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HGF-mediated Up-regulation of PHLDA2 Is Associated With Apoptosis in Gastric Cancer

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Abstract. *Background/Aim: Expression of pleckstrin homology-like domain family A member 2 (PHLDA2) has been reported to be suppressed or activated in several cases of malignant tumors. However, its apoptotic regulatory mechanism and role in gastric cancer are not understood. This study examined the role of PHLDA2 in apoptosis in gastric cancer. Materials and Methods: We used cell culture, western blotting, semiquantitative reverse transcription polymerase chain reaction, MTT assays, and PHLDA2 knockdown with short hairpin RNA (shRNA). Results: To identify the pathway associated with HGF-induced PHLDA2 up-regulation, the cells were treated with PI3-kinase inhibitor (LY294002), MEK inhibitor (PD098059), or p38 inhibitor (SB203580) and then analyzed by western blotting. HGF-mediated changes in PHLDA2 protein levels were only decreased by LY294002. PHLDA2-shRNA cells showed decreased levels of p53 and increased levels of pAKT. Furthermore, HGF-induced cell proliferation and in vitro invasion were increased in PHLDA2 knockdown cells and HGF-induced cell apoptosis was increased in PHLDA2 knockdown cells. Conclusion: PHLDA2 plays a role in gastric cancer tumorigenesis by inhibiting apoptosis through the PI3K/AKT pathway.*

Since 2018, gastric cancer has been the most common neoplasm in South Korea (1) and the main cause of cancer-related mortality worldwide (2). We studied the role of hepatocyte growth factor (HGF) and its associated functions in gastric cancer proliferation and invasion.

HGFs are a group of factors affecting angiogenic ability; they are described as heparin-binding growth factors that bind the N-methyl-N'-nitroso-guanidine human osteosarcoma transforming gene (c-MET) tyrosine kinase receptor (3). HGF is produced by mesenchymal cells and affects endothelial and epithelial cells in a paracrine or autonomic fashion. HGF/MET signaling is well known to be involved in oncogenesis and cancer progression in several cancers and accelerates aggressive cell invasiveness, which is strongly associated with tumor metastasis (4-6).

The pleckstrin homology-like domain family A member 2 (*PHLDA2*) gene is a homolog of the mouse *TDAG51* gene, an apoptosis-related gene that functions during normal development. It is located within the tumor suppressor region of 11p15 (7).

PHLDA2 inhibition has been reported to promote tumor proliferation and invasion in several malignancies. In osteosarcoma, PHLDA2 inhibition promotes tumorigenesis and metastasis *via* the PI3K/AKT/mTOR pathway (8). It was also reported that PHLDA2 silencing resulted in the reduction of invasion and proliferation of breast cancer cells (9) and inhibited cancer cell aggressiveness in pancreatic cancer (10). A recent study also showed that PHLDA2 promotes invasion and proliferation in colorectal cancer (11). The mechanism by which PHLDA2 acts depending on the tumor type is unclear. However, osteosarcoma is a mesenchymal tumor, while breast and pancreatic cancer are epithelial malignancies. In addition, one study reported that treatment with the epidermal growth factor receptor (EGFR) up-regulated PHLDA2 *via* the AKT pathway in lung cancer cell lines (12). However, the mechanism of tumorigenesis is not clear, and PHLDA2 has not been investigated in gastric cancer.

In this study, we selected genes that were up-regulated in gastric cancer by HGF treatment by using a human complementary DNA (cDNA) microarray and explored the function of the genes with respect to HGF in the pathogenesis of gastric cancer using gastric cancer cell lines (NUGC3 and NKN28 cells). PHLDA2 was identified as one of the genes up-regulated by HGF treatment. Because *PHLDA2* is known as an apoptosis-related gene, this study focused on identifying the function and the pathways of HGF-up-regulated PHLDA2 associated with apoptosis in gastric cancer.

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Key Words: Gastric cancer, hepatocyte growth factor, PHLDA2.

Materials and Methods

Cell culture. Two human gastric cell lines (NUGC3, MKN28) were obtained from the Korea Cell Line Bank. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco/ThermoFisher Scientific, Waltham, MA, USA) and 50 U/ml penicillin/streptomycin (Gibco) at 37˚C in 5% CO_2 incubator.

Reagents and antibodies. Recombinant human HGF was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were purchased from Bio-Rad Laboratories (Philadelphia, PA, USA). Antibodies against PHLDA2 and p53 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The antibody against Bcl2 was purchased from BD Biosciences (San Jose, CA, USA). PD98059 was purchased from Biomol Research Laboratories, Inc. (Butler Pike, PA, USA). SB203580 and LY 294002 were purchased from Calbiochem Inc. (San Diego, CA, USA).

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR). TRIzol was used to isolate RNA from cells. Complementary DNA (cDNA) was generated by reverse transcription using MMLV reverse transcriptase (Promega Corp., Madison, WI, USA) and the oligo (dT) priming method in a 10-μl reaction mixture. PCR was performed in a 10-μl reaction volume containing 10 mM Tris-HCl pH 8.5, 50 mM KCl, 1 μl cDNA, 200 μM dNTPs, 1 mM $MgSO_4$, 1 U platinum pfx Taq polymerase, and 2 μM primer. The reaction cycles were as follows: an initial denaturation at 95˚C for 4 min; 30 cycles at 94˚C for 15 s, 60˚C for 15 s, and 72˚C for 30 s; and a final extension at 72˚C for 10 min. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide and visualized on an ultraviolet transilluminator (13).

Complementary DNA microarray analysis. The cDNA microarray, containing a set of 17,448 sequence-verified human cDNA clones, was provided by Genomic Tree Inc. (Daejeon, Republic of Korea). cDNA microarray experiments were conducted as described below (14). In summary, total RNA (100 μg) was reverse transcribed in the presence of Cy3-dUTP or Cy5-dUTP (25 mM stock, NEN Life Science Products, Boston, MA, USA) at 42˚C for 2 h. The labeled cDNA was then hybridized with the cDNA microarray at 65˚C for 16 h. We washed the hybridized slides, scanned them with an axon 4000 B scanner (Axon Instruments, Molecular Devices, San Jose, CA, USA), and analyzed the hybridized slides using GenePix Pro 4.0 (Axon Instruments-software, San Jose, CA, USA). Raw data were normalized and analyzed using GeneSpring 6.0 (Silicon Genetics-software, Redwood City, CA, USA). The genes were filtered according to their intensities in the control channel. When the control channel values were below 80 in all of the samples, we considered the results unreliable. Intensity-dependent normalization (LOWESS) was performed when the ratio was reduced to the residual of the LOWESS fit of the intensity *versus* the ratio curve. The average normalized ratios were calculated by dividing the average normalized signal channel intensity with the average normalized control channel intensity (15). Welch's ANOVA test was performed for p-values ≤0.1 and ≤0.05 to identify differentially expressed genes. Correlation analysis was conducted using Pearson's correlation (–1 to 1). Spots which changed ≥2 fold were considered significant.

Western blot analysis. To detect the levels of cellular proteins, cells were harvested and incubated with a lysis buffer containing protease inhibitors. Proteins (50 μg) were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in TTBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% tween-20) for 1 h at room temperature, followed by incubation with a primary antibody overnight at 4˚C. After washing, the membrane was incubated with secondary antibody (Horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies) for 1 h and 30 min at 4˚C. Immune complexes were detected using western blot analysis luminol reagent. Protein bands were visualized with Fujifilm LAS-4000 image system (Stanford, CT, USA).

MTT assay. Cell proliferation was measured by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Control and transfected cells (1,500/well) were seeded in 96-well plates. The cells were then serum-starved for 24 h and treated for 72 h with or without HGF (10 ng/ml). After treatment, 1 mg/ml MTT solution was added to the cells for 4 h. The medium was aspirated and the formazan product was solubilized with 100 μl dimethyl sulfoxide. Viability was assessed by measuring absorbance at 570 nm with a Bio-Rad multiscan plate reader (Hercules, CA, USA).

Apoptosis. The cell cycle profiles were measured by staining with propidium iodide. Trypsinized cells were resuspended in 70% ethanol and incubated at -20˚C for at least 1 h. Subsequently, the pellets were washed twice with ice-cold PBS and resuspended in 0.5ml ice-cold PBS containing RNase (50 μg/ml) and propidium iodide (50 μg/ml) and incubated at 37˚C for 30 min. After the cells were analyzed by fluorescence-activated cell sorting (FACS; Becton-Dickinson, San Jose, CA, USA) at an excitation wavelength of 480 nm (16).

PHLDA2 knockdown with short hairpin RNA. The human PHLDA2-specific short hairpin RNA (PHLDA2-shRNA, RHS4741-EG7262) was purchased from Open Biosystems (Huntsville, AL, USA). NUGC3 and MKN28 cells were transfected with PHLDA2-shRNA using Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies Inc., Gaithersburg, MD, USA). Cells were cultured with puromycin (25 μg/ml) and, subsequently, were diluted serially. Repeating these processes, we identified the stably transfected clones with low expression of the target gene by using western blot analysis.

Results

Up-regulation of PHLDA2 levels after HGF treatment. To validate PHLDA2 up-regulation by HGF treatment, western blot and reverse transcription polymerase chain reaction (RT-PCR) analyses were performed in NUGC3 and MKN28 gastric cell lines. RT-PCR showed that the expression levels of PHLDA2 messenger RNA (mRNA) were increased by treatment with HGF (Figure 1A). Also, western blot analysis showed that PHLDA2 protein levels were increased 1 h after HGF treatment (Figure 1B). These results showed that HGF treatment of gastric cancer cells increased the expression levels of PHLDA2 mRNA and protein.

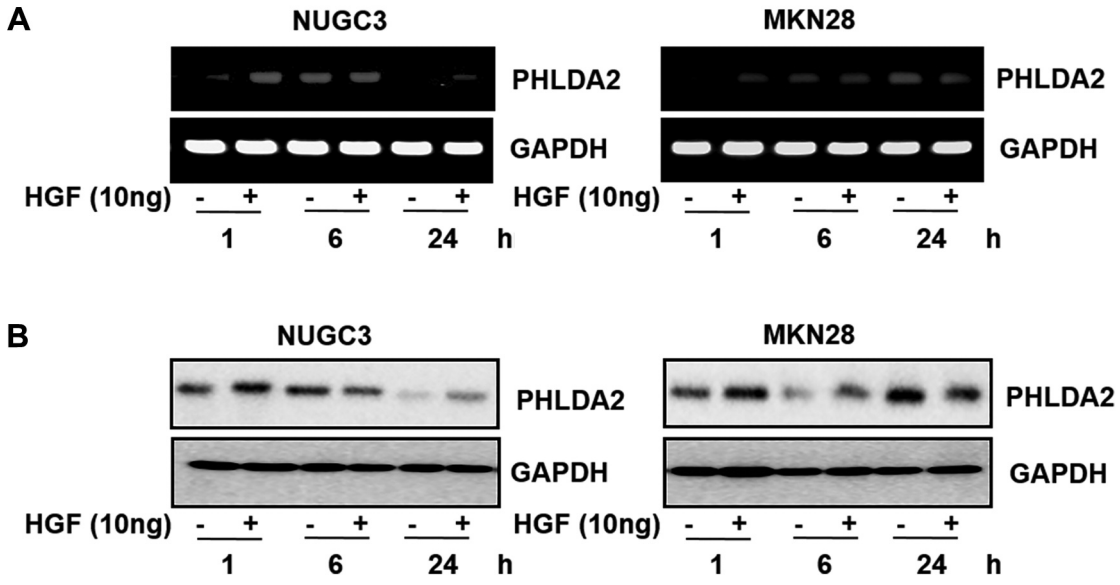

Figure 1. *Effects of HGF on the expression levels of PHLDA2 in NGUC-3 and MKN-28 cells. Cells were serum-starved for 24 h, treated with/without 10 ng/mL HGF for the indicated times and harvested. The expression levels of PHLDA2 mRNA and protein were confirmed by reverse transcription-polymerase chain reaction and western blot analysis. Representative data from three independent experiments are shown. HGF: Hepatocyte growth factor; PHLDA2: pleckstrin homology-like domain family A member 2.*

Dose-dependent effects of HGF on PHLDA2. The dose-dependent effects of HGF on PHLDA2 were analyzed by western blotting. PHLDA2 protein expression was found to increase with increasing concentrations of HGF (0, 10, and 40 ng/ml) (Figure 2A). Several studies reported that HGF has a role in anti-apoptotic signaling in cancer cells (17-19). For the experiment examining apoptosis associated with HGF-mediated changes in PHLDA2 expression, the well-known apoptosis gene, *p53,* and anti-apoptotic gene, *Bcl2*, which regulates cell death by inhibiting apoptosis, were analyzed by western blotting. p53 protein expression decreased with increasing HGF (0, 10, and 40 ng/ml). In contrast, the Bcl2 protein expression increased with increasing HGF (0, 10, and 40 ng/ml) (Figure 2B).

Effect of LY294002, PD098059, and SB203580 on PHLDA2 expression. To identify the pathway through which HGF regulates PHLDA2, we treated the cells with a phosphoinositide 3-kinase (PI3K) inhibitor (LY294002), mitogen-activated protein kinase (MEK) inhibitor (PD098059), or p38 inhibitor (SB203580) and then performed western blotting. The HGF-induced protein levels of PHLDA2 were decreased by treatment with LY294002 (Figure 3). However, treatment with PD098059 and SB203580 did not affect PHLDA2 expression in either NUGC-3 or MNK28 cell lines. These results indicate that HGF-induced changes in PHLDA2 expression may be regulated by the PI3K pathway.

Knockdown of PHLDA2 affects apoptosis. To determine the association of PHLDA2 with apoptosis, we examined the effect of PHLDA2 knockdown on apoptosis markers, including P53 and bcl2. PHLDA2 knockdown resulted in decreased levels of PHLDA2 in both cell lines. We analyzed the effect of PHLDA2 knockdown on HGF-mediated p53 and bcl2 regulation using western blotting. The expression levels of p53 were decreased in both PHLDA2 knockdown cell lines. On the other hand, the levels of bcl2 were increased in both PHLDA2 knockdown cell lines. We also measured the HGF-mediated changes in p-AKT expression and found that the levels of p-AKT were increased in both PHLDA2 knockdown cell lines. These results indicate that PHLDA2 is associated with apoptosis, and PHLDA2 may be regulated by the AKT pathway (Figure 4).

Effect of PHLDA2 knockdown on HGF-mediated proliferation. To identify the effect of PHLDA2 on HGF-induced proliferation of gastric cell lines, we treated PHLDA2 shRNA cells and control cells with HGF and measured cell proliferation after 72 h by using the MTT assay. The results showed that HGF-mediated proliferation was lower in PHLDA2 knockdown cells than in control cells in both gastric cancer cell lines ($p<0.05$) (Figure 5).

Effect of PHLDA2 knockdown on HGF-mediated cell invasion. To determine the role of PHLDA2 in cell invasion, an *in vitro* invasion assay was performed using Matrigel-coated migration

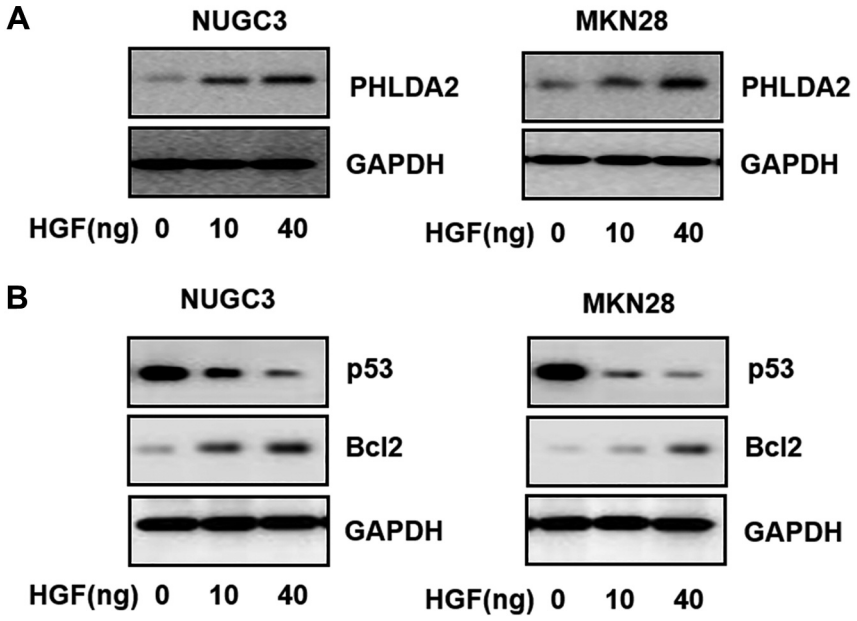

Figure 2. *Dose-dependent effect of HGF on the expression of PHLDA2. Serum-starved cells were treated with 0, 10, 40 ng/ml HGF for 1 h and harvested. The expression levels of PHLDA2, p53, Bcl2 and Bag3 were confirmed by western blotting. Representative data from three independent experiments are shown. HGF: Hepatocyte growth factor; PHLDA2: pleckstrin homology-like domain family A member 2; P53: tumor protein p53; Bcl2: B-cell lymphoma 2.*

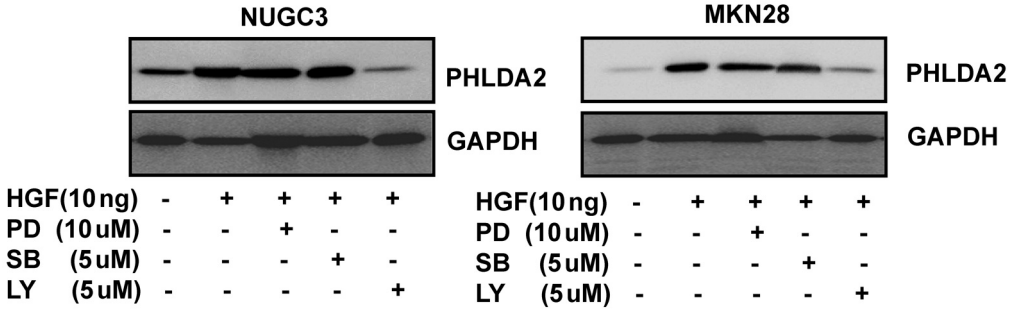

Figure 3. *Effects of LY, PD, SB on HGF-induced expression of PHLDA2. The cells (1×10^6/well) were plated overnight in complete medium, starved for 24 h, and then treated with or without LY, PD, SB for 45 min prior to incubation with or without 10 ng/ml of HGF for 15 min. PHLDA2 expression was analyzed by western blotting. Representative data from three independent experiments are shown. HGF: Hepatocyte growth factor; PHLDA2: pleckstrin homology-like domain family A member 2; LY: LY294002; PD: PD098059; SB: SB203580.*

chambers. PHLDA2 shRNA cells and control cells were treated with HGF and used for the assay. After 72 h incubation, the data showed that HGF-mediated cell invasion decreased in PHLDA2 shRNA cells of both cell lines ($p<0.05$) (Figure 6).

Effect of PHLDA2 knockdown on HGF-mediated cell apoptosis. Figure 4 shows that the apoptosis-related genes bcl2 and p53 are associated with PHLDA2 using knockdown cells. To confirm the role of PHLDA2 in HGF-mediated apoptosis, we treated PHLDA2 shRNA cells and control cells with HGF and measured cell apoptosis after 30 min using FACScan. The data showed that HGF-mediated cell apoptosis increased in PHLDA2 shRNA cells compared with the control cells ($p<0.05$) (Figure 7).

Discussion

HGF, a paracrine cellular growth factor that functions through binding to its cognate receptor c-MET, is known to play an important role in cancer progression (6). HGF and c-MET receptors are involved in multiple biological activities, including proliferation, motility, and survival (20-

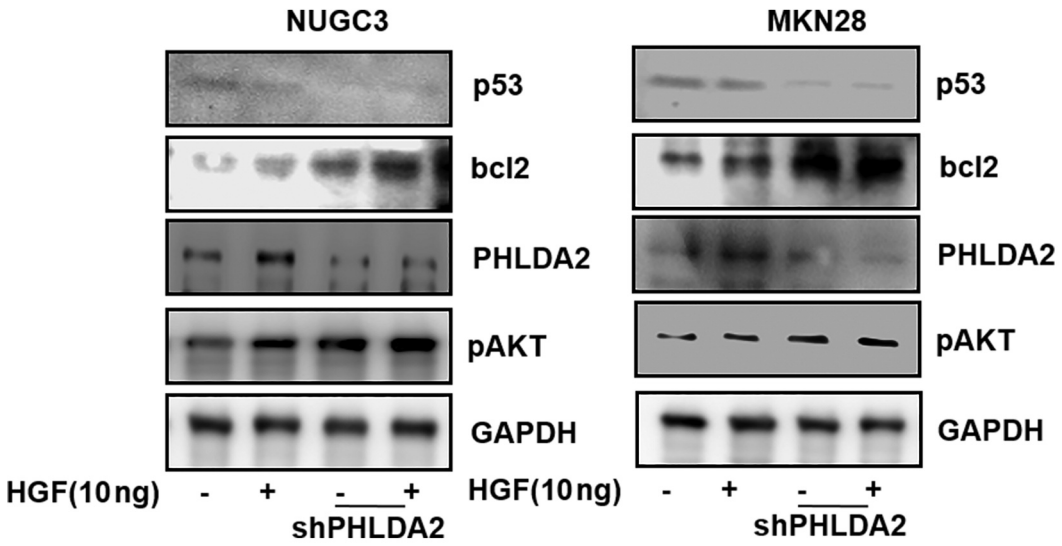

Figure 4. *The levels of p53, Bcl2, pAKT in shPHLDA2 stable cell lines. Non-transfected control and PHLDA2-shRNA transfected cells were serum-starved and treated with/without HGF (10 ng/ml). PHLDA2, p53, Bcl2and pAKT expression levels were confirmed by western blot analysis. Representative data from three independent experiments are shown. HGF: Hepatocyte growth factor; PHLDA2: pleckstrin homology-like domain family A member 2; P53: tumor protein p53; Bcl2: B-cell lymphoma 2; pAKT: phosphorylated protein kinase B.*

22). MET and HGF persist throughout life, and the fact that up-regulation of HGF is observed after liver, kidney or heart injury suggests that this pathway is activated to protect against tissue damage and to promote repair and renewal (23, 24). Under normal conditions, HGF-induced MET tyrosine kinase activation is tightly regulated by multiple mechanisms, including paracrine ligand transmission, ligand activation at the target receptor, and receptor internalization and degradation by ligand activation (25-27). Despite multiple mechanisms of regulation, dysregulation of the HGF/c-MET pathway occurs in a variety of neoplasms (28, 29). Many genes are up-regulated by HGF and affect HGF/c-MET signaling, which is associated with tumor proliferation and progression as well as metastasis (30, 31). Other oncogenic pathways in HGF/c-MET involve persistent kinase activation upon MET over-expression or mutation (21).

We explored HGF/c-MET signaling and the function of the associated up-regulated genes in gastric cancer. In previous studies, we identified up-regulated genes as early as 6 h following HGF/c-MET activation. Among the genes up-regulated by HGF were those that associated with tumor progression and invasion downstream of the HGF/c-MET pathway in gastric cancer. We identified that the HGF/c-MET pathway facilitates oncogenesis through MAPK, STAT, PI3K-AKT cascades, and nuclear factor kappa-chain-enhancer of activated B cells (NF-kB), which are known to participate in oncogenic signal activation, cell proliferation, and invasion in gastric cancer.

We confirmed that HGF up-regulated PHLDA2 using western blotting and RT-PCR. HGF-induced PHLDA2 expression was observed after 1 h of treatment and was also dose-dependent. Some studies have reported that PHLDA2 may be regulated through the PI3/AKT pathway (12, 32). We also found that HGF induced PHLDA2 *via* the PI3/AKT pathway by showing that the levels of PHLDA2 were decreased after LY (PI3 kinase inhibitor) treatment and that the expression levels of pAKT increased in PHLDA2 knockdown cells.

We also investigated the role of PHLDA2 in the tumorigenesis of gastric cancer. The proliferation of NUGC3 and MKN28 PHLDA2 knockdown cells was increased. Invasion was also increased in PHLDA2 knockdown cells compared to control cells. Thus, HGF/c-MET-induced PHLDA2 promotes cell proliferation and invasion and may affect tumor formation and metastasis of gastric cancer. We hypothesized that the mechanism of PHLDA2 tumorigenesis involves inhibition of apoptosis, and we determined the change in the expression levels of p53 and Bcl2 in PHLDA2 knockdown gastric cancer cell lines.

It is well known, that *p53* is a tumor suppressor gene, and *Bcl2* (B-cell lymphoma 2) is a gene that inhibits apoptosis (33, 34). We confirmed that the levels of p53 were decreased and those of Bcl2 were increased in PHLDA2 knockdown cells. These findings indicate that HGF/c-MET-induced PHLDA2 expression inhibits apoptosis, which may promote progression of gastric cancer. To confirm the role of PHLDA2 in apoptosis, we conducted FACS. We found that

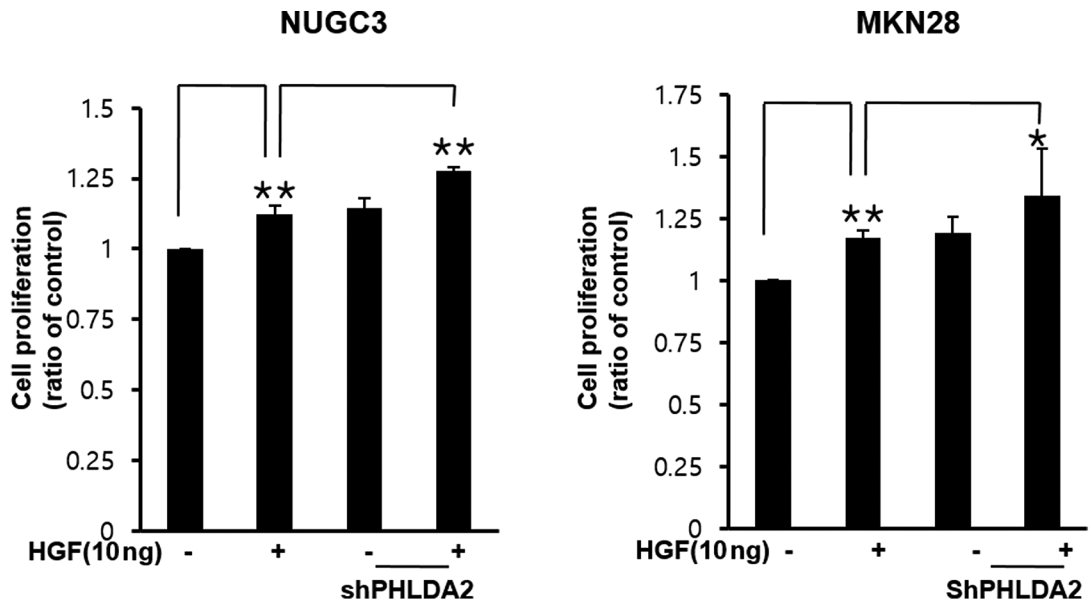

Figure 5. *The role of PHLDA2 in HGF-induced cell proliferation. Control (1,000/well) and stable PHLDA2-shRNA cells were seeded in 96-well plates with DMEM media supplemented with 5% FBS and incubated for 24 h. After serum-starvation for 24 h, cells were treated with or without 10 ng/ml of HGF for 72 h. Cell proliferation was measured by MTT assays and expressed as a percentage of HGF-untreated control cells. Values are the means±SD of three independent experiments performed in triplicate. HGF: Hepatocyte growth factor; PHLDA2: pleckstrin homology-like domain family A member 2; DMEM: Dulbecco's modified Eagle's medium.*

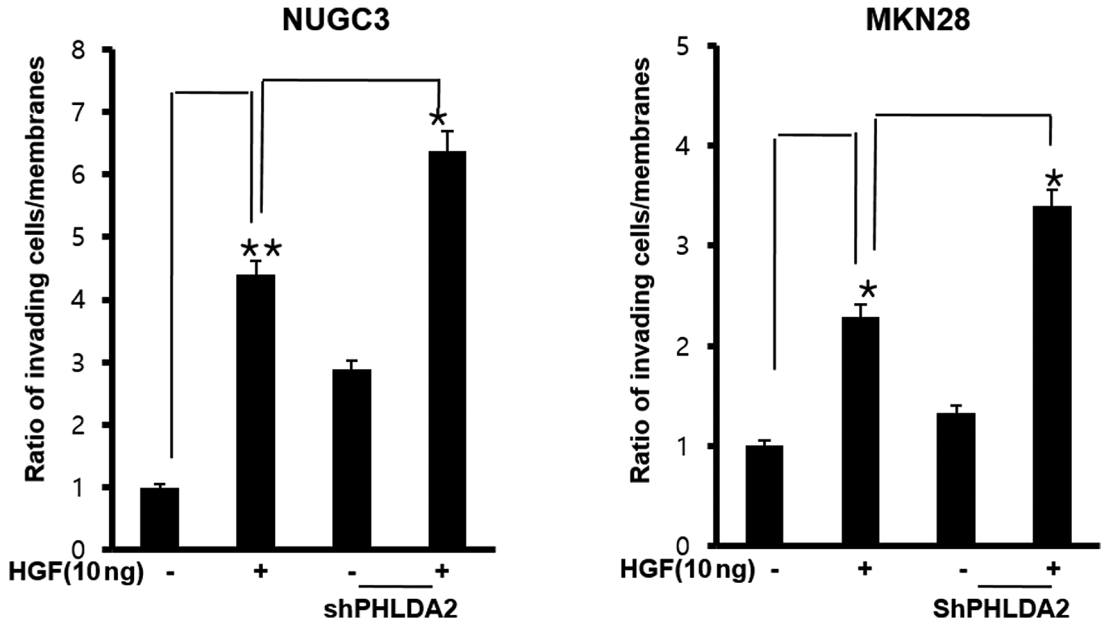

Figure 6. *Role of PHLDA2 in HGF-induced cell invasion. Stable PHLDA2-shRNA and control cells were treated with/without 10 ng/ml HGF for 48 h. Cell invasion capacity was measured using the standard two chamber invasion assay with Matrigel migration chambers. Values are means±SD of three independent experiments. HGF: Hepatocyte growth factor; PHLDA2: pleckstrin homology-like domain family A member 2; shRNA: short hairpin RNA.*

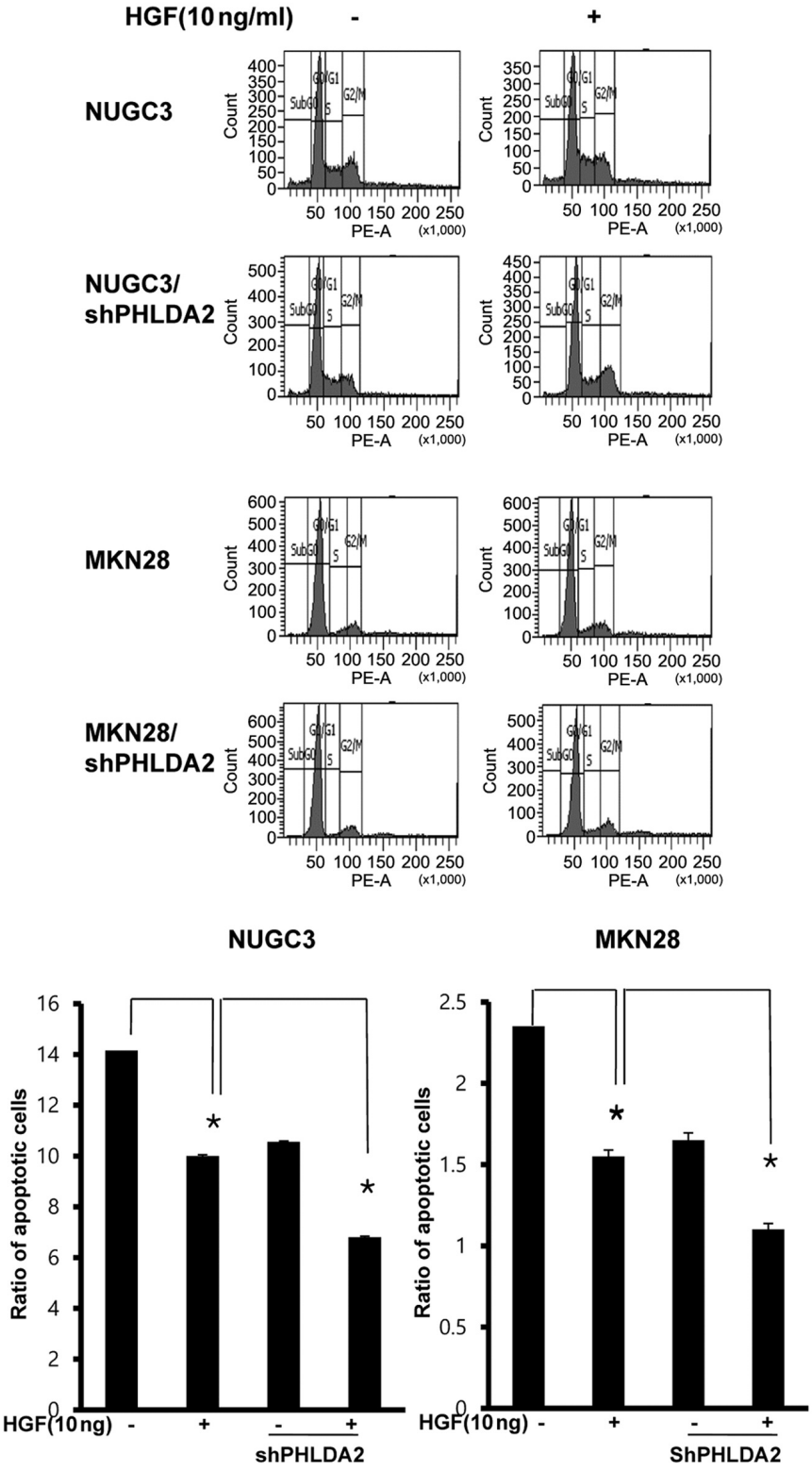

Figure 7. *Effect of HGF on apoptosis of shPHLDA2 stable cells. Stable shPHLDA2 cells were treated in the presence or the absence of HGF (10 ng/ml) and then cell pellets were fixed and stained with PBS containing 10 μg/ml propidium iodide and analyzed by FACS. Representative data from three independent experiments are shown. HGF: Hepatocyte growth factor; PHLDA2: pleckstrin homology-like domain family A member 2; FACS: fluorescence-activated cell sorting.*

the numbers of the PHLDA2 knockdown gastric cancer cell lines were decreased. Thus, PHLDA2 plays a role in tumor progression by inhibiting HGF-induced apoptosis.

However, the pathway of the direct regulation of bcl2 or p53 by PHLDA2 has not been identified. In addition, this study is only limited in *in vitro* data regarding PHLDA2, and additional studies are warranted to identify the exact role of HGF/c-MET-mediated PHLDA2 in gastric cancer proliferation and invasion, including *in vivo* experiments with knockout mice.

In conclusion, we demonstrated that HGF/c-MET-regulated PHLDA2 is a gastric cancer promoter. HGF/c-MET-induced PHLDA2 expression promoted cell proliferation and invasion of gastric cancer cell lines. In addition, we showed that up-regulation of PHLDA2 decreased apoptosis activity. In addition, we revealed that HGF/c-MET regulates PHLDA2 *via* the PI3K/AKT pathway. Additional studies are needed to confirm the role of PHLDA2 in HGF-induced processes and determine how PHLDA2 could be used as a potential therapeutic target in gastric cancer.

Conflicts of Interest

The Authors declare that there are no conflicts of interest in relation to this study.

Authors' Contributions

Conception and design: S.A. Koh and K.H. Lee. Development of methodology: S.A. Koh and K.H. Lee. Acquisition of data: S.A. Koh and K.H. Lee. Analysis and interpretation of data: S.A. Koh and K.H. Lee. Writing, review, and/or revision of the article: S.A. Koh. Administrative, technical, or material support: S.A. Koh and K.H. Lee. Study supervision: K.H. Lee.

Acknowledgements

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