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NCAPH Is Required for Proliferation, Migration and Invasion of Non-small-cell Lung Cancer Cells

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Abstract. *Background/Aim: Non-structural maintenance of chromosomes condensin I complex subunit H (NCAPH) is implicated in correct chromosome condensation and segregation during mitosis. However, the functional role of NCAPH in the pathogenesis of non-small-cell lung cancer (NSCLC) remains unclear. The aim of this study was to elucidate the role of NCAPH in NSCLC cells. Materials and Methods: A549 and H1299 NSCLC cells were transfected with small-interfering RNA (siRNA) against NCAPH. Subsequently, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, colony-formation assay and flow cytometry analysis were performed to reveal the role of NCAPH in NSCLC cells. In addition, migration and invasion assay were also performed. Results: NCAPH knockdown inhibited cell proliferation, induced cell-cycle arrest at G_2/M phase, and prevented colony formation, migration and invasion by NSCLC cells. Conclusion: NCAPH is involved in NSCLC progression and development, and may be a potential therapeutic target for NSCLC treatment.*

Condensins are large protein complexes that play a role in chromosomal assembly and segregation during mitosis and meiosis (1). There are two different types of condensin complexes in eukaryotes, condensin I and II (2). Both complexes contain the same pair of core subunits, the structural maintenance of chromosomes (SMC) proteins, SMC2 and SMC4. Condensin I and II also contain three non-SMC subunits, NCAPD2, NCAPG, and NCAPH in condensin I; and NCAPD3, NCAPG2, and NCAPH2 in condensin II (3). SMC2 and SMC4 belong to a larger family of chromosomal ATPases and the SMC2–SMC4 heterodimer adopts a V-shaped structure with two long coiled-coil arms. The non-SMC subunits, CAP-D2, CAP-G, CAP-D3 and CAP-G2 contain HEAT repeats, while the CAP-H and CAP-H2 subunits belong to the kleisin family of proteins (4). The HEAT repeat domains of the condensin non-SMC subunits are involved in DNA binding, resulting in the interaction of the condensin complexes with chromosomes (5). Condensins play different roles in chromosomal structure depending on their subunits. Condensin I is involved in the formation of the compact loops of mitotic chromosomal axes, while condensin II is required for the rigidity of those axes (6, 7).

In addition to the functions associated with mitotic chromosomal condensation and segregation of non-SMC subunits, recent studies have also reported that non-SMC condensin I subunits are implicated in the regulation of human cancer progression (8-10). In fact, NCAPD2 is involved in breast cancer progression *via* p53 signaling pathway-related cell-cycle arrest. Furthermore, NCAPG has been identified as a novel mitosis-related gene required for hepatocellular cancer (HCC) cell proliferation and migration. Another non-SMC subunit, NCAPH, has also been implicated in the pathogenesis of several types of cancer. NCAPH plays a role in mitotic cell death, a major form of cell death in cancer cells, to prevent cancer cell proliferation with uncontrolled cell cycles (11). Furthermore, studies have shown that NCAPH expression is involved in the pathogenesis and prognosis of colonic cancer (CC) and HCC (12, 13). NCAPH is highly expressed in CC and HCC tissues compared to normal non-cancerous tissues, and is associated with reduced survival rates. Moreover, NCAPH inhibits CC and HCC cell proliferation and migration, as well as xenograft tumor formation. Intriguingly, recent bioinformatics analyses in a study of potential molecular mechanisms associated with lung and prostate cancer led to the identification of *NCAPH* as a key gene involved in lung tumorigenesis (14-16).

Lung cancer is the most common malignant tumors. It is histopathologically classified into small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC). In

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particular, NSCLC is responsible for about 80-85% of all lung cancer diagnoses (17). Although up-regulation of NCAPH expression has been associated with NSCLC pathogenesis, the functional and molecular mechanisms behind this remain unclear. Herein, we sought to elucidate the oncogenic effect of NCAPH in human lung cancer cell lines.

Materials and Methods

Cell culture. Human NSCLC cell lines (A549, Calu-1, Calu-3, H358, H460, H1299, HCC827, and SK-MES-1) and the normal lung fibroblast cell line (MRC5) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). MRC5 cells were cultured in the Eagle's minimum essential medium (EMEM; Welgene, Daegu, Korea). Calu-1 and Calu-3 cell lines were cultured in the McCoy's 5A and Dulbecco's modified Eagle's medium (DMEM; Welgene), respectively. All other cells were cultured in RPMI1640 medium (Welgene). The media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; GibcoBRL Life Technologies, Rockville, MD, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA, USA). All cells were maintained in a humidified incubator at 37˚C with 5% CO_2.

