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석사학위 논문

Delphinidin induced cell cycle arrest and apoptosis on Human pharyngeal squamous carcinoma cells

조선대학교 대학원

치의학과

Le Thanh Do

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도경부 암세포에 대한 delphinidin 의 세포주기억제 및 세포사멸 연구

2009 년 8 월 25 일

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Abstract

Delphinidin induced cell cycle arrest and apoptosis on Human pharyngeal squamous carcinoma cells

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Delphinidin, one of sugar free counterparts of anthocyanin, can be found in many daily foods and drinks. In FaDu cells treated with delphinidin, the expression of p27, a cell cycle inhibitor, was increased. The expression of both cdk1 (cdc2) and cyclin B, the key regulators in G_2/M phase, was decreased in western blotting analysis. In addition, the decreased expression of G_1/S regulators, cyclin D, cdk2 and cdk4, were confirmed. FACs analysis, after treating with delphinidin for 24 hr, showed a dramatic increase of DNA content (22 % to 60 %) at G_2/M checkpoint with the decrease of DNA content at G_1/S phase. The cleaved form of caspase 3 and caspase 7 and the inactivated fragment p85 of PARP were detected. Fluorescence microscopic analysis of nuclear condensation stained with DAPI showed chromatin condensation. An apoptotic DNA ladder bands were detected. Protein expression analysis of anti-apoptotic Bcl-2 and pro-apoptotic Bad and Bax, using western blotting

analysis, supported the apoptosis phenomena. Delphinidin inhibited AKT (PKB) phosphorylation in a dose dependent manner and up-regulated the phosphorylation of p38 MAP kinase. Overall, our data support that delphinidin induces apoptosis on FaDu hypopharyngeal cancer cells by suppressing the cell cycle progression.

국문초록

도경부 암세포에 대한 delphinidin 의 세포주기억제 및 세포사멸 연구

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무당체 Anthocyanin 의 하나인 delphinidin 은 일상적인 많은 음식과 음료에 함유되어 있다. 두경부 암세포에 Delphinidin 을 처리했을 때 세포주기 억제제인 p27 발현이 대조군에 비해 2 배이상 증가하였다. Western blot 분석에서 G2/M 기에서의 주 조절자인 cdk1(cdc2)와 cyclin B 의 발현이 delphinidin 처리 후 감소하였다. G1/S 주기 조절자인 cyclin D, cdk2 와 cdk4 발현의 감소도 확인하였다. FACS 분석에서 delphinidin 조작 24 시간 후에 G2/M 기에서 DNA 양의 증가와 함께 G1/S 기에서 DNA 양 감소를 확인하였다. Caspase3 과 caspase7 의 활성과 PARP 의 비활성분절인 p85 들이 delphinidin 세포처리 후 증가되었다. DAPI 염색에 의한 형광현미경 분석에서는 chomatin 의 응결이 관찰되었다. 또한 DNA apoptotic ladder band 의 형성을 전기영동실험에 의해 증명하였다. Western blot 분석에 의한 Anti-apoptotic Bcl2 와 pro-apoptotic Bad 와 Bax 의 단백질 발현 분석에서 Bad/Bcl-율과 Bax/Bcl-2 율과 비교했을 때는 1 보다 더 높았다. 용량의존성에서 Delphinidin 은 AKT(PKB) 인산화를 억제하였고 p38 MAP kinase 인산화를 증가시켰다. 종합하면 delphinidin 이 FaDu 인두암 세포에서 apoptosis 를 유도하고 세포주기 진행을 억제하는 것을 확인하였다.

I. Introduction

Anthocyanins are common plant pigments, which are components in many daily foods. They were identified as natural compounds with a capacity to prevent tumor growth. Delphinidin is one of anthocyanidins, which are sugar-free counterparts of anthocyanins. It inhibits vascular endothelium growth factor (VEGF) phosphorylation and inhibits (VEGF)-induced bovine aortic endothelial cell proliferation by inhibiting ERK1/2 phosphorylation [24, 25]. Study by Laure Favot showed that Delphinidin inhibited cell proliferation on HUVECs by down-regulating cyclin A, cyclin D1, up-regulating p27, and arresting cell cycle at G1/S checkpoint [12]. Maria C. M. also found that Delphinidin induce apoptosis on different human cell lines. They detected the cleaved form of PARP, DNA fragment and apoptotic cell death [13]. Delphinidin was confirmed to be a potent inhibitor of the epidermal growth-factor receptor [15].

The cell cycle is consist of interphase and mitotic phase. They are both regulated by cyclins and cyclin dependent kinases (Cdk). Cyclins are synthesized and degraded frequently during the cell cycle process. There are 5 cyclin families: A, B, C, D, and E, with some members in each family and the expression of each of these is a tissue dependent [18]. For the cell to complete the division, it has to pass G_1 checkpoint to synthesize DNA and pass G_2 checkpoint to do division. In mammalian cells, passage through G_1 is

controlled by the ordered expression of D and E types of cyclins, which associate with cdk4/6 and cdk2 [18]. The cyclin D associated cdk4/6 starts cell cycle by phospholating retinoblastoma protein family (Rb), following to release transcription factors (E2F) from inactive Rb-E2F complexes and indirectly up regulating cyclin E. The functional cyclin E/cdk2 requires for G_1 progression, and the continued Rb inactivation during this period contributes to synthesis of cyclin A necessary for upcoming S-phase [18]. DNA replication takes place in S-phase. Cdk2 is the only Cdk known to regulate G_1/S phase transition and progression through S phase in association with cyclin E and later with cyclin A. Mitosis is then initiated by cdc2/cyclin B complexes, also known as M phase promoting factor (MPF). Cdc2/cyclin A complexes also contribute to the preparation for mitosis in G_2 phase. Cyclin B1 is an essential cell cycle gene. It translocates to nucleus at the end of G_2 phase. It has been demonstrated that nuclear cyclin B1/Cdc2 complexes are responsible for nuclear envelope breakdown, chromosome condensation and mitotic spindle assembly. The cdk inhibitor $p27^{kip1}$ (p27) participates in cell cycle exit and helps maintain the G_0 state by ensuring that cyclin/cdk complexes remain inactive. High p27 levels in quiescent cells establish an inhibitory threshold that must be reduced for cell cycle re-entry. Cyclin D1/cdk4/6 plays an important role in this process by sequestering p27 away from cyclin E/Cdk2 in early G_1. In a satisfying twist, as a feedback respond, cyclin E/Cdk2

phosphorylates p27 later in G_1 and indirectly marks it for proteasomal degradation [18].

Apoptosis, a programmed cell death, is an important counterpart to mitosis for the regulation of cell numbers during development, homeostatic cell turnover in the adult, and many other settings. Apoptosis is characterized by a series of distinct morphological and biochemical alterations to the cell such as DNA fragmentation, chromatin condensation, cell shrinkage, and membrane blebbing. Apoptotic cells then break into small membrane surrounded apoptotic bodies that are removed by phagocytosis [16].

During the last two decades, the identification and characterization of cellular factors in the apoptotic signal pathways has been an intensive research field. Activation of apoptotic signaling is achieved by either an extrinsic or an intrinsic pathway. The extrinsic pathway is triggered by ligation of cell surface death receptors with their specific ligands, resulting in the activation of caspase 8. The activated caspase 8 then cleaves and activates caspase-3, and -7. The intrinsic pathway is activated when the cells are under severe stresses such as growth factor deprivation, oxidants and DNA-damaging agents by leaking cytochrome c from mitochondria. The leakage of cytochrome c into cytosol results in the activation of caspase-9 and then the activation of caspase-3, -6, and -7 [16]. Caspases, a family of cysteinyl aspartate-specific proteases, are central mediators of apoptotic and inflammatory pathways. Caspases are synthesized as zymogens with a prodomain of variable length

followed by a large subunit and a small subunit. The large prodomains contain protein recruitment motifs that consist of six or seven antiparallel amphipathic α-helices. These prodomains allow recruitment and proximity-induced activation in protein complexes. The caspases are activated through proteolysis at specific Asp residues residing between the prodomain and the subunits. This results in the generation of mature tetrameric caspases, containing two large subunits and two small subunits [14]. Poly (ADP-ribose) polymerase (PARP) is the key enzyme in DNA repair process. It binds to the damaged strand and repairs the damage. During apoptosis process, executor caspase 3 and 7 cleave PARP, producing 85 kDa and 25-29 kDa parts, inactivating PARP and producing an apoptotic marker protein (85 kDa) [26].

Akt, also known as Protein kinase B (PKB), regulates essential cellular functions such migration, proliferation, differentiation, apoptosis and metabolism. Akt is activated by phosphorylation. The last step of Akt activation is the phosphorylation of Serine^{473} residue [22]. It inhibits cell cycle inhibitors and proapoptotic proteins. Activation of Akt frequently observed in human cancers. It overcomes G_2/M cell cycle checkpoint [24]. In opposition, a mitogen-activated protein kinase, p38 MAPK mediates apoptosis induced by many anti-cancer agents. It is activated by phosphorylation at some specific amino acid residues, such as Threonine 180 and Tyrosine 182 [17].

Because of chemo-preventive benefits of anthocyanidins, many researches have been active. But there are few publications on the effects of delphinidin,

and the cell signaling mechanism triggered by delphinidin remains unclear. The pharyngeal cancer is the 20th common cancer in the world. About 65000 cases were found annually [27]. Among the pharyngeal cancers, the hypo-pharyngeal cancer is the most dangerous type with the five-year survival rate relatively low as 20 %. Fadu, an epithelial cell line from a squamous carcinoma cell of the hypo-pharynx, was established in 1968 from a Hindu patient [21]. For these reasons, the study on the delphinidin was chosen to understand how delphinidin triggers the cell signal and how such a dangerous cancer cells responds to a delphinidin treatment.

II. Materials and experimental procedures

1. Delphinidin

Delphinidin chloride was purchased from Extrasynthese, France. Delphinidin was dissolved in ethanol and stock solution was prepared at a concentration of 5 mg/ml. The stock solution was divided in small equal parts and stored at -20 ^{0}C. The treating solution was prepared just before use by diluting the stock solution in cell seeding media.

2. Cell culture

Fadu cells were seeded in MEM medium (Welgene Inc., Korea) with 10 % FBS (Welgene Inc., Korea) and 1 X antibiotics (Invitrogen, USA). Cells were cultured in humid air with 5 % CO_2 at 37 ^{0}C. The media was changed every 24 hours.

3. Cytotoxicity

Toxicity of delphinidin on cells was investigated by using MTT assay. Cells were cultured in 96-well plate and treated with various concentrations of delphinidin. After 24 hours of treatment, the old media with drug was removed using 200 μl multi-channel pipette. MTT1 working solution was prepared just before use from the stock solution (5 mg/ml in PBS, Sigma, USA) by diluting one part of stock solution into nine parts of seeding media. The MTT1 working solution was added to the wells (200 μl per well) and the cell dish was incubated further for 4 h. The MTT1 solution was removed (200 μl multi-

channel pipette was set to 150 µl to remove MTT1 solution, the 100 µl pipette was set to 70 µl and the protein loading tips were used to remove the remaining solution in each well to avoid loosing cells) after 4 hours incubation and 200 µl of lysis buffer (10 % sodium dodecylsulfate, 0.1 N HCl) was added into each well. The cell dish was incubated for 1 more hour and the absorbance of each well was read at the wavelength of 540 nm. All pipette handling was done at the same site of the wells and never added solution directly to the well surface (instead, lightly on the wall of the wells). The data was analyzed using GraphPad Prism software, version 4.

4. Cell cycle analysis

Cells were treated with 70 µg/ml delphinidin for 24 hours. After treatment, cells were harvested by briefly trypsinizing and centrifuging at 300 *xg*. Cell pellet was washed once in ice-cold 1 X PBS and centrifuged. The cells then were fixed in 75 % ethanol (the cell pellet was resuspended in 250 µl of 1 X PBS, and then 750 µl of absolute ethanol was added into the cell suspension) for 2 hours at -20 ^{0}C. After fixing, cell suspension was centrifuged at 350 *xg* for 5 minute at 4 ^{0}C and ethanol was removed. Fixed cells were stained with 250 µl of PI solution (20 µg/ml Propidium Iodide, 200 µg/ml DNase-free RnaseA in 1 X PBS) for 15 minutes at 37 ^{0}C. DNA content was analyzed in Beckman Coulter system with the excitation wavelength of 488 nm.

5. DNA apoptosis ladder bands

Procedure for detecting DNA ladder was modified from Hugh J.M. Brady (2004). Cells at growth phage were harvested by briefly trypsinizing and centrifuging at 300 *x g*. The cell pellet was resuspended in seeding media (1 ml) and cell concentration was determined using Neubauer counting chamber. 3.2 x 10^6 cells were transferred to each 100 mm dish and incubated for 20 hours with the same seeding conditions. The cells then were treated with 25 and 70 µg/ml of delphinidin for 24 hours. After treatment, floating (dead) cells in media and adhesive cells were harvested into the same tube and centrifuged. Cell pellets were washed once with ice cold 1 X PBS and centrifuged. 150 µl of lysis buffer (200 mM HEPES, pH 7.5, 2% (v/v) Triton X-100, 40 mM NaCl and 20 µM EDTA, pH 8.0) [All components were prepared separately as stock solutions and the working solution was prepared just before use by mixing the stock solutions] was added into each tube containing the cell pellet and pipetting several times to resuspend cells completely. The cell tubes then were kept on ice for 30 minutes to lyse cells. The cell lysates were centrifuged at 20000 *x g* for 10 minutes at 4 ^{0}C, the supernatants were saved to new tubes. Proteinase K was added to each tube to the final concentration of 100 µg per ml and after incubation at 50 ^{0}C for 1 hour, the tubes were cooled down on ice for 5 min. DNase-free RNaseA then was added into each tube to final concentration of 200 µg/ml and the tubes were incubated for further 1 hour at 37 ^{0}C to break down RNA. 75 µl of phenol and 75 µl of chloroform / isoamyl alcohol mixture (24: 1) were added to each tube (all solutions was kept at 4 ^{0}C

before use), vortex the tubes gently and centrifuge them at 20000 *x g* at 25 ^{0}C for 10 minutes. The top layers of supernatants containing DNA fragments were carefully saved into new 2 ml tubes. 100 µl of TE buffer was added to the tubes containing middle and bottom layer, vortex the tubes gently and repeated centrifuging to harvest more DNA fragments. The top layers were saved in combining with the top layers of previous step (about 300 µl in total). 250 µl of chloroform / isoamyl alcohol mixture then was added to each tube, mixing and centrifuging steps were repeated and the top layer was carefully saved to new 2 ml tubes. 50 µl of 3 M sodium acetate and 1400 µl of absolute ethanol were added to each tube. The tubes were mixed gently and kept at -20 ^{0}C for 3 hours. DNA pellets were saved after centrifuging the tubes at 20000 *x g* for 15 minutes at 4 ^{0}C. The pellets were washed once with 1 ml of 70 % ethanol and repeated centrifugation. The tubes containing DNA pellets were kept at room temperature in clean bench for about 5 to 15 minutes with the caps opened to dry the DNA pellets briefly. The DNA pellets were dissolved in 30 µl of TE buffer and kept at -20 ^{0}C for agarose electrophoresis. DNA ladder was separated by electrophoresis with 1.5 % agarose gel for 2.5 hours under 50 voltages at room temperature. Pictures were taken using Biorad UV photographer.

6. Immunoblotting

Lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1 % Triton, 2.5 mM sodium pyrophosphate, 1 mM β-

glycerophosphate, 1 mM Na_3VO_4, 1 μM leupeptin) was purchased from Cellsignaling, 1 mM PMSF was added at prior of use.

Anti-p27, bax, bad, Bcl2, cdc2/cdk1, cdk4, procapase-3 and caspase-3 cleaved form, beta-actin and all secondary antibodies were purchased from SantaCruz, USA. Antibodies for cleaved form of caspase-7 (p20) were purchased from Cellsignaling, USA. Anti-PARP-1 (p85) came from Epitomics, USA. And anti-phospho-p38 MAPK (Thr180/Tyr182) was from Abfrontier, Korea. Other chemicals were obtained from Sigma (USA) or Merck (Germany).

Cells were seeded in 100 mm dishes, and then transferred to 60 mm dishes at growth phase. After 16 hours, the cells were treated with 70 μg/ml of delphinidin chloride in seeding medium for 24 hours. The media was collected and centrifuged at 300 *x g* to harvest floating cell, the cell pellets were washed once with ice-cold 1 X PBS and centrifuged again. The cell dishes were washed twice with ice-cold 1 X PBS; 300 μl of lysis buffer was added to each dish, and the dishes were left on ice for 3-5 min, using scraper to harvest adhesive cells into the tubes containing the dead cells. The cell suspensions were homogenized by pushing up and down 20 times by using 200 μl micropipette. The tubes was kept on ice for 15 more minutes, and sonicated for 2 minutes. The tubes were then centrifuged at 20000 *x g* for 20 minutes. The supernatants were saved as the total protein for western blotting. Total protein concentration was detected by using BCA^{TM} protein assay kit from (PIERCE). Protein samples were prepared in equal small parts (4 loading dose in each

tube) by adding appropriated volume of 5 X loading dye to total protein sample, boiling at 95 ^{0}C for 7 minutes and then cooling down on ice. The prepared sample tubes were kept at -80 ^{0}C. A sufficient amount of total protein was loaded on gel to detect interested proteins. Polyvinylidene Fluoride membrane (Pall Corporation, USA) and Bio-Rad transfer system were used for protein transfer. The transferring buffer was prepared as 10 x stock solution and kept at room temperature to avoid precipitation. The transfer working solution (1 X) containing 10 % methanol was prepared at least 2 hours before use to cool down at 4 ^{0}C. The membranes after transferring were blocked by incubating in 5 % skim milk in 1 X TBS for 2 hours at room temperature. Primary antibody and secondary antibody dilution was followed the commendation of manufactures. After incubating with primary antibody, membranes were washed with 1 X TBS three times for 18 minutes in total. After incubation of secondary antibodies, washing step was repeated, but there was a difference between desired proteins, with high expression level protein (or strong antibody, ex. beta actin), instead of using 1 X TBS in the second wash, we used 1 X TBS supplemented 0.1 % tween-20. The proteins on membrane were detected by Welgene detection kit (Welgene, Korea). Duration of film exposure was adjusted depending on the proteins detected.

Density of protein bands were analyzed using Biorad Image Master Software. The value was normalized with the band intensity of beta actin in the same lane.

Control samples were used as standards (1 unit of density) to calculate the normalized band intensity of the others.

7. Cytoplasm cytochrome c assay

Cells were seeded in 6 well plate with 5 x 10^5 cells/well. The cells were treated with 0, 25, and 70 μg/ml of delphinidin for 24 hours.

Cytochrome c that released from mitochondria into cytoplasm was detected using BMS263 kit (Bender Medsystems, USA). Working solutions were prepared just before use; the needed volume of each solution was calculated in milliliter. Washing buffer was prepared by diluting 1 part of stock solution in 19 parts of distilled water to the total volume, following the equation: 0.3 x 11 x number of wells. The he final solution was adjusted to pH 7.4. The volume of assay buffer was prepared: 0.1 x number of wells by diluting the stock solution in distilled water (X 20) and mixing gently to avoid bubble formation. Biotin-conjugate working solution was prepared by diluting the stock solution (X 100) in assay buffer: 0.05 x number of wells. Cytochrome c standard solution was prepared by adding distilled water and shaking to ensure solubilization. Streptavidin-HRP stock solution was diluted 200 times in assay buffer to produce working solution: 0.1 x number of wells. 5 ml of lysis buffer concentrate was diluted with 45 ml distilled water, mixed gently and stored at room temperature.

After treatment, the dead cell and adhesive cells were harvested by trypsinization and centrifugation. 200 μl of lysis buffer was added into each

sample and incubated for one hour at room temperature with gentle shaking. The cell lysates were centrifuged at 1000 *x g* for 15 minutes at 4 ^{0}C. The supernatants were saved as cytosol cytochrome-c samples (divided in equal small parts and kept at -80 ^{0}C). The samples were diluted (X 50) in assay buffer for the assay.

All reagents were mixed thoroughly prior to use. The well trips were inserted into a new case, washed twice with approximately 300 µl washing buffer per well (through aspiration of microwell contents between washes, be careful not to scratch the surface of the microwells). After washing, microwell trips were emptied and tapped on paper towel to remove remaining washing buffer (do not allow wells to dry). 100 µl of assay buffer was added in duplicate to all standard wells and blank wells. Standard dilutions were prepared by diluting with 100 µl cytochrome c standard stock solution in assay buffer. 100 µl of each pre-diluted sample was added, in duplicate, to the designated wells. 50 µl of diluted biotin-conjugate was added to all wells, including the blank wells. The microwell trips were sealed with the plate cover and incubated at room temperature for 2 hours. After incubation, plate cover was removed and the wells were emptied. Microwell strips were washed 3 times with 300 µl washing buffer per well. 100 µl of diluted streptavidin-HRP was added to all wells, including the blank wells. The wells were sealed with new plate cover and incubated at room temperature for 1 hour. After incubation, washing step was repeated. 100 µl of TMB substrate solution was added to all wells and

incubated the microwell strips at room temperature for about 10 minutes (cover the well trips with aluminum foil). When the highest standard was developed to a dark blue color (OD of 0.6 – 0.65 is reached), the enzyme reaction was stopped by quickly adding 100 µl of stop solution into each well. The absorbance of each well was read immediately on ELISA system, using 450 nm as the primary wavelength (optionally 620 nm as the reference wave length). The data was analyzed using Microsoft Excel to produce the ratio of cytosol cytochrome c level to the total protein.

8. Akt phosphorylation assay

The phosphorylation of Akt^{S473} was detected using CASE^{TM} kit (SABiosciences, USA). 9 x 10^4 cells at growth phase were transferred to 96 well plate in two sets of experiment (one for Akt phosphorylation, the other for total phosphorylated protein). The cells were grown in seeding media (supplemented 10 % FBS) for 24 hours, and starved for the next 12 hours (overnight). The cells were activated by seeding media containing 10 % FBS and treated with 0, 30, 50 and 70 µg/ml of delphinidin or 50 µg/ml of Akt phosphorylation specific inhibitor LY294002 (SABiosciences, USA) for 24 hours. After treatment, the media was removed; the cells were fixed and blocked on the dish. The cells in one set were incubated with phosphorylated Akt antibody, the other set with total phosphorylated protein antibody, and left at room temperature for 1 hour. After incubation, primary antibody solutions

were removed; the dish was washed and the secondary antibody was added to the plate. After incubating with secondary antibody for 30 minutes, the washing step was repeated; the developing solution was added to the plate and kept there for 10 minutes at room temperature before adding the stop solution. The absorbance at 450 nm (OD_{450}) was read immediately on ELISA plate reader (the values present for the phosphorylated Akt or total phosphorylated protein). The cells were washed and stained with staining buffer and lyszed in 1 % SDS; the absorbance at 595 nm was read (OD_{595}; the values represent the number of cells in each well). Finally, Akt phosphorylated level was normalized with the relative cell number, and then with total phosphorylated protein. The data was produced using GraphPad Prism software, version 4.

III. Results

1. Cell cytotoxic assay

The toxicity on Fadu cells of main anthocyanidins was investigated. Result in figure 1 showed that delphinidin was the most toxic one.

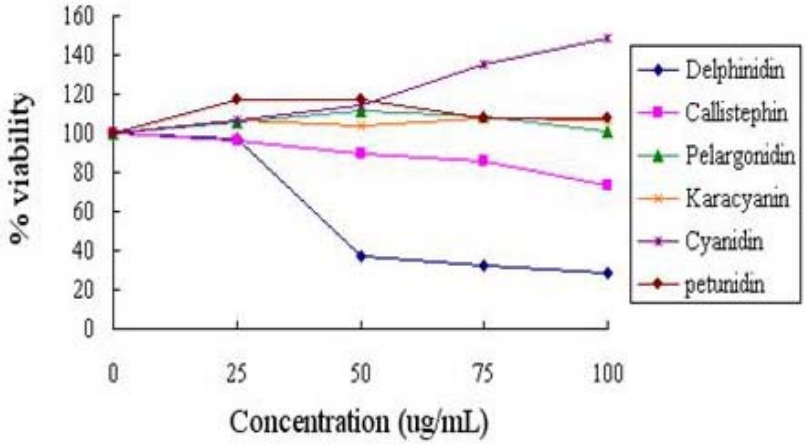

Figure 1. Cytotoxity of anthocyanidins on Fadu cells

2. Cell morphology

Cells at growth phase were treated with 25 or 70 μg/ml delphinidin for 24 hours; the images were recorded at X40 and X100 magnification. Delphinidin inhibited cell growth, even with the low dose (25 μg/ml). The cell confluence was not changed even after treating with 25 μg/ml of delphinidin for 24 hours. In higher dose (70 μg/ml treatment), delphinidin induced cell death, showing floating dead cell body and empty space when compared with control dishes or the early stage of the treatment. The survived cells under delphinidin treatment was changed their morphology to thinner and longer shape (Figure 2).

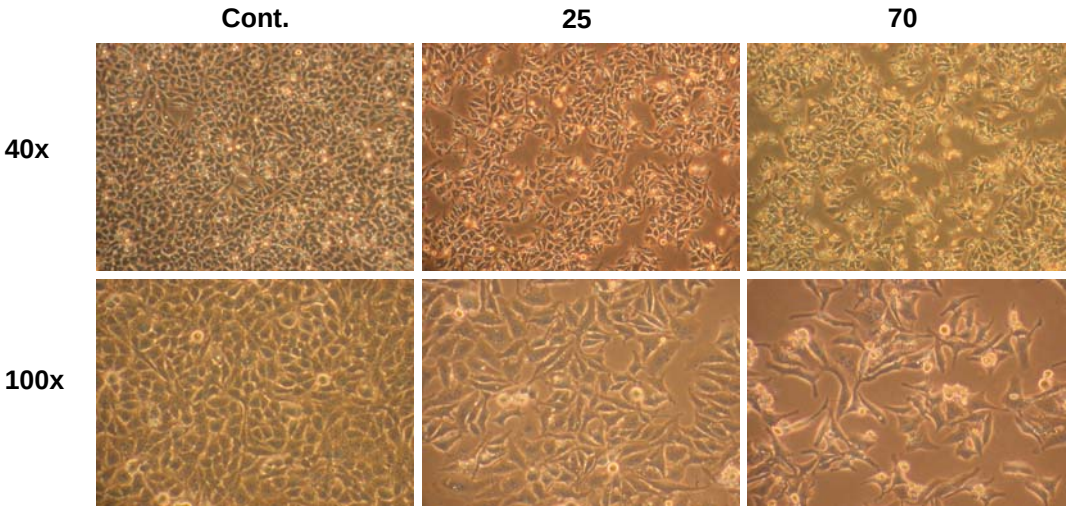

Figure 2. Cell death and morphological changes after treatment with delphinidin. Upper pictures showed the changes of cell confluences and amount of dead cells; in comparing with treatment beginning confluences, the confluences of the control dishes were increased; the cell confluences were not changed during 24 hours treatment with 25 μg/ml of delphinidin. With 70 μg/ml of delphinidin, the confluences were decreased and cell death was dramatically increased. The lower images show the change of cell morphology to thinner and longer shape when delphinidin concentration increased.

3. Cell cycle proteins

P27 is a well known inhibitor of cyclin dependent kinase activation and formation of cdk/cyclin complexes [11, 23]. Over expression of p27 inhibits cell cycle progression. In most cases, p27 inhibits the activation of cdk2/cylin E, and cdk4/cyclin D complexes. Expression of p27 was found to be down-regulated in many oral cancers [28]. After treating with 70 μg/ml of delphinidin, p27 expression in fadu cells was increased, nearly twice in comparing with the control (Figure 3).

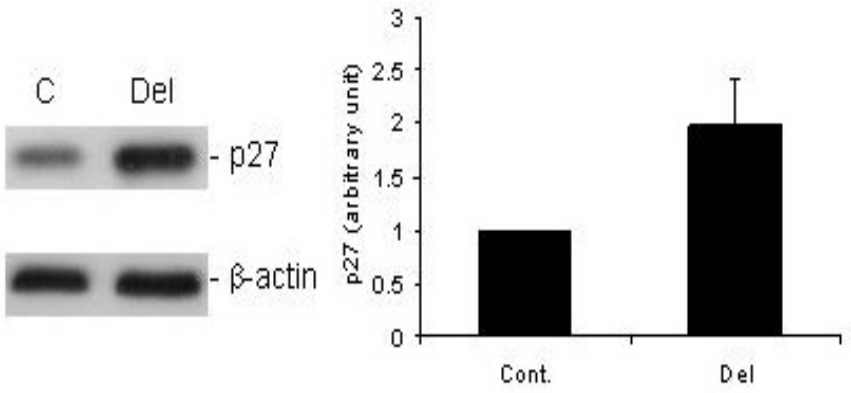

Figure 3. P27 expression after delphinidin treatment. Cells were treated with 70 μg/ml of delphinidin for 24 hours, p27 level increased almost 2 times in treated samples comparing with control samples.

Cdk1 is an essential cdk in mammalian cells [3]. In our data, the expression of cdk1 decreased about 40 % in comparing with control samples (Figure 4a). Similarly, cdk2 decreased dramatically after treatment (Figure 4b). Unlike cdk1 and cdk2, cdk4, a starter of cell cycle, decreased only moderately (Figure 4c). The same behavior of cylin B1 and cylin D1 was observed (Figure 5a, b). They both dramatically decreased but cyclin E1 was not changed after treatment (data not show). Cdk2 and cyclin D1 were inhibited, but cdk4 and cyclin E1, by some ways, might complete the functions of interphase cdk/cyclin complexes to drive cell cycle into and pass through the S phase.

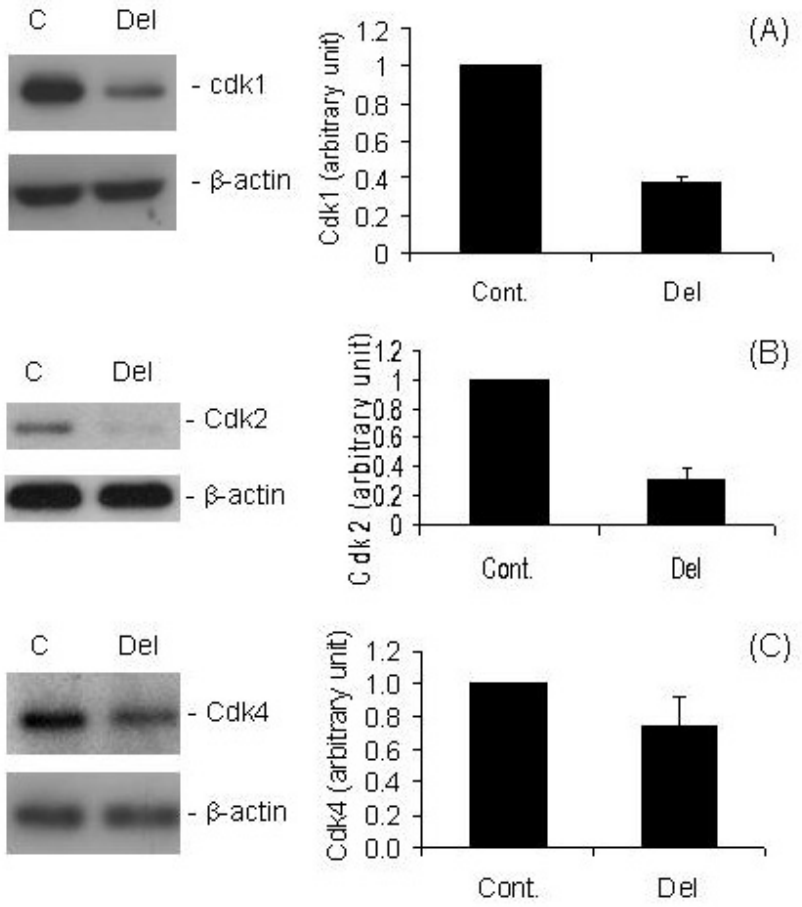

Figure 4. The expression of Cdks after delphinidin treatment. Cells were treated with 70 μg/ml of delphinidin for 24 hours. After treatment cells were lysed and protein levels were investigated by western blotting analysis. Cdk1 and cdk2 dramatically decreased after treatment while cdk4 lightly decreased moderately.

Cyclin B1 and cdk1 were proteins those ensure G_2 checkpoint passing and complete the mitosis [5, 8 and 20]. They were decreased after treatment with delphinidin, indicating that the cell cycle was arrested at G_2/M checkpoint.

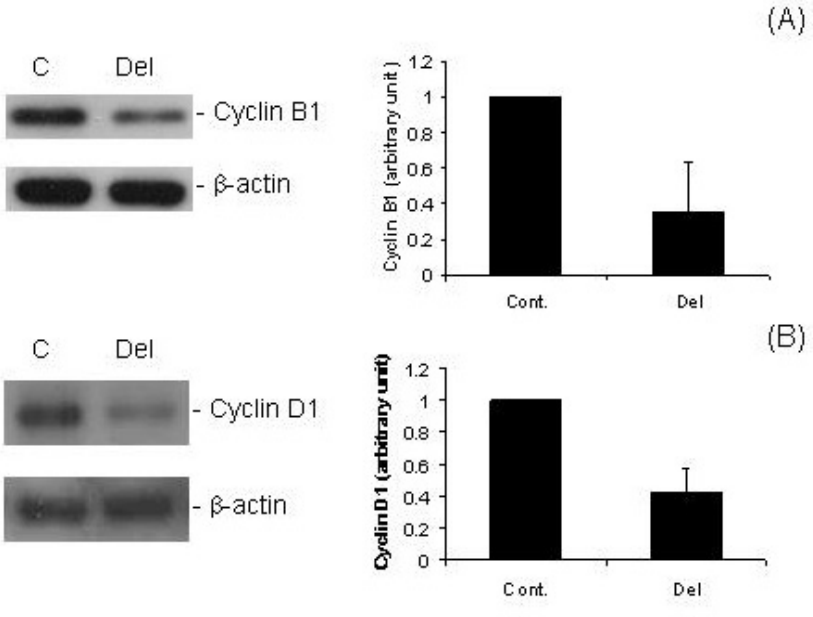

Figure 5. Cyclin B1 and cyclin D1 expression after delphinidin treatment.

4. Cell cycle arrest (FACs analysis)

After treatment, the cell population at G_1/S phase was reduced. Cdk4 can not bind to cyclin E so how cdk4 and cyclin E drove cells into S phase and completed the DNA synthesis (the cells at G_1/S were reduced from 59.6% to 31 %) was not clear. The number of cells at G_2/M phase was dramatically increased from 22% to 60.4%. This was an expected result because the expression of cdk1 and cyclin B1, the proteins functioning at G_2 checkpoint and during mitosis, was inhibited. This data confirmed that delphinidin arrested cells at G_2/M phase (Figure 6).

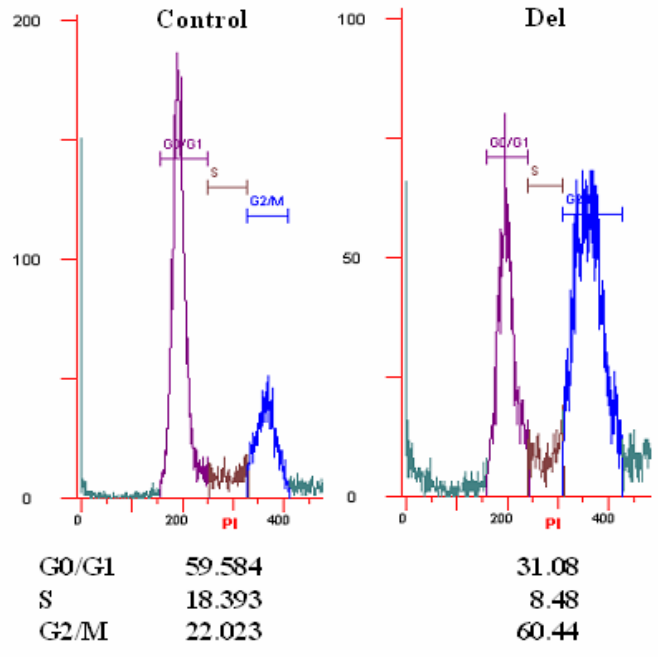

Figure 6. Delphinidin induced cell cycle arrest at G_2/M phase. The number of cells at G_2/M phase was dramatically increased in treated sample (right) in comparing with the control (left).

5. Apoptosis

The apoptotic proteins were checked in western blot. The most important apoptotic proteins were executor caspases (caspase-3, -7) and poly ADP ribose polymerase (PARP). Once executor caspases are activated, they cleave a number of proteins that lead to the destruction of cellular organelles [14]. One substrate of executor caspases was PARP. Physiological function of PARP is repairing DNA damages. When the cells undergo apoptosis, executor caspases cleave PARP, generating inactive fragments of PARP [2]. Figure 7

shows that caspase 3, -7 were activated and the inactive fragment, p85, of PARP was detected (Figure 7).

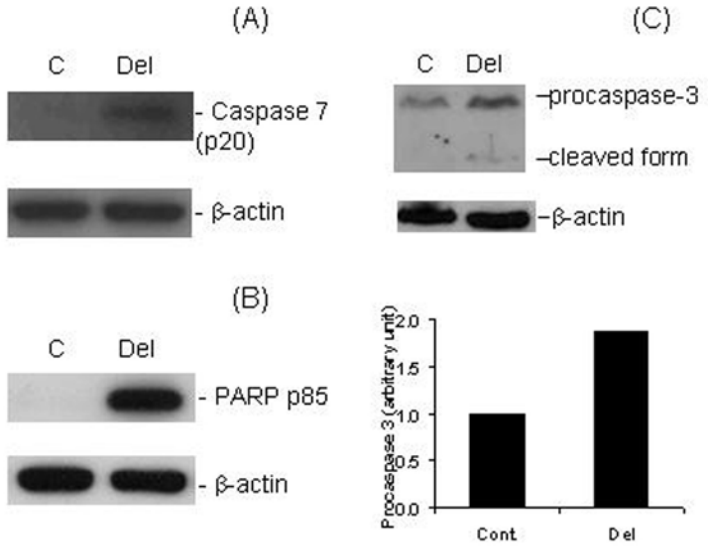

Figure 7. Apoptotic evidence after delphinidin treatment. Cleaved active form of caspase -7 (A). Procaspase 3 and its cleaved active form (C), procaspase 3 also increased after treatment. Inactive fragment, p85, of PARP in (B).

In late apoptosis, the cells were fragmented and produced the apoptotic bubbles. Nuclei were fragmented along with the destruction of cellular skeleton. Cells stained with DAPI after treatment with 70 μg/ml delphinidin for 24 hours were observed under fluorescence microscope. In fluorescent image, chromatin in nuclei of the cells treated with delphinidin was condensed. The nuclei of delphinidin treated cells were smaller and condensed (Figure 8b) while the nuclei in control cells showed up a round large shape with low signal (Figure 8a).

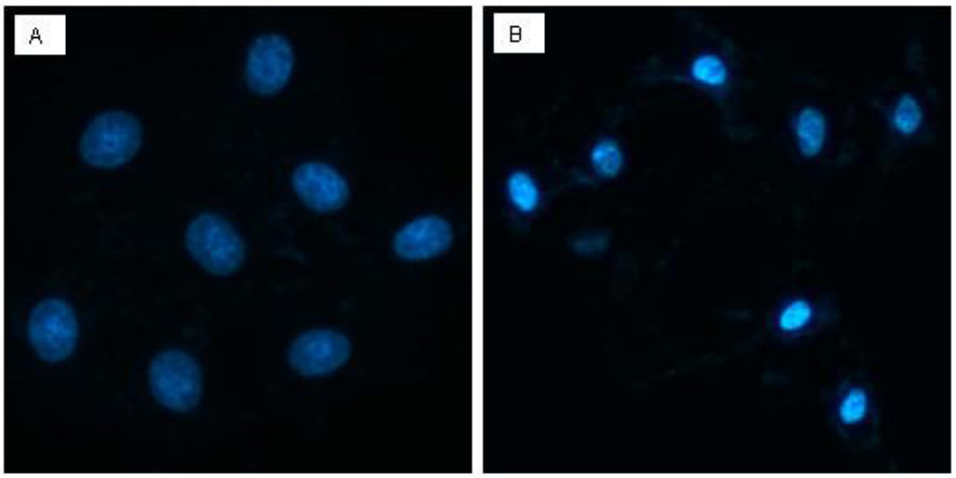

Figure 8. Apoptotic evidence by fluorescence microscopy after delphinidin treatment. Chromatin in cell treated with delphinidin was condensed. A) Control sample; B) cells treated with 70 μg/ml of delphinidin for 24 hour. Cells were fixed in 75 % ethanol, stained with DAPI and stimulated at wavelength of 488 nm. Pictures were taken at the same magnification.

In dying cells, DNA was cleaved by an endonuclease that fragments the chromatin into nucleosomal units, which were multiples of about 180-bp oligomers and appeared as a DNA ladder run on agarose gel. After treated with two concentrations of delphinidin, 25 or 70 μg/ml, the cells were harvested and DNA was purified using phenol, chloroform and separated on 1.5% agarose gel under 50 voltages for 2 hours. The DNA ladder was recognizable even at the low dose of 25 μg/ml and became clear when treated with 70 μg/ml of delphinidin (Figure 9).

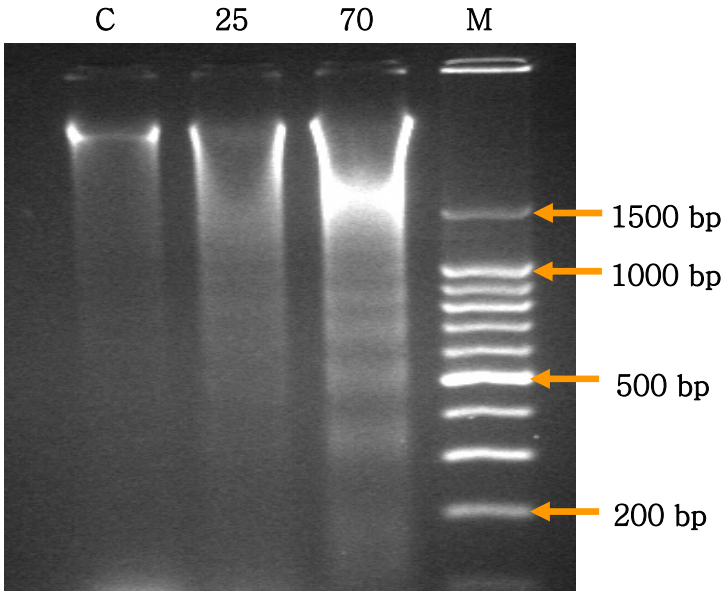

Figure 9. Apoptotic DNA ladder bands. Cells were treated with 25 or 70 μg/ml of delphinidin for 24 hours, DNA was then isolated with phenol/ chloroform.

With the data showed above we concluded that delphinidin induced apoptosis on fadu cells. The question is which pathways are involved in the cell death. We examined the protein level of cytochrome c, Bcl2 proteins, phosphorylation of MAPK p38 and Akt proteins. After treating cells with 25 or 70μg/ml delphinidin, the release of cytochrome c from mitochondrial inner membrane into cytosol was investigated using an ELISA kit. Cytochrome c level in cytosol was increased (Figure 10 a). The ratios of pro-apototic members of Bcl2 protein family to anti-apoptotic Bcl2 (bax/bcl2 and bad/bcl2) increased after treatment (Figure 10b).

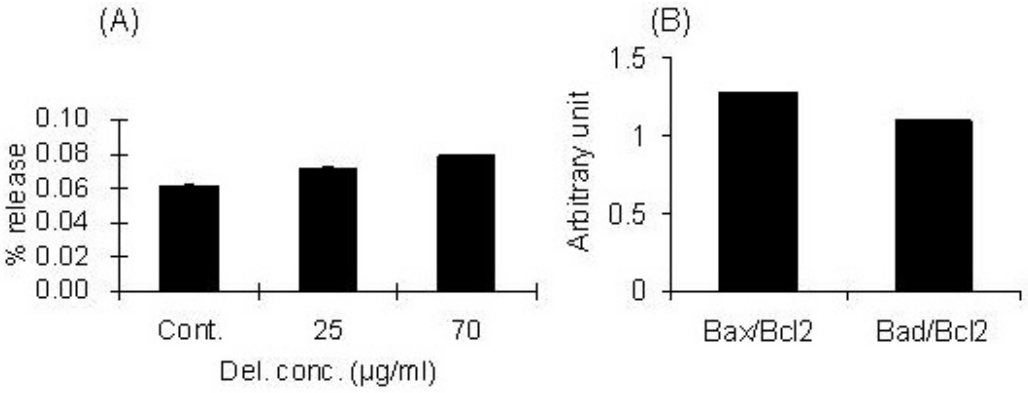

Figure 10. Cytochrome c release and the ratios of Bax/Bcl2 and Bad/Bcl2 after delphinidin treatment.

We investigated the phosphorylation level of p38 (p-p38$^{Thr180/Tyr182}$) and Akt (Akt^{ser473}). Phosphorylation of Akt at serine residue 473 was inhibited by delphinidin (Figure 11a). This might be for cell cycle arrest and frees the pro-apototic proteins. We found that p-p38 increased after treated cells with delphinidin (Figure 11b). This demonstrated that delphinidin induced apoptosis through p38 MAPK. But the p38 pathway specific inhibitor SB203580 did not prevent cell death on fadu cells by delphinidin (data not show).

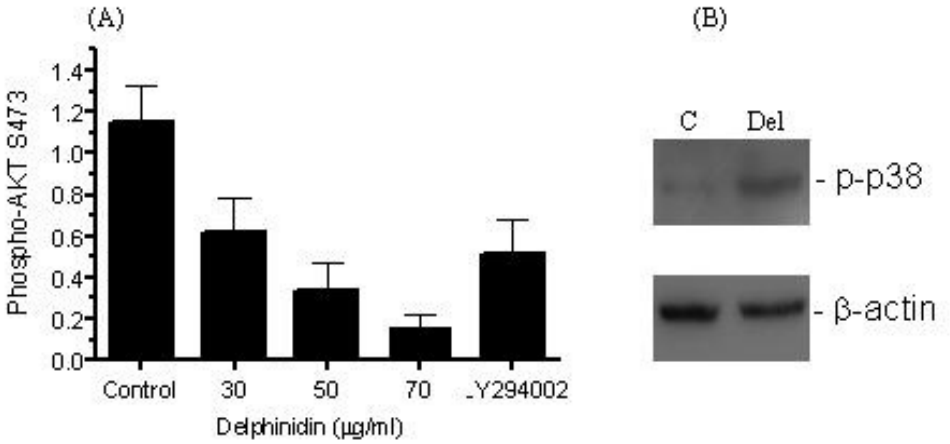

Figure 11. The effect of delphinidin on phosphorylation of Akt and MAPK p38.

IV. Discussion and conclusion

In the cell morphology study, based on cell confluences and cell death (floating cells), delphinidin inhibited Fadu cell growth at low dose and induced cell death at a high dose as 70 μg/ml. Two questions here: 1) which phases of the cell cycle was arrested? 2) Did delphinidin induce Fadu cell death? For the first question, we checked cell cycle proteins and DNA content at the cell cycle phase. The second was answered by collecting apoptotic evidence.

P27Kip1 is the cell cycle suppressor. It binds to interphase cylin/cdk complexes and inhibits their functions. Cyclin E type is one regulator of p27, they interfere the functions of each other. In our data, p27 level increased but the level of cylin E1 was unchanged (data not show). This may suggest that p27 does not effect on the expression of cyclin E but effects on its function. The expression level of cdk1 and cdk2 was dramatically decreased but cdk4 level was just slightly decreased. Cdk4 may have the function to drive the cell into S phase. And also cdk2, even with low level, still can complete S phase. P27 inhibitory effect on cell cycle was not strong enough to arrest cell cycle completely at G1/S or S phase. But it may delay the G1/S and S phase transition. The idea that p27 may bind to either cdk1 or cyclin B type, will be investigated in near future. It is very well known that the activity of cyclin B/cdk1 complex ensures passing G_2/M checkpoint and completing cell division, called as maturation-promoting factor (MPF). In our data showed that

delphinidin suppressed both cdk1 and cyclin B1. Further study on cell cycle inhibitors will be necessary. The G_2/M phase arrest of cell cycle was confirmed up in FACs analysis study, showing that the cell population at G2/ M phase increased dramatically from 22 % to 60%. Therefore we concluded that delphinidin induced cell cycle arrest at G_2/M phase by up-regulating p27Kip1 and down-regulating Cdk1 (cdc2) and Cyclin B1.

The cleaved form of caspase-3 and caspase-7 were produced after treatment with delphinidin. Caspase-3 activates caspase-7 and the activated caspase-7 cleaves more substrates than active caspase-3, such as a PARP, the enzyme functioning in DNA repaire. Since PARP can not complete DNA repaire, the cells can not pass through G2/ M check-point to divide and alaternatively decide to go apoptosis [14]. PARP1 inactivated fragment, p85, was detected clearly after delphinidin treatment. The chromatin was condensed and the apoptotic DNA ladder was appeared on agarose gel electrophoresis, which are the strong evidence for apoptosis phenomenon.

It is not clear yet what pathway(s) was the main pathway in the apoptosis induction by delphinidin. In our data, cytochrome c was increased, but the ratios of bax and bad to bcl2 were only slightly increased, indicating mitochondria pathway might not be the only the pathway involved in the process. P38 mitogen-activated protein kinase involves in membrane blebbing, nuclear condensation and caspase activation [6, 19] through the activation of Bim protein [1]. Its activation requires several steps of phosphorylation with

the final phosphorylation at threonine 180 and serine 182. And the activation process is inhibited by SB203580. Our result showed the increase of phosphorylation at those amino residues. This meant p38 was activated by delphinidin and involved in apoptosis.

Since Akt involves in many different survival pathways, we checked its phosphorylation at Serine 473. Delphinidin was a strong inhibitor of Akt phosphorylation. Further study on this pathway will be continued in future.

Overall, we found that delphinidin suppressed cell cycle process at G2/M phase and induced apopotosis on Fadu cells. The mitochondria, Akt and p38 involved in this process. The cell signal summarizing our current data was described in figure 12.

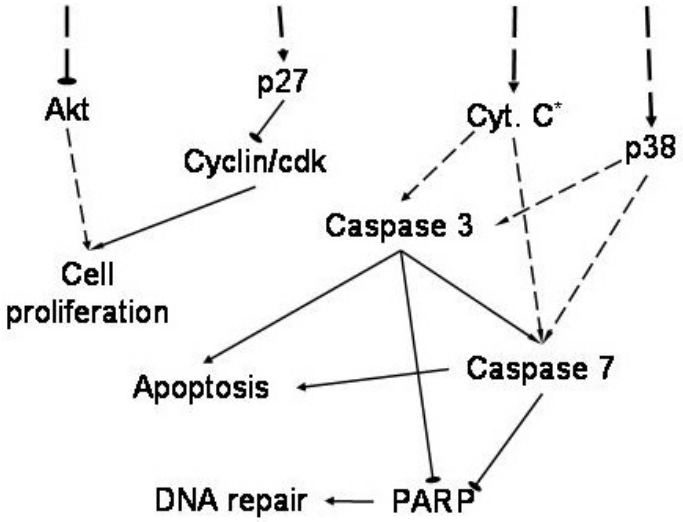

Figure 12. Cell signals induced by delphinidin on Fadu cells: solid lines mean directly effect, dash lines mean multi-step, arrows mean activation, block lines mean inhibition.

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논문제목	한글: 도경부 암세포에 대한 delphinidin 의 세포주기억제 및 세포사멸 연구 영문: Delphinidin induced cell cycle arrest and apoptosis on Human pharyngeal squamous carcinoma cells				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함

2. 위의 목적을 위하여 필요한 범위 내에서의 편집・형식상의 변경을 허락함.
다만, 저작물의 내용변경은 금지함.

3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.

4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.

5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.

6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음

7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

2009 년 8 월

저작자: Le Thanh Do (서명 또는 인)

조선대학교 총장 귀하

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