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Master's Thesis

5-Fluorouracil Resistance Mechanism and Resistance Reversal in SNU-C5/5-FU, Fluorouracil-Resistant Human Colon Cancer Cells

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February, 2013

CONTENTS

LIST OF ABBREVIATIONS

Abbreviations	Full name
5-FU	5-fluorouracil
TS	Thymidylate synthase
PTEN	Phosphatase and tensin homolog deletion on chromosome ten
PI3K	Phosphatidylinositol 3-kinase
GSK-3β	Glycogen synthase kinase-3β
Wnt	Wingless-type MMTV integration site family member
Cox-2	Cyclooxygenase-2
NF-κB	Nuclear factor- kappa B
IκB	Inhibitory kappa B
IKK	IκB kinase
PGs	Prostaglandins
IAP	Inhibitor of apoptosis proteins
PARP	Poly-(ADP-ribose) polymerase
SD	Standard deviation

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1. ABSTRACT

Recent studies reported that overexpression of phospho-AKT leads to resistance of apoptosis via phospho-AKT modulation of a variety of cellular processes. In this study, we investigated the resistance mechanism of SNU-C5/5-FU, fluorouracil-resistant human colon cancer cells, and the possible involvement of phospho-AKT for acquired resistance to 5-fluorouracil (5-FU). First, we confirmed that SNU-C5/5-FU overexpress phospho-AKT compared with SNU-C5/WT and SNU-C5/OXT. Interestingly, the loss of PTEN induced phosphorylation of AKT in SNU-C5/5-FU. When treated with LY294002 (PI3 kinase inhibitor), we could observe the expression of E-cadherin increased and the phospho-GSK-3β decreased, which are known proteins downstream of phospho-AKT. Thereby, it decreased the nuclear translocation of β-catenin. Furthermore, the NF-κB signaling was activated by the increased AKT phosphorylation and promoted the formation of COX-2. In cytoplasm, COX-2 contributes to the stabilization of survivin, an anti-apoptotic protein that directly interacts with procaspase-3. Also, the treatment of LY294002 down regulated NF-κB signaling and the COX-2. Treatment with LY294002 or Vioxx (COX-2 inhibitor) reduced the interaction between survivin and procaspase-3. Under combination treatment with LY294002

and 5-FU, the apoptotic characteristics, such as apoptotic bodies, cleavage of procaspase-9, cleavage of procaspase-3 and cleavage of poly(ADP-ribose) polymerase (PARP), were observed in SNU-C5/5-FU. Furthermore, apoptosis was also induced by combination treatment with Vioxx and 5-FU. These findings provide evidence that overexpression of phosphorylated AKT is an important mechanism of resistance in SNU-C5/5-FU. The results suggest that inhibition of phosphorylated AKT may overcome fluorouracil-resistant in the SNU-C5/5-FU cells.

2. INTRODUCTION

Colon cancer is one of the most prevalent cancers in the United States, and incidence rates of colon cancer have been increasing steadily worldwide (Jemal et al. 2008). Moreover, because of the change to Westernized dietary pattern, incidence rates of colon cancer also have steadily increased in Korea (Park et al. 2012).

In recent years, because of increased colon cancer incidence, a variety of treatments have been developed. However, 80% of cancer-related deaths were related to resistance to anticancer-drugs (Luqmani 2005, Ichihashi, Kitajima 2001). Thus, we need to examine closely the mechanisms of resistance.

5-Fluorouracil (5-FU) is an anti-cancer drug that prevents DNA synthesis by targeting thymidylate synthase (TS). TS catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) in DNA synthesis. The well-known resistance mechanism of 5-FU is increased TS (Jette et al. 2008). But recent studies report that SNU-C5/5-FU colorectal resistance cancer cells which have acquired resistance to 5-FU did not increase TS (Jung 2006, Kim et al. 2005).

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway regulates a variety of cellular

processes, such as cell growth and cell survival (Kim, Chung 2002). In addition, it has been

reported that this signal promotes the proliferation and survival of colon cancer cells (Bao et

al. 2004). Phosphorylation of AKT inhibits GSK-3β (Do et al. 2008) and induces

instability of E-cadherin expression by activating mdm2, an anti-apoptotic protein (Zhou et

al. 2009). Also, activation of AKT modulated NF-κB signaling promotes survival and

resistance apoptosis in cancer cells, via activation of IκB kinase (IKK) (Ahn, Aggarwal 2005,

Dan et al. 2008, Wang et al. 2009). The activation of AKT is strongly related to inactivation

of PTEN, tumor suppressor gene, in breast cancer cells (Clark et al. 2002). Therefore, the

activation of AKT contributes to maintaining immortality in cancer cells via regulating the

various apoptosis factors, which involved apoptosis, even they were exposed to stressful

conditions such as anticancer drug treatment (Clark et al. 2002, Seal et al. 2012).

Phosphatase and tensin homolog deletion on chromosome ten (PTEN) is a well-

recognized negative regulator of PI3K/AKT pathway (Gupta, Dey 2012, Kerr et al. 2006).

PTEN suppresses the PI3K/AKT cell survival pathway via dephosphorylation of

phosphatidylinositol 3,4,5-triphosphate (PIP3)(Tamura et al. 1999). Unfortunately, recent

studies reported that it showed the loss of PTEN expression in colon cancer (Colakoglu et al.

2008, Sawai et al. 2008). In addition, loss of PTEN expression led not only to increasing risk

of relapse of colon cancer (Colakoglu et al. 2008), but also metastasis of colorectal cancer

and poor survival in colon cancer patients (Sawai et al. 2008). Interestingly, low expression of PTEN was associated the resistance of treatment with anticancer drug (Oki et al. 2005, Sherbakova et al. 2008).

E-cadherin is a transmembrane glycoprotein which has an important function involving cell-cell adhesion. Generally, the cytoplasmic domain of E-cadherin is combined with β-catenin, α-catenin and several ancillary proteins (Fanelli et al. 2008, Mohamet, Hawkins & Ward 2011, Huber, Weis 2001). When E-cadherin was loosed, β-catenin was translocated to the nucleus and activated the capacity of transcription factor (Huber, Weis 2001).

In the nucleus, β-catenin forms a transcription factor complex with Tcf/Lef proteins (Sparks et al. 1998, Akiyama 2000). The transcription factor complex activates the expression of target genes such as cyclin D1 and c-myc, which have important roles of cell growth, proliferation and survival (Sparks et al. 1998, Akiyama 2000, Li et al. 2005, Gotoh et al. 2003). Interestingly, recent studies reported that β-catenin was associated with resistance to apoptosis by modulating the c-myc, cyclin D1 and other proteins, which are involved with cell cycle and apoptosis, in various cancer cells (Yeung et al. 2010, Woodward et al. 2007, Cui et al. 2012).

In wnt/β-catenin signaling, glycogen synthase kinase-3β (GSK-3β) led to ubiquitination and degradation of β-catenin in the absence of wnt signal. However, in the presence of Wnt

signal, β-catenin accumulated at a high-level in cytoplasm because of inactivated GSK-3β and translocated to the nucleus (Akiyama 2000, Woodward et al. 2007, Cui et al. 2012).

Nuclear factor kappa B (NF-κB) is a protein transcription factor that is associated with oncogenesis (Ahn, Aggarwal 2005, Mayo, Baldwin 2000), cell survival and proliferation (Ahn, Aggarwal 2005, Yang et al. 2012). In addition, it has been reported that activated NF-κB contributed to resistance for anti-cancer drugs in colon cancer cells (Wang et al. 2009). Interestingly, NF-κB was activated by phosphorylated AKT that promoted activation of IκB kinase (IKK). Thereby, inhibition of NF-κB (IκB), which is combined with a NF-κB, was phosphorylated and then degraded by activated IKK. Sequentially, activated NF-κB was translocated to the nucleus and acted as a transcription factor that induced gene expression of anti-apoptotic proteins and enzymes including COX-2 (Ahn, Aggarwal 2005, Dan et al. 2008).

Cyclooxygenase-2 (COX-2) is an enzyme that forms prostaglandins (PGs) from arachidonic acid. Generally, COX-2 is an inducible form generated by inflammation and tumors (Abrahao et al. 2010, Asting et al. 2011). Various studies showed that overexpression of COX-2 was involved in cell survival, development of tumors (Grosch et al. 2006, Cervello, Montalto 2006) and resistance to apoptosis (Redondo et al. 2011, Chen et al. 2010). It has been reported that COX-2 is overexpressed and PGE_2 increased in SNU-C5/5-FU

(Choi et al. 2011). Furthermore, when co-treated 5-FU with COX-2 inhibitor, the viability of SNU-C5/5-FU cells decreased by co-treatment of 5-FU in a dose dependent manner (Choi et al. 2011). Recent studies reported that survivin was stabilized by PGE_2, which suppressed the ubiquitination of survivin that is anti-apoptotic protein (Krysan et al. 2004).

Survivin, one of the IAP (inhibitor of apoptosis proteins) family, suppressed apoptosis and promoted development of tumor via modulated microtubule dynamics (Pennati, Folini & Zaffaroni 2007). Survivin is mainly present in the nucleus, but is simultaneously present in the nucleus and cytoplasm in carcinogenic cells (Samuel et al. 2005). In cytoplasm, survivin directly inhibits caspase-3 and caspase-7 (Shin et al. 2001, Chai et al. 2001). Over-expressed survivin shows resistance to anti-cancer drug and radiation therapy (Pennati, Folini & Zaffaroni 2007).