Small interfering RNA (siRNA) transfection. A549 and H1299 cells were transfected with siRNA oligonucleotide duplexes against human *NCAPH* using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The siRNA sequences targeting *NCAPH* (*NCAPH* siRNA-1: 5'-GAGUUCAGGAGCUGGAAGG-3'; *NCAPH* siRNA-2: 5'-ACCACAGGGAAGCUGGAAA-3'; *NCAPH* siRNA-3: 5'- UCAGAGAUUCUUAAACAGAAA) were designed and synthesized for transient transfection. AccuTarget Control siRNA (Bioneer, Daejeon, Republic of Korea) was used as a negative control.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis. Total RNA was isolated from NSCLC cells using the Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed using SYBR Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan) on a CFX96 real-time PCR Detection System (Bio-Rad, CA, USA). Next, cDNA was amplified as follows: 94˚C for 2 min, 94˚C for 5 s, 60˚C for 10 s, and 72˚C for 20 s. Steps 2 through 4 were repeated for 40 cycles. Each reaction was performed in triplicate, and the results of three independent experiments were used for statistical analysis. Relative mRNA expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method (18). Primer sequences were as follows: *NCAPH* forward 5'- AAACAACCTCAATGTCTCCGAAG-3', reverse 5'- ACAACCTAACTCT GGCAACTCG -3'.

Western blot analysis. NSCLC cells were lysed in RIPA buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate] containing ethylenediaminetetra-acetic acid (EDTA)-free protease inhibitor cocktail tablets (Roche, Basel, Switzerland). Equal amounts of proteins were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 1 h in TBS-T [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20] containing 5% skim milk at room temperature, and then incubated with primary antibodies to NCAPH (Novus Biologicals, Littleton, CO, USA), phospho-histone H3 (Cell Signaling Technology, Danvers, MA, USA), histone H3 (Cell Signaling Technology) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4˚C. After washing the membranes, appropriate peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Inc., West Grove, PA, USA) were applied for 1 h at room temperature. The blots were then detected by EZ-Western Detection Kit (DoGEN Bio, Seoul, Republic of Korea).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A549 and H1299 cells were seeded in 96-well plates at a density of 3×10^4 cells/well. Next, 5 mg/ml (MTT) solution (Sigma, St. Louis, MO, USA) was added to each well and the plates were incubated for 4 h at 37˚C. After removing the medium, dimethyl sulfoxide (Sigma) was added to each well at 37˚C to solubilize formazan crystals. Absorbance values were measured at 570 nm.

Colony-formation assay. A549 and H1299 cells were seeded in 60 mm dishes and incubated at 37˚C in 5% CO_2 for 14 days to allow for colony formation. Colonies were fixed with 95% methanol for 10 min and stained with 2% methylene blue in 50% ethanol. For colony formation in soft-agar, the culture dishes were prepared to be separated into a lower layer containing 0.6% agar and an upper layer containing 0.3% agar, then cells were seeded onto culture dish and incubated for 3 weeks. Colonies were imaged and counted.

Cell-cycle analysis. A549 and H1299 cells were trypsinized, washed twice with phosphate-buffered saline (PBS) and fixed in 70% ethanol. The fixed cells were washed with PBS, incubated with 100 μg/ml RNase at 37˚C for 30 min, stained with propidium iodide (50 μg/ml) and analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The proportion of cells in each cell-cycle phase were analyzed using Cell Quest Pro (BD Biosciences) and ModFit LT™ (Verity Software House, Topsham, ME, USA).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Cell death was determined by *in situ* cell death detection kit (Roche Molecular diagnostics, Mannheim, Germany), based on labeling DNA strand breaks by TUNEL assay according to the manufacturer's protocol. Briefly, A549 and H1299 cells were seeded on coverslips, washed twice with PBS, and then fixed with 4% paraformaldehyde. The slips were incubated in permeabilisation solution (0.1% Triton X-100 in 0.1 % sodium citrate) for 2 min on ice, and were stained with TUNEL reaction mixture for 1 h at 30˚C in the dark. The coverslips were mounted with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). TUNEL-positive cells were identified under a Zeiss LSM 510 Meta microscope (Carl Zeiss, Jena, Germany) and were analyzed by Zen Pro software (Carl Zeiss).

