

The effects of cell cycle arrest  
by [6]-Gingerol, a major phenolic  
compound of ginger,  
on pancreatic cancer cells

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This certifies that the Master's Thesis  
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ABSTRACT

The effects of cell cycle arrest by [6]-Gingerol, a major phenolic compound of ginger, on pancreatic cancer cells

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(Directed by Professor Si Young Song)

Many of the pungent plants, including the ginger (*Zingiber officinale* Roscoe, Zingiberaceae) family, possess anti-carcinogenic activity. However, the molecular mechanisms by which plants exert anti-tumorigenic effects are largely unknown. The purpose of this study was to investigate the action of [6]-Gingerol, phenolic compound derived from ginger plant, on two human pancreatic cancer cell lines, BxPC-3 and Hpac. In result, [6]-Gingerol inhibited the cell growth and G1 cell cycle arrest in both cell lines. Induction of apoptosis was observed in BxPC-3. Western blot analyses indicated that [6]-Gingerol decreased both cyclin A and cdks expression levels. It also induced the expression of p21<sup>cip1</sup>, an important cell cycle inhibitor in G1 and

S phase. Consequently phosphorylation of Rb proteins was reduced followed by blocking of S phase entry. The p53 expression was decreased rapidly by [6]-Gingerol treatment in both cell lines, which indicates that the increase of p21<sup>cip1</sup> was caused by p53-independent pathway. [6]-Gingerol also increased the phosphorylation of Akt in Hpac but not in BxPC-3. It might represent the resistance of Hpac against the apoptosis induction. This study first suggests that [6]-Gingerol has anti-cancer activities due to the effects of cell cycle arrest in G1 phase.

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Key words : [6]-Gingerol, pancreatic cancer, G1 cell cycle arrest, apoptosis

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## I . INTRODUCTION

It has been identified that various plant-derived compounds or phytochemicals have ability to interfere with carcinogenesis and tumorigenesis. They are also known to possess potential chemopreventive properties.

Plant of ginger (*Zingiber officinale* Roscoe, Zingiberaceae) family is one of the most highly consumed dietary substances in the world. In China and Malaysia, the rhizome of ginger has been used in traditional oriental herbal medicine for the management of common cold, digestive disorders, rheumatism, neuralgia, colic and motion-sickness<sup>1</sup>. The oleoresin from

rhizome of ginger contains pungent ingredients including Gingerol, Shoagol, and Zingerone<sup>2</sup>. Recently, these phenolic substances have been found to possess many interesting pharmacological and physiological activities. Especially, there are many laboratory data supporting that [6]-Gingerol (1-[4'-hydroxy-3'-methoxyphenyl]-5-hydroxy-3-decanone) (Fig. 1), the major pungent principle of ginger, has anti-oxidant, anti-inflammation and anti-tumor promoting activities<sup>2</sup>.

It shows anti-cancer and/or chemoprevention activities throughout various studies. For instance, [6]-Gingerol inhibited pulmonary metastasis in mice bearing B16F10 melanoma cells through the activation of CD8+ T cells<sup>3</sup>. And it also inhibited tumor promotion of ICR mice induced skin tumor by 12-O-tetradecanoyl phorbol-13-acetate (TPA)<sup>4</sup>, and blocked the azoxymethane-induced intestinal carcinogenesis in rodents<sup>5</sup>. In other case, [6]-Gingerol interfered with EGF-induced transformation of mouse epidermal JB6 cell line, and reduced the activation of Activator Protein-1 (AP-1), which plays a critical role in tumor promotion<sup>6</sup>. Moreover, [6]-Gingerol exerted inhibitory effects on the cell viability and DNA

synthesis, also induced apoptosis of human promyelocytic leukemia (HL-60) cells<sup>7</sup>. Recently, Mahady, G. B. et. al<sup>8</sup> showed the data on ginger root extracts and Gingerol inhibit the growth of *Helicobacter pylori* CagA5+ strains, which has a specific gene linked to the development of premalignant and malignant histological lesions in stomach. So they suggested that ginger and gingerol have effects of chemoprevention to the gastric-intestinal cancers.

There are many evidences that many phytochemicals ,such as epigallocatechin gallate(EGCG), genestine, tangeretin, silymarin, silibinin, and quercetin<sup>9-13</sup>, can inhibit the proliferation or survival of various cancer cell lines and also induce cell cycle arrest. Although anticancer activities of ginger extract and constituents have been examined, the underlying mechanism has not been researched. Therefore we examined the anticancer activities of [6]-Gingerol, and investigated its mechanism in two different pancreatic cancer cell lines, especially the effects on the cell cycle progression.

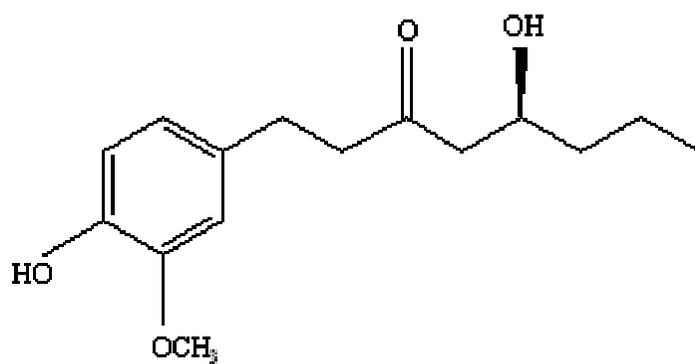


Figure 1. Chemical structure of [6]-

## II. MATERIAL AND METHODS

### Chemicals and Cell culture

A purified preparation of [6]-Gingerol (>98.0% pure) was purchased from Wako Pure Chemical Industries, LTD (Osaka, Japan). It was dissolved in sterile DMSO (dimethyl sulphoxide) and diluted with media.

