박사 학위논문

The Inhibitory Effect of Nicotinamide on Human Intrahepatic Cholangiocarcinoma Cells

2017 년 2 월

서울대학교 대학원

의학과 병리학전공

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니코틴아마이드(NA)의 종양 억제 효과

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지도 교수 장자준

이 논문을 의학박사 학위논문으로 제출함

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서울대학교 대학원
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WANG YUE

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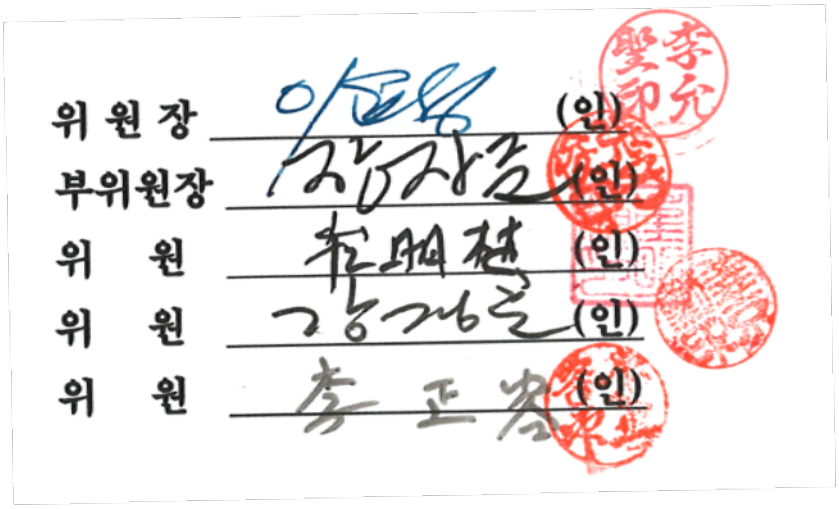

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The Inhibitory Effect of Nicotinamide on Human Intrahepatic Cholangiocarcinoma Cells

by
Wang Yue

A Thesis Submitted to the Department of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Medicine Science (Pathology) at Seoul National University College of Medicine

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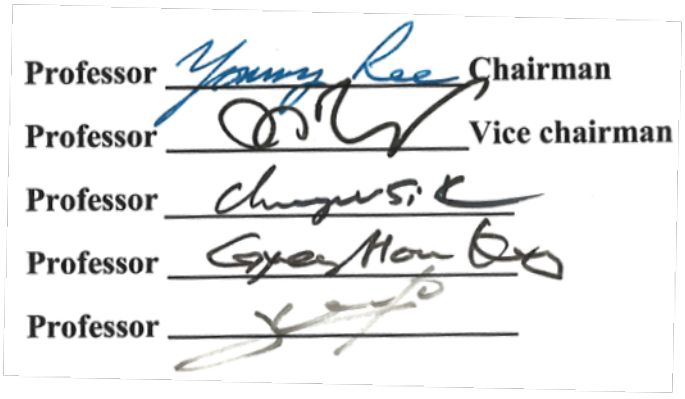
Approved by Thesis Committee:



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② 본인의 논문을 디지털화하여 인터넷 등 정보통신망을 통한 논문의 일부 또는 전부의 복제.배포 및 전송 시 무료로 제공하는 것에 동의합니다.

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논문제목 : 인체 담관암세포에서 니코틴아마이드(NA)의 종양 억제 효과

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Abstract

The Inhibitory Effect of Nicotinamide on Human Intrahepatic Cholangiocarcinoma Cells

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Background: Intrahepatic cholangiocarcinoma (iCCA) is a devastating malignancy with no effective treatment; it is associated with a high mortality rate. Nicotinamide (NA, the amide form of vitamin B3) has been shown to be effective in the treatment of various diseases. However, the effects of NA in iCCA have not been studied.

Materials and Methods: Four human iCCA cell lines (HuCCT1, JCK, OZ and Cho-CK) were used to test the inhibitory effect of NA. Cell proliferation was assessed by WST1 and BrdU assays, cell cycle was evaluated by flow cytometry using propidium iodide, apoptosis was detected by Annexin V-FICT assay and the invasive potential of iCCA cells was tested by invasion assay. Western blotting was used to detect the changes at protein level. Besides, the p53 specific siRNA was used to knockdown the p53 expression in iCCA.

Results: NA significantly inhibited cell viability and induced

apoptosis in all four cell lines. It arrested cell cycle in G1 phase, decreased Cyclin D1 and Cdk4 protein expression levels and increased p16 level. NA increased the levels of cleaved caspases 3 and 9, but had no effect on caspase 8. In HuCCT1 and OZ cell lines, NA treatment significantly impaired the invasion abilities and inhibited epithelial-mesenchymal transition (EMT)-like changes, such as increase in epithelial marker E-cadherin expression, decrease in mesenchymal marker vimentin and EMT transcription factors Slug expression.

Conclusions: We showed for the first time that NA markedly inhibited cell proliferation, induced apoptosis and attenuated invasiveness in human iCCA. Our findings provide the experimental basis for using NA as a potential anticancer agent against human iCCA in the future.

* Some of the work are published in Molecular & Cellular Toxicology.

(Wang Y. *et al*, 2017)

Keywords: Intrahepatic cholangiocarcinoma, Nicotinamide, apoptosis, cell cycle, invasion.

Student Number: 2013-31359

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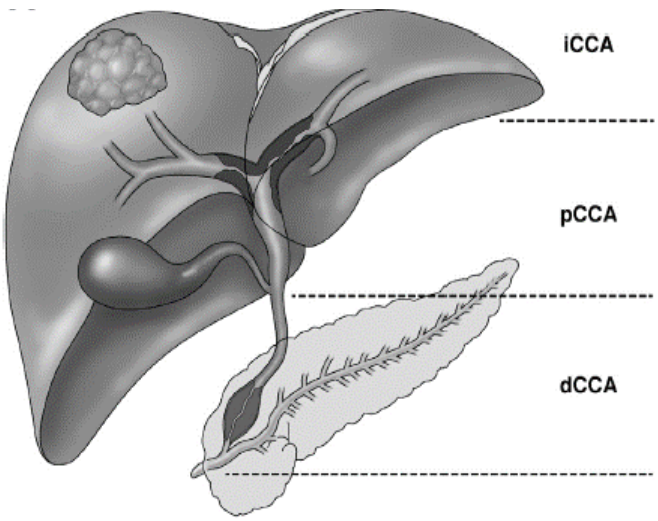
Introduction

Intrahepatic cholangiocarcinoma (iCCA) is the second most common primary liver cancer worldwide. Over the past 3 decades, the overall incidence of iCCA appears to have increased, and the percentage of patients who lived at least 5 years after diagnosis has decreased [1].

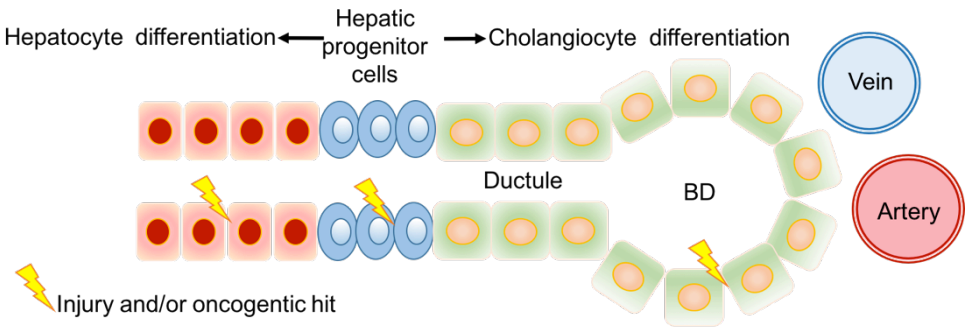
The iCCA has a distinct clinical presentation , and was anatomically distinguished as perihilar cholangiocarcinoma (pCCA) and distal cholangiocarcinoma (dCCA) [40] (Graph 1).