Migration and invasion assay. A549 and H1299 cells were seeded in 6.5-mm transwell plates with an 8.0-μm pore polycarbonate membrane insert (Corning, Bedford, MA, USA) in 150 μl serum-free media. The lower chamber was filled with 700 μl of medium containing 10% FBS. After 24 h, migrant cells that had attached to the lower surface were fixed with 100% methanol for 10 min and

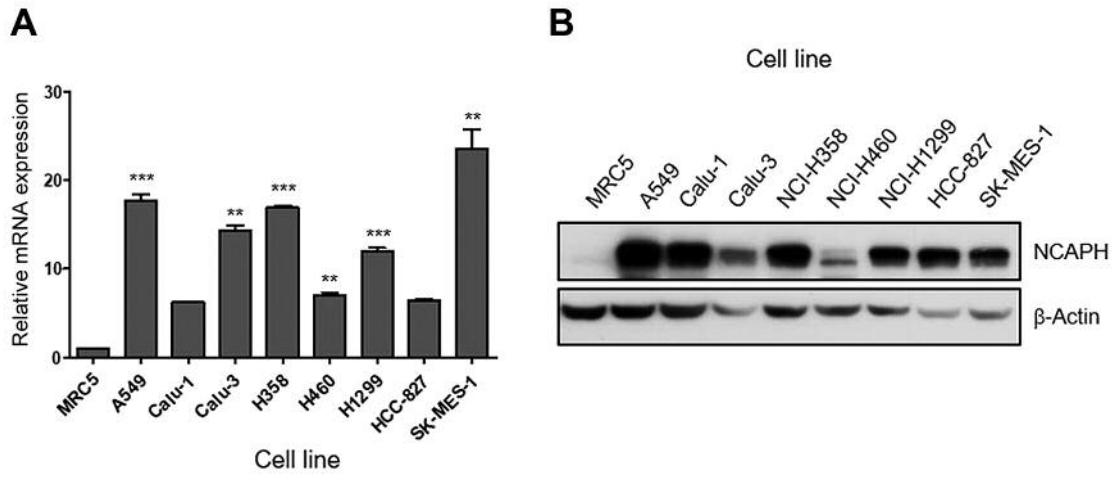

Figure 1. *mRNA expression of non-structural maintenance of chromosomes condensin I complex subunit H (NCAPH) in non-small-cell lung cancer (NSCLC) cell lines and normal lung cells. A: NCAPH mRNA expression was measured in a normal lung cell line (MRC5) and different human NSCLC cell lines by quantitative real time-reverse transcription polymerase chain reaction. This experiment was repeated three times and the data shown represent the mean±SD. Significantly different at **p<0.01 and ***p<0.001 compared with IMR90 cell. B: NCAPH protein expression was detected by western blotting using the same cell lines. Cell lysates were probed with NCAPH antibody and β-actin was used as a loading control.*

stained for 10 min with Hoechst (Sigma). Cells in five random different fields of view at ×20 magnification were counted using an IN Cell Analyzer 2500HS imaging system (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

Statistical analysis. All experiments were performed at least three times. The data are presented as the mean ± standard deviation (SD). Statistical comparisons were carried out using unpaired *t*-tests and *p*-values of less than 0.05 were considered to be statistically significant. All statistical analyses were performed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA).

Results

NCAPH is highly expressed in lung cancer cell lines. Recent bioinformatics analyses have shown that *NCAPH* is up-regulated in lung cancer (15, 16). However, its functional role in lung cancer remains unclear. To evaluate the role of NCAPH in lung cancer, we analyzed NCAPH expression in different NSCLC cell lines (namely A549, Calu-1, Calu-3, H358, H460, H1299, and HCC-827) and in a normal lung fibroblast cell line (MRC5). Our qRT-PCR data show that *NCAPH* mRNA expression was significantly up-regulated in six out of eight NSCLC cell lines compared to normal MRC5 cells (Figure 1A). We also found that NCAPH protein expression was also up-regulated in NSCLC cell lines compared to MRC5 cells (Figure 1B). These results suggest that NCAPH is linked to lung cancer cell progression. Thus, lung adenocarcinoma cell lines, A549 and H1299 cells, which are cell lines commonly used to study the functional mechanisms involved in lung cancer, were chosen for further studies.

NCAPH-targeted siRNAs reduce NCAPH expression in NSCLC cells. To elucidate the role of NCAPH in human NSCLC, we transfected three *NCAPH* siRNAs into A549 and H1299 cells, and validated their knockdown efficiency through measurement of mRNA and protein expression. qRT-PCR analysis showed approximately 80-90% mRNA knockdown efficiency in A549 and H1299 cell lines (Figure 2A). Next, we confirmed NCAPH protein expression by western blot analysis. NCAPH protein expression was also down-regulated in siRNA-treated A549 and H1299 cells (Figure 2B).

NCAPH knockdown inhibits NSCLC cell proliferation. We performed MTT assay and colony-formation assays to determine the effect of *NCAPH* knockdown on A549 and H1299 cells. MTT assay data showed that all of the siRNAs targeting NCAPH significantly reduced A549 and H1299 growth rates (Figure 3A). Next, colony-formation assays revealed that both *NCAPH* siRNA-treated cell lines formed fewer cell colonies compared to control cells (Figure 3B). These results suggest that *NCAPH* knockdown significantly reduced NSCLC cell proliferation.

NCAPH knockdown induces cell-cycle arrest at the G_2/M phase and increases apoptosis. To understand whether NCAPH is essential for cell proliferation, we analyzed the effect of *NCAPH* knockdown on the cell-cycle distribution of A549 and H1299 lung cancer cells by cell-cycle analysis. We found that the cell-cycle distribution of *NCAPH* siRNA-transfected cells was different from that of control cells. The

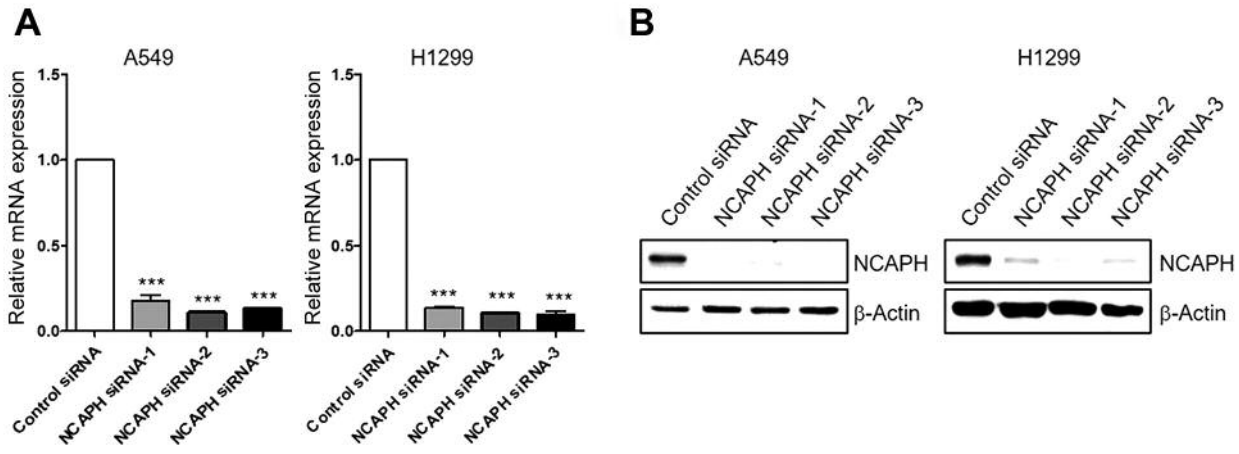

Figure 2. *Knockdown of non-structural maintenance of chromosomes condensin I complex subunit H (NCAPH) by small interfering (siRNA) treatment in non-small-cell lung cancer (NSCLC) cells. A: A549 and H1299 cells were transfected with three different siRNAs for NCAPH (NCAPH siRNA-1, -2, and -3), as well as a negative control siRNA. After 48 h, NCAPH mRNA expression was measured by quantitative real time-reverse transcription polymerase chain reaction. The data are presented as the mean±SD from three independent experiments. ***Significantly different at p<0.001 compared with control siRNA-transfected cells. B: NCAPH protein expression was assessed by western blot analysis. Cell lysates were probed with antibody to NCAPH, and β-actin was used as a loading control.*

results showed that *NCAPH* siRNA-transfected cells displayed no significant change in the G_0/G_1 and S phases. However, cell numbers in the G_2/M phase were significantly higher in the *NCAPH* knockdown A549 (Figure 4A) and H1299 cells (Figure 4B). Cell-cycle arrest at G_2/M phase by *NCAPH* knockdown was further demonstrated by expression of phosphorylated histone H3, as a cell cycle-related marker of mitotic phase using western blot analysis (Figure 4C). As expected, phosphorylation of histone H3 in *NCAPH* knockdown A549 and H1299 cells was relatively high compared to that of control cells. Collectively, these results suggest that NCAPH expression is significantly associated with the cycle distribution in lung cancer cells.

Next, in order to determine whether the inhibition of cell proliferation by *NCAPH* knockdown was attributed to cell death, we measured apoptosis of A549 and H1299 cells with *NCAPH* knockdown using TUNEL assay. As shown in Figure 4D, apoptosis of A549 and H1299 cells was increased by *NCAPH* siRNA treatment.

NCAPH knockdown inhibits anchorage-independent growth, migration, and invasion by NSCLC cells. Soft-agar assays were performed to evaluate the effect of *NCAPH* siRNA on tumorigenicity in lung cancer cells. *NCAPH* knockdown in both the A549 and H1299 cell lines resulted in fewer colonies compared to the control siRNA-transfected cells (Figure 5A). We also studied cell migration and invasion by A549 and H1299 cells using a transwell assay. The number of *NCAPH*-silenced A549 and H1299 cells that managed to migrate were lower compared to the control cells (Figure 5B). The invasive ability of A549 and H1299 cells with *NCAPH* knockdown was also notably hampered in comparison to the control cells (Figure 5C). Taken together, these results indicate that *NCAPH* knockdown reduced anchorage-independent growth and motility, as well as the invasive properties of NSCLC cells.

Discussion

Bioinformatics studies aimed at identifying potential diagnostic and therapeutic markers for lung cancer have shown that many genes, primarily those involved in the regulation of the cell cycle, cell proliferation, and cell division, are associated with tumorigenesis or metastasis (14, 16). Normal cells undergo growth, DNA replication and division, and the cell cycle includes a mechanism for correcting errors that occur during this process. One of the hallmarks of cancer is abnormal cell proliferation, in which the process of cell-cycle regulation malfunctions due to genetic mutation. Thus, cell-cycle dysregulation is closely linked to carcinogenesis and tumor progression (19). Recent studies have shown that condensin subunits, being involved in chromosome condensation and segregation during the cell cycle, are associated with cancer and may be potential anticancer therapeutic targets (20). For example, knockdown of the D2 subunits of NCAPD2 results in inhibition of cell proliferation, arrest at G_2/M, increased apoptotic cell death, and reduced invasiveness of triple-negative breast cancer cells (10). NCAPG regulates proliferation, is involved in regulation of the cell cycle and migration of HCC cells (9). Moreover, growing evidence has shown that NCAPH is a mitosis-regulating gene involved in

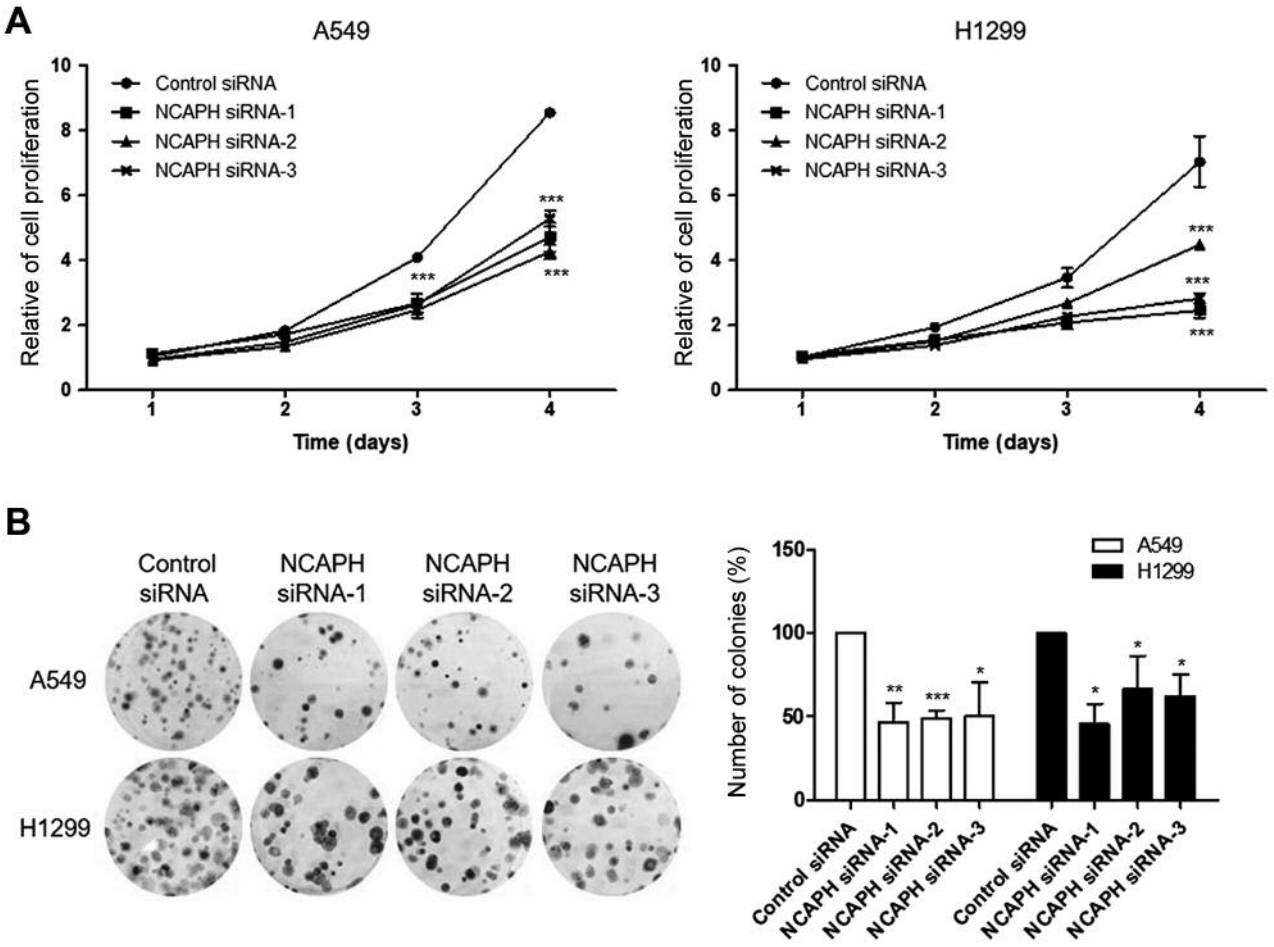

Figure 3. *Effects of knockdown of non-structural maintenance of chromosomes condensin I complex subunit H (NCAPH) on non-small-cell lung cancer (NSCLC) cell proliferation. A: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed to determine the proliferative effect of NCAPH knockdown in A549 and H1299 cells. Growth curves were generated to determine the number of cells grown 24, 48, 72 and 96 h after NCAPH siRNA transfection. B: Colony formation assays of A549 and H1299 cells treated with either control siRNA or NCAPH siRNAs (NCAPH siRNA-1, NCAPH siRNA-2). Cells were grown for 14 days after transfection. Colonies were subsequently fixed with methanol, stained with methylene blue, counted, and imaged. The data are presented as the mean±SD from three independent experiments. Significantly different at *p<0.05, **p<0.01 and ***p<0.001 compared with control siRNA-treated cells.*

tumorigenesis and cancer progression. NCAPH expression is up-regulated in various cancer types including of the colon, prostate, liver, and pancreas (12, 13, 15, 21). Additionally, NCAPH overexpression is associated with poor patient prognosis. Taken together, this suggests that NCAPH plays an important role in cancer progression and development. In fact, according to bioinformatics analyses from The Cancer Genome Atlas datasets, NCAPH is significantly overexpressed in NSCLC (14). However, functional studies of the role of NCAPH in NSCLC have not been sufficiently explored.

In this study, we demonstrated the oncogenic effect of NCAPH in NSCLC. Our results indicate that knockdown of *NCAPH* by siRNAs suppressed cell growth, colony formation, migration, and invasion of A549 and H1299 cell lines. Furthermore, cell-cycle analysis showed that *NCAPH* knockdown in A549 and H1299 cells led to an accumulation of cells in the G_2/M phase and increased cell death. This suggests that reduced cell proliferation following *NCAPH* knockdown is due to impaired cell-cycle progression and increased cell death. Furthermore, *NCAPH* knockdown significantly reduced anchorage-independent growth, migration, and invasion in A549 and H1299 cells. These data suggest that NCAPH is an important oncogenic factor involved in the regulation of proliferation and cell cycle in NSCLC cells. Indeed, previous studies have shown that *NCAPH* knockdown induces G_2/M arrest in pancreatic cancer (21) and CC cells (12). In pancreatic cancer cells, *NCAPH* knockdown was found to induce arrest at the S and

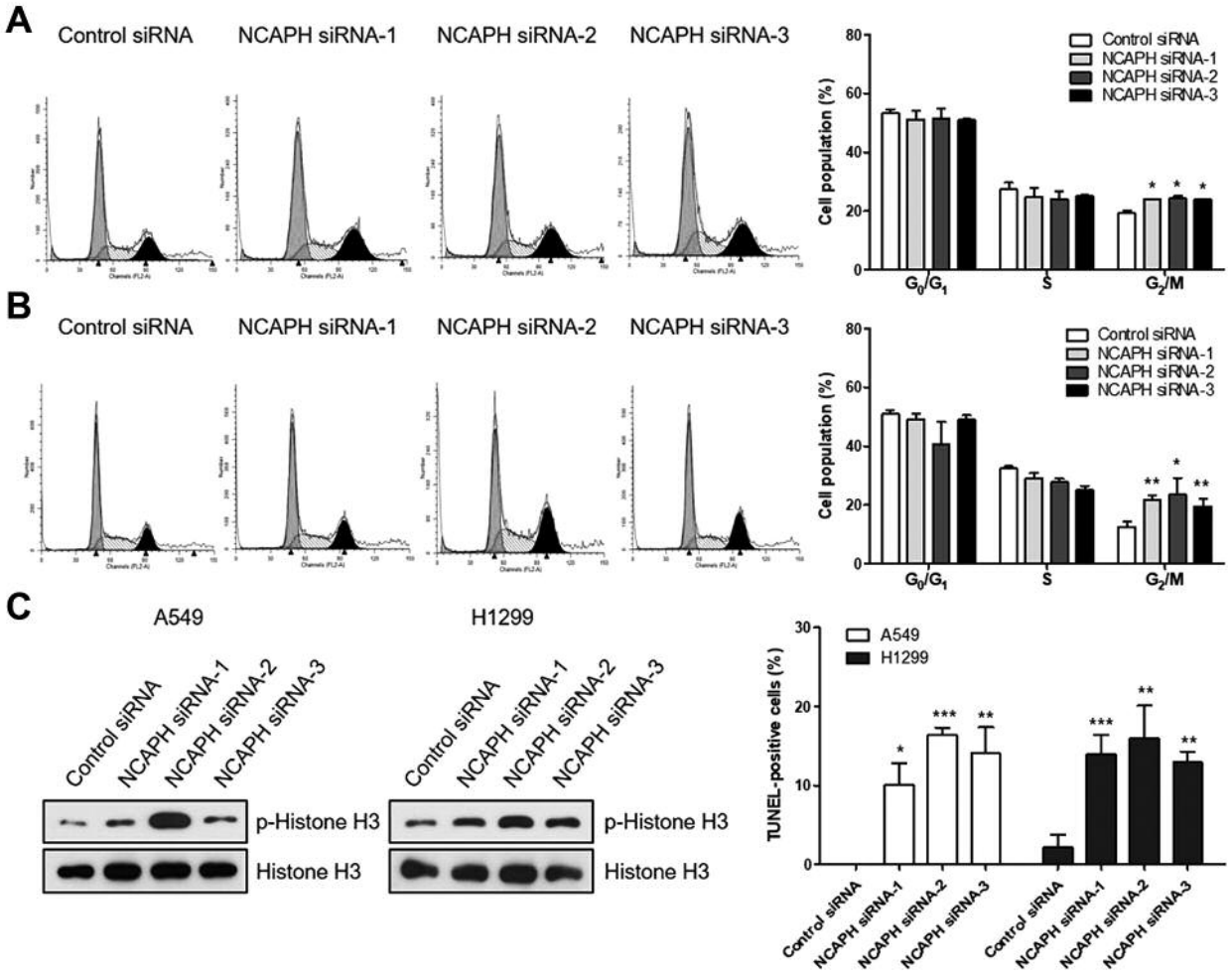

Figure 4. *Effects of knockdown of non-structural maintenance of chromosomes condensin I complex subunit H (NCAPH) on cell-cycle progression in A549 and H1299 non-small-cell lung cancer cells. A549 (A) and H1299 (B) cells were transfected with control siRNA or NCAPH siRNAs and all cells were analyzed by flow cytometry. The proportion of cells in each cell-cycle phase was analyzed using Modfit LT software and is represented as histogram plots. The bar graph shows the proportion of cells in the G_0/G_1, S and G_2/M phases. C: The expression of phospho-histone H3 was tested by western blot analysis in these cells, and anti-histone H3 antibody was used as internal control. D: Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to measured apoptotic cells. The bar graph represented the percentage of TUNEL-positive cells. Data was shown as the mean±SD from three independent experiments. The significant difference was determined by mean±SD. Significantly different at *p<0.05, **p<0.01 and ***p<0.001 compared with control siRNA-treated cells.*

G_2/M phases of the cell cycle, thereby preventing proper chromosome condensation. This suggests that NCAPH is required during cell-cycle progression for the development of mature condensed chromosomes from poorly condensed chromosomes. In general, when an abnormal cell division which cannot be corrected occurs, the affected cells will die. *NCAPH* knockdown in pancreatic cancer cells results in chromosomal aberrations and DNA damage, leading to apoptotic cell death. Therefore, NCAPH may be a potentially powerful therapeutic target for human cancer.

In conclusion, our data confirm that *NCAPH* knockdown suppresses cell proliferation and induces cell cycle arrest at G_2/M in NSCLC cells. In addition, *NCAPH* knockdown reduces the transforming ability, motility, and invasiveness of NSCLC cells. Based on our knowledge of cell cycle-targeted therapies for cancer, this study suggests that NCAPH may be a potential therapeutic target for NSCLC. However, to support this result, further studies will be needed to elucidate the detailed functional roles of NCAPH in NSCLC.

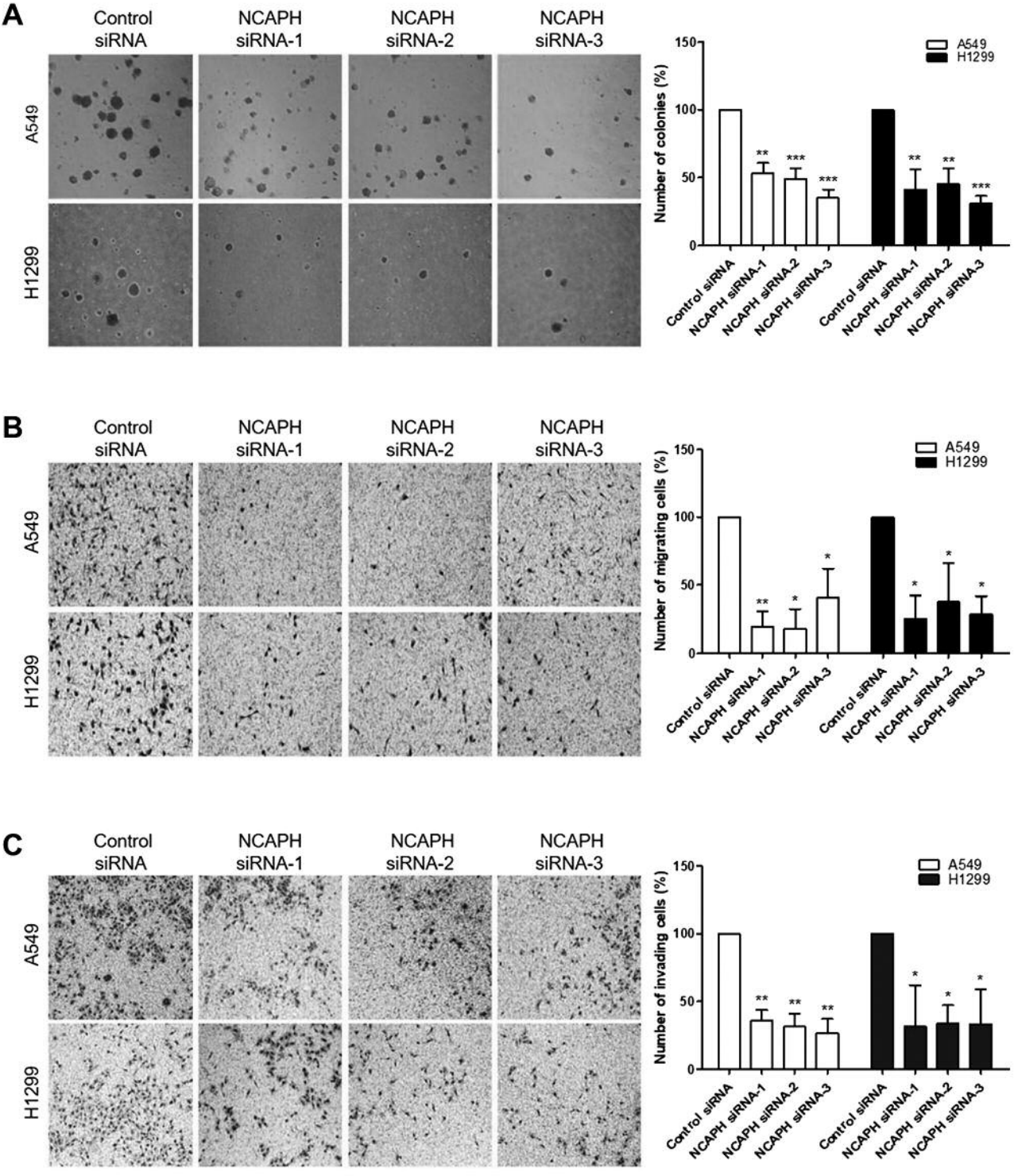

Figure 5. *Effects of knockdown of non-structural maintenance of chromosomes condensin I complex subunit H (NCAPH) on anchorage-independent growth, migration and invasion of A549 and H1299 non-small-cell lung cancer cells. A: Following transfection of A549 and H1299 cells with control siRNAs or NCAPH siRNAs, soft-agar colony-formation assays were performed to determine the transforming ability of these cells. After 3 weeks of cultivation, the colonies were imaged and counted under a microscope. B: Migration assay assessment of NCAPH siRNA-transfected A549 and H1299 cells. C: Invasion assay was performed using both cell lines. The data are represented as the mean±SD of three independent experiments. Significantly different at *p<0.05, **p<0.01 and ***p<0.001 compared with control siRNA-treated cells.*

Conflicts of Interest

The Authors declare no conflicts of interest.

Author's Contributions

Seok Won Kim and Seon-Joo Park designed the study, analyzed the data and prepared the article. Byeol Kim and Ji-Yeon Lim performed the experiments and collected the data. All Authors participated in the writing and revision of the final article.

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