Human pancreatic cancer cells (BxPC-3, Hpac) were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI1640 and DMEM/F12 medium respectively, containing 10% fetal bovine serum. Both cell line were incubated in a 5% CO<sub>2</sub> atmosphere at 37°C.

### Assessment of cell viability

The viability of the cells was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma, St. Louis, MO, USA) assay. The cells were seeded into 96-well plates, after 24hours they were treated with various concentration of [6]-Gingerol. After incubation for indicated times, MTT solution was added to the plate at a final

concentration of 0.5mg/ml and the cells were incubated for 4 hours in dark. The resulting MTT-products were dissolved by DMSO and determined by measuring the absorbance at 570nm with ELISA reader (Molecular Devices, Sunnyvale, CA, USA). Each point represents the mean of triplicate wells.

### **Cell cycle analysis**

The cells were seeded into 100-mm dishes, after 24 hours treated with the indicated concentrations of [6]-Gingerol for indicated hours. The cells were trypsinized, and washed twice with cold PBS (pH 7.4), and centrifuged. The pellet was fixed with cold ethanol for 12 hours at 4°C. The pellets were washed with cold PBS. They were incubated with RNase (200µg/ml final concentration) and stained with Propidium Iodide (100µg/ml final concentration) for 1 hour and analyzed by flow cytometry. Flow cytometry was performed on a FACS Caliber system equipped with argon-ion laser (Becton-Dickinson Immunocytometry system, San Jose, CA, USA). Percentages of cells in each phase were calculated using Cell Modfit software programs (Becton-Dickinson).

## Detection of Apoptotic Cells

To identify cells undergoing apoptosis, Annexin V-FITC Apoptosis Detection Kit (Becton-Dickison Biosciences Pharmingen, Franklin Lakes, NJ, USA) was used. Cells were harvested at different intervals after [6]-Gingerol treatment, containing floating and adherent cells. After washing with cold PBS (pH 7.4), the cells were stained and analyzed by the flow cytometry. The quantitative analysis was performed with WINMDi 2.8 program. Each analysis included at least 10,000 events.

To detect apoptotic cells, we also performed the terminal transferase uridyl nick end labelling (TUNEL) assay (R&D Systems, Inc., Minneapolis, MN, USA). TUNEL assay was used to detect genomic DNA fragment with double stranded breaks which are the general feature of apoptotic cells. The assay was preceded as following. BxPC-3 and Hpac cells were seeded onto 8-well chamber slide. After 24 hours the cells were treated with 400 $\mu$ M of [6]-Gingerol by indicated times. The cell samples were fixed by 3.7% formaldehyde and stained as labeling manual. To make the DNA accessible to the labeling

enzyme, the cell membranes are permeabilized with cytonin reagent. Next, terminal deoxynucleotyl transferase (TdT) is added and nucleotides are biotinylated to the 3'-ends of the DNA fragments. Straptavidin-conjugated Fluorescein (FITC) specifically binds to the biotinylated DNA fragments. Also the cell samples were incubated with DAPI solution (1:4000 dilutions) which stains nucleus. Then the sample slide was covered with Vectashield mounting media (Vector Labortoties, Burlinghame, CA, USA). Images of FITC and DAPI were observed under Olympus BX51 microscopy (Olympus, Tokyo, Japan).

### **Protein extraction and Western blot analysis**

Whole cell lysates, used to determine the levels of various proteins, were prepared following method. In brief, the pancreatic cancer cells were seeded onto culture-dishes, after 24 hours incubated with [6]-Gingerol for the indicated times. The cells were washed twice with cold PBS (pH 7.4) and added cell lysis buffer (70mM beta-glycerolphosphate (pH 7.2), 0.6 mM Na vanadate, 2M MgCl<sub>2</sub>, 1mM EGTA, 1mM DTT, 0.5%

Triton X-100, 0.2mM PMSF, 1X Protease Inhibitor; Leupeptin, Pepstatin, Aprotinin, and antipain each 5µg/ml). The cell lysate was centrifuged at 13,200 rpm for 30 min at 4°C. The protein concentrations were measured by the Bradford dye-binding protein assay, using bovine serum albumin (Sigma) as a standard. For Western blot analysis, protein samples were solubilized by boiling in sample buffer and subjected to SDS-PAGE followed by electrotransfer onto nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK). And we probed with following primary antibodies: rabbit polyclonal anti-cyclin A, rabbit polyclonal anti-cyclin D1, rabbit polyclonal anti-cyclin E, rabbit polyclonal anti-cdk 2 and rabbit polyclonal anti-cdk 4 (Delta Biolabs, Campbell, Ca, USA), goat or rabbit polyclonal anti-pRb(Ser780) (Bio Source, Camarillo, CA, USA), mouse monoclonal anti-Rb, rabbit polyclonal anti-cdk 6, rabbit polyclonal anti-p21, mouse monoclonal anti-p53, rabbit polyclonal anti-PI3K p85α, goat polyclonal anti-ERK1, mouse monoclonal anti-pERK, mouse monoclonal anti-pJNK, goat or chicken polyclonal anti-Akt1, rabbit polyclonal anti-pAkt1/2/3(Ser 473)-R, and mouse monoclonal anti-K-Ras

(Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The membrane was washed and incubated with horseradish peroxidase-conjugated species-appropriate secondary antibodies (Santa Cruz Biotechnology Inc.). Then it developed with enhanced chemiluminescence reagents (Amersham Life Science), and exposed to films in a dark room.

### III. RESULTS

#### *[6]-Gingerol inhibited cell growth and induces cell cycle arrest in G0/G1 phase.*

To assess the cytotoxic effects of [6]-Gingerol on two pancreatic cancer cells, dose-dependent curves were generated by MTT assay. The result shows that [6]-Gingerol exhibited cytotoxic effects on both BxPC-3 and Hpac cell lines proportional to the dosage (Fig. 2). Compared with  $IC_{50}$  value, there are no significant difference between BxPC-3 cells (387.4 $\mu$ M) and Hpac cells (405.3 $\mu$ M). This result is based on three independent experiments.

We next examined the effects of [6]-Gingerol in cell cycle perturbations. Cell cycle analysis was carried out by DNA flow cytometry (Fig. 3). BxPC-3 and Hpac cells were treated with 400 $\mu$ M (approximately  $IC_{50}$  of day3) of [6]-Gingerol. In our data, [6]-Gingerol treatment definitely caused cell cycle arrest of G0/G1 phase in both cell lines. The treatment of [6]-Gingerol arrested and accumulated more than 60% of cells in G0/G1 phase, maintained up to 48 hours. Consequently the fractions of

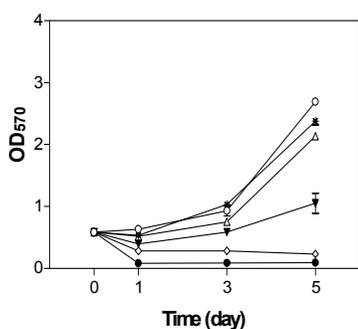
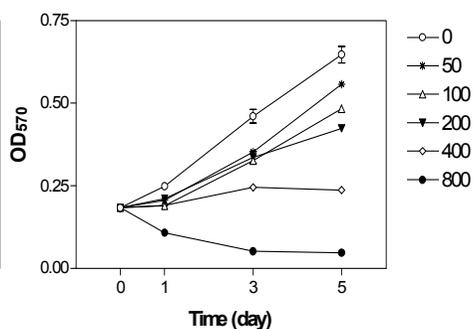
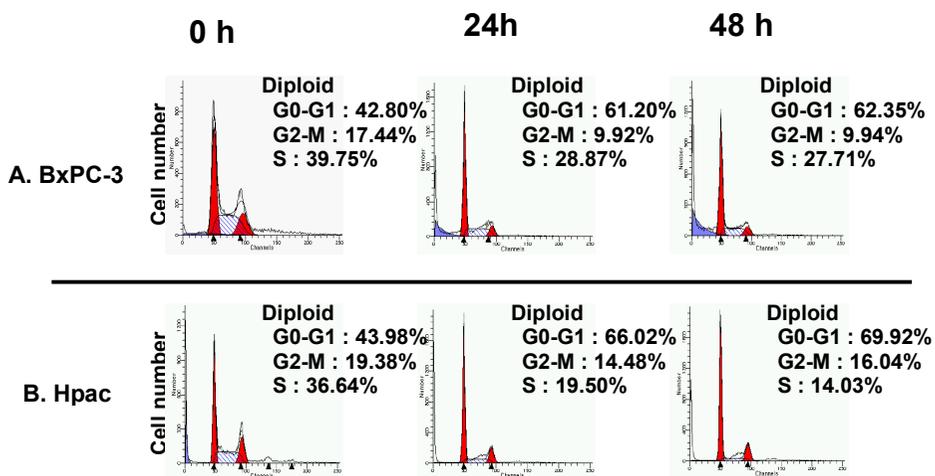
**A. BxPC-3****B. Hpac**

Figure 2. Cytotoxic effects of [6]-Gingerol on BxPC-3 (A) and Hpac (B) cells

Both BxPC-3 and Hpac were treated with certain concentration (in micro molar) of [6]-Gingerol, and cell viabilities were determined by MTT assay. The result shown here is based on three independent experiments. Each point represents the average of triplicate wells; bars, SEM.



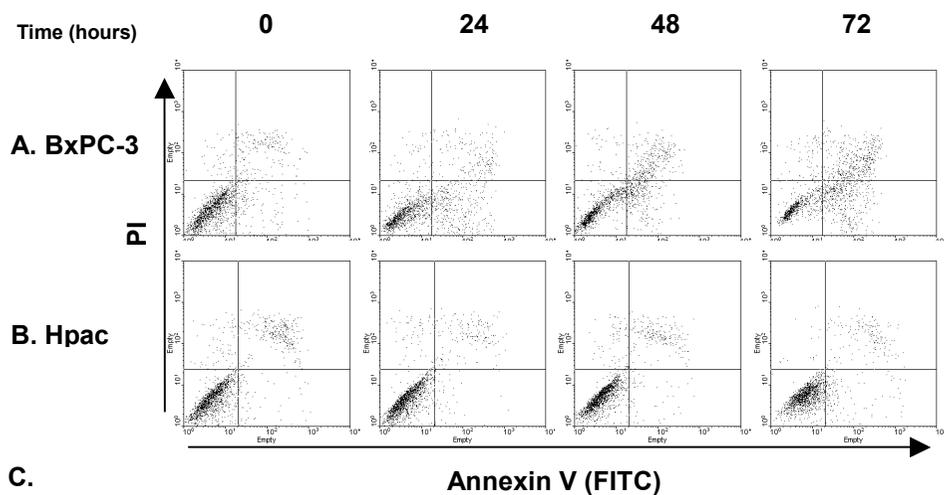
**Figure 3.** The effects of [6]-Gingerol on cell cycle in BxPC-3 and Hpac cell lines

Cells were plated and cultured in 10% FBS included medium over 24hours then treated with 400µM [6]-Gingerol. With various incubation times, the cells were fixed with ethanol and stained with Propidium Iodide. Then they were analyzed by flow cytometry. Percentages were calculated by using ModFit LT software.

S and G2/M phases were decreased after treatment. At 24 hours after treatment, there was a significant increase in the sub-G1 population (26.58%) suggesting apoptosis in BxPC-3 but not in Hpac cells (3.57%).

*[6]-Gingerol induces apoptosis in BxPC-3 cell.*

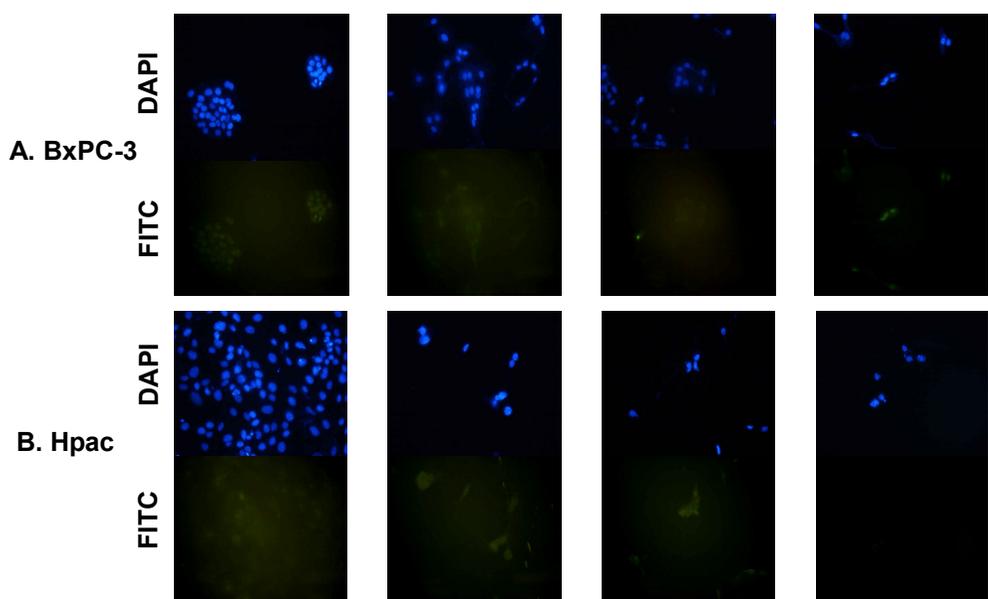
Upon a previous research, [6]-Gingerol induced apoptosis in leukemia cells<sup>7</sup>. We examined whether [6]-Gingerol also induce apoptosis in pancreatic cancer cells. The cell undergoing apoptotic process presented many phosphatidylserine, early apoptotic marker, on its surface. To exam the external cellular environment exposing phosphatidylserine, cells were treated with Annexin-V/FITC, having a high affinity for phosphatidylserine, and analysed by flow cytometry (Fig. 4). It was shown that about 30% of BxPC-3 cells treated with [6]-Gingerol were found in the early apoptosis stage (Annexin V-FITC positive and PI negative) after 24 hours, and the cells were shifted to the late apoptosis stage (Annexin V-FITC and PI positive) after 48 hours. After treatment for 72 hours, more than 50% of BxPC-3 cells were display apoptotic cell death.



		Control	24h	48h	72h
BxPC-3	Live	75.93 %	59.37%	61.01%	39.32%
	Early Apoptosis	12.17%	28.68%	20.55%	34.17%
	Late Apoptosis	8.90%	9.62%	15.79%	22.45%
Hpac	Live	76.39 %	85.65%	83.17%	77.02%
	Early Apoptosis	5.52%	4.13%	4.58%	13.67%
	Late Apoptosis	14.95%	7.66%	9.03%	8.15%

**Figure 4. Effects of [6]-Gingerol on apoptosis**

Using the Annexin V-FITC apoptosis detection kit, we examined apoptosis in two cell lines induced by [6]-Gingerol. The X and Y axis represents annexin V-FITC and Propidium Iodide (PI) fluorescence respectively. Population in down-left part (Annexin V-FITC and PI negative) is viable, and down-right part (Annexin V-FITC positive and PI negative) is undergoing apoptosis. Cells observed in Annexin V-FITC and PI positive indicates either late stage of apoptosis or dead cells. (A) BxPC-3 and (B) Hpac cells were treated with 400 $\mu$ M of [6]-Gingerol for the indicated time (top). The population percentage of each part is calculated in table (C).



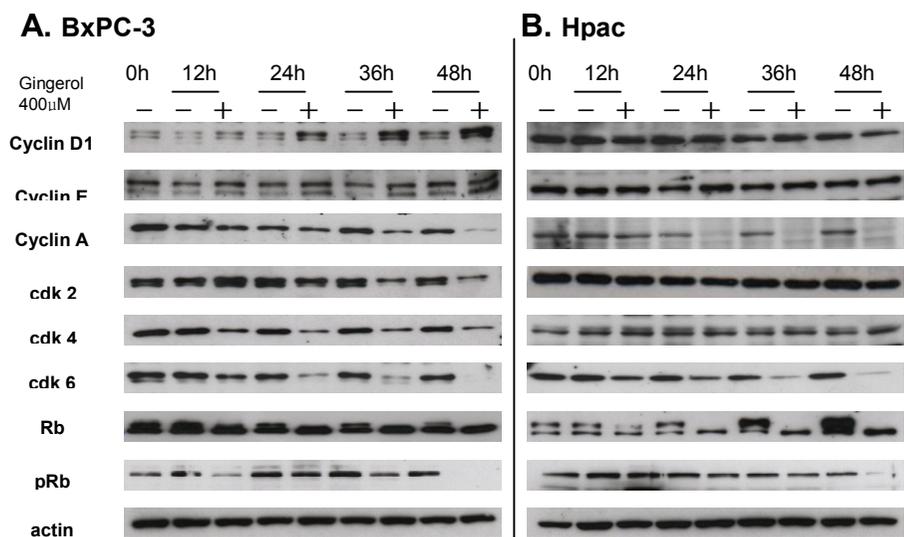
**Figure 5. TUNEL assay to detection apoptotic cells**

BxPC-3 (A) and Hpac (B) were incubated with 400 $\mu$ M of [6]-Gingerol and fixed by formaldehyde. DNA fragmentation, which is the feature of apoptotic cells, was detected by TUNEL assay. Nucleus detected with DAPI staining (blue), and DNA fragment visualized with FITC (green). Photomicroscopy was carried out by using same exposure time at 400X magnification.

This transition is a typical apoptotic process. Since Hpac cells displayed few early apoptosis until 72 hours indicates that this cell line is highly resistance to [6]-Gingerol induced apoptosis. This observation is corresponds to the DNA flow cytometry result with minimal sub-G1 population only in Hpac.

Also we performed TUNEL assay to observe DNA fragmentation. During apoptosis, specific endonucleases cleave genomic DNA creating fragments with double-stranded breaks. DNA fragmentation in individual apoptotic cells is visualized by detection of biotinylated nucleotides incorporated onto the free 3'-hydroxyl residues of these DNA fragments. This DNA fragments are specifically bound to Straptavidin-conjugated Fluorescein (FITC) and it can be detected with fluorescence microscope. The result is showed in Fig. 5. The blue spots indicate individual nucleus and the green light is FITC, which bind to DNA fragmentation. By contraries, there was no detection of FITC in both cell lines.

*[6]-Gingerol caused changes in various cell cycle related protein expressions.*



**Figure 6. Western blot analyses of the cell cycle regulatory proteins**

Both BxPC-3 and Hpac cells were treated with 400µM of [6]-Gingerol, and harvested every 12 hours. (A) indicates the levels of proteins expression in the BxPC-3 and (B) in Hpac. The upper band of Rb indicates phosphorylated form. pRb antibody detects specifically on Ser-780 phosphorylated Rb. Actin was used as a loading control.

Based on the previous experiment cell cycle analysis, we examined the effect of [6]-Gingerol on the levels of G0/G1 phase-related proteins by Western blot analyses (Fig. 6). Both BxPC-3 and Hpac cells were treated with 400 $\mu$ M of Gingerol and harvested every 12 hours. In BxPC-3 and Hpac cells, expression levels in various proteins were changed by treating [6]-Gingerol. In BxPC-3, [6]-Gingerol induced decrease in cyclin A, cdk 2, cdk 4 and cdk 6 expression and increase in cyclin D1 expression. In Hpac, [6]-Gingerol decreased the cyclin A and cdk 6 expression. The level of phosphorylated Rb protein was decreased in both BxPC-3 and Hpac cells. In Fig.6, the upper band of Rb protein, phosphorylated form, was disappeared gradually and definitely disappeared after 48 h. Upon the various changes, [6]-Gingerol induced G1 cell cycle arrest in both pancreatic cancer lines.

*Effect of [6]-Gingerol on the expression of p21<sup>cip1</sup> was caused by p53-independent pathway.*

In both BxPC-3 and Hpac cells, the expression of p21<sup>cip1</sup>, a cyclin dependent kinase inhibitor, was increased by [6]-Gingerol

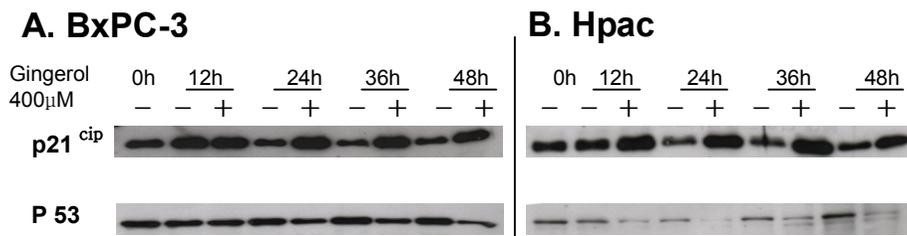
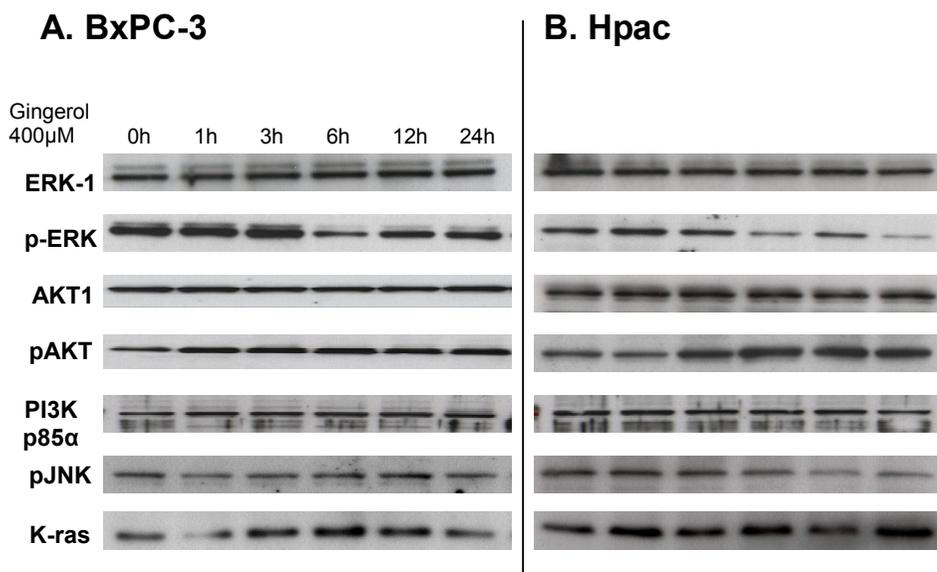


Figure 7. Effects of [6]-Gingerol on the expression of p21<sup>cip</sup> and p53

p21<sup>cip1</sup>, a cyclin dependent kinase inhibitor, was increased by [6]-Gingerol in BxPC-3 (A) and Hpac cells (B). Also, the treatment of



**Figure 8. Western bolt analysis for MAP kinases and PI3K/Akt pathway**

The BxPC-3 (A) and Hpac (B) were treated with 400µM of Gingerol. Each phosphorylated proteins are considered as activated form. p-ERK specially reacts with Thy-204 phosphorylated ERK1 and ERK2. pAKT can detect Ser 473 phosphorylated Akt1 and phosphotyalted Akt2 and Akt3. pJNK reacts with phosphorylated JNK1/2/3.

(Fig. 7). The overexpression of p21<sup>cip1</sup> could accelerate the cell cycle arrest in G1 phase by [6]-Gingerol.

It is well known that induction of p21<sup>cip1</sup> is associated with the expression of p53. In addition, the BxPC-3 and Hpac cells are comparable in their genotype in p53; BxPC-3 is p53 mutant and Hpac is wild. Based on the fact, we examined whether p53 affect on the action of [6]-Gingerol or not (Fig. 7). The treatment of [6]-Gingerol reduced the p53 level in the both cell lines and it implies that the over-expression of p21<sup>cip1</sup> by [6]-Gingerol was caused by p53-independent pathway.

#### ***[6]-Gingerol affects MAPKs and PI3K/Akt pathway.***

There are differences between Bxpc-3 and Hpac cell lines in induction of apoptosis and changes in expression of cell cycle controlling proteins. The difference in [6]-Gingerol response may be caused by the altered mitogen-activated protein kinases (MAPKs) and PI3K/Akt pathways, which play important roles in cell cycle progression and cell survival. Therefore we examined the levels of various proteins by Western blot analyses (Fig. 8). In BxPC-3 cells, [6]-Gingerol

decreased the phosphorylation of ERK protein at 6 hours and increased afterward. On the other hands, phospho-Akt protein did not change in 24 hours. In Hpac cells, phospho-ERK protein was decreased after 6 hours, however phospho-Akt was increased. On the other, phosphorylated JNK(c-Jun N-terminal kinase) decreased only in Hpac cells. In addition, there were slight alteration of K-ras levels but no change of PI3(1-Phosphatidylinositol 3)-kinase p85 $\alpha$  levels in both cell lines.

#### IV. DISCUSSION

When cell in G1 phase receives extracellular signals, cell decides either to proliferate or to withdraw the cycle into a resting state, G0 phase. Unlike S, G2 and M phases, G1 phase progression depends on the stimulation by mitogens and it can be blocked by anti-proliferative factors. Generally, cancer cells control this process abnormally that cells remains in the cycle uninterruptedly. When the cells remains in the cell cycle, maturation and differentiation activities takes place and that may contribute to carcinogenesis and cancer progression<sup>14</sup>. To enter the S phase, there is a restriction point, G1-S checkpoint in the cell cycle that mammalian cells become committed and complete the cell cycle. The cell cycle mechanism is controlled mainly by the phosphorylation of proteins participated in each stage. These proteins are called cyclins and cyclin-dependent protein kinases (Cdks). Passage through the restriction point and entry into S phase is sequentially regulated by cyclin D, E, and A. Cdk activity is influenced by the following factors; binding of cyclins, both positive and negative regulatory

phosphorylations, and constraint of Cdk inhibitory protein(CKI). CKI is consisted of INK4 and CIP1/KIP1 families. In all eukaryotic cells, cyclin-mediated Rb hyperphosphorylation induces a release of E2F-1, and E2F-1 which then activates transcription of DNA synthesis-related gene<sup>15</sup>. During mid G1 phase, complex of cyclin D-cdk4/cdk6 phosphorylates Rb protien. In late G1 phase, another type of G1 cyclin-dependent kinase complex, cyclin E-Cdk2, becomes rapidly activated which then catalyzes additional phosphorylation of Rb to hyperphosphorylated form and suppress function of Rb. As a result E2F-responsive genes are expressed and the cell cycle stage moves onto S phase<sup>16</sup>. When cell enters S phase, expression of cyclin A is increased and newly translated cyclin A forms complex with Cdk2.

Phenolic compounds comprise one of the largest and most ubiquitous groups of plant metabolites. They are formed to protect the plant from photosynthetic stress, reactive oxygen species (ROS), wounds, and herbivores. The most commonly contained ones in foods are flavonoids and phenolic substances. Hence phenolic compounds takes important parts of the human

diet<sup>17</sup>. In addition, current interest is raised up by many observations that dietary phenolic compounds have various activities such as antioxidant, anti-inflammation and anti-carcinogenesis.

In this present study, we first investigated the effects of [6]-Gingerol which is a phenolic substance derived from ginger roots, on two pancreatic cancer cell lines. We found that [6]-Gingerol inhibited the cell growth, disrupted the cell cycle progression in both cell lines, and also induced apoptosis in BxPC-3 cells. Interestingly, it is noticeable that normal cell showed highly resistance to the cytotoxicity of [6]-Gingerol. For instance, RIE (rat interstitial epithelial cell) showed 50% growth inhibition at over 900 $\mu$ M (data not shown). This selectivity may be the great advantage of [6]-Gingerol for the further therapeutic or chemoprevention use.

The data indicates that cell death induced by [6]-Gingerol in both cell lines was associated with the disruption of cell cycle progression. When G1 arrested cells induced by serum starvation were treated with [6]-Gingerol, significant G0/G1 phase arrest was maintained in the both cells (data was not

shown). On the contrary, cells without [6]-Gingerol treatment were released from G0/G1 phase. Nevertheless there were some results that various phenolic substances induce cell cycle arrest in some phases, this is the first report that reveals on [6]-Gingerol effect upon cell cycle in cancer cell lines.

Western blot analyses indicated that [6]-Gingerol causes a decrease in the expressions of cyclin A and several cdks including cdk 2, cdk4, and cdk6 in BxPC-3. Corresponding to BxPC-3, cyclin A and cdk 6 expression levels were decreased in Hpac. Thus, the reduction of cyclin or cdk expressions results the blocking of cyclin-cdk complexes formation and that lowers the level of phospho-Rb rapidly. As if Rb proteins remain in unphosphorylated form, E2F cannot be activated and the cells fail to enter the S phase. However, the cyclin D1 level was increase in BxPC-3 and it is assumed as a feedback response to G1 arrest.

A number of phytochemicals, including EGCG (Epigallocatechin-3-Gallate)<sup>18</sup>, tangeretin<sup>11</sup>, genestein and silymarin<sup>12</sup> have been shown to induce a cell cycle arrest accompanied by increased p21<sup>cip1</sup> expression, an important cell

cycle inhibitor in G1 and S phase. [6]-Gingerol also increased the level of p21<sup>Cip1</sup> in both cell lines. Finally the cancer cells treated with [6]-Gingerol fail the progression of cell cycle, presumably it suppresses their growth.

The signal transduction that regulates cell growth and cell cycle is unclear, but there are much evidences that Ras pathway and c-Myc play an important role in cyclins or cdks control related with the G1, S phase<sup>12,15,19-21</sup>. *Ras* is oncogene and involved in growth signal transmission into the cytoplasm. Activating point mutations in the *K-ras* oncogene are frequent in pancreatic carcinomas compare to other types of tumor. About 90% of pancreatic cancers harbor activating point mutations in codon 12 of *K-ras*<sup>22</sup>. The Hpac cells expressed a mutant K-Ras, whereas the BxPC-3 cells expressed a wild type K-Ras. This difference on genetic information may cause distinct effects of the sensitivity to this chemical either by change of protein expression in two cell lines.

Based on *in vitro* and *in vivo* studies, the Cip/Kip family including p21<sup>Cip1</sup>, p27<sup>Kip1</sup> and p57<sup>Kip2</sup> was initially thought to interfere with the activation of cyclin D-, E- and A-dependent

kinases. More recent study, however, has altered this view and revealed that even the Cip/Kip proteins are specific inhibitors of cyclin E- and A-dependent Cdk2, they act as positive regulators of cyclin D-dependent kinases<sup>23</sup>. p21<sup>cip1</sup> is a representative member of the Cip/Kip proteins. As it is mentioned above, the level of p21<sup>cip1</sup> protein was up-regulated in both cell lines. Upon the recent studies, it is assume that the overexpression of p21<sup>cip1</sup> influenced the increase of cyclin D1 level in BxPC-3. Transcription of p21<sup>cip1</sup> is regulated by tumor suppressor protein, p53. The BxPC-3 cells have point mutated p53 proteins and the Hpac cells have the wild type p53 proteins. In the both cell lines, the level of p53 is decreased by [6]-Gingerol. Probably induction of p21<sup>cip1</sup> was not associated with expression of p53. It has been reported Ras signaling through the Raf/MAPK pathway also elevates levels of p21<sup>cip1</sup> in some cell types<sup>23-25</sup>. However it is still unclear whether the over-expression of p21<sup>cip1</sup> by [6]-Gingerol is resulted by Ras signaling activations.

The anti-cancer activities of [6]-Gingerol could be associated with a control of signal transduction of MAP kinases

and PI3K/Akt pathway, so we investigated the change of associated proteins. The expressions of K-Ras proteins showed inconsiderable change by [6]-Gingerol in the both cell lines. The ERK, that is the downstream kinases of Ras proteins, was shown alterations of phosphorylation. JNK, a subfamily of the MAPK pathway, was not changed amount of the phosphorylation in BxPC-3 but decreased in Hpac.

While, the PI-3K/Akt pathway has an important role in preventing cells from undergoing apoptosis and contributing to the pathogenesis of malignancy<sup>26</sup>. More recently evidences have suggested that this pathway is also associated with the regulation of cell cycle progression<sup>27</sup>. In the both cells [6]-Gingerol could not affect the expression of PI-3K p85 $\alpha$ , the PI3-kinase regulatory subunit. However Akt, which is regulated by PI3-kinase, was increased phosphorylation by this chemical in only the Hpac cells. Activated Akt through its phosphorylation promotes the cell survival by anti-apoptotic mechanism. Activated Akt decreases the transcription of death genes, such as Fas ligand, IGFBP-1, Bim by phosphorylation forkhead family transcription factors, which promote their

sequestration by 14-3-3 proteins in the cytoplasm. Meanwhile, it increases the transcription of survival genes by activation of NF- $\kappa$ B and CREB transcription factors. Additionally activated Akt also phosphorylate and inactivate the proapoptotic protein, BAD<sup>26-28</sup>. The increase of phospholated Akt by [6]-Gingerol could protect apoptosis, despite of cell cycle arrest in Hpac. While in the BxPC-3 cells there was no change in phosphorylation of Akt by it, then BxPC-3 could undergo apoptotic cell death.

## V. CONCLUSION

In the present study, we found that [6]-Gingerol inhibited cell growth and caused G1 arrest of the cell cycle in two different pancreatic cancer cell lines, also induced apoptosis. It appears that BxPC-3 cells are slightly more sensitive to inhibitory effects of [6]-Gingerol than Hpac.

There are many evidences that ginger has anti-cancer/tumor activities, based on the extensive amount of laboratory and epidemiology data. But we first report that [6]-Gingerol protect cancer by causing cell cycle disruption and/or induction of apoptosis. Also it is inexpensive originated from natural product and appears to be safe for long period of time with adverse side effect. . This compound is more useful though modification of its structure or combination treatment with other therapy and so on. Thus, the purpose of this study was to clarify molecular mechanism of [6] Gingerol on two pancreatic cancer cell lines.

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ABSTRACT(IN KOREAN)

췌장암 세포에서 [6]-Gingerol 의 세포주기 억제 효과

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박 연 정

최근 생강과(*Azingiber officinale* Roscoe, Zingiberace)를 포함한 많은 식물들이 항암 효과를 가진다는 사실이 알려지고 있다. 하지만 이러한 식물의 종양 억제효과에 대한 분자 수준의 작용 기전은 거의 알려져 있지 않다. 이 연구에서는 생강의 주요한 페놀성 물질인 [6]-Gingerol 의 작용과 그 기전을 인간 유래의 두 췌장암 세포 (BxPC-3, Hpac)에서 관찰하였다. 그 결과 [6]-Gingerol 은 두 세포의 성장을 저지하였으며, G1 기에서의 세포주기 억제 효과도 나타냈다. 또한 BxPC-3 세포를 세포사멸로 유도하였다. Western Bolt 분석 결과 [6]-Gingerol 은 cyclin A 와 여러 cdk 의 발현 수준을 저하시켰고, G1 과 S 기의 강력한 세포주기 억제자인 p21<sup>cip</sup> 의 발현을 증가시켰다. 이러한 작용들은

결국 Rb 단백질의 인산화를 감소시켰고 이로 인하여 BxPC-3 와 Hpac 세포의 S 기 진행이 저지되었다. 한편 p53 의 발현 변화를 관찰한 결과, 두 세포 모두에서 [6]-Gingerol 은 p53 의 발현이 저하되었다. 이를 통해 p21<sup>cip</sup> 의 증가가 p53 과 비의존적 경로로 유래되었음을 추론할 수 있다. [6]-Gingerol 은 MAPK 와 PI3K/Akt 의 세포신호전달체계에도 영향을 주었다. 특히 Hpac 에서만 Akt 의 인산화를 증가시켰지만, BxPC-3 에서는 변화가 주지 못했다. 이것은 Hpac 에서 [6]-Gingerol 로 인한 세포사멸 유도가 실패한 이유로 생각된다. 이 연구는 처음으로 G1 기의 세포주기 억제 효과에서 기인한 [6]-Gingerol 의 항암 작용을 증명하였다.

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핵심되는 말 : [6]-Gingerol, 췌장암, G1 기의 세포주기 억제, 세포 사멸