Here, we demonstrated the characteristic of SNU-C5/5-FU, fluorouracil resistant human colon cancer cells, on 5-FU resistance mechanism. We examined the resistance reversal in SNU-C5/5-FU.

3. MATERIALS AND METHODS

3.1. Reagents

5-Fluorouracil (5-FU), Oxaliplatin (OXT) and trypan blue were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Mouse monoclonal anti-E-cadherin, anti-PTEN, anti-α Tubulin and anti-Ub, rabbit polyclonal anti-caspase-3, anti-β-catenin and anti-IκB-α and goat polyclonal anti-COX-2 and anti-survivin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotech, CA, USA); rabbit monoclonal anti-p-NF-κB, anti-GSK-3β and anti-cleaved caspase-3, Rabbit polyclonal anti-phospho-GSK-3β, anti-Akt, anti-phospho-Akt, anti-cleaved caspase-9, anti-phospho-mTOR, anti-mTOR and anti-poly (ADP-ribose) polymerase (PARP) antibodies were purchased from Cell Signaling Technology (Cell signaling Technology, Beverly, MA, USA); mouse monoclonal anti-Cyclin D1 antibodies were purchased form BD biosciences (BD biosciences, USA); mouse monoclonal β-actin was purchased from Sigma; PI3 kinase inhibitor (LY294002) was purchased from Calbiochem (Merck KGaA, Germany); COX-2 inhibitor (Vioxx) was purchased from Santa Cruz Biotechnology (Santa Cruz Biotech, CA, USA); Dynabeads® Protein G was purchased from NOVEX® (Invitrogen, Norway), Aprotinin, leupeptin, Nonidet P-40 were obtained

from Roche (Roche Applied Science, Indianapolis, IN); Western blotting reagent, West-zol

enhanced chemilumin, was obtained from Intron (iNtROn Biotechnology, Korea).

3.2. Cell culture

SNU-C5/WT, a human colon cancer cell line, was obtained from the Korean Cell Line

Bank (KCLB). SNU-C5/5-FU and SNU-C5/OXT, a human resistant colon cancer cell line,

were obtained the Research Center for Resistant Cells. SNU-C5/WT,SNU-C5/5-FU and

SNU-C5/OXT cells cultured in RPMI 1640 (Hyclone, UT, USA) medium supplemented with

10% heat inactivated fetal bovine serum (Hyclone, UT, USA), 100 U/mL penicillin and 100

mg/mL streptomycin (GIBCO Inc, Grand Island, NY, USA) at 37°C in a humidified

atmosphere with 5% CO_2. After 2 days, SNU-C5/5-FU and SNU-C5/OXT cells were

changed Medium, RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine

serum, 100 U/mL penicillin, 100 mg/mL streptomycin and 140 μM of 5-FU or 7.14 μM of

OXT.

3.3. Cell viability assay (trypan blue staining)

The effect of 5-FU, oxaliplatin or combined treatment of LY294002 (PI3 kinase inhibitor)

and 5-FU on the growth of SNU-C5/WT, SNU-C5/5-FU and SNU-C5/OXT cells was

evaluated using the trypan blue staining (Comes et al. 2007).Cells were seeded at 2×10^5

cells/mL in 1 ㎖ on 24-well plates at 37°C in 5% CO_2 gas to allow cell attachment. After 24

h, cells were treated with 5-FU (1, 10, 50, 100 and 200 μM) or combined treatment of

LY294002 (20μM)and 5-FU (1, 10, 50, 100 and 200 μM)for 72 h. At the end of

experimental incubation, cells were detached using 0.25% trypsin-EDTA. Cell pellets were

then suspended with PBS. 100 μL of suspended cells were mixed with identical volume of

0.01% trypan blue solutions for 4 min. Unstained cells (viable cells) in the mixture were

counted using a hemacytometer.

3.4. Morphological analysis of apoptosis by Hoechst 33342 staining

SNU-C5/5-FU cells were seeded at 2×10^5 cells/mL in 1 mL on 24-well microplates. After

24 h of incubation, cells were treated with LY294002 (20 μM) and/or 5-FU (100 μM) for 24

h. The cells were incubated in a Hoechst 33342 (10 μg/ml medium at final) at 37°C for 30

min. SNU-C5/5-FU cells were observed with an inverted fluorescent microscope equipped

with an IX-71 Olympus camera and photographed (magnification ×200).

3.5. Western blot analysis

SNU-C5/WT, SNU-C5/5-FU and SNU-C5/OXT cells were seeded at 2×10^5 cells/mL.

After 24h, cells were lysed with lysis buffer (50 mMTris-HCl [pH 7.5], 150 mMNaCl, 2 mM

EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mMNaF, 1 mMdithiothreitol, 1 mM

Phenylmethylsulfonylfluoride, 25 μg/mL aprotinin, 25 μg/mL leupeptin, 1 mM DTT, 1%

Nonidet P-40) for 30min at 4°C. SNU-C5/5-FU cells were seeded 2×10^5 cells/mL for 24h

and treated with LY294002 (20μM) and/or 5-FU (10, 50 and 100μM) for 15min ~ 24h. After

treatment, SNU-C5/5-FU cells were lysed with lysis buffer for 30min at 4°C. The lysates

were centrifuged at 15,000 rpm, 4°C for 15min. Protein content was determined according to

the method of Bradford assay (Bradford 1976). The cell lysates were separated by 6~15%

SDS-PAGE gels and then transferred used to polyvinylidene fluoride (PVDF) membrane

(BIO-RAD, Hercules, CA, USA) by glycine transfer buffer (192 mM glycine, 25 mM Tris-

HCl [pH 8.8], and 20% MeOH [v/v]) at 200mA for 2 h. After blocking with 5% skim milk

solution, the membrane was incubated with primary antibody against PARP (1:2000),

caspase-3 (1:1000), cleaved caspase-3 (1:1000), caspase-9 (1:1000), cleaved caspase-9

(1:1000), Akt (1:1000), phospho-Akt (1:1000), GSK-3β (1:1000), phosphor-GSK-3β

(1:1000), β-catenin (1:2000), E-cadherin (1:1000), mTor (1:1000), phospho-mTor (1:1000),

phohpho-NF-κB (1:1000), IκB (1:1000), COX-2 (1:1000), Survivin (1:1000), PTEN

(1:1000), Ub (1:1000), Cyclin D1 (1:1000), α-Tubulin (1:1000) and β-actin (1:5000)

antibodies at 4°C overnight and incubated with a secondary HRP antibody (1:5000; Vector

Laboratories, Burlingame, VT, USA) at room temperature for 1h. Protein bands were detected using a WEST-ZOL® plus Western Blot Detection System (iNtRON., Gyeonggi-do, Korea) with subsequent exposure to X-ray films (AGFA, Belgium).

3.6. Co-immunoprecipitation assay

SNU-C5/5-FU cells were seeded 2×10^5 cells/mL for 24h and treated with LY294002 (20μM) for 24h. After treatment, SNU-C5/5-FU cells were lysed with lysis buffer for 30min at 4°C. The lysates were centrifuged at 15,000 rpm, 4°C for 15min. 50 μL of Dynabeads® Protein G transfer to tube and remove the supernatant by placing the tube on the magnet to separate the beads. Separated beads were added directly to antibody in 200 μL PBS with 0.02% Tween-20 and incubated with rotation for 10 min at room temperature. The supernatant was then removed. The beads ‑ antibody complex was washed using 200 μL PBS with 0.02% Tween-20 and remove the supernatant. The beads ‑ antibody complex was added directly to the cell lysates and incubated with rotation for 10 min at room temperature. The supernatant was removed and the beads ‑ antibody ‑ Ag complex was washed using 200 μL PBS with 0.02% Tween-20 at 3 times and the supernatant removed. The beads – antibody – Ag complex was added 20 μL of elution buffer (50 mM Glycine [Ph 2.8]) and 10 μL of NuPAGE LDS Sample buffer and then heated for 100min at 70 °C. The supernatant was

separated from the beads using a magnet and loaded onto an SDS-PAGE gel.

3.7. Confocal microscopy

SNU-C5/WT, SNU-C5/5-FU and SNU-C5/OXT cells were fixed in 3.5% formaldehyde for 30 min. The fixed cells permeabilized in 0.1% triton X-100. Cells were blocked in 3% BSA for 1h at room temperature. Cells were treated with primary antibodies (1:100) overnight at 4°C. Immunofluorescences stain of primary antibodies was stained with Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 rabbit anti-goat IgG secondary antibody. The fluorescence was identified using confocal microscopy (FV500, OLYMPUS) and the images were acquired at constant PMT, gain, offset, magnification (40X oil immersion objective with zoom factor of 4) and resolution.

3.8. Statistical analysis

Results are shown as means ± standard deviation (SD) from three independent experiments. Student's t-test was used to determine the data with the following significance levels: *$p<0.05$, **$p<0.01$, ***<0.001. All assays were performed with at least three independent experiments.

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Table 1. Antibodies used in Western blot analysis.

