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Pine needle hexane extract promote cell cycle arrest and premature senescence via p27^{KIP1} upregulation gastric cancer cells

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Abstract *Pinus densiflora sieb. et zucc.*(pine needle) is a traditional medicine used in several East Asian countries. However, the efficacy of pine needle has rarely been reported. In this study showed that the anti-proliferative effects and the mechanisms of hexane layer of pine needle MeOH extract (PNH) on gastric cancer cells. At first, PNH inhibited the proliferation of gastric cancer cells in a dose-dependent manner. Moreover, PNH treatment induced G1 phase cell cycle arrest through the increased p27^{KIP1} expression and decreased cyclin dependent kinase (CDKs) activity. Furthermore, PNH treatment induced premature senescence without oncogenic stress, through the expression of p27^{KIP1} and Skp2. Taken together, these results showed that PNH inhibited gastric cancer cell proliferation through the induction of G1-cell cycle arrest and premature senescence via induced p27^{KIP1} expression, as controlled by Skp2 reduction. Also, PNH could be a candidate for anti-gastric cancer treatment and may be useful in the development of anti-gastric cancer drugs.

Keywords Pine needle hexane extract (PNH) · p27 · Skp2 · Gastric cancer

Introduction

Gastric cancer is the most common cancer around the world. Recently, although the incidence and mortality rate of gastric cancer has decreased, it still remains a common cancer (Lee et al., 2002; Torre et al., 2015). Surgical resection is a major curative treatment in gastric cancer, but the 5-year survival rate for all patients is as low as 15% to 35% (Lim et al., 2005). Therefore, studies on adjunctive therapies such as microarray studies, gene profiling and new therapeutic targets agents to treat gastric cancer are continuing. Recent studies on chemotherapy of gastric cancer have focused on cancer cell growth, apoptosis, cell cycle, and cellular senescence of gastric cancer (Wen et al., 2016). Also, premature senescence is a novel potential therapeutic target for cancer (Ewald et al., 2010; Schmitt, 2007).

According to hallmarks of cancer, unlimited cellular proliferation was involved in various cancer progression and low survival of patients including gastric cancer (Hanahan and Weinberg, 2000). The cell cycle is an essential regulator of the processes of cell proliferation and

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growth and cancer cells are not stop at points of the cell cycle because of loss of checkpoint. This is confirmed by altered expression and activity of cell cycle related proteins (Feitelson et al., 2015). In normal cells, cyclins and cyclin-dependent kinase (CDK) complexes continuously regulatory act together in G1 to S phase transition and in G2 to mitosis transition (Steller, 1995). When CDKs are not controlled, it causes cancer and cell cycle inhibitors such as the $p21^{CIP/WAF}$ and $p27^{KIP1}$, Cip/Kip proteins are tightly regulated to prevent it (Sherr, 1996). If abnormal proliferation and DNA damage occurs in cells, $p21^{CIP/WAF}$ and $p27^{KIP1}$ bind to cyclin-CDK complexes and generate cell cycle arrest to block their catalytic activity (Toyoshima and Hunter, 1994). CDK is suppressed by the binding of $p27^{KIP1}$ and cyclin E—CDK2 complexes, as a result G1-cell cycle arrest is induced (Coqueret, 2003). Therefore, various anti-cancer drugs were targeting above gene targets (Waldman et al., 1997).

In recent study confirmed that cellular senescence plays a defense to oncogene-mediated transformation in vitro and in vivo (Braig et al., 2005). In other words, premature senescence induced by various factors plays tumor-suppressive barrier in tumorigenesis (Braig and Schmitt, 2006). S-phase kinase-associated protein 2 (Skp2), which is one of the constituent of ubiquitin–proteasome system acts as the substrate specific factor for the Skp1–Clu1–F-box (SCF) complex (Wei et al., 2013). Also, Skp2 deficiency lead to induction of cell cycle inhibitors $p27^{KIP1}$ and $p21^{CIP/WAF}$, which can contribute to senescence response (Wang et al., 2012).

Red Pine trees (*Pinus densiflora sieb. et zucc.*) belongs a family of *pinaceae* are widely distributed in East-Asia, such as Korea, Japan and China. In Korea, Pine trees (needles, cones, barks etc.) were widely used for food or folk medicine for disease prevention (Kim and Shin, 2005). According to folk remedies, *Pinus densiflora* have been used as a medicine for rheumatitis, hemorrhage, gastroenteric trouble, hypertension and asthma (Bae, 2000). Recent studies have shown that *pinus densiflora* has anti-nociceptive and anti-inflammatory (Choi, 2007), antioxidant, anti-mutagenic, and anti-cancer effects (Kwak et al., 2006).