Graph 1. Anatomic localization of three CCA subtypes

(Graph adapted from Sumera Rizvi *et al.* 2013)

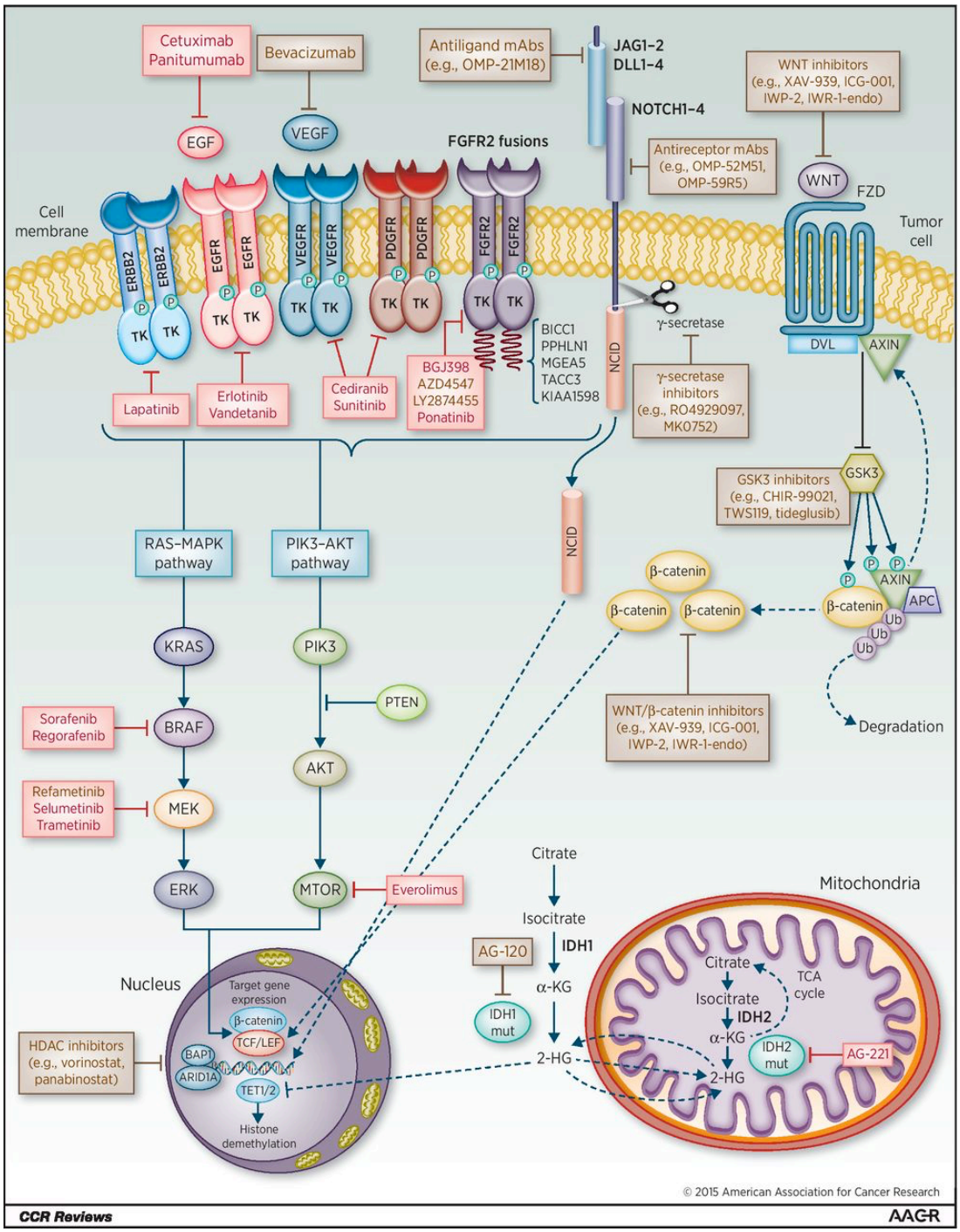
Roskams T *et al.* suggested that iCCA possibly origins from multiple cells, because iCCA includes a group of histologically heterogeneous tumors with diverse cellular phenotypes and cell markers[2]. In addition, the group of Akiba J *et al.* found the existence of mixed hepatocellular cholangiocarcinoma (HCC-iCCA) tumors[3], which points out the presence of a possible common cell of origin. Thus, iCCA is currently believed to derive from biliary epithelial cells (cholangiocytes) of the intrahepatic biliary tract, hepatic progenitor cells (HPC), or even mature hepatocytes, which depending on the initial triggering mutation and/or environmental insult (Graph 2).



Graph 2. Schematic representation of multiple cells of origin in liver cancers.

Future studies about iCCA cell origin are needed to fully define these routes to iCCA, and to understand their molecular underpinnings as well as their relevance to different iCCA subtypes.

In clinical, the feasible treatment for iCCA is surgical resection, whereas liver transplantation remains controversial. On the other hand, so far no molecular targeted therapy has been proven effective for either iCCA or other biliary tract cancers. Due to the limited understanding of the biology of iCCA and the uncertainty of "oncogenic addiction" (dependency status of cancer cell on the activation or loss of specific genes), the development of effective targeted therapies [4] was hindered. Thus, examining the molecular pathogenesis of iCCA become more and more important. Agrin Moeini et al. reviewed and reported that the complex process involving multiple genomic alterations and signaling pathway deregulations (Graph 3). However, the more accurate mapping of the genomic landscape of iCCA need the application of new technologies.



Graph 3. Current and potential targeted therapies in iCCA

(Graph 3 adapted from Moeini *et al.*2016)

Tyrosine kinase receptor signaling: several growth factor signaling pathways (i.e., EGF/EGFR) have been reported to be aberrantly activated in iCCA. The specific binding of growth factors results in oligomerization and autophosphorylation of their receptors, followed by signaling through the RAS–MAPK and PI3K–AKT effector cascades.

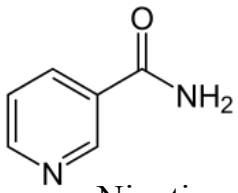
FGFR2 fusions: The presence of fusion partners in the cytoplasmic domain of FGFR2 results in constitutively active receptors that induce signaling through downstream signaling pathways.

NOTCH signaling: Binding of ligands on the surface of neighboring cells to the extracellular domain of NOTCH receptors (NOTCH-R) induces proteolytic cleavage of the receptor, releasing its intracellular domain (NICD), which then translocates to the nucleus and regulates expression of target genes.

WNT/β-catenin signaling: activation of frizzled (FZD) receptors by WNT ligands triggers the displacement of the regulatory

APC/Axin/GSK3-complex, accumulation of β-catenin and induction of target genes.

IDH signaling: Mutated IDH enzymes acquire the capacity to synthesize 2-hydroxygluterate (2-HG) from α-ketoglutarate (α-KG). 2-HG alters the activity of α-KG–dependent dioxygenase enzymes involved in multiple cellular processes, including cell differentiation, survival, and DNA methylation. Molecular targeted therapies have also been highlighted; drugs currently assessed in phase II clinical trials (red) and those evaluated in early clinical trials or preclinical studies (brown) are shown.



Nicotinamide (NA) is a water-soluble amide form of niacin (nicotinic acid or vitamin B3). Both niacin and NA are widely available in plant and animal foods. It is commercially used in vitamin supplements, skin preparations [5], and in the treatment of a broad spectrum of diseases, e.g. Alzheimer's disease[6]. Over the years, NA has been shown to exert a number of anticancer effects such as effective inhibition of the growth and progression of bladder tumours [7], and the inhibition of the development of preneoplastic lesions and well-differentiated HCC during hepatocarcinogenesis in mice[8]. Moreover, a drug safety evaluation on long-term NA treatment of up to 3 g daily [9], showed no side effects. Earlier studies using considerably higher doses (1–12 g daily for months) rarely reported gastrointestinal side effects such as nausea [9-14]. Notably, all possible effects of NA may be attributable to its role as a major NAD^+ precursor, a direct inhibitor of four classes of enzymes, including poly (ADPribose) polymerase-1

(PARP1) [6,15], SIRT1 [16-19], an inhibitor of inducible NO synthase (iNOS) [20,21] and a free radical scavenger [22]. Although the effects of NA have been partially elucidated, little is known about its effect on human iCCA.

Therefore, this study was designed to determine the effects of NA on cell cycle, apoptosis and invasive ability of human iCCA cell lines and to reveal the underlying mechanisms.

Materials and Methods

Cell lines and cell culture

The iCCA cell lines, JCK and Cho-CK, were kindly provided by the Division of Gastroenterology and Hepatology of Chonbuk National University Hospital (Jeonju, Republic of Korea). HuCCT1 and OZ cell lines were purchased from Japanese Collection of Research Bioresources Cell Bank (lbaraki, Japan). JCK and Cho-CK [23] were maintained in Dulbecco's Modified Eagle Medium (GIBCO-BRL, Carlsbad, CA, USA). HuCCT1 and OZ cells were maintained in RPMI 1640 medium (GIBCO-BRL, Carlsbad, CA, USA) and William's E medium (GIBCO-BRL, Carlsbad, CA, USA), respectively. Each medium was supplemented with 10% foetal bovine serum (GIBCO-BRL, Carlsbad, CA, USA) and 1% penicillin-streptomycin (10,000 units/l of penicillin and 10,000 μg/mL of streptomycin).

Reagents

Nicotinamide（NA, M.W.=122.12）was purchased from Sigma (St.

Louis, MO, USA). NA powder was stored at 4℃ . Before NA treatment, the 1.12g NA was dissolved in 1ml culture medium to make the stock solution (concentration of 1M).

Cell proliferation and DNA synthesise analysis

HuCCT1, OZ, JCK and Cho-CK cells at 200 μL ($1x10^2$/mL) per well were seeded in 96-well plates. After incubation for 18 h, the cells were pre-incubated with NA (0, 10, 20 and 30 mM) and assayed at 0, 24, 48 and 72 h. Cell proliferation was evaluated using a PreMix WST-1 Cell Proliferation Assay System (Takara Bio Inc., Japan), and the DNA synthesis in the four cell lines incubated with NA for 48 h (0, 10, 20 and 30 mM) was detected using ELISA BrdU colorimetric kit (Roche, Mannheim, Germany). The results are presented as mean $\pm$ SE absorbance of triplicate assays.

Analysis of cell cycle

HuCCT1, OZ, JCK and Cho-CK cells were plated in 6-well plates and incubated at 37℃ for 18 h. The cells were further incubated with

NA (0, 10, 20 and 30mM) for 48 h. They were then harvested by trypsinization, and suspended in staining buffer (0.1% triton X-100 and 0.1 mM EDTA [pH 7.5]) with 50 μg/mL of RNase (Sigma, St. Louis, MO, USA) and 50 μg/mL of propidium iodide (PI, Sigma, St. Louis, MO, USA). Within 2 h, the cells were analysed using a BD FACS Calibur Flow Cytometer (BD Biosciences, MA, USA), and the percentage of cells in the different cell cycles was determined.

Apoptosis assays

NA-induced apoptosis was determined using the MEBCYTO Apoptosis Kit (MBL Medical and Biological Laboratories Co., Nagoya, Japan). After incubation with NA (0, 15, 30 mM) for 48 h, the cells were trypsinized and resuspended in 300 μL of binding buffer. Thereafter, 5 μL of annexin V-FITC and 2.5 μL of PI were added to 300 μL of the cell suspension. The samples were kept in dark for 30 min and analysed using a BD FACS Calibur Flow Cytometer (BD Biosciences, MA, USA).

Western blot analysis

Total proteins were extracted with a protein extraction kit (T-PER Tissue Protein Extraction Reagent, Thermo Scientific, USA). The protein extracts (50 μg/sample) were subjected to electrophoretic separation by 10-20% Tris-glycine SDS-PAGE and were transferred onto PVDF membranes (Millipore) after being mixed with 5X loading buffer and boiled at 96°C for 8 min. The PVDF membranes were blocked in Tris-buffered saline, 0.5% Tween-20 (TBST) containing 3-8% skim milk for 2 h and incubated overnight at 4°C with primary antibodies to p53 (1:1000; CST), vimentin (1:1000), Snail (1:1000), E-cadherin (1:1000), and Slug (1:1000) (all from Abcam) in TBST that contains 5% skim milk. The other used primary antibodies has been list below (Table 1). Anti-mouse or anti-rabbit IgG was used as the secondary antibody (BD Biosciences, MA, USA). The membranes were then visualized by an ECL (SuperSignal West Dura Extended Duration Substrate, Thermo Scientific, USA). All WB detections were

performed in triplicate.

Table 1. The usage of the primary antibodies in detail.

Target proteins	Pro. Size	Source	Dilution	Total Pro	Brand:Cat. No
CyclinA	54	R	1:500	20	SANTACRUZ,SC-751
Cyclin D1	36	M	1:500 in TBST	50	BD
Cyclin E	43	M	1:100 in TBST	100	SANTACRUZ
Cdk2	34	R	1:200	20	SANTACRUZ,SC-748
Cdk4	34	R	1:500	20	SANTACRUZ,SC-260
Cdk6	40	M	1:100 TBST	50	SANTACRUZ,SC-56362
p53	53	R	1:500	20	Cell Signaling,2525
P21	27	M	1:500 in TBST	50	SANTACRUZ
P16	16	R	1:500 in TBST	50	SANTACRUZ
BAD	23	R	1:500 in TBST	50	Cell Signaling,9292
Bax	23	R	1:500	50	SANTACRUZ,sc493
Bax	21	M	1:500 TBST	50	BIOVISON,3331-100
Bcl-2	26	M	1:500	50	SANTACRUZ,sc509
Bcl-2	28	R	1:500 TBST	50	Cell Signaling,2876
Caspase-3	35	R	1:1000 in 3% skim milk	50	Cell Signaling
Cleaved-c3	17,35	R	1:100 in TBST	50	Cell Signaling
Caspase-8	50/55	M	1:500	20	MBL,M058-3
Caspase-8	50/55	M	1:500	20	BD,551244
Caspase-9	46/10	R	1:500 TBST	50	SANTACRUZ,SC-7885
Cleaved caspase-3	17,19	R	1:500 TBST	50	Cell Signaling,9661
Cleaved caspase-8	10	M	1:500	50	Cell Signaling,9748
Rb	107	R	1:2000 in 5% skim milk	50	SANTACRUZ
PCNA	36	M	1:40000 in 3% skim milk	20	SANTACRUZ, SC-56
Sirt1	127	R	1:1000 in 3% skim milk	20	SANTACRUZ

R: anti-rabbit; M: Anti-mouse; TBST: 50 mM Tris, 150 mM NaCl and 0.05% Tween 20

Invasion assay

The invasiveness of human iCCA cells was assayed using transwell membranes coated with 500 ng/μL of Matrigel (BD Biosciences, San

Jose, CA, USA)[24]. After the bottom chamber was filled with 750 μL of the complete growth medium, the prepared cells (incubated for 48 h with 0, 15, 30 mM NA) were seeded at 2 x10^4 cells per well in 500 μL of serum-free opti-MEM medium (GIBCO-BRL, Carlsbad, CA, USA) in the upper compartment of the transwell and allowed to invade the polycarbonate membrane for 48 h. After incubation, these cells were fixed with 100% ethanol and stained with haematoxylin and eosin (H&E). The cells were counted in 5 microscopic fields by using a phase-contrast microscope (Nikon Eclipse 80i, Japan), and the pictures were taken using a Digital Sight DS-Fi1 camera (Nikon) attached to a microscope.

p53 siRNA and transfection

A small interfering RNA specific for human p53 was used to knock down p53 in HuCCT1 and OZ cells[25]. The sequence for the p53 siRNA was GACUCCAGUGGUAAUCUAC (Cosmo genetech, Seoul, Korea). A human wild-type p53 expression vector (pCMV-neo-BAM)

and control vector (pCMV-tag2B) were kindly provided by Prof. Sang Hoon Kim (Kyung Hee University, Seoul, Korea). The Lipofectamine® RNAiMAX Transfection Reagent was purchased from Invitrogen (Carlsbad, CA, USA).

Cells were seeded in 6 well, and transfection started when 70% confluence. Final p53-specific siRNA usage was 50pM per well. After transfection, HuCCT1 and OZ cells were collected and analyzed at 24 h and 48 h, respectively.

Statistical analysis

All statistical analyses were performed using Origin 9.0. Data are expressed as the mean ± SE and representative of at least three independent experiments. The control and treated groups were compared using Student's t test. Differences were considered statistically significant when the P value was less than 0.05 (*$p<0.05$, **$p<0.001$).

Results

1.1 NA inhibits proliferation of human iCCA cells

The cell proliferation (WST1) data showed that NA effectively inhibited growth of all cell lines tested in a time- and dose-dependent manner (Fig. 1A *$p<0.05$). After 24 h of NA incubation, the growth of HuCCT1 and JCK was clearly inhibited by a relatively high dose of 40 mM. In addition, after 48 h of exposure to NA, even 20 mM NA could inhibit growth of these four cell lines. The IC_{50} values after 48 h of exposure to the drug was 23.5, 15.3, 25 and 28.8 mM for HuCCT1, JCK, OZ and Cho-CK cells, respectively. Moreover, the dose dependence was more obvious after 72 h of NA treatment.

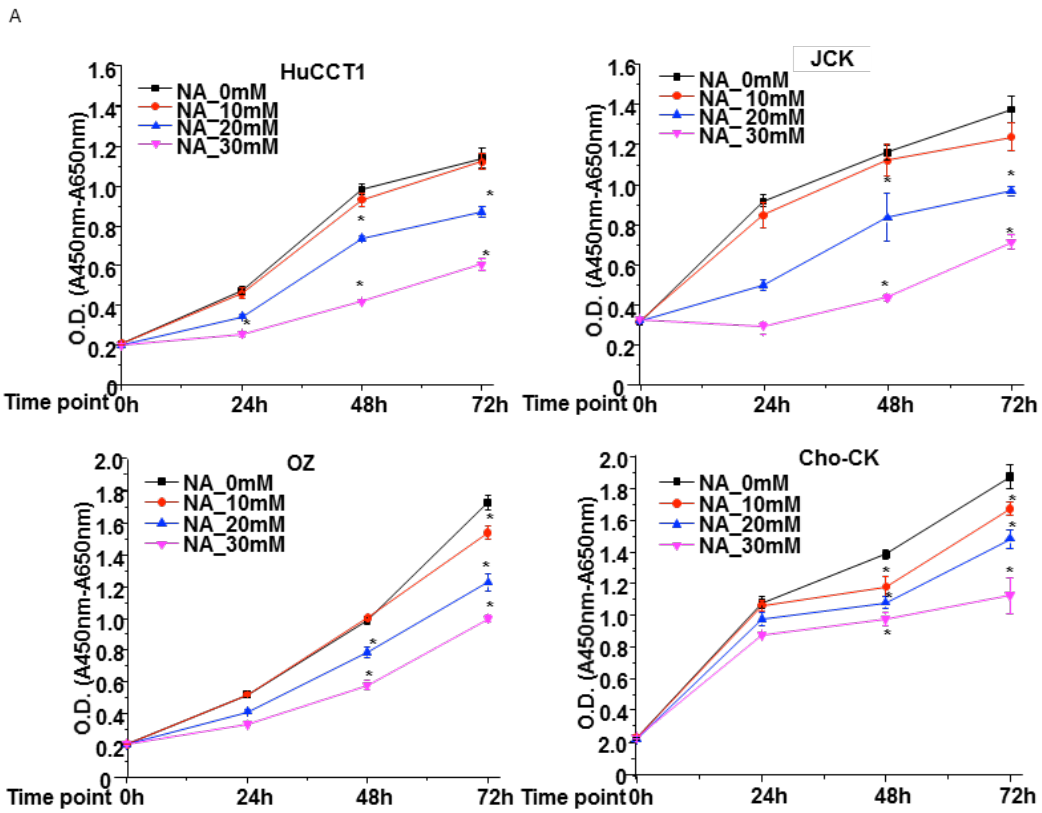


Figure 1. (A) The cells (HuCCT1, OZ, JCK and Cho-CK) were treated by NA at different doses (0, 10, 20, 30mM) for 0, 24, 48, and 72 h and then the cell proliferation was determined by WST-1. The values were measured by the ELISA reader at 450nm with a reference wave length of 650nm.

1.2 NA inhibits DNA synthesis of human iCCA cells

The cells were treated with various concentrations of NA for 48 h prior to BrdU incorporation assay (Fig. 1B, *$p<0.05$). Consistent with WST-1 data, the data from BrdU assay showed that DNA synthesis of iCCA cells was efficiently inhibited in a dose-dependent manner by NA.

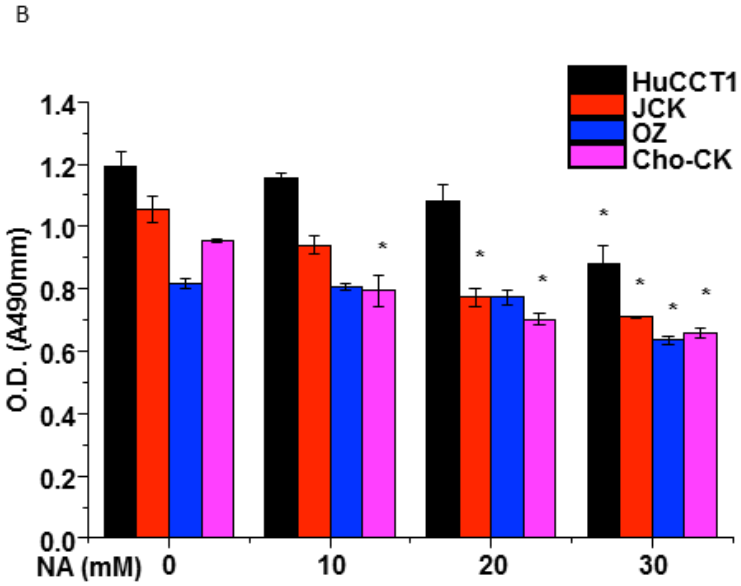


Figure 1. (B) After treated by NA at different doses (0, 10, 20, 30mM) for 48 h, the DNA synthesis was detected by BrdU assay. The values were measured by the ELISA reader at 490nm.

2. NA treatment induces G1 cell cycle arrest

To explore the mechanism underlying the inhibition of iCCA cell growth by NA, cell cycle analysis was performed in all four cell lines. The results revealed that the iCCA cell cycle was significantly arrested in the G1 phase after 48 h of NA treatment, in a dose-dependent manner (Fig. 2A *$p<0.05$). The percentage of G1 phase HuCCT1, JCK, OZ and Cho-CK cells increased from 49 to 72, 60 to 84, 56 to 82 and 60 to 80%, respectively.

Moreover, in all four cell lines, the expression of the cell cycle inhibitor, p16 increased, and the expression levels of G1-phase proteins, including Cyclin D1 and cdk4, decreased after NA treatment in a dose-dependent manner. Notably, in HuCCT1 and OZ cell lines, the expression levels of Cyclin E and ckd2 also decreased after an increase in the expression of p21 and p53 (Fig. 2B).

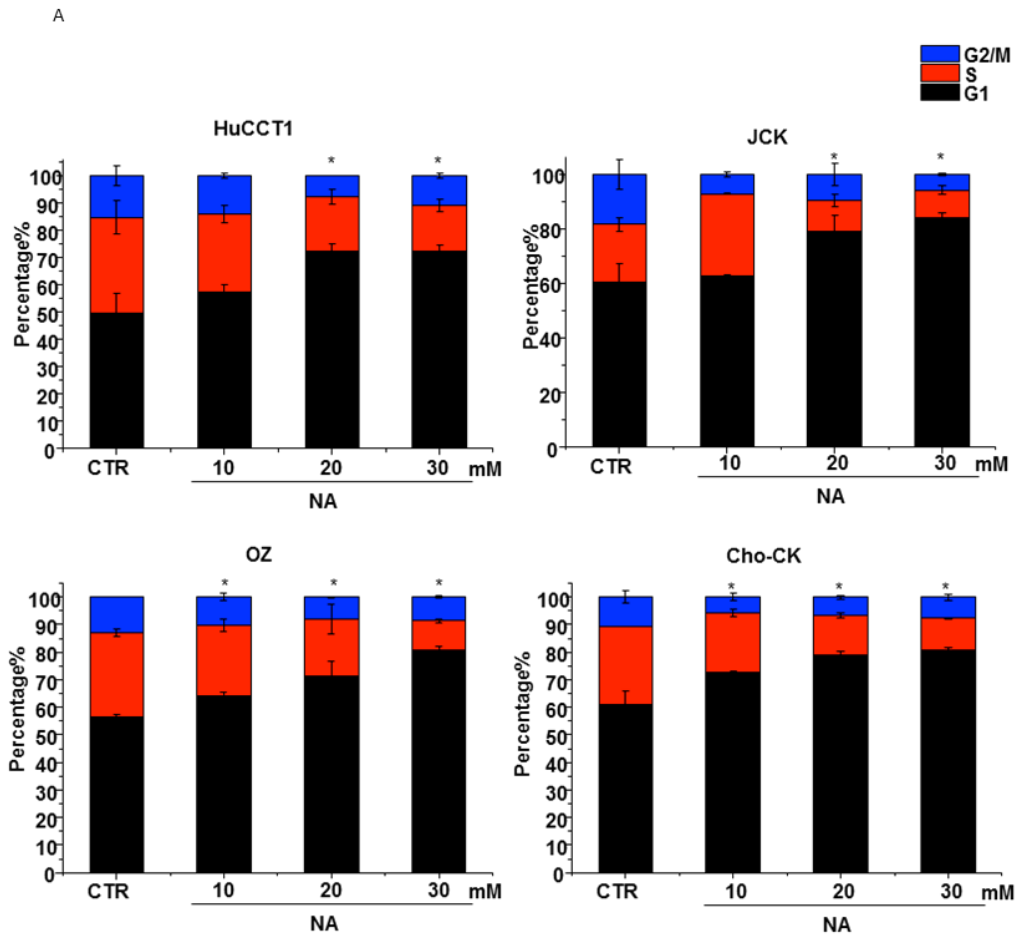


Figure 2. (A) The cell cycle phase (G2/M, S and G1) distribution histograms of PI-stained cells

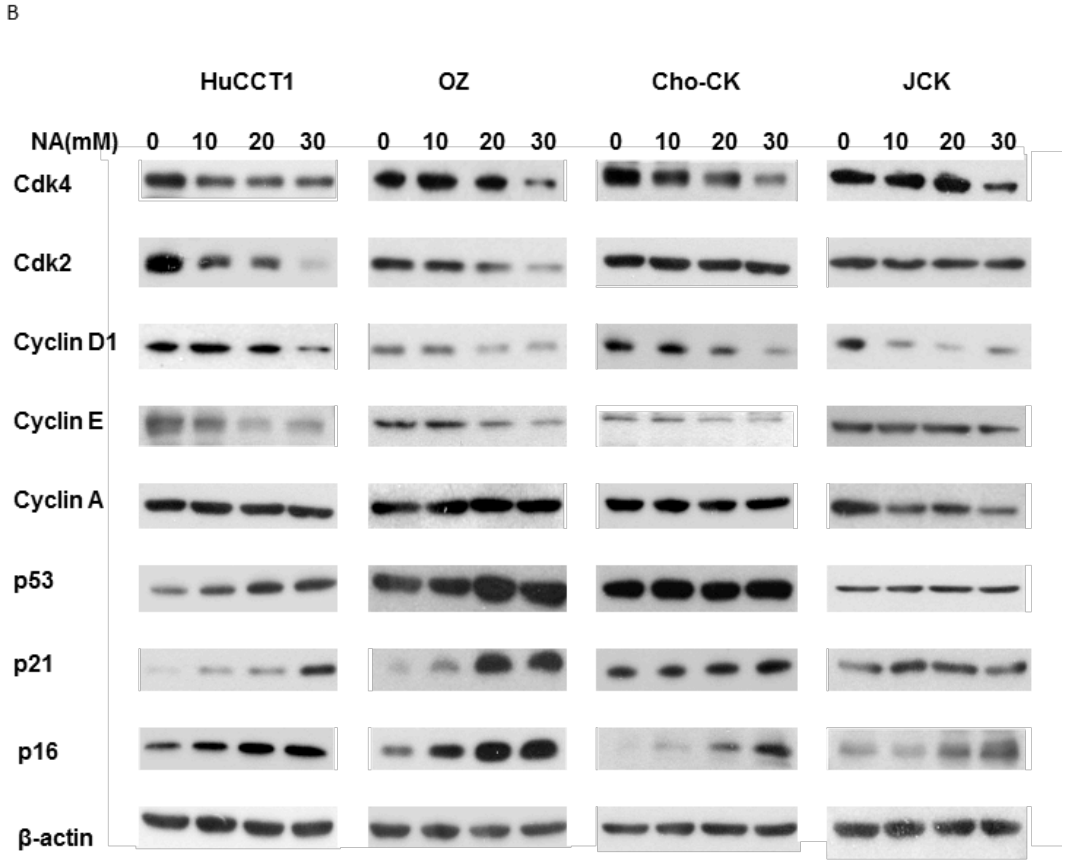


Figure 2. (B) Western blot detection of G1 phase-related proteins (Cdk4, Cdk2, CylinD, CyclinA, p53, p21 and p16) in iCCA cells by NA treatment at different doses(0, 10, 20, 30mM) for 48 h. β-actin was used as an internal control.

3.1 NA induces apoptosis in human iCCA cells.

Apoptosis was significantly induced by 30 mM NA in all the cell lines tested (Fig. 3 *$p<0.05$, **$p<0.001$). In Cho-CK cells, apoptosis was induced by NA dose-dependently. Although the JCK cell number in the right quadrant, which presents apoptosis, was small, it was significant.

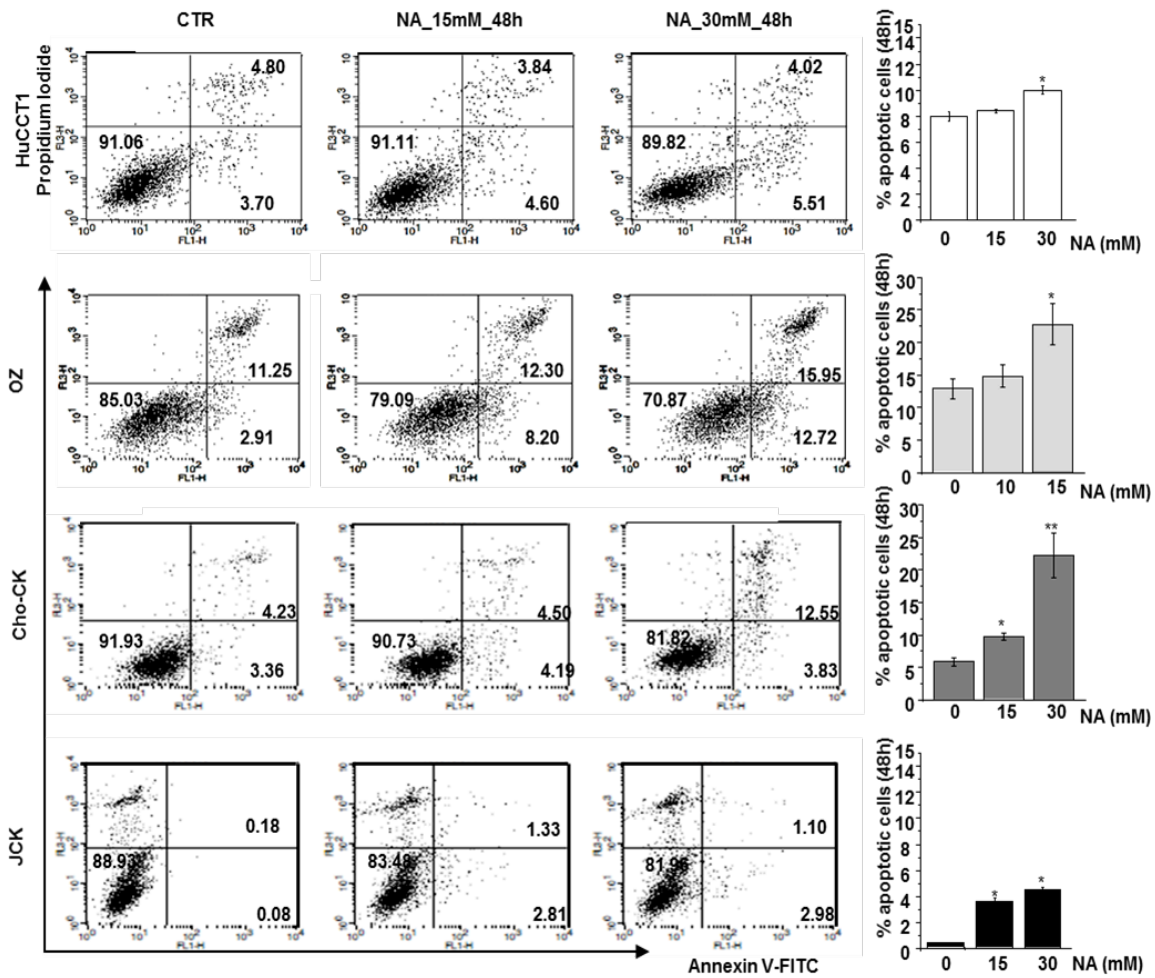


Figure 3. HuCCT1, OZ, JCK and Cho-CK cells were incubated with different doses of NA (0, 15 and 30mM) for 48 h, they were then stained with Annexin V-FITC and Propidium Iodine. The apoptotic cells was quantified by flow cytometry and reported in bar graph.

3.2 NA induces cell death via the intrinsic mitochondrial pathway.

Next, we explored the effect of NA on apoptotic pathways by western blotting (Fig. 4). NA potently increased cleaved caspase-3 protein levels, which is a hallmark of apoptosis. In addition, we observed decreased levels of Bcl-2 expression and increased levels of BAX expression. The apoptotic cascade was activated by NA owing to an increase in the levels of cleaved caspase-9. However, caspase-8 was not affected by NA. These results indicate that NA induced apoptosis via the intrinsic mitochondrial pathway in human iCCA cells.

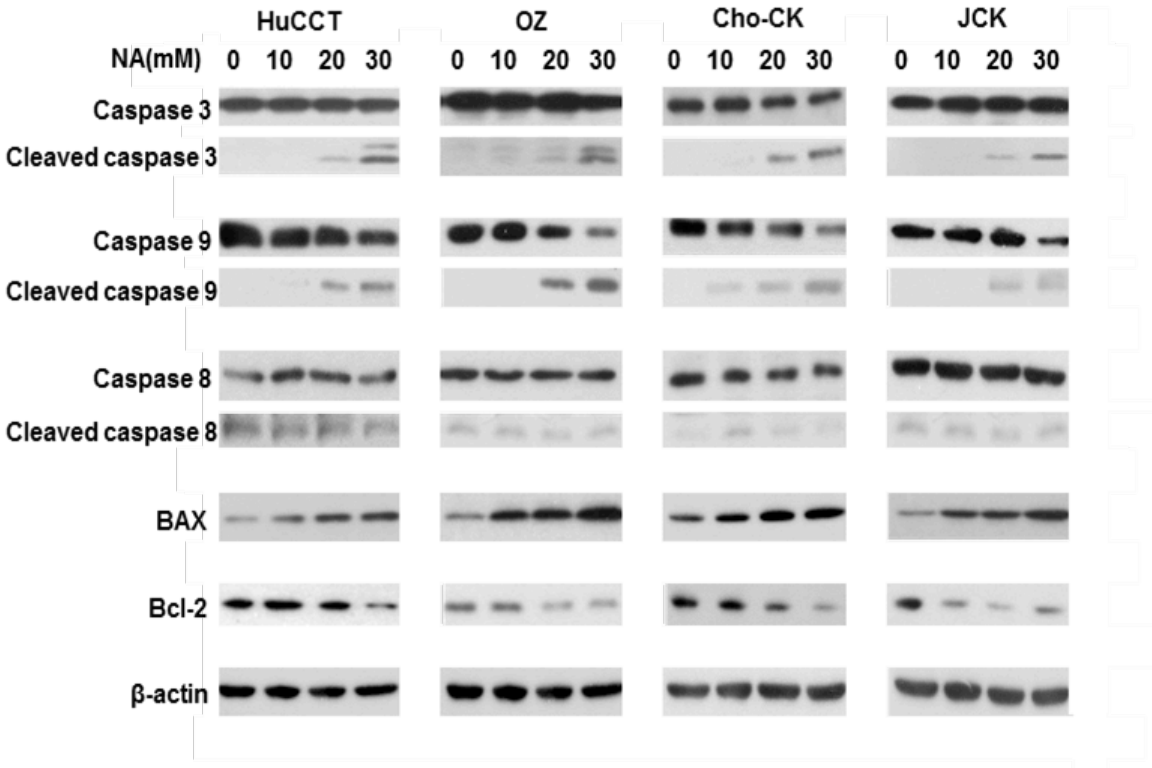


Figure 4. The apoptosis-related proteins (Caspase-3, cleaved Caspase-3, Caspase-9, cleaved Caspase-9, Caspase-8, cleaved Caspase-8, Bax and Bcl-2) were detected by western blotting analysis after NA treatment at different doses (0, 10, 20, 30Mm) in iCCA cells (HuCCT1, OZ, JCK and Cho-CK). β-actin was used as an internal control.

4.1 NA weakened the invasive potential of human iCCA cells

After 48 h of incubation with NA, the invasive characteristics of HuCCT1, OZ, JCK and Cho-CK cells were analysed. In Cho-CK and JCK cells, no significant change was observed at the different concentrations treated (Fig. 5C, D); however, the HuCCT1 and OZ cell population (30 mM NA treatment) that moved into the Matrigel was significantly smaller than that of the control group (Fig. 5A, B *$p<0.05$). This suggested that NA weakened the invasion ability of HuCCT1 and OZ cells. As shown in Fig 5. E, the expression levels of invasion-associated epithelial cell markers E-cadherin were increased by NA treatment; the expression of mesenchymal cell marker vimentin and the EMT-inducing transcription factor Slug was decreased by NA treatment. However, NA had no effect on Snail, another EMT-inducing transcription factor .

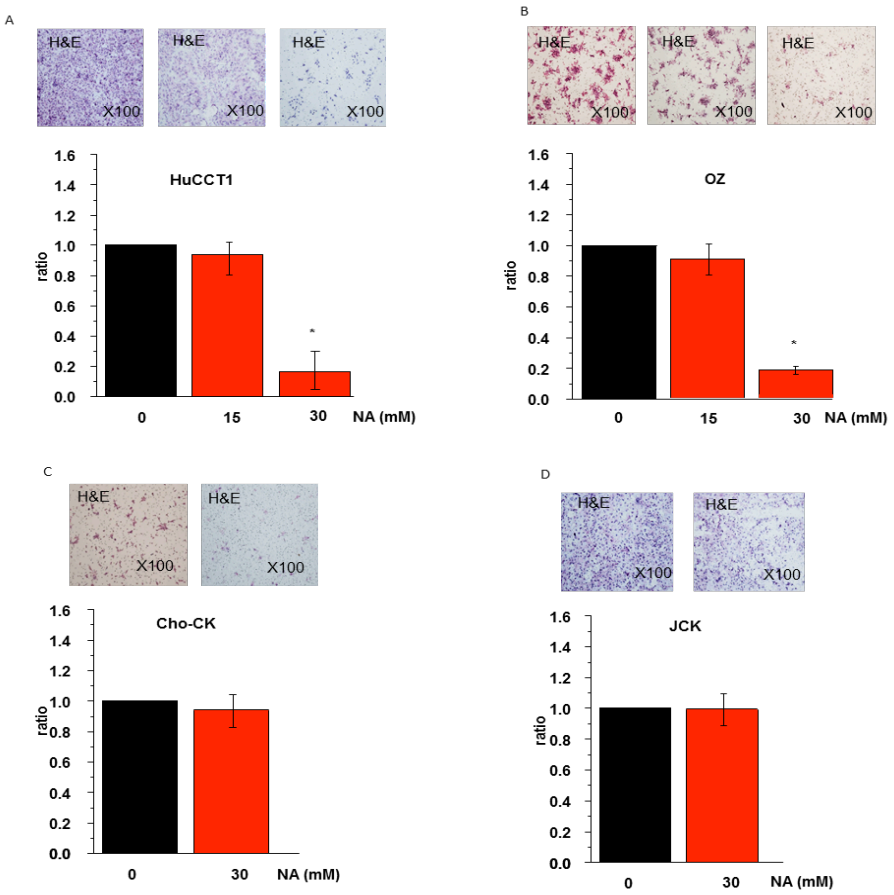


Figure 5. The invasion ability of (A) HuCCT1, (B) OZ, (C) Cho-CK and (D) JCK was assessed by the invasion assay (magnification, x100, H&E staining) with different dose of Nicotinamide (NA, 0, 15 and 30mM) treatment for 48 h; the ratio of cell (compared with 0mM NA group) was counted and reported in bar graph.

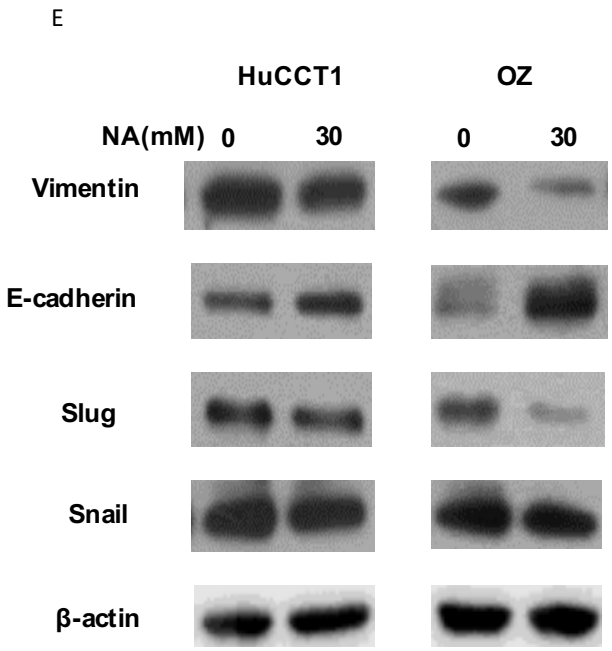


Figure 5. (E) Markers of 'EMT-like changes' (Vimentin, E-cadherin, Slug and Snail) were detected by western blotting in HuCCT1 and OZ cells with or without 30 mM NA treatment for 48 h. β-actin was used as an internal control.

4.2 p53 involved in the inhibition of NA on invasive potential in human iCCA cells

p53 may involve in the impaired invasion ability of HuCCT1 and OZ cell lines by NA treatment. In order to test this hypothesis, we used siRNA specific for human p53 to knock down p53 in both cells. Western blot analyses demonstrated that p53-target siRNA effectively reduced the expression of p53 in HuCCT1 and OZ cells after 24 or 48 h, respectively. (Fig. 6A). Then we found that p53 siRNA treatment abolished this effect of NA on HuCCT1 and OZ cell invasion (Fig. 6B). Indeed, the data from western blot showed that p53 siRNA treatment significantly reduced expression levels of epithelial cell markers (E-cadherin), and increased mesenchymal cell markers (vimentin) and Slug expression (transcription factor of EMT) levels. In addition, NA treatment did abolish these effects (Fig. 6C). These results suggest NA inhibited iCCA invasive potential in a p53-dependent way.

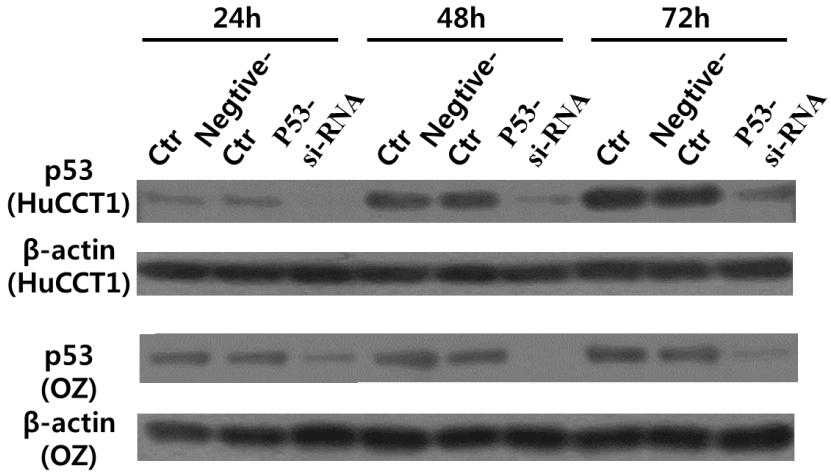


Figure 6. (A) Expression of p53 were detected by western blotting in HuCCT1 and OZ cells with or without p53-targeted siRNA treatment for 24, 48 and 72 h. β-actin was used as an internal control.

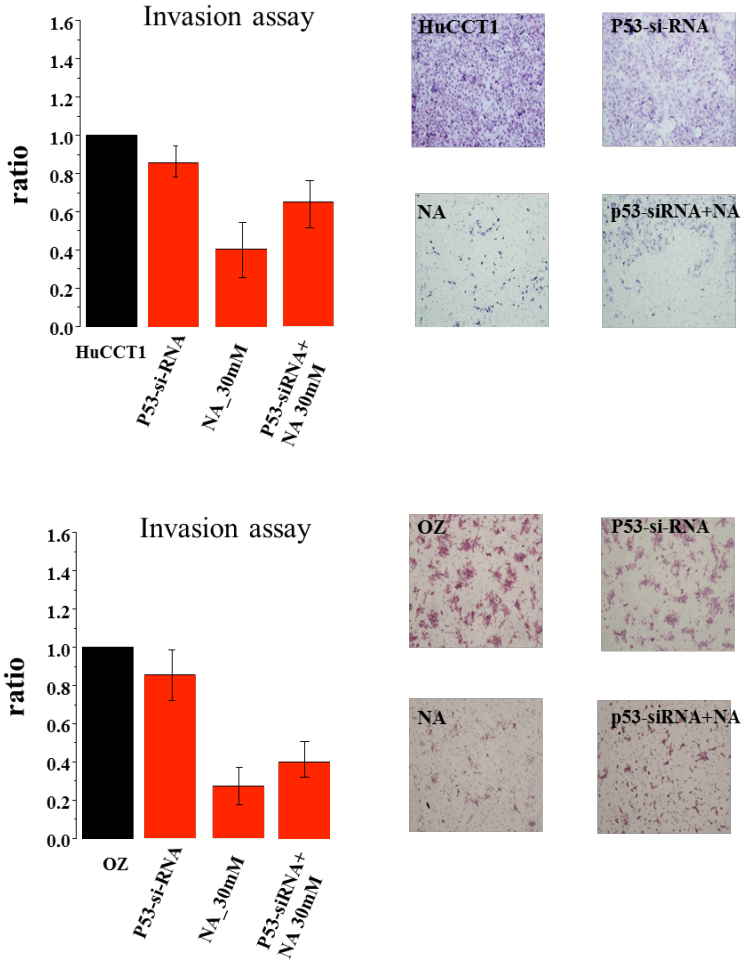


Figure 6. (B) The invasion ability of HuCCT1, OZ was assessed by the invasion assay (magnification, x100, H&E staining) after p53-targeted siRNA with or without 30 mM NA treatment for 48 h; the ratio of cell (compared with 0mM NA group) was counted and reported in bar graph.

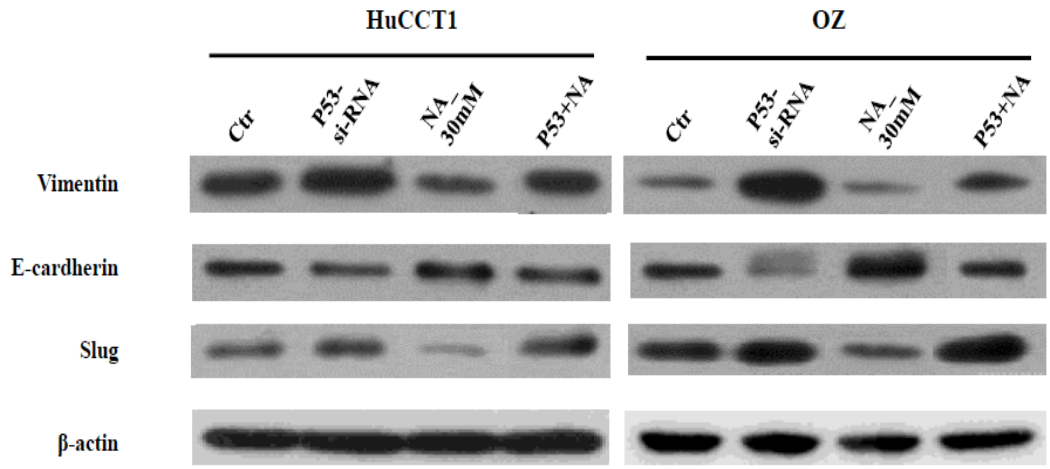


Figure 6. (C) Markers of 'EMT-like changes' (Vimentin, E-cadherin and Slug) were detected by western blotting in HuCCT1 and OZ cells after p53-targeted siRNA with or without 30 mM NA treatment for 48 h. β-actin was used as an internal control.

Discussion

Investigating the potential role of well-known natural products as anticancer agents in cancer development or treatment has intrigued scientists and clinicians for decades. The present study examined the mechanisms underlying the anticancer effects of nicotinamide (NA) in four human iCCA cell lines (HuCCT1, OZ, JCK and Cho-CK). NA potently inhibited human iCCA cell proliferation, arrested the cell cycle, induced apoptosis and attenuated the invasive ability of the cells. To our knowledge, this is the first report to focus on the inhibitory effect of NA treatment in human iCCA.

It is well known that cellular proliferation of tumours can be suppressed either by the interruption of cell cycle or by inducing cell apoptosis. In this study, we showed for the first time that NA treatment slows down the cell cycle by arresting the growth of human iCCA cells in the G1 phase. Two classic cell-cycle inhibitor families, the cip/kip (CDK interacting protein/kinase inhibitory protein) family and the

INK4a/ARF (inhibitor of kinase 4/alternative reading frame) family, are known to play important roles in the regulation of cell cycle [26]. This study suggested that NA treatment significantly increased the expression levels of p16, which belongs to the INK4a/ARF family, in all four cell lines. p16 acts as a tumour suppressor by binding to CDK4/6 and preventing its interaction with Cyclin D [27,28]. Notably, in HuCCT1 and OZ cell lines, NA treatment also increased the expression levels of p53 [29] and p21, which belongs to cip/kip family. p21 in turn inhibits Cdk2 activity by interacting with Cyclin E. This interaction ultimately inhibits the downstream activities of transcription factors such as E2F1 and arrests cell proliferation in G1 phase [30]. In addition, the differences in the cell cycle regulatory mechanisms among the cell lines may be possibly due to the differences in genetic background (e.g. JCK cells expressed no functional p53 [31]). The mechanisms mentioned above are illustrated in Graph 4 A.

Secondly, apoptosis can be initiated by two conventional pathways, the extrinsic and intrinsic [29,32,33]. Here, we demonstrated that NA induced significant apoptosis in all iCCA cell lines tested; however, it showed no effect on caspase-8, suggesting that NA had no effect on the extrinsic apoptotic pathway. In contrast, the expression levels of pro-apoptotic BAX were increased by NA treatment, while those of anti-apoptotic BCL-2 decreased. In addition, NA activated the apoptotic cascade by increasing the levels of cleaved caspases 3 and 9. It can be concluded that in iCCA cells, NA treatment led to the activation of programmed cell-death via the intrinsic mitochondrial pathway. The mechanisms mentioned above are illustrated in Graph 4 B.

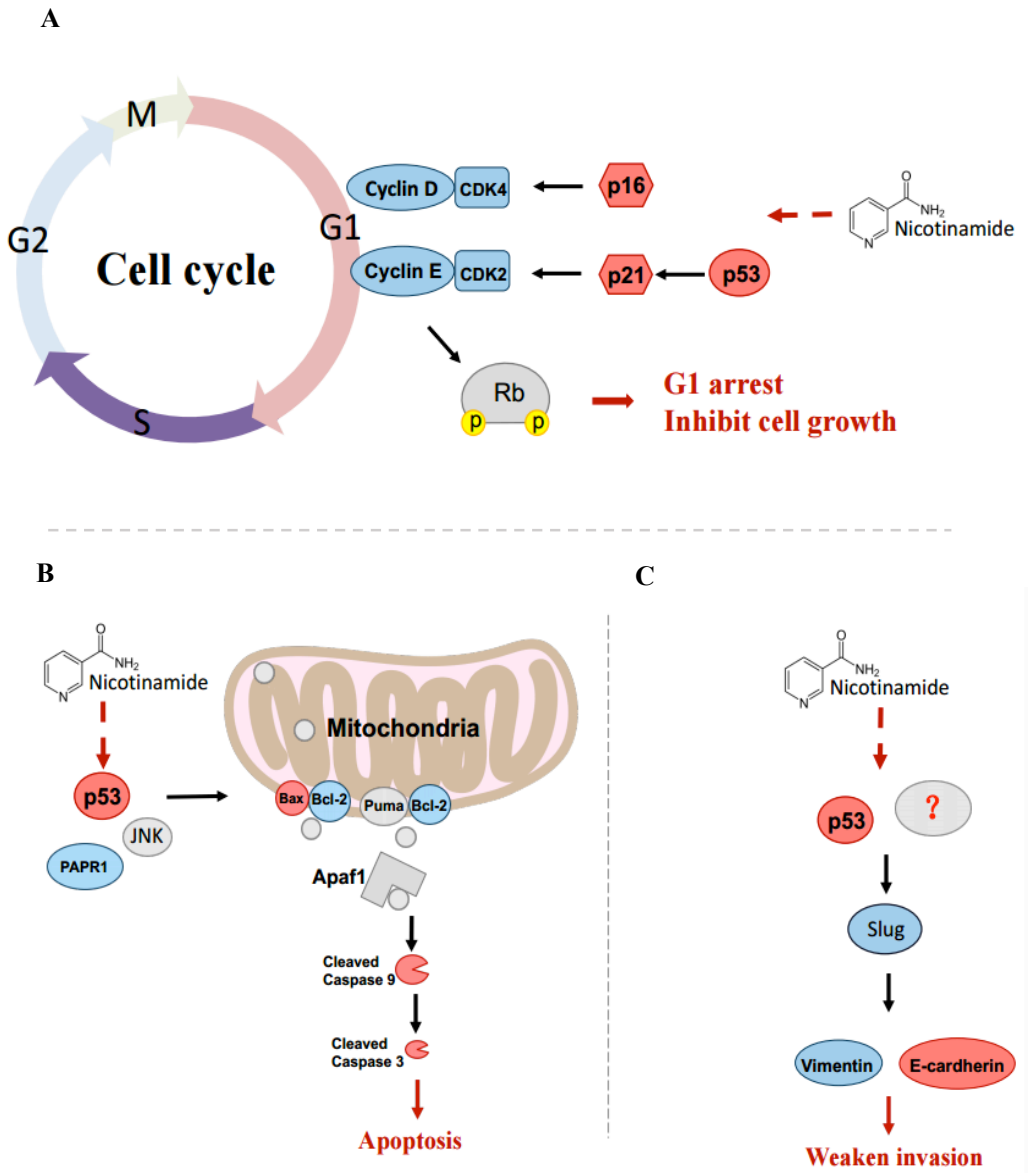
Recently, many studies have been conducted to investigate the mechanisms involved in iCCA local invasion and distant metastasis [24,34,35]. EMT (epithelial-mesenchymal transition)-like changes are considered to be closely related to local invasion and distant metastasis

of human cancer[36,37]. Here, we revealed that NA treatment weakened the invasive ability of HuCCT1 and OZ cells and inhibited the EMT-like changes of iCCA at protein level. Firstly, the expression of EMT-inducing transcription factor, Slug, was decreased by NA. Then, NA treatment contributed to the increase in expression levels of the hallmark of EMT, E-cadherin. However, the levels of vimentin decreased after treatment with NA.

In addition, the relationship between p53 and invasion in iCCA cells is intriguing. Although the predominant regulatory factor in EMT-like changes is still not clear, it has been reported in many previous studies that p53 acted as an inhibition factor in the regulation of EMT process in cancer [38,39]. Consistent with this hypothesis, our present results revealed that NA weakened the invasion ability only in HuCCT1 and OZ cells in which p53 has been activated. Moreover, after the expression of p53 was knocked down by the siRNA treatment in both cell lines, the inhibitory effect of NA on invasive ability was partially

reversed in both cell lines. In addition, the effects on the markers of "EMT" after NA treatment was also abolished by the p53 knocking down. This suggested that NA could weaken invasive ability of iCCA cells partially in a p53 dependent manner. Besides, p53 was not the only "brake" of "EMT like changes" in iCCA. NA, a broad-spectrum inhibitor, may have effect on the other key regulator of iCCA cell invasion potentials. (Graph 4C)

In summary, this is the first report on the anticancer role of NA in human iCCA *in vitro*, and it highlights the different inhibition mechanisms of NA among the four cell lines. This study will aid the further development of NA as an anticancer regimen against human iCCA.



Graph 4. The possible mechanisms of inhibitory effect of NA on iCCA cells**.** The upregulation of protein expression is showed in red, downregulation in blue, unknown in gray

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국문 초록

간내 담관암은 원발성 간암종으로 현재까지 적절한 치료법이 없어 높은 사망률을 보이는 치명적인 암종이다. 니코틴아마이드 (Nicotinamide, the amide form of vitamin B3, NA)는 종양억제 유전자로 알려진 RUNX3 의 발현을 증가시키고 안정성을 높여 발암억제 효과를 보인다는 연구 결과가 있다. 마우스 간암모델에서 NA 는 간암 전암병변인 변이소와 선종 및 초기 간세포암 발생을 현저히 감소시키는 효과를 보였다. 그러나 간내 담관암에 관한 발암억제 효과 및 그 구체적 작용 기전에 관한 연구는 없는 실정이다.

본 연구에서는 4 종류의 사람 담관암세포주 (HuCCT1, JCK, OZ, Cho-CK)를 이용하여 NA 의 간내 담관암 발생 억제효과와 그 기전을 알아보고자 하였다. 세포증식능에 미치는 영향을 알아보고자 WST1, BrdU 분석법을, 세포주기 분석은 PI 염색 후 유세포분석기를 이용하였다. 세포자멸사는 Annexin V-FICT 염색 후 유세포분석기를 사용하였고, 세포침윤도는 침윤도 측정기, 관련 단백질의 변화는 웨스턴블롯으로 측정하였다.

NA 는 사람 담관암세포주의 증식을 용량 의존적으로 억제하고 세포 자멸사를 증가 시켰다. NA 는 Cyclin D1, Cdk4 발현을 억제하고, p16 발현을

증가시켜 G1 세포주기 억제효과를 보였다. NA 투여에 의해 분리형 caspase3, 9 발현이 증가해 세포자멸사를 유도하였다. 또한 NA 는 p53 발현을 증가시키고 상피-간질 변환 (epithelial-mesenchymal transition) 관련 단백질을 감소 시켜 세포 침윤성을 억제하였다. 이는 NA 가 난치성 간내 담관암의 억제제 효과를 보일 가능성을 시사하고 있는 결과였다.

주요어: 인체 담관암, 니코틴아미드, 세포 자멸사, 침윤성

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