Antibody	Origin	Company
Akt	rabbit polyclonal	Cell signaling Technology
phospho-Akt	rabbit polyclonal	Cell signaling Technology
mTor	rabbit polyclonal	Cell signaling Technology
phospho-mTor	rabbit polyclonal	Cell signaling Technology
GSK-3β	rabbit monoclonal	Cell signaling Technology
phospho-GSK-3β	rabbit polyclonal	Cell signaling Technology
phospho-NF-κB	rabbit monoclonal	Cell signaling Technology
caspase-9	rabbit polyclonal	Cell signaling Technology
Cleaved caspase-9	rabbit polyclonal	Cell signaling Technology
Cleaved caspase-3	rabbit monoclonal	Cell signaling Technology
poly-(ADP-ribose) polymerase (PARP)	rabbit polyclonal	Cell signaling Technology
E-cadherin	mouse monoclonal	Santa Cruz Biotechnology
β-catenin	rabbit polyclonal	Santa Cruz Biotechnology
COX-2	goat polyclonal	Santa Cruz Biotechnology

IκB-α	rabbit polyclonal	Santa Cruz Biotechnology
survivin	goat polyclonal	Santa Cruz Biotechnology
Caspase-3	rabbit polyclonal	Santa Cruz Biotechnology
Ub	mouse monoclonal	Santa Cruz Biotechnology
PTEN	mouse monoclonal	Santa Cruz Biotechnology
α-Tubulin	mouse monoclonal	Santa Cruz Biotechnology
Cyclin D1	mouse monoclonal	BD biosciences, USA
β-actin	mouse monoclonal	Sigma

4. RESULTS

4.1. SNU-C5/5-FU stably acquired resistance on 5-fluorouracil

To evaluate the effect of 5-FU on the viability in SNU-C5/WT and SNU-C5/5-FU, cell viability was demonstrated using trypan blue staining assay. SNU-C5/WT and SNU-C5/5-FU were treated with 5-FU (1, 10, 50, 100 and 200μM) for 72h,SNU-C5/WT cells were decreased significantly cell viability (Figure 1; 1μM, 54.3%; 10μM, 18.5%; 50μM, 13.3%; 100μM, 11.09% and 200μM, 6.8%), but SNU-C5/5-FU cells did not decrease significantly compared to SNU-C5/WT cells (Fig.1; 1μM, 85.9%; 10μM, 84.7%; 50μM, 74.8%; 100μM, 67.8% and 200μM, 46.1%). IC_{50} of SNU-C5/5-FU was more than 40 times higher (Fig.1; IC50 of SNU-C5/WT, 4.84 μM and IC_{50} of SNU-C5/5-FU, 182.66 μM).
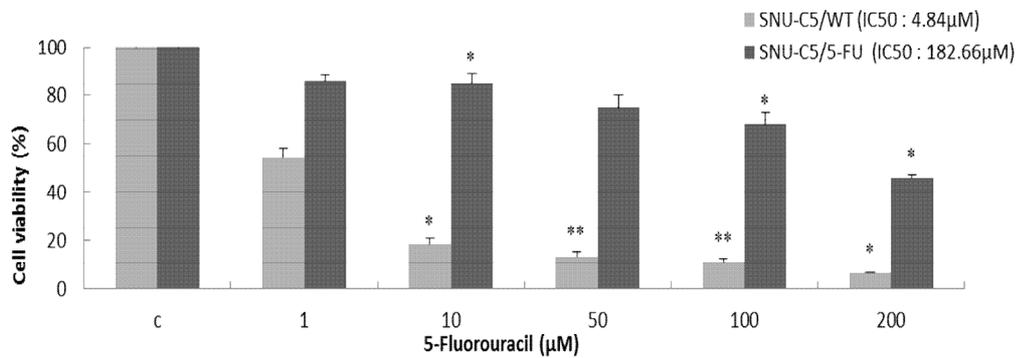
Figure 1. Cytotoxicity of 5-Fluorouracil on SNU-C5/WT and SNU-C5/5-FU SNU-C5/WT and SNU-C5/5-FU cells were plated in 6-well ($2X10^5$cells/ml) and treated with 5-FU (1, 10, 50, 100 and 200 μM) for 72h. The results are expressed as percentages of viable cells compared with control by trypan blue staining. The data are presented as the mean ± SD from experiments representative of three independent trials. *p<0.05, **p<0.01, and ***p<0.001 compared with the control.

4.2. SNU-C5/5FU has the characteristic of overexpression of phospho-AKT.

To determine that exceptional characteristic of SNU-C5/5-FU cells, we compared with SNU-C5/WT, SNU-C5/5-FU and SNU-C5/OXT. Interestingly, phospho-AKT was overexpressed in SNU-C5/5-FU (Figure 2A). Furthermore, over-phosphorylated AKT of SNU-C5/5-FU cells were identified at confocal microscopy (Figure 2B). Also, mTor, a protein modulated by phospo-AKT (LoPiccolo et al. 2008), was over-phosphorylated in SNU-C5/5-FU (Figure 3).

4.3. SNU-C5/5-FU down-regulates E-cadherin and up-regulates phospho-GSK-3β

Recent studies reported that phosphorylated AKT leads to decreased E-cadherin (Zhou et al. 2009) and phosphorylation of GSK-3β (Son et al. 2012). Therefore, we investigated expression of E-cadherin and phospho-GSK-3β in SNU-C5/5-FU cells, which has over-phosphorylated AKT. As a result, expression of E-cadherin was suppressed (Figure 4) and expression of phospho-GSK-3β was increased (Figure 5) in SNU-C5/5-FU compared with SNU-C5/WT and SNU-C5/OXT.

4.4. Phosphorylated AKT led to down-regulation of E-cadherin and phosphorylation of GSK-3β in SNU-C5/5-FU

To establish whether expression of E-cadherin (Figure 4) and phospo-GSK-3β (Figure 5) depended on overexpression of phospho-AKT, SNU-C5/5-FU cells were treated with LY294002 (PI3 kinase inhibitor). As a result, E-cadherin expression was increased (Figure 6) and phospho-GSK-3β expression was decreased (Figure 7) by treatment of LY294002 in time dependent manner. Furthermore, treatment of LY294002 induced down-regulation of β-catenin and cyclin D1 (Figure 7). These results suggested that phospho-AKT regulates GSK-3β and E-cadherin in SNU-C5/5-FU.

(A)

(B)
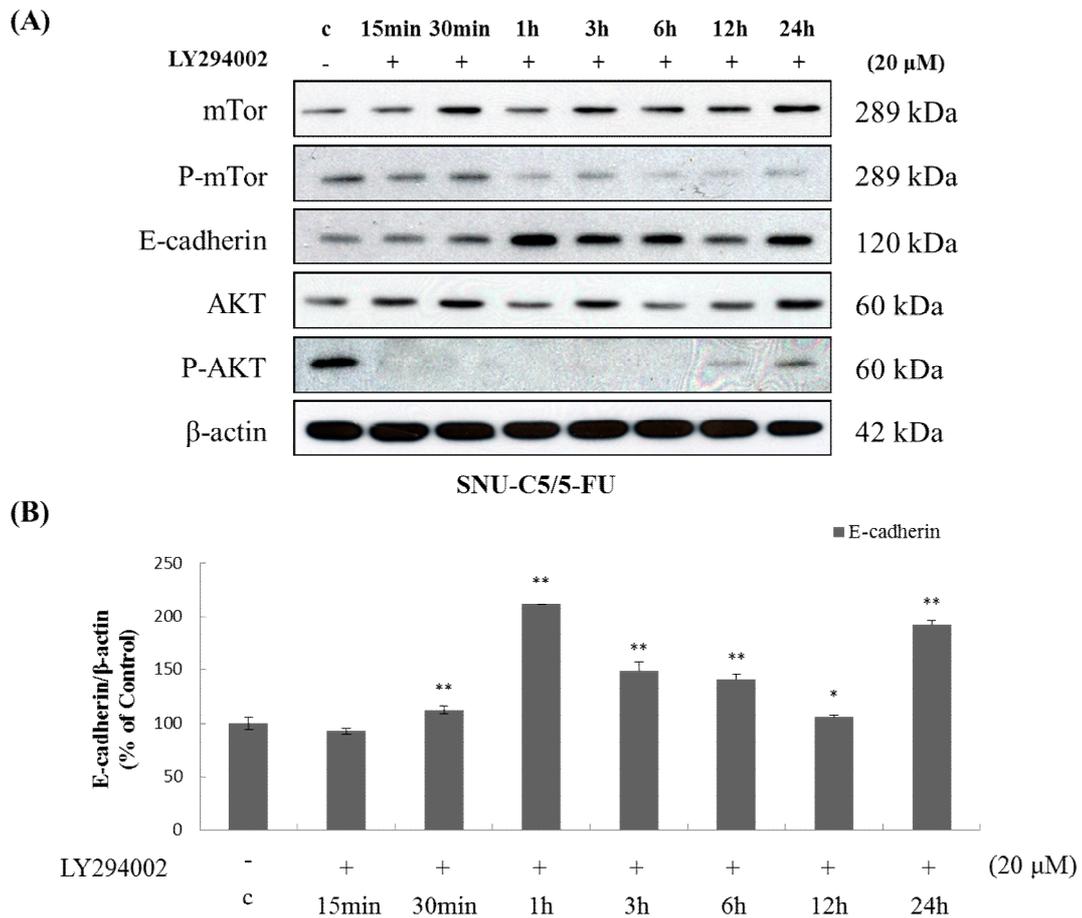
Figure 6. The effect of LY294002 on the expression of E-cadherin in SNU-C5/5-FU (A)
The SNU-C5/5-FU cells (2 x 10^5 cells/mL) were pre-incubated for 24h and then cells were treated with LY294002 (20 μM) for the indicated time. Lysates were prepared from these cells, and the expression of AKT, Phospho-AKT, mTor, phospho-mTor and E-cadherin measured by Western blot analysis using specific antibodies. (B) Data represent the percentage of E-cadherin expression in SNU-C5/5-FU cells. The data are presented as the mean ± SD from experiments representative of three independent trials. *p<0.05, **p<0.01, and ***p<0.001 compared with the control.