In this study, we confirmed the molecular mechanism that pine needle hexane extract (PNH) induced G1-cell cycle arrest against human gastric cancer cell lines. Also, PNH induced G1-cell cycle arrest via correlation of $p27^{KIP1}$ and Skp2 in human gastric cancer cells. Furthermore, PNH induce premature senescence without oncogenic stress and is therefore involved in anti-proliferative activity of gastric cancer cells.

Materials and methods

Preparation of pine needle n-Hexane fraction (PNH)

Pinus densiflora Sieb. et Zucc (Needles of red pine) had been picked up from Gokseong, South Korea. Harvested needles of red pine were cleaned with tap water and removed water. The washed pine needle was extracted with 80% MeOH (v/v) at 69 °C for 3 h. This crude extract was further partitioned with n-hexane, EtOAc, and n-BuOH. The n-Hexane fraction was found to be the most active among the solvent fractions.

Cell culture

Human gastric cancer cell lines (AGS, YCC-2, MKN28, SNU-216, SNU-601 and SNU-668) obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea) were cultured in RPMI-1640 medium (Welgene, Daegu, Korea) supplemented with 5% fetal bovine serum (FBS) (Corning Costar, New Work, USA) and 1% antibiotic–antimycotic (Gibco, Auckland, NZ, USA). Cell cultures were maintained at 37 °C in an atmosphere of 5% CO_2.

Cell proliferation detection assays

AGS, YCC-2, MKN28, SNU-216, SNU-601 and SNU-668 cells were plated in 96-well culture plates (1×10^4 per well). After incubation for 24 h, Cells were treated with various concentrations of PNH ($1 \sim 60$ μg/ml). The PNH was dissolved in dimethyl sulfoxide (DMSO: Sigma-Aldrich, St. Louis, MO, USA). After treatment for 24 or 48 h, EZ-cytox kit (WST-1 solution: Daeil, Seoul, Korea) was added to each well. After 1 h of additional incubation, the absorbance was measured on an U.V spectrophotometer (SPARK, Tecan, Switzerland) at a wavelength of 450 nm. Inhibition of cell proliferation by PNH alone or in combination with 5-FU (TargetMol, Wellesley Hills, MA, USA) or Paclitaxel (PTX) (TargetMol) was measured using the WST-1 assay same method.

Crystal violet staining assay

AGS and SNU-668 cells were plated in 6-well culture plates. After incubation for 24 h, the cells were treated with PNH (40 μg/ml) for 48 h. Washing the cells with 1X PBS and fixing by 10 min exposure to 1% glutaraldehyde (Sigma-Aldrich). After fixation, washing with 1X PBS. Cells were stained with 0.5% Crystal violet (Sigma-Aldrich) for 10 min at room temperature.

SA-β-galactosidase assay

The β-galactosidase assay for senescence was performed using a senescence detection kit (BioVision, Milpitas, CA, USA). Briefly, cells were plated in 6-well plate. After incubation for 24 h, the cells were treated with PNH (40 μg/ml) for 48 h. After incubation, the cells washed once with phosphate-buffered saline (PBS; Welgene, Daegu, Korea), and fixed with a fixation solution for 15 min at room temperature. Cells were washed twice with PBS and incubated with the staining solution overnight at 37 °C before microscopic analysis.

Cell cycle analysis

AGS and SNU-668 cells were plated in culture plates and treated with PNH (40 μg/ml) for time-dependent course (24 and 48 h). Cells were harvested and washed with cold PBS, and then resuspended cells in 5 ml 70% EtOH overnight at − 20 °C. After fixation, the cells were washed twice with cold PBS and resuspended in Propidium Iodide (PI: Sigma-Aldrich) solution (RNaseA (Sigma-Aldrich) 50 μg/ml and PI 50 μg/ml in PBS) and transferred to FACS tubes. Cell cycle distribution after PNH (40 μg/ml) treatment was measured by PI staining using CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA).

Reverse transcription polymerase chain reaction (RT-PCR)

The cells were collected by centrifugation and total RNA was isolated from Pine needle extracts-treated cells using RiboEX (GeneAll, Seoul, Korea) according to protocol. To synthesize cDNA, 0.5 μg of total RNA was primed with oligo dT and reacted with mixture of Hyperscript (GeneAll). To measure the mRNA level of target genes, cDNA was amplified using PTC-200 (Bio-Rad, Hercules, CA, USA), AmpONE^{TM} (GeneAll) mixture and the primers. The primers used were: 5′-ATGAAATTCACCCCCT TTCC-3′ (sense) and 5′-CCCTAGGCTGTGCTCACTTC-3′(anti-sense) for human p21$^{CIP/WAF}$ (galectin-3) gene; 5′-AGATGTCAAACGTGCGAGTG-3′ (sense) and 5′-T CTCTGCAGTGCTTCTCCAA-3′ (anti-sense) for human p27^{KIP1} gene; 5′-GGCTGCTTTTAACTCTGGTA-3′ (sense) and 5′-ACTTGATTTTGGAGGGATCT-3′ (anti-sense) for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as a normalization control.

