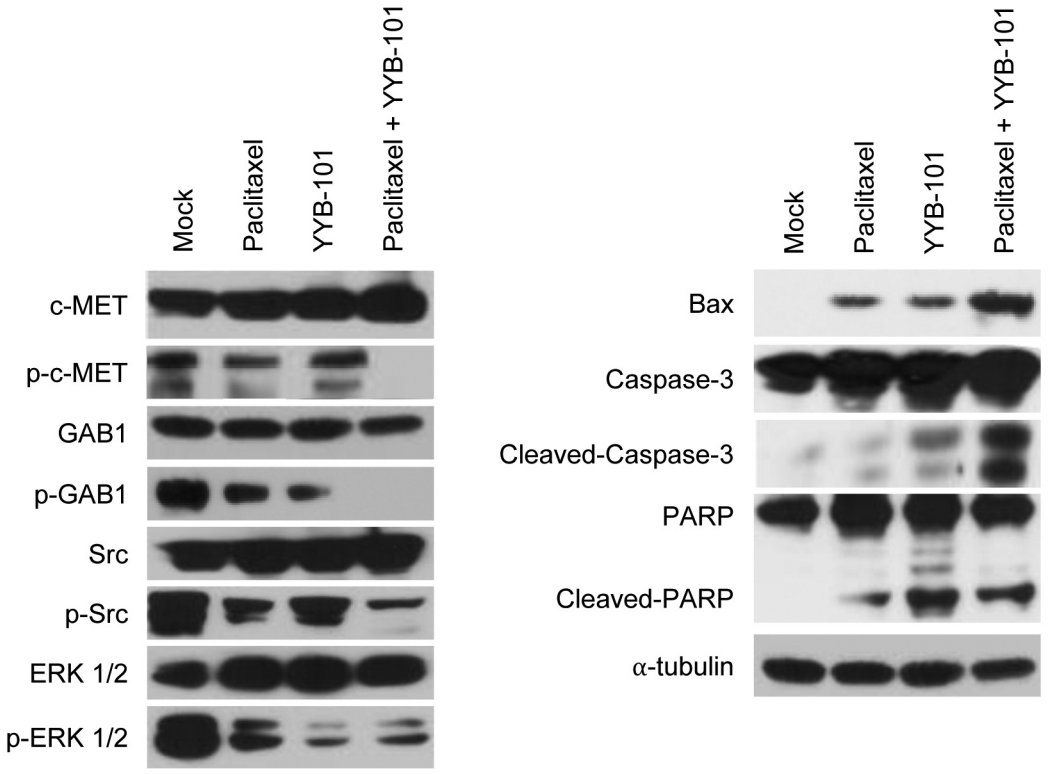

Figure 4. *The YYB-101 effect on c-MET signaling and apoptosis signaling in orthotopic ovarian cancer mouse xenografts. A2780/luc cells (1×10^5) were injected into the right ovaries of mice. One week after tumor implantation, mice were treated with paclitaxel (10 mg/kg once weekly), YYB-101 (40 mg/kg twice a weekly), or paclitaxel with YYB-101. After 30 d, the ovary tumor tissue was isolated and total proteins were subjected to western blot analysis to detect c-MET molecules (c-MET, p-c-MET, GAB1, p-GAB1, Src, p-Src, ERK 1/2, and p-ERK 1/2) and apoptosis-related molecules (Bax, caspase-3, cleaved caspase-3, PARP, and cleaved PARP).*

cancer, pulmonary adenocarcinoma, or non-small cell lung cancer (22-25). Unlike these three antibodies that bind to the HGF β-chain, YYB-101 effectively blocks c-MET signaling by binding to the HGF α-chain involved in the high-affinity binding of c-MET . Since YYB-101 is effective in various tumors when administered in combination with chemotherapy agents, and is under clinical development for melanoma and colorectal cancer, we examined whether YYB-101 could act as an effective OC therapeutic.

In cancer cells, EMT is activated by actin-cytoskeleton rearrangement and induces cancer cell metastasis. Actin-cytoskeleton reorganization enables cell elongation, thereby promoting cell migration, invasion, and focal adhesion (FA) formation. Actin is an important part of the cytoskeleton and is involved in cell movement and division (26, 27). HGF activates small GTPases that regulate actin rearrangement by signaling pathways, such as RhoA, Rac1, and Cdc42. Consequently, HGF-regulated actin rearrangement results in increased cancer cell migration (28-30). Likewise, FAs acts as a mechanical link to the extracellular matrix (ECM), so their dynamic assembly and disassembly play a central role in cell migration. Among them, paxillin is involved in regulating cell FAs and is a signaling adaptor protein, which is localized at FA sites. HGF regulates actin and FAs by a PI3K-related mechanism in several carcinoma cells to increase their motility (31). In particular, HGF induces paxillin phosphorylation, which mediates ERK kinase or PKC kinase activation to regulate cell FA contact to the ECM (32). All of these actions play an important role in cell morphology, migration, and invasion. In this study, we examined the signaling mechanisms affected by YYB-101 treatment in HGF-induced OC cell migration and invasion. In particular, YYB-101 treatment inhibited paxillin and F-actin phosphorylation, which indicated that YYB-101 disrupted the cytoskeleton structure (Figure 2). Moreover, ERK and Src signaling, which are involved in cell migration and invasion, were inactivated following treatment with the combination of YYB-101 and paclitaxel (Figure 3). These results indicated that YYB-101 inhibits the motility of ovarian cancer cells by inducing cytoskeleton rearrangement and by affecting

signaling molecules. Especially, combination treatment with paclitaxel has been shown to synergistically reduce the phosphorylation of downstream molecules of c-MET and thereby, ehance anticancer efficacy.

Cancer cell survival is associated with the HGF/c-MET axis. It has been shown that themechanism involves caspase and PARP cleavage, and that the absence of c-MET ligand is associated with caspase and PARP cleavage (33). Our *in vitro* and *in vivo* results demonstrated that caspase and PARP cleavage increased when cells were treated with YYB-101 only or in combination with paclitaxel (Figure 3C and 4). Similarly, YYB-101 and paclitaxel combination treatment significantly reduced OC cell proliferation (Figure 4A and B). YYB-101 induced apoptosis in OC cells by upregulating the apoptotic signaling pathway.

In conclusion, our results showed that the HGF/c-MET axis is inhibited by YYB-101; inactivates the c-MET signaling pathway and effectively inhibits cancer cell progression. Also, YYB-101 inhibits cell proliferation by activating the apoptotic signaling pathway. In particular, the YYB-101 and paclitaxel combination treatment showed a synergistic anticancer effect compared to the single YYB-101 or paclitaxel treatments. Therefore, our findings suggest that YYB-101 and its combination with standard chemotherapeutics could be a new potential treatment strategy for OC treatment.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

H.J.K and K.H designed and wrote the manuscript. H.J.K performed experiments and data analysis.

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