(A)

SNU-C5/5-FU

(B)
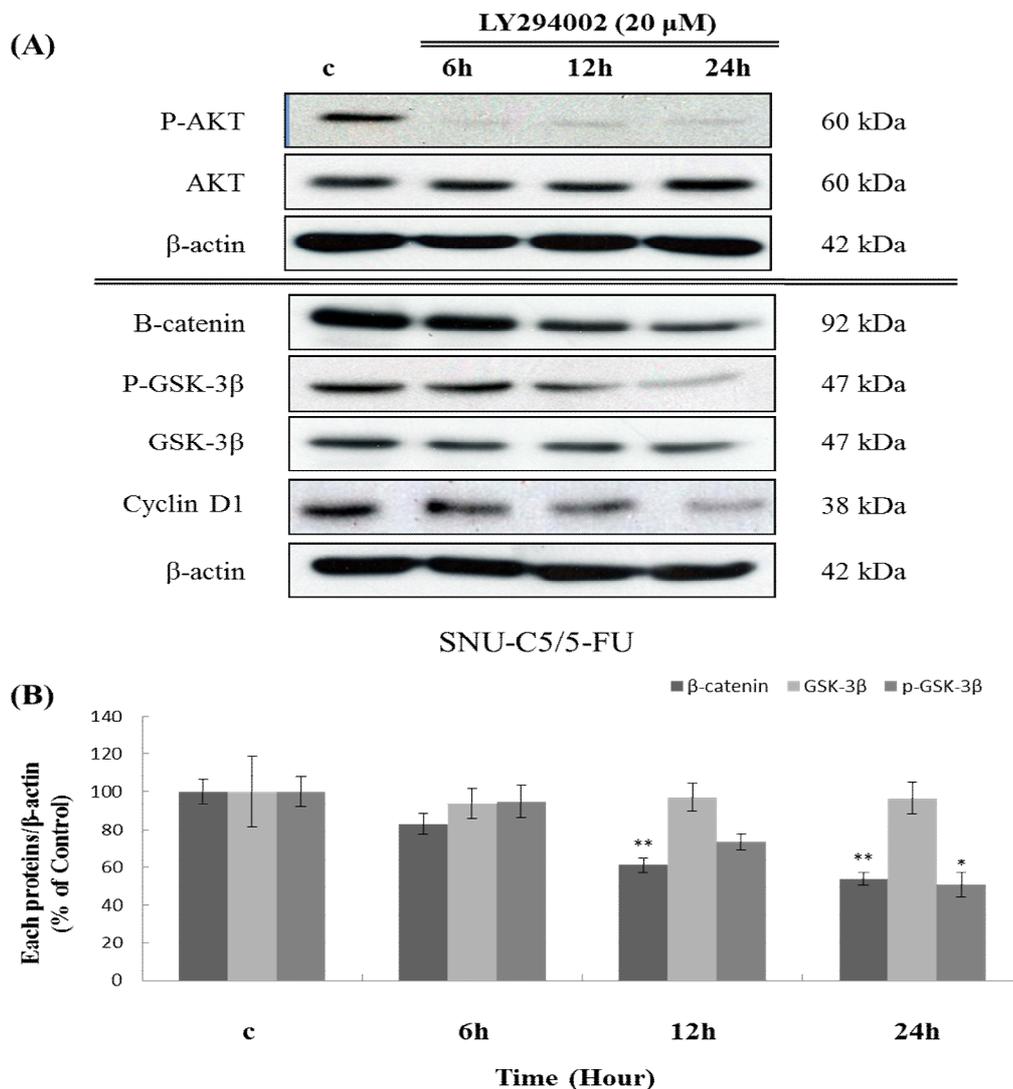
Figure 7. The effect of LY294002 on the expression of GSK-3β, phospho-GSK-3β, cyclin D1, and β-catenin in SNU-C5/5-FU (A) The SNU-C5/5-FU cells (2 x 10^5 cells/mL) were pre-incubated for 24h and then cells were treated with LY294002 (20 μM) for the indicated time. Lysates were prepared from these cells, and the expression of AKT, Phospho-AKT, phospho-GSK-3β, GSK-3β, cyclin D1, and β-catenin measured by Western blot analysis using specific antibodies. (B) Data represent the percentage of β-catenin, phospho-GSK-3β, and GSK-3β expression in SNU-C5/5-FU cells. The data are presented as the mean ± SD from experiments representative of three independent trials. *p<0.05, **p<0.01, and ***p<0.001 compared with the control.

4.5. SNU-C5/5-FU has characteristic to translocation of β-catenin in cytoplasm and nucleus

Various studies showed that β-catenin was involved the cell-cell adhesion by interaction of E-cadherin in plasma membrane (Fanelli et al. 2008, Mohamet, Hawkins & Ward 2011). But, loss of E-cadherin led to discharge of β-catenin into the cytoplasm (Huber, Weis 2001). β-catenin was degraded by activated GSK-3β in cytoplasm. If GSK-3β was inactivated, β-catenin acted as a transcription factor in the nucleus (Akiyama 2000). In other words, activated GSK-3β and stabilization of E-cadherin prevents β-catenin from acting on transcription factor. Thus, we determined the expression and location of β-catenin. As a result, we found no significant difference on expression of β-catenin (Figure 8A). However, β-catenin located the cytoplasm and nuclear in the SNU-C5/5-FU comported with SNU-C5/WT and SNU-C5/OXT (Figure 8B).

4.6. Phospho-AKT led to the nuclear translocation of β-catenin

We determined whether localization of β-catenin in cytoplasm and nucleus were associated with down-regulation of E-cadherin and inactivated GSK-3β by over-phosphorylated AKT. E-cadherin was increased in plasma membrane and phospho-GSK-3β was decreased by treatment of LY294002 (Figure 9). Interestingly, treatment of LY294002 induced not only decreased expression of β-catenin in the cytoplasm and nucleus but also increased expression of β-catenin in plasma membrane (Figure 9). Therefore, we determined that the interaction of E-cadherin and β-catenin were regulated by phospho-AKT. β-catenin was decreased but the interaction of β-catenin and E-cadherin was increased by treatment of LY294002 in a dose dependent manner (Figure 10). This result indicated that phospho-AKT led to the nuclear translocation of β-catenin via loss of E-cadherin and inactivated GSK-3β (Figure 11A). As a resultant, inhibition of phosphorylated AKT prevents β-catenin from entering the nucleus for cell survival (Figure 11B).

(A)

| LY294002 | - | 10 | 15 | 20 | (μM) |

IP : β- catenin / IB: E-cadherin ▇▇▇▇ 120kDa

IP : β- catenin / IB: β- catenin ▇▇▇▇ 92 kDa

SNU-C5/5-FU

(B)
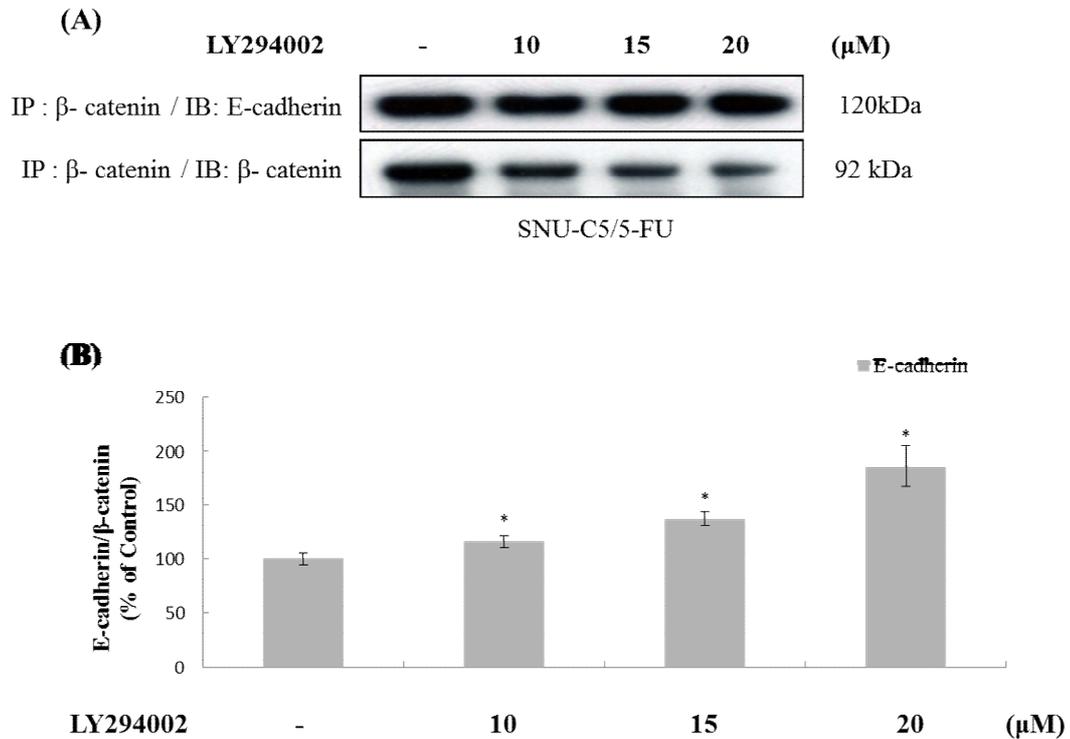
Figure 10. The effect of LY294002 on β-catenin interacts with E-cadherin in SNU-C5/5-FU (A) The SNU-C5/5-FU cells (2 x 10^5 cells/mL) were pre-incubated for 24h and then cells were treated with LY294002 (10, 15 and 20 μM) for 24h. The lysate of SNU-C5/5-FU cells was immunoprecipitated with anti-β-catenin antibody, and was immunoblotted with anti-β-catenin and anti-E-cadherin antibody. (B) Data represent the percentage of E-cadherin expression in SNU-C5/5-FU cells. The data are presented as the mean ± SD from experiments representative of three independent. *p<0.05, **p<0.01, and ***p<0.001 compared with the control.