Transfection of Skp2 construction

Transfection of human Skp2 plasmid DNA into the AGS and SNU-668 gastric cancer cells were using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. Human Skp2 was cloned into the pLECE3 vectors. Those pLEECE3-Skp2 construction was reported (Kim et al., 2014).

Western blotting

Cell lysate extractions were prepared with RIPA buffer 20 mM Tris–HCL (pH 7.5), 150 mM NaCl, 1 mM Na_2-EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pytophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 1 μg/ml leupeptin and a protease inhibiter cocktail. The protein concentration was measured using the BCA protein assay kit (Thermo, Waltham, MA, USA). The protein was resolved in SDS-PAGE(sodium dodecyl sulfate–polyacrylamide gel electrophoresis) gels and electro transferred to PVDF membranes, and then blocked in 5% skim milk in 1X TBS-T (1X Tris buffered saline and 0.1% Tween 20). The membranes were incubated over-night at 4 °C with all primary antibody (1:2000 dilution in 5% Bovine serum albumin (BSA) in 1X TBS-T buffer). All secondary antibodies (1:5000 dilution in 5% BSA in 1X TBS-T buffer) were incubated for 2 h at room temperature. Antibodies used in this study were p21, p27, p53, Skp2, Rb, phospho-Rb (Ser 807/811), Cyclin D1, CDK2, CDK4, GAPDH and anti-mouse, anti-rabbit polyclonal immunoglobulins that were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes that were probed with primary antibodies and secondary antibodies were detected by ECL solution (Biosharp, Hefei, China).

Statistical analysis

Statistical analysis were performed by using GraphPad Prism 5 (San Diego, CA, USA). Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) and student's *t* test. Values of $p < 0.05$ were considered statistically significant.

Results and discussion

Effect of PNH extract on cell proliferation of gastric cancer cells

Traditionally, *Pinus densiflora* have been used for alternative medicine in East Asia such as Korea, China and Japan to treat gastroenteric trouble, hypertension and asthma (Bae, 2000). Recently, mechanisms for various biological activities of *pinus densiflora* have been studied (Kwak et al., 2006; Lee et al., 2018). However, *Pinus densiflora* is not well studied of anti-cancer effect in gastric cancer. Therefore, we demonstrated role of PNH in anti-

gastric cancer effects. To examine the anti-proliferative activity of pine needle extract, we initially extracted with 80% MeOH and further partitioned with n-hexane, EtOAc, and n-BuOH. After that we check about gastric cancer cell viability those crude extract (Fig. S1). Among of crude extract, we selected n-hexane extract, because gastric cancer cell viability was decreased by n-hexane extract (PNH). We also checked the effect of PNH on cell proliferation of six human gastric cancer cell lines (Fig. 1). The data showed that treatment of PNH decrease in the cell proliferation using WST assay, with prominent inhibitory effect at a concentration of > 40 μg/ml. Through the Table 1, the half maximal inhibitory concentration of gastric cancer cell growth (IC_{50}) by PNH was about 40 μg/ml. The results showed that PNH inhibited gastric cancer cell proliferation in a dose dependent manner. As followed, we performed mechanism study of 40 μg/ml PNH concentration in gastric cancer cells.

Table 1 IC_{50} values for PNH in viability assays with gastric cancer cell lines

Gastric cancer cell lines	IC_{50} (μg/ml)
SNU216	35.31 ± 2.28
SNU601	39.82 ± 2.59
SNU668	42.62 ± 2.04
AGS	40.88 ± 1.97
MKN28	30.45 ± 2.27
YCC2	45.69 ± 1.66

Determination of IC_{50} value of PNH in six gastric cancer cells after 48 h incubation: results are mean ± SD values for three independent experiments

PNH suppresses the cell proliferation and induction of G1-cell cycle arrest in gastric cancer cells

Among of cancer hallmarks, unlimited cellular proliferation because of uncontrolled expression levels of key factors regulating proliferation and survival are called cancer (Hanahan and Weinberg, 2000). The cell cycle is an essential regulator of the processes of cell proliferation and growth and cancer cells are not stop at points of the cell cycle because of loss of checkpoint. The reason why important of targeting the cell cycle in anticancer therapy has been based on results of the cell cycle arrest by CDK inhibition could induce apoptosis in cancer (Schwartz and Shah, 2005). Among of 6 gastric cancer cell lines, we selected AGS and SNU-668, as a high effect on PNH, for examine the molecular mechanism. At first, we performed that growth inhibition was associated with inhibition of cell proliferation. After PNH treatment decrease of AGS and SNU-668 density were observed by WST assay (Fig. 2A) and crystal violet staining (Fig. 2B). Also, we confirmed that cell cycle arrest was associated with inhibition of cell proliferation. We then identified cell cycle population through PI staining (Fig. 2C, D). We analyzed the cell cycle phase through treatment of PNH for 24 and 48 h in AGS (Fig. 2C) and SNU-668(Fig. 2D). AGS treated with PNH (40 μg/ml) for 24 h resulted in an increase in G1-cell cycle phase (62.1%) higher than none treated cells G1-cell cycle phase (48.0%). Furthermore, G1-cell cycle phase (69.6%) were more increased after 48 h. SNU-668 treated with PNH (40 μg/ml) for 24 h resulted in an increase in G1-cell cycle phase (63.6%) higher than none treated cells G1-cell cycle phase (49.0%). Furthermore, G1-cell cycle phase (77.0%) were more increased after 48 h. Those data showed that PNH induced G1-cell cycle phase is significantly increased in a time-dependent manner and S and G2/M phase decreased.

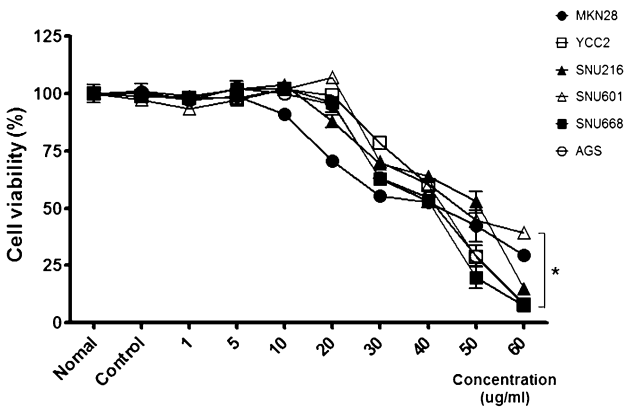

Fig. 1 Treatment with PNH in gastric cancer cell lines reduces cell viability in a dose-dependent manner. WST assays were performed to detect the cell viability by a dose-dependent PNH treatment for 48 h in AGS, MKN28, YCC2, SNU216, SNU601 and SNU-668. The error bars indicate 95% confidence intervals; $*P < 0.0001$ using one-way ANOVA

Increased premature senescence by PNH without oncogenic stress

Recent studies have confirmed that an irreversible cell growth arrest state of cellular senescence as anti-proliferative activity mechanism (Beausejour and Campisi, 2006; Shay and Wright, 2007). Therefore, we confirmed that examine whether premature senescence induced by PNH in AGS and SNU-668 for 24 h treatment, using by SA-β-gal activity, as an universal marker of cellular senescence (Fig. 3A, B). Because, cellular senescence can cause limit excessive or aberrant cellular proliferation for protects against the development of cancer (Collado et al., 2007). Observation with a microscope (Fig. 3A), cellular senescence significantly increased in both AGS and SNU-668

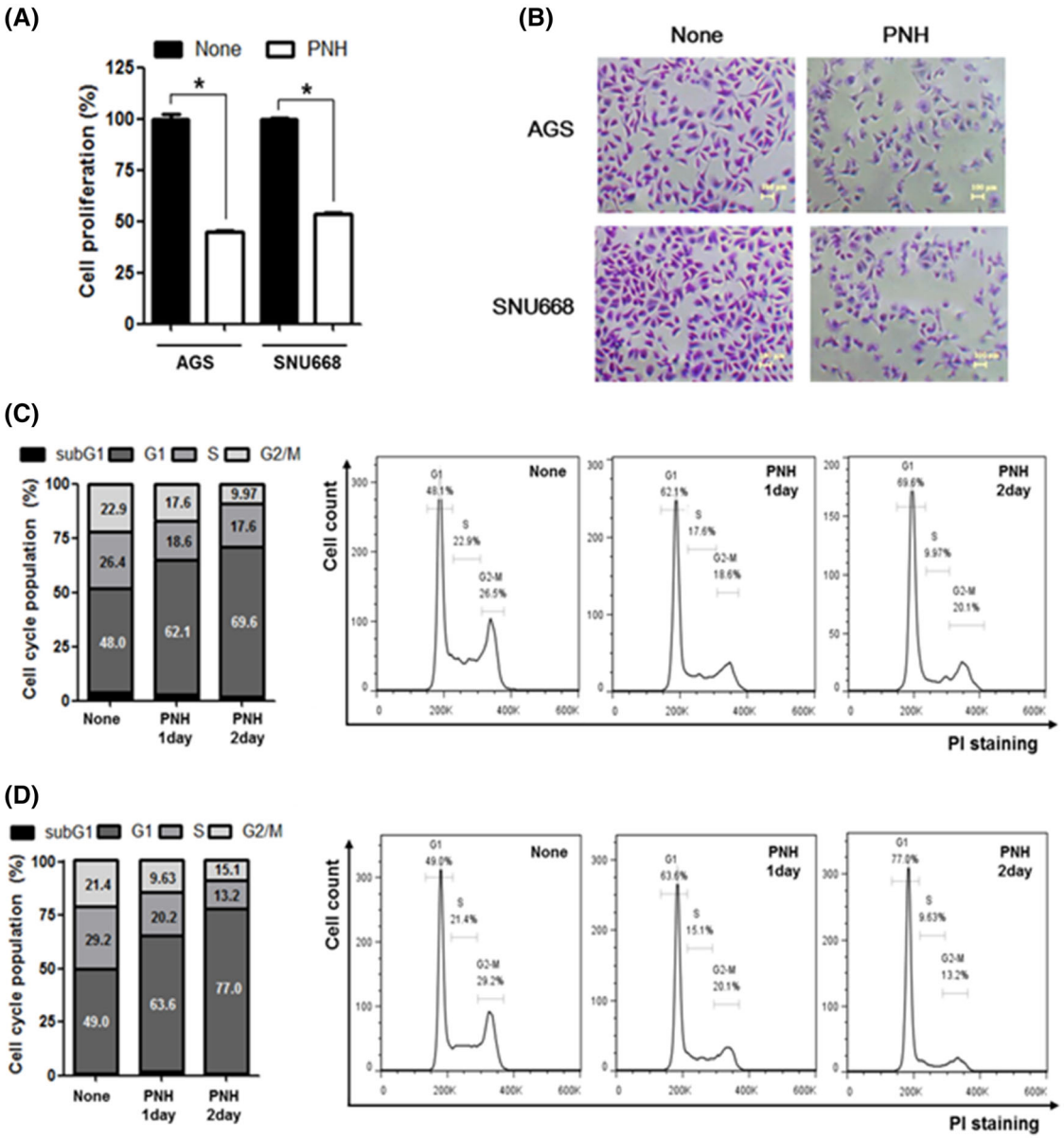

Fig. 2 PNH reduces the proliferation and induces cell cycle arrest in gastric cancer cells. **A** Cell proliferation after PNH (40 μg/ml) treatment for 48 h in AGS and SNU-668. The error bars indicate 95% confidence intervals; $*P < 0.0001$ using the two-sided *t*-test. **B** After PNH treatment for 48 h, morphology and density of four gastric cancer cells were assessed by microscope and crystal violet staining, respectively. Scale bar represents 100 μm. **C**, **D** Cell cycle populations were examined by PI staining after PNH (40 μg/ml) treatment for 1 and 2 days in AGS and SNU-668. A quantitative graph (left panel) and histogram (right panel) are generated

cells lines (36% in AGS cells and 31% in SNU-668 cells) compared to none treatment group (Fig. 3B). Taken together, these results demonstrate that PNH induce premature senescence in the gastric cancer cells.

PNH induces G1/S cell cycle arrest and premature senescence through the $p27^{KIP1}$ protein expression

In order to confirm G1-cell cycle arrest and premature senescence in PNH treated gastric cancer cells, we examined expression levels of cell cycle and senescence related genes, such as $p21^{CIP/WAF}$ and $p27^{KIP1}$ using by RT-PCR (Fig. 3C). Through the mRNA expression of $p21^{CIP/WAF}$ and $p27^{KIP1}$ were not change. However, interestingly, when we checked the protein expression of $p27^{KIP1}$ and $p21^{CIP/WAF}$ were not same with mRNA expression data (Fig. 3D). Usually, high levels of p53 induce G1 phase arrest by increasing $p21^{CIP/WAF}$ expression (Osman et al., 1999). However, the expression levels of $p21^{CIP/WAF}$ and p53 decrease by the treatment with PNH

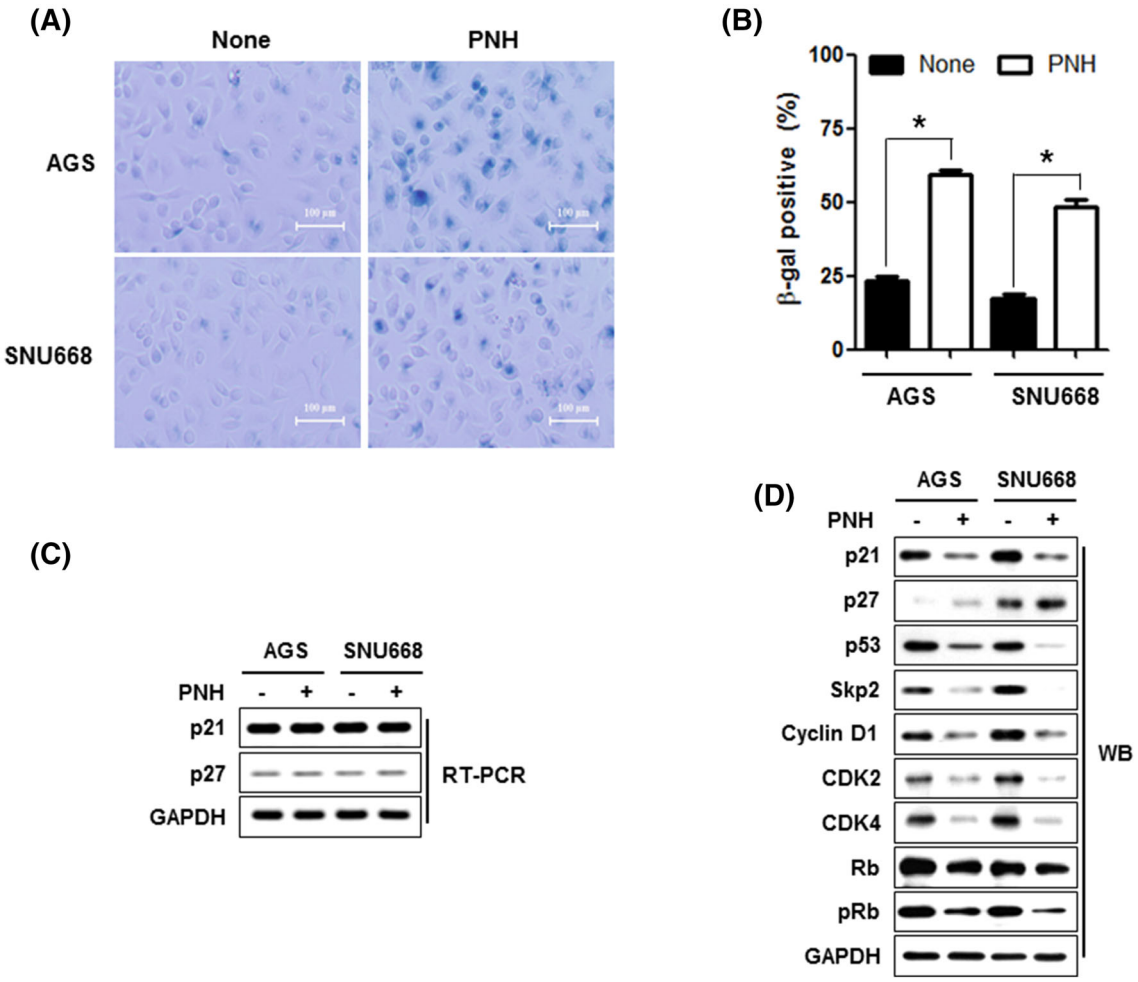

Fig. 3 Increased premature senescence and expression of G1 phase-associated regulatory proteins in gastric cancer cells treated with PNH. **A** Premature senescence was detected by β-galactosidase activity after PNH (40 μg/ml) treatment for 48 h in AGS and SNU-668. **B** The graph shows the percentage of β-galactosidase-positive cells. The error bars indicate 95% confidence intervals; $*P < 0.0001$ using the two-sided *t*-test. **C**, **D** Cell lysates of AGS and SNU-668 after PNH (40 μg/ml) treatment for 48 h were prepared and analyzed by RT-PCR using primers specific for $p21^{CIP/WA}$ and $p27^{KIP1}$. GAPDH served as the normalization control. Protein levels of $p21^{CIP/WAF}$, $p27^{KIP1}$, p53, Skp2, Cyclin D1, CDK2, CDK4, Rb and pRb (Ser 807/811) were also detected by western blotting. GAPDH was used as the normalization control

both AGS and SNU-668 cells for 24 h. These results showed that p53 and $p21^{CIP/WAF}$ were not affected in G1 cell cycle arrest by PNH treatment. Also, Fig. 3D data showed that $p27^{KIP1}$ was increased in by the treatment with PNH both AGS and SNU-668 cells for 24 h. In addition, we have previously reported that p27KIP1 was involved in premature senescence of gastric cancer cells and that it was regulated by Skp2 level in gastric cancer (Kim et al., 2014). Therefore, we were confirmed that Skp2 expression in AGS and SNU-668 cells with PNH treatment, which contributed to the progression of cancer by Skp2–mediated $p27^{KIP1}$ degradation acted as an oncogene in human gastric cancer cells (Kim et al., 2014; Wen et al., 2016). The data showed that, PNH decrease Skp2 protein expression levels and induce premature senescence through induction of $p27^{KIP1}$ protein expression. Also followed, PNH increase G1 cell cycle arrest through the induced $p27^{KIP1}$ (Fig. 3D).

Generally, G1 phase of the cell cycle is regulated by the sequential assembly and activation cyclin-CDK complexes (Viglietto et al., 2002). $p27^{KIP1}$ plays an important role in the regulation of cyclin-CDK complexes. The expression of CDK2, CDK4 and cyclin D1 decreased following treatment with PNH. Also, we confirmed that retinoblastoma (Rb) protein expression as a cell cycle regulator, phosphorylated Rb is known as inhibit cell cycle progression with E2F transcription factor binding (Nevins, 2001). Phosphorylated Rb decreased by PNH treatment (Fig. 3D). Therefore, PNH induced inhibition of cell cycle progression through regulation of cyclin-CDKs and Rb.

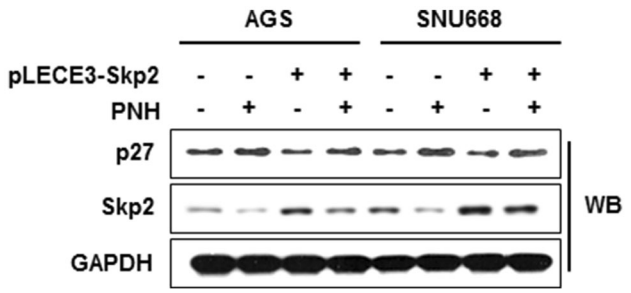

Fig. 4 Protein regulation of PNH in Skp2 overexpressed gastric cancer cells. Cell lysates of Skp2 overexpressed AGS and SNU-668 after PNH (40 μg/ml) treatment for 48 h were prepared and protein levels were detected using antibodies against $p27^{KIP1}$ and Skp2 by western blotting. GAPDH was used as the normalization control

PNH induced $p27^{KIP1}$ protein expression via Skp2 down-regulation

To examine the regulation of abnormal expression of Skp2 by PNH, we performed transfection experiments using Skp2 overexpression vector. When Skp2 was overexpressed in AGS and SNU-668, the expression of $p27^{KIP1}$ was decrease. And also, we were confirmed increased $p27^{KIP1}$ expression resulting from PNH treatment in AGS and SNU-668 cells was significantly diminished by Skp2 overexpression. These data suggest that PNH significantly induced $p27^{KIP1}$ through the Skp2 expression in gastric cancer cells (Fig. 4).

Combined treatment with PNH and anti-cancer agents, 5-Fluorouracil and Paclitaxel, enhanced the therapeutic effects on gastric cancer cells

We also confirmed that clinical potential of PNH when combination treatment of anti-cancer drugs as a 5-Fluorouracil (5-FU) and Paclitaxel (PTX). 5-FU is one of the most widely used anti-cancer drugs. It is a pyrimidine analog and works through irreversible inhibition of thymidylate synthase (Longley et al., 2003). PTX is an anti-microtubule agent and one of the anti-cancer chemotherapy drugs. It is a cytoskeletal drugs and induce

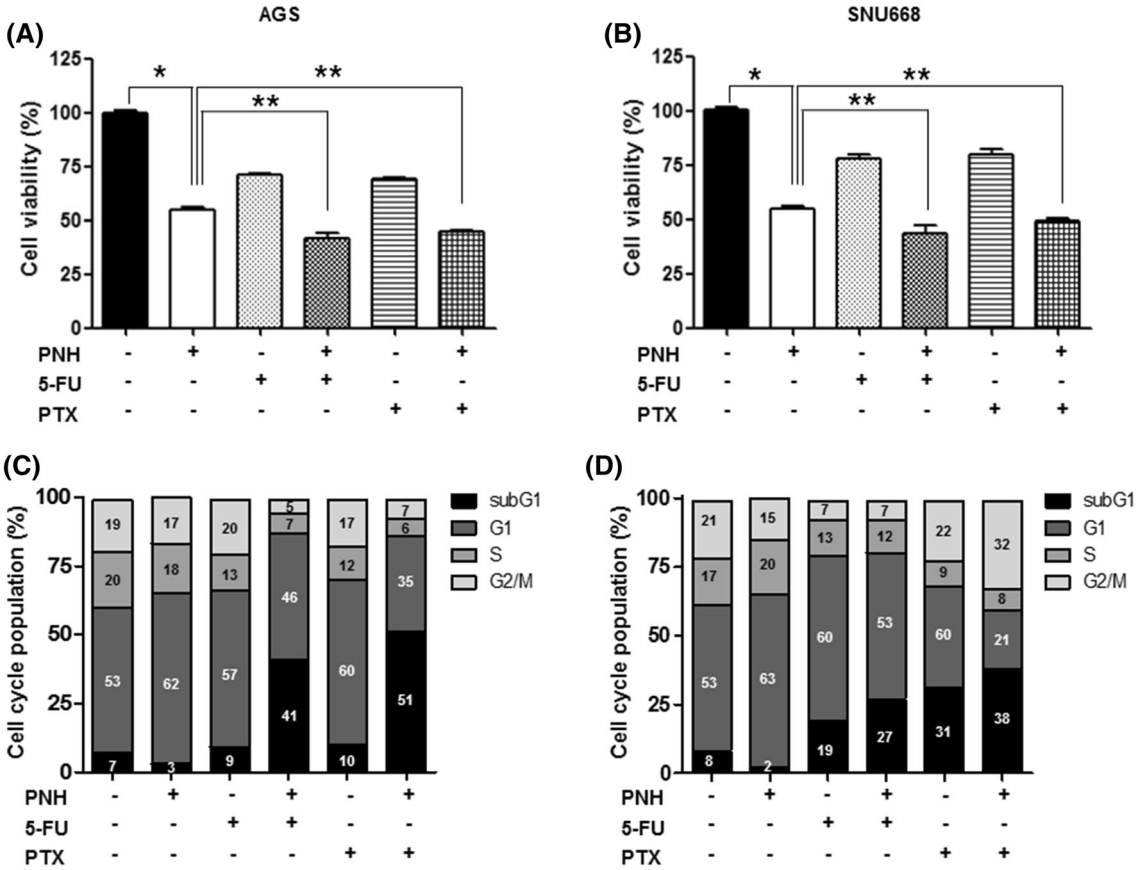

Fig. 5 The effect of PNH and anti-cancer agent combination treatment on gastric cancer cells. **A**, **B** Combined treatment with PNH alone (40 μg/ml) and anticancer agents 5-FU alone (2 μM in AGS, 6 μM in SNU-668), PTX alone (2 nM in AGS, 10 nM in SNU-668 or a combination of half concentration, enhanced the therapeutic effects on AGS(**A**) and SNU-668(**B**) cells. The error bars indicate 95% confidence intervals; $*P < 0.0001$ using the two-sided *t*-test and $**P < 0.005$. **C**, **D** Cell cycle populations were examined by PI staining after 5-FU, PTX and PNH alone or combination treatment for 48 h in AGS(**C**) and SNU-668(**D**) cells

fault in mitotic spindle assembly and cell division (Wang et al., 2003). Therefore, to confirm the practical applications of PNH in gastric cancer therapy, we conducted a combination treatment with PNH and anti-cancer agents, as a 5-fluorouracil (5-FU) and paclitaxel (PTX) in gastric cancer cells (Kim et al., 2016). AGS and SNU-668 were treated with PNH alone (40 μg/ml), 5-FU alone (2 μM in AGS, 6 μM in SNU-668), PTX alone (2 nM in AGS, 10 nM in SNU-668) or a combination of PNH and 5-FU or PNH and PTX (each at half concentration). After 48 h, we performed WST assays for cell viability and each combination treatment showed more inhibition of cell viability than treatment with the single agents alone in both cells (Fig. 5A, B). Through the PI stating, the combination treatments (PNH with 5-FU or PTX) were increased sub-G1 population (41% or 51%) more than the single treatments (7–10%) in AGS cells (Fig. 5C). Also, SNU-668 cells showed similar aspect of sub-G1 population induction (Fig. 5D). These data supported that treatment of PNH was help to increase apoptosis with anti-cancer drugs, as a 5-FU and PTX (Fig. 5C, D). Also, these data propose that PNH enhanced the therapeutic effects on gastric cancer cells when it was used in combination treatments anti-cancer drugs.

However, we did not identify any single compound in PNH to be responsible for the anti-cancer effect, which is a limitation of this study and the subject of future investigations. In addition, we reasoned that PNH was a better candidate for determining the mechanism underlying PNH-mediated cell cycle regulation and premature senescence in gastric cancer cells. Taken together, we demonstrated that PNH inhibited gastric cancer cell proliferation by inducing G1 phase arrest and premature senescence by increasing $p27^{KIP1}$ and decreasing Skp2 expression levels. Therefore, we suggest that PNH could be a candidate for anti-proliferative activity in gastric cancer.

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Compliance with ethical standards

Conflict of interest The authors have no conflicting interest.

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