Figure 11. The characteristics of SNU-C5/5-FU on E-cadherin, GSK-3β, and β-catenin

(A) phosphorylated AKT led to nuclear translocation of β-catenin via loss of E-cadherin and inactivated GSK-3β. (B) Inhibition of phospho-AKT prevents nuclear translocation of β-catenin

4.7. SNU-C5/5FU has characteristic to overexpression of Cyclooxygenase-2

To find other properties of SNU-C5/5-FU cells, we compared with SNU-C5/WT, SNU-C5/5-FU and SNU-C5/OXT. SNU-C5/5-FU cells markedly overexpress COX-2 compared to SNU-C5/WT and SNU-C5/OXT (Figure 12A). Moreover, overexpression of COX-2 was identified by confocal microscopy (Figure 12B).

4.8. Over-activated NF-κB signaling led to overexpression of COX-2

NF-κB signaling modulated COX-2 expression (Abrahao et al. 2010). Therefore, we examined whether that COX-2 was overexpressed by NF-κB signaling in SNU-C5/5-FU. Interestingly, IκB-α was suppressed and phospho-NF-κB was overexpressed in SNU-C5/5-FU (Figure 13). Thus, we investigated the involvement of over-activated NF-κB signaling and over-expressed COX-2. The treatment of TPCK (IκB protease inhibitor) decreased the COX-2 level in dose dependent manner (Figure 14). These data suggest that over-activated NF-κB signaling led to over-expression of COX-2 in SNU-C5/5-FU cells.

(A)

TPCK   -   10   15   20   (μM)

Cox-2   70~72 kDa

β-actin   42 kDa

SNU-C5/5-FU

(B)
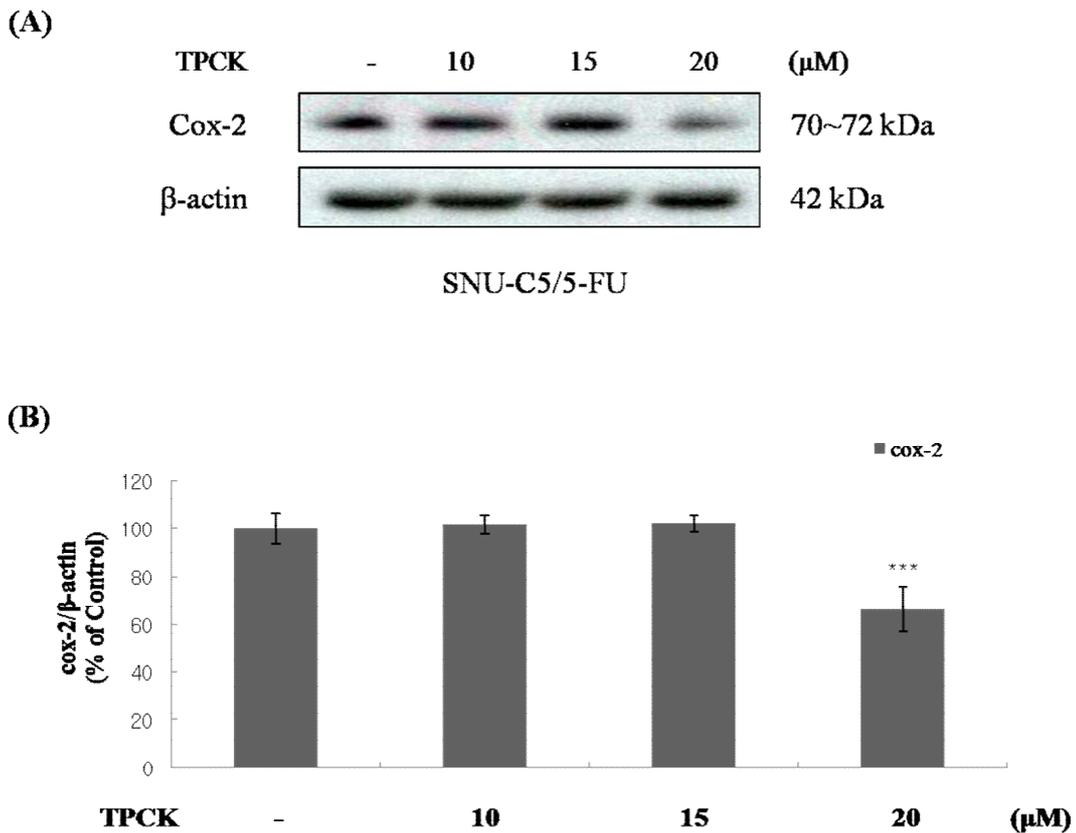
Figure 14. The effect of TPCK on the expression of COX-2 in SNU-C5/5-FU (A) The SNU-C5/5-FU cells (2×10^5 cells/mL) were pre-incubated for 24h and then cells were treated with TPCK (10, 15 and 20 μM). Lysates were prepared from these cells, and the expression of COX-2 measured by Western blot analysis using specific antibodies. (B) Data represent the percentage of COX-2 expression in SNU-C5/5-FU cells. The data are presented as the mean ± SD from experiments representative of three independent. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with the control.

4.9 phospho-AKT led to activation of NF-κB signaling in SNU-C5/5-FU

IκB kinase (IKK) was activated by phospho-AKT and it act NF-κB signaling by phosphorylation of IκB (Ahn, Aggarwal 2005, Dan et al. 2008). Thus, we examined whether that phosphorylation of AKT modulates the NF-κB signaling in SNU-C5/5-FU. As a result, COX-2 and phospho-NF-κB were decreased by treatment of LY294002 in a dose and time dependent manner (Figure 15). These data indicated that phospho-AKT modulates NF-κB signaling in SNU-C5/5-FU.

4.10. phospho-AKT led to stabilization of survivin via overexpression of COX-2

Previous studies reported that SNU-C5/5-FU was showed with overexpression of PGE_2 by

overexpression of COX-2 (Choi et al. 2011). In addition, it has been reported that PGE_2

affect the resistance to apoptosis via inhibiting ubiquitination of survivin in lung cancer cells

(Krysan et al. 2004). Thus, we confirmed that COX-2 contributes to the stabilization of

survivin expression in SNU-C5/5-FU. Survivin showed overexpression in SNU-C5/5-FU

(Figure 16). Also, it was decreased by treatment with LY294002 or Vioxx (COX-2 selective

inhibitor) in a dose and time dependent manner (Figure 17A and B). Furthermore, treatment

of LY294002 or Vioxx led to the ubiquitination of survivin in a dose dependent manner

(Figure 17C). These results indicated that phospho-AKT induces the stabilization of survivin

by up-regulation of COX-2.

(A)

	c	15min	30min	1h	3h	6h	12h	24h	20μM
LY294002	−	+	+	+	+	+	+	+	

Survivin   16.5 kDa

β-actin   42 kDa

	c	15min	30min	1h	3h	6h	12h	24h	20μM
Vioxx	−	+	+	+	+	+	+	+	

Survivin   16.5 kDa

β-actin   42 kDa

SNU-C5/5-FU

(B)

LY294002	−	10	15	20	(μM)
Survivin					16.5 kDa
β-actin					42 kDa

SNU-C5/5-FU

Vioxx	−	10	15	20	(μM)
Survivin					16.5 kDa
β-actin					42 kDa

SNU-C5/5-FU

■survivin

■survivin

(C)

LY294002	−	10	15	20	(μM)

IP : survivin / IB: Ub

IP : survivin / IB: survivin   16.5 kDa

SNU-C5/5-FU

Vioxx	−	10	15	20	(μM)

IP : survivin / IB: Ub

IP : survivin / IB: survivin   16.5 kDa

SNU-C5/5-FU

Figure 17. The effects of LY294002 and Vioxx on the expression of survivin in SNU-C5/5-FU (A and B) The SNU-C5/5-FU cells (2 x 10^5 cells/mL) were pre-incubated for 24h and then cells were treated with LY294002 and Vioxx (10, 15 and/or 20 μM) for the indicated time or 24h. Lysates were prepared from these cells, and the expression of survivin measured by Western blot analysis using specific antibodies. (C) The SNU-C5/5-FU cells (2 x 10^5 cells/mL) were pre-incubated for 24h and then cells were treated with LY294002 and Vioxx (10, 15 and 20 μM) for 24h. The lysate of SNU-C5/5-FU cells was immunoprecipitated with anti-survivin antibody, and was immunoblotted with anti-survivin and anti-ubiquitin antibody.

4.11 phospho-AKT led to expression of survivin in cytoplasm via up-regulation of COX-2

Recent studies reported that nucleus expression of survivin regulated cell cycle but, cytoplasm expression of survivin induced resistance on apoptosis ((Krysan et al. 2004)). Interestingly, we identified that survivin expression was increased in the cytoplasm (Figure 15) and that it was stabilized via COX-2 (Figure 17). Thus, we observed that cytoplasm expression of survivin was involved with COX-2. When treated with 5-FU, we could not observe a change in expression on survivin. But treatment with Vioxx or LY294002 decreased expression of survivin in cytoplasm (Figure 18). These results indicated that cytoplasm expression of survivin was modulated by COX-2 and phospho-AKT. Consequentially, phospho-AKT induces the cytoplasm expression of survivin via overexpression of COX-2.

4.12 Phospho-AKT led to resistance on apoptosis via stabilization of survivin

In cytoplasm, stabilized survivin contributes the resistance on apoptosis by interaction

with caspase-3 or caspase-7 (Shin et al. 2001, Chai et al. 2001). Thus, we examined whether

survivin was associated with caspase-3. While treatment of 5-FU did not increase the

activation of caspase-3, co-treatment with Vioxx and 5-FU increased the activation of

caspase-3 (Figure 19A). In addition, treatment of LY294002 or Vioxx reduced the

interaction of porcaspase-3 with survivin and increased the interaction of cleaved-caspase-3

with survivin (Figure 19B). This indicated that survivin avoids apoptosis by direct

interaction of caspase-3 in SNU-C5/5-FU. Consequentially, phospho-AKT induced the

expression of COX-2 via activation of NF-κB signaling. COX-2 contributes to stabilization

of survivin, leading to resistance of apoptosis by direct interaction of caspase-3 in SNU-

C5/5-FU. kinase (Figure 20A). Therefore, inhibition of COX-2 or inhibition of phospho-

AKT induced apoptosis by degradation of survivin (Figure 20B).

(A)

| | 5-FU | - | 10 | 50 | 100 | - | 10 | 50 | 100 | (μM) |
| | Vioxx | - | - | - | - | 20 | 20 | 20 | 20 | (μM) |

AKT — 60 kDa

P-AKT — 60 kDa

Cox-2 — 70-72 kDa

Caspase 3 — 37kDa

Cleaved-Caspase 3 — 17kDa

β-actin — 42 kDa

SNU-C5/5-FU

(B)

| | LY294002 | - | 10 | 15 | 20 | (μM) |

IP : survivin / IB: pro-caspase 3 — 37 kDa

IP : survivin / IB: cleaved-caspase 3 — 17 kDa

IP : survivin / IB: survivin — 16.5 kDa

SNU-C5/5-FU

| | Vioxx | - | 10 | 15 | 20 | (μM) |

IP : survivin / IB: pro-caspase 3 — 37 kDa

IP : survivin / IB: cleaved-caspase 3 — 17 kDa

IP : survivin / IB: survivin — 16.5 kDa

SNU-C5/5-FU

Figure 19. The effect of LY294002 and Vioxx on survivin interacts with caspase-3 in SNU-C5/5-FU (A) The SNU-C5/5-FU cells (2 x 10^{5} cells/mL) were pre-incubated for 24h and then cells were treated with 5-FU (10, 50 and 100 μM) and/or Vioxx (20 μM) for the indicated time or 24h. Lysates were prepared from these cells, and the expression of AKT, p-AKT, COX-2, and caspase-3 measured by Western blot analysis using specific antibodies.

(B) The SNU-C5/5-FU cells (2 x 10^5 cells/mL) were pre-incubated for 24h and then cells were treated with LY294002 or Vioxx (10, 15 and 20 μM) for 24h. The lysate of SNU-C5/5-FU cells was immunoprecipitation with anti-survivin antibody, and was immunoblotted with anti-survivin and anti-caspase-3 antibody.

Figure 20. The characteristics of SNU-C5/5-FU on NF-κB signaling and survivin (A) phosphorylated AKT led to activation of NF-κB signaling via activation of IκB kinase. COX-2, which is formation by NF-κB signaling, contributes stabilization of survivin. (B) Inhibition of phospho-AKT prevents stabilization of survivin in cytoplasm.

4.13 phospho-AKT contributes resistance on 5-fluorouracil in SNU-C5/5-FU

We examined whether that overexpression of phospho-AKT contributes the resistance on 5-FU in SNU-C5/5-FU. Treatment of 5-FU did not showed characteristics of apoptosis (Figure 21A, B and C). However, co-treatment with 5-FU and LY294002 showed the features of apoptosis, for example, nuclear morphologic changes (Figure 21A), increased apoptosis related proteins such as cleavage of proaspase-9, cleavage of procaspase-3 and cleavage of poly(ADP-ribose)polymerase (PARP) (Figure 21B and C). Furthermore, when co-treated 5-FU with LY294002, the viability of SNU-C5/5-FU cells decreased by co-treatment of 5-FU in a dose dependent manner (Fig.21D; 5-FU 1μM, 85.3%; 10μM, 63.3%; 50μM, 54.12%; 100μM, 45.87% and 200μM, 38.35%) compared with treatment of 5-FU (Figure 1). In addition, combination treatment of 5-FU and Vioxx also were decreased cell viability (Fig.21D; 5-FU 20 1μM, 90.17%; 10μM, 83.23%; 50μM, 66.14%; 100μM, 49.69% and 200μM, 44.53%). In addition, IC_{50} of combination treatments in SNU-C5/5-FU was significantly reduced (IC_{50} of co-treatments with LY294002 and 5-FU, 76.342 μM and IC_{50} of co-treatments with Vioxx and 5-FU, 97.427 μM). These data indicated that over-expressed phospho-AKT contributes resistance on 5-FU. Moreover, survivin, which was one of the downstreams of phospho-AKT, was a major resistance mechanism on 5-FU.

Figure 21. The effect of LY294002 or Vioxx on induction of apoptosis in SNU-C5/5-FU

(A) The SNU-C5/5-FU cells (2 x 10^5 cells/mL) were pre-incubated for 24h and then cells were treated with 5-FU (10, 50 and 100 μM) and/or LY294002 (20 μM) for the indicated time or 24h. The cells were stained with Hoechst 33342 (10 μg/ml medium at final) apoptotic bodies were observed with an inverted fluorescent microscope equipped with an IX-71 Olympus camera and photographed (magnification ×200). (B) The SNU-C5/5-FU cells (2 x 10^5 cells/mL) were pre-incubated for 24h and then cells were treated with LY294002 (20 μM) and/or 5-FU (10, 50 and 100 μM) for 24h. Lysates were prepared from these cells, and the expression of caspase-9, caspase-3, PARP, AKT and phospho-AKT measured by Western blot analysis using specific antibodies. (C) The SNU-C5/5-FU cells (2 x 10^5 cells/mL) were pre-incubated for 24h and then cells were treated with LY294002 (20 μM) and/or 5-FU (100 μM) for 24h. Immunofluorescent stain of cleaved caspase-3 and cleaved caspase-9 was performed with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody and the fluorescence was identified using confocal microscopy (FV500, OLYMPUS). (D) SNU-C5/5-FU cells were plated in 6-well (2 X 10^5 cells/ml) and treated LY294002 or Vioxx (20 μM) and/or 5-FU (1, 10, 50, 100 and 200 μM) for 72h. The results are expressed as percentages of viable cells compared with control by trypan blue staining. The data are presented as the mean ± SD from experiments representative of three independent trials. *p<0.05, **p<0.01, and ***p<0.001 compared with the control.

4.14 Loss of PTEN was cause of the over-phosphorylated AKT in SNU-C5/5-FU

Previous studies reported that loss of PTEN promotes the phosphorylation of AKT in colon cancer (Colakoglu et al. 2008, Sawai et al. 2008). Therefore, we confirmed the PTEN level of SNU-C5/5-FU. As a result, PTEN expression was down-regulated in SNU-C5/5-FU compared with SNU-C5/WT and SNU-C5/OXT (Figure 22). This result suggested that loss of PTEN led to over-phosphorylation of AKT in SNU-C5/5-FU.

Finally, in SNU-C5/5-FU, overexpression of phospho-AKT, which by loss of PTEN, modulates a variety of downstream effects such as nuclear translocation of β-catenin via loss of E-cadherin, inactivation of GSK-3β and it led to stabilization of survivin in cytoplasm via activated NF-κB signaling. Thus, stabilized survivin inhibits apoptosis by interaction of caspase-3 (Figure 23A). Consequently, inhibition of phospho-AKT was increased sensitive on apoptosis (Figure 23B).

5. DISCUSSION

In this study, we investigated the resistance mechanism and resistance reversal in SNU-C5/5-FU, 5-fluorouracil (5-FU) resistance human colon cancer cells. SNU-C5/5-FU has characteristic that over-phosphorylation of AKT. In SNU-C5/5-FU, phospho-AKT regulates a variety of cellular processes such as down-regulation of E-cadherin, inactivation of GSK-3β and activation of NF-κB signaling. Moreover, treatment of LY294002 (PI3 kinase inhibitor) led to not only reversal of these cellular processes but also increased apoptosis by 5-FU. 5-FU is an anti-cancer drug that prevents DNA synthesis by inhibiting the biosynthesis of thymine. Variety studied showed that increased thymidylate synthase (TS) led to resistance of 5-FU (Jette et al. 2008, Peters et al. 2002, Van der Wilt et al. 1992). Interestingly, TS expression did not increase in SNU-C5/5-FU (Jung 2006, Kim et al. 2005).

The PI3K/AKT pathway regulates cell growth and survival in cancer cells (Kim, Chung 2002). Moreover, phospho-AKT was induced resistance of apoptosis via modulate other cellular processes (Kim, Chung 2002). The phospho-AKT led to unstable E-cadherin by activation of mdm2 that known is anti-apoptotic protein (Zhou et al. 2009). E-cadherin is a transmembrane protein, which is involved in cell-cell adhesion. Cytoplasmic domain of E-cadherin has been binding β-catenin and other proteins (Fanelli et al. 2008, Mohamet,

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Hawkins & Ward 2011, Huber, Weis 2001). Thus, loss of E-cadherin led to release of β-catenin and it could act as transcription factor on cell survival and proliferation (Huber, Weis 2001). However, activated GSK-3β induced degradation of β-catenin in cytoplasm. Unfortunately, phospho-AKT led to inactivation GSK-3β (Akiyama 2000, Woodward et al. 2007, Cui et al. 2012). In other words, phospho-AKT induced that down-regulation of E-cadherin and inactivation of GSK-3β. Resultantly, phosphorylated AKT induced activation to β-catenin transcription factor via down-regulation of E-cadherin and inactivation of GSK-3β. Actually, we confirmed the previous result that β-catenin was discharged by loss of E-cadherin (Figure 4 and 8). In addition, because of inactivated GSK-3β (Figure 5), it did not degrade β-catenin in cytoplasm, translocates into the nucleus and acts as a trans-factor on cell survival (Figure 8 and 9). The upper mechanism on these series of events was excessive phosphorylation of AKT (Figure 9 and 11A). Therefore, inhibition of phosphorylation of AKT can suppresses that β-catenin discharge to the cytoplasm by increased of E-cadherin and it also promoted the degradation of β-catenin via activated GSK-3β. Conclusively, inhibition of phosphorylated AKT suppressed β-catenin action as a trans-factor in SNU-C5/5-FU cells (Figure 9 and 11B).

Various studies show that phospho-AKT promotes the activation of NF-κB signaling via stimulation of IκB kinase (Ahn, Aggarwal 2005, Dan et al. 2008, Wang et al. 2009).

Activated NF-κB induced enzymes such as COX-2 and variety proteins (Ahn, Aggarwal 2005). Our result indicated the overexpression of cox-2 in SNU-C5/5-FU compared with SNU-C5/WT and SNU-C5/OXT (Figure 12). As expected, NF-κB signaling was over-activated in SNU-C5/5-FU and blocked by treatment of LY294002 as well as expression of COX-2 (Figure 13 and 15). It has been reported that COX-2 modulates the resistance to apoptosis by inhibition to ubiquitination of survivin, anti-apoptotic protein (Krysan et al. 2004). Therefore, activation of NF-κB by phospho-AKT presumably regulated resistance of apoptosis via stabilization of survivin. We confirmed the overexpression of survivin (Figure 16). Thus, we identified whether that survivin is regulated by COX-2 and phospho-AKT. Treatment with LY294002 or Vioxx decreases survivin expression in time- and dose-dependent manner (Figure 17A and B). Moreover, the ubiquitination of survivin was induced by LY294002 and Vioxx (Figure 17C). Previous studies showed that survivin modulates the cell cycle in the nucleus. However, in carcinogenic cells, survivin is located in the nucleus and cytoplasm (Samuel et al. 2005). In the cytoplasm, survivin directly suppresses the caspase-3 and caspase-7 leading to resistance of apoptosis (Shin et al. 2001, Chai et al. 2001). Unfortunately, PGE2 contributes the remaining survivin after half-life in cytoplasm (Krysan et al. 2004). SNU-C5/5-FU has over-expressed survivin in cytoplasm (Figure 16). Therefore, we guessed that overexpression of survivin in cytoplasm was an indirect cause of over-

expressed COX-2 that was modulated by phospho-AKT. The over-expressed survivin in the

cytoplasm was decreased by LY294002 or Vioxx compared with treatment of 5-FU (Figure

18). These results suggested that COX-2 and phospho-AKT led to stabilization of survivin.

Thus, we examined whether survivin was associated with caspase-3 expression. Inhibition of

COX-2 was induced the cleavage of caspase-3 (Figure 19A). Moreover, treatment of Vioxx

not only decreased the interaction of survivin and procaspase-3 but also increased the

interaction of survivin and cleaved caspase-3. Also, treatment of LY294002 decreased the

interaction of survivin and procaspase-3 (Figure 19B). This result indicates that inhibition of

COX-2 induces the cleavage of caspase-3 via ubiquitination of survivin. In SNU-C5/5-FU,

phospho-AKT indirectly modulates COX-2 expression via activation of NF-κB signaling.

Over-expressed COX-2 regulated PGE2 expression and contributed the stabilization of

survivin. In cytoplasm, accumulated survivin controlled the resistance of apoptosis through a

correction with procaspase-3 (Figure 20A) Therefor the inhibition of phospho-AKT

prevented the resistance to apoptosis by promotes to degradation of survivin (Figure 20B).

Eventually, SNU-C5/5-FU avoids apoptosis that was induced by 5-FU. Thus, we

investigated whether that overexpression of phospho-AKT is associated with the resistance

of 5-FU. Combination treatment of LY294002 and 5-FU led to characteristics of apoptosis

such as nuclear morphologic changes (Figure 21A) and expression of apoptosis-related

proteins (Figure 21B and C). Moreover, combination treatment of LY294002 and 5-FU

significantly inhibited cell viability (Figure 21D). Interestingly, combination treatment of

Vioxx and 5-FU showed the inhibited cell viability (Figure 21D). These results suggested

that over-expressed AKT contributes to resistance to apoptosis by 5-FU in SNU-C5/5-FU. In

addition, survivin, which was one of the downstream of phospho-AKT, was an important

mechanism of resistance by phospho-AKT.

Previous studies reported that loss of PTEN could explain the phosphorylation of AKT in

colorectal cancer cells (Tamura et al. 1999, Colakoglu et al. 2008, Sawai et al. 2008) and in

this study, we confirmed that expression of PTEN was down-regulated in SNU-C5/5-FU

compared with SNU-C5/WT and SNU-C5/OXT (Figure 22). This result indicated that loss

of PTEN led to over-phosphorylation of AKT in SNU-C5/5-FU.

In summary, SNU-C5/5-FU has over-expressed phospho-AKT by loss of PTEN. Thus,

over-phosphorylation of AKT contributes to resistance to 5-FU by regulating a variety of

downstream such as E-cadherin, GSK-3β and NF-κB signaling (Figure 23A). Therefore,

inhibition of phospho-AKT increase sensitivity on 5-FU (Figure 23B). Taken together, these

results demonstrate that 5-FU resistance mechanism in SNU-C5/5-FU and the inhibition of

phospho-AKT may be a therapeutic target in SNU-C5/5-FU, 5-fluorouracil resistance colon

cancer treatment.

6. REFERENCES

Abrahao, A.C., Castilho, R.M., Squarize, C.H., Molinolo, A.A., dos Santos-Pinto, D.,Jr & Gutkind, J.S. 2010, "A role for COX2-derived PGE2 and PGE2-receptor subtypes in head and neck squamous carcinoma cell proliferation", *Oral oncology,* vol. 46, no. 12, pp. 880-887.

Ahn, K.S. & Aggarwal, B.B. 2005, "Transcription factor NF-kappaB: a sensor for smoke and stress signals", *Annals of the New York Academy of Sciences,* vol. 1056, pp. 218-233.

Akiyama, T. 2000, "Wnt/beta-catenin signaling", *Cytokine & growth factor reviews,* vol. 11, no. 4, pp. 273-282.

Asting, A.G., Caren, H., Andersson, M., Lonnroth, C., Lagerstedt, K. & Lundholm, K. 2011, "COX-2 gene expression in colon cancer tissue related to regulating factors and promoter methylation status", *BMC cancer,* vol. 11, pp. 238.

Bao, S., Ouyang, G., Bai, X., Huang, Z., Ma, C., Liu, M., Shao, R., Anderson, R.M., Rich, J.N. & Wang, X.F. 2004, "Periostin potently promotes metastatic growth of colon cancer by augmenting cell survival via the Akt/PKB pathway", *Cancer cell,* vol. 5, no. 4, pp. 329-339.

Bradford, M.M. 1976, "A rapid and sensitive method for the quantitation of microgram

quantities of protein utilizing the principle of protein-dye binding", *Analytical Biochemistry,* vol. 72, pp. 248-254.

Cervello, M. & Montalto, G. 2006, "Cyclooxygenases in hepatocellular carcinoma", *World journal of gastroenterology : WJG,* vol. 12, no. 32, pp. 5113-5121.

Chai, J., Shiozaki, E., Srinivasula, S.M., Wu, Q., Datta, P., Alnemri, E.S. & Shi, Y. 2001, "Structural basis of caspase-7 inhibition by XIAP", *Cell,* vol. 104, no. 5, pp. 769-780.

Chen, W., Bai, L., Wang, X., Xu, S., Belinsky, S.A. & Lin, Y. 2010, "Acquired activation of the Akt/cyclooxygenase-2/Mcl-1 pathway renders lung cancer cells resistant to apoptosis", *Molecular pharmacology,* vol. 77, no. 3, pp. 416-423.

Choi, C.H., Lee, T.B., Lee, Y.A., Choi, S. & Kim, K.J. 2011, "Up-regulation of cyclooxygenase-2-derived prostaglandin E(2) in colon cancer cells resistant to 5-fluorouracil", *Journal of the Korean Surgical Society,* vol. 81, no. 2, pp. 115-121.

Clark, A.S., West, K., Streicher, S. & Dennis, P.A. 2002, "Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells", *Molecular cancer therapeutics,* vol. 1, no. 9, pp. 707-717.

Colakoglu, T., Yildirim, S., Kayaselcuk, F., Nursal, T.Z., Ezer, A., Noyan, T., Karakayali, H. & Haberal, M. 2008, "Clinicopathological significance of PTEN loss and the phosphoinositide 3-kinase/Akt pathway in sporadic colorectal neoplasms: is PTEN loss predictor of local recurrence?", *American Journal of Surgery,* vol. 195, no. 6, pp. 719-

725.

Comes, F., Matrone, A., Lastella, P., Nico, B., Susca, F.C., Bagnulo, R., Ingravallo, G., Modica, S., Lo Sasso, G., Moschetta, A., Guanti, G. & Simone, C. 2007, "A novel cell type-specific role of p38alpha in the control of autophagy and cell death in colorectal cancer cells", *Cell death and differentiation,* vol. 14, no. 4, pp. 693-702.

Cui, J., Jiang, W., Wang, S., Wang, L. & Xie, K. 2012, "Role of Wnt/beta-catenin signaling in drug resistance of pancreatic cancer", *Current pharmaceutical design,* vol. 18, no. 17, pp. 2464-2471.

Dan, H.C., Cooper, M.J., Cogswell, P.C., Duncan, J.A., Ting, J.P. & Baldwin, A.S. 2008, "Akt-dependent regulation of NF-{kappa}B is controlled by mTOR and Raptor in association with IKK", *Genes & development,* vol. 22, no. 11, pp. 1490-1500.

Do, T.V., Kubba, L.A., Antenos, M., Rademaker, A.W., Sturgis, C.D. & Woodruff, T.K. 2008, "The role of activin A and Akt/GSK signaling in ovarian tumor biology", *Endocrinology,* vol. 149, no. 8, pp. 3809-3816.

Fanelli, M.A., Montt-Guevara, M., Diblasi, A.M., Gago, F.E., Tello, O., Cuello-Carrion, F.D., Callegari, E., Bausero, M.A. & Ciocca, D.R. 2008, "P-cadherin and beta-catenin are useful prognostic markers in breast cancer patients; beta-catenin interacts with heat shock protein Hsp27", *Cell stress & chaperones,* vol. 13, no. 2, pp. 207-220.

Gotoh, J., Obata, M., Yoshie, M., Kasai, S. & Ogawa, K. 2003, "Cyclin D1 over-expression

correlates with beta-catenin activation, but not with H-ras mutations, and phosphorylation of Akt, GSK3 beta and ERK1/2 in mouse hepatic carcinogenesis", *Carcinogenesis,* vol. 24, no. 3, pp. 435-442.

Grosch, S., Maier, T.J., Schiffmann, S. & Geisslinger, G. 2006, "Cyclooxygenase-2 (COX-2)-independent anticarcinogenic effects of selective COX-2 inhibitors", *Journal of the National Cancer Institute,* vol. 98, no. 11, pp. 736-747.

Gupta, A. & Dey, C.S. 2012, "PTEN, a widely known negative regulator of insulin/PI3K signaling, positively regulates neuronal insulin resistance", *Molecular biology of the cell,* vol. 23, no. 19, pp. 3882-3898.

Huber, A.H. & Weis, W.I. 2001, "The structure of the beta-catenin/E-cadherin complex and the molecular basis of diverse ligand recognition by beta-catenin", *Cell,* vol. 105, no. 3, pp. 391-402.

Ichihashi, N. & Kitajima, Y. 2001, "Chemotherapy induces or increases expression of multidrug resistance-associated protein in malignant melanoma cells", *The British journal of dermatology,* vol. 144, no. 4, pp. 745-750.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T. & Thun, M.J. 2008, "Cancer statistics, 2008", *CA: a cancer journal for clinicians,* vol. 58, no. 2, pp. 71-96.

Jette, L., Bissoon-Haqqani, S., Le Francois, B., Maroun, J.A. & Birnboim, H.C. 2008, "Resistance of colorectal cancer cells to 5-FUdR and 5-FU caused by Mycoplasma

infection", *Anticancer Research,* vol. 28, no. 4B, pp. 2175-2180.

Jung, K. 2006, *Synergistic effect of combined betulinic acid with anticancer drugs in anticancer drug-resistant colon cancer cells*, Chosun university.

Kerr, F., Rickle, A., Nayeem, N., Brandner, S., Cowburn, R.F. & Lovestone, S. 2006, "PTEN, a negative regulator of PI3 kinase signalling, alters tau phosphorylation in cells by mechanisms independent of GSK-3", *FEBS letters,* vol. 580, no. 13, pp. 3121-3128.

Kim, K.J., Min, Y.D., Choi, C.H. & Lee, T.B. 2005, "The role of selective COX-2 blocker on the 5-FU, irinotecan and oxaliplatin resistant colon cancer cell line", *J Kor Soc Colpoproctol,* vol. 21, pp. 93.

Kim, D. & Chung, J. 2002, "Akt: versatile mediator of cell survival and beyond", *Journal of biochemistry and molecular biology,* vol. 35, no. 1, pp. 106-115.

Krysan, K., Merchant, F.H., Zhu, L., Dohadwala, M., Luo, J., Lin, Y., Heuze-Vourc'h, N., Pold, M., Seligson, D., Chia, D., Goodglick, L., Wang, H., Strieter, R., Sharma, S. & Dubinett, S. 2004, "COX-2-dependent stabilization of survivin in non-small cell lung cancer", *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* vol. 18, no. 1, pp. 206-208.

Li, Y.J., Wei, Z.M., Meng, Y.X. & Ji, X.R. 2005, "Beta-catenin up-regulates the expression of cyclinD1, c-myc and MMP-7 in human pancreatic cancer: relationships with carcinogenesis and metastasis", *World journal of gastroenterology : WJG,* vol. 11, no.

14, pp. 2117-2123.

LoPiccolo, J., Blumenthal, G.M., Bernstein, W.B. & Dennis, P.A. 2008, "Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations", *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy,* vol. 11, no. 1-2, pp. 32-50.

Luqmani, Y.A. 2005, "Mechanisms of drug resistance in cancer chemotherapy", *Medical principles and practice : international journal of the Kuwait University, Health Science Centre,* vol. 14 Suppl 1, pp. 35-48.

Mayo, M.W. & Baldwin, A.S. 2000, "The transcription factor NF-kappaB: control of oncogenesis and cancer therapy resistance", *Biochimica et biophysica acta,* vol. 1470, no. 2, pp. M55-62.

Mohamet, L., Hawkins, K. & Ward, C.M. 2011, "Loss of function of e-cadherin in embryonic stem cells and the relevance to models of tumorigenesis", *Journal of oncology,* vol. 2011, pp. 352616.

Oki, E., Baba, H., Tokunaga, E., Nakamura, T., Ueda, N., Futatsugi, M., Mashino, K., Yamamoto, M., Ikebe, M., Kakeji, Y. & Maehara, Y. 2005, "Akt phosphorylation associates with LOH of PTEN and leads to chemoresistance for gastric cancer", *International journal of cancer.Journal international du cancer,* vol. 117, no. 3, pp. 376-380.

Park, B., Choi, K.S., Lee, Y.Y., Jun, J.K. & Seo, H.G. 2012, "Cancer screening status in Korea, 2011: results from the Korean National Cancer Screening Survey", *Asian Pacific journal of cancer prevention : APJCP,* vol. 13, no. 4, pp. 1187-1191.

Pennati, M., Folini, M. & Zaffaroni, N. 2007, "Targeting survivin in cancer therapy: fulfilled promises and open questions", *Carcinogenesis,* vol. 28, no. 6, pp. 1133-1139.

Peters, G.J., Backus, H.H., Freemantle, S., van Triest, B., Codacci-Pisanelli, G., van der Wilt, C.L., Smid, K., Lunec, J., Calvert, A.H., Marsh, S., McLeod, H.L., Bloemena, E., Meijer, S., Jansen, G., van Groeningen, C.J. & Pinedo, H.M. 2002, "Induction of thymidylate synthase as a 5-fluorouracil resistance mechanism", *Biochimica et biophysica acta,* vol. 1587, no. 2-3, pp. 194-205.

Redondo, S., Ruiz, E., Gordillo-Moscoso, A., Navarro-Dorado, J., Ramajo, M., Rodriguez, E., Reguillo, F., Carnero, M., Casado, M. & Tejerina, T. 2011, "Overproduction of cyclo-oxygenase-2 (COX-2) is involved in the resistance to apoptosis in vascular smooth muscle cells from diabetic patients: a link between inflammation and apoptosis", *Diabetologia,* vol. 54, no. 1, pp. 190-199.

Samuel, T., Okada, K., Hyer, M., Welsh, K., Zapata, J.M. & Reed, J.C. 2005, "cIAP1 Localizes to the nuclear compartment and modulates the cell cycle", *Cancer research,* vol. 65, no. 1, pp. 210-218.

Sawai, H., Yasuda, A., Ochi, N., Ma, J., Matsuo, Y., Wakasugi, T., Takahashi, H., Funahashi, H., Sato, M. & Takeyama, H. 2008, "Loss of PTEN expression is associated with

colorectal cancer liver metastasis and poor patient survival", *BMC gastroenterology,* vol. 8, pp. 56.

Seal, S., Chatterjee, P., Bhattacharya, S., Pal, D., Dasgupta, S., Kundu, R., Mukherjee, S., Bhattacharya, S., Bhuyan, M., Bhattacharyya, P.R., Baishya, G., Barua, N.C., Baruah, P.K., Rao, P.G. & Bhattacharya, S. 2012, "Vapor of Volatile Oils from Litsea cubeba Seed Induces Apoptosis and Causes Cell Cycle Arrest in Lung Cancer Cells", *PloS one,* vol. 7, no. 10, pp. e47014.

Sherbakova, E.A., Stromskaia, T.P., Rybalkina, E.I., Kalita, O.V. & Stavrovskaia, A.A. 2008, "Role of PTEN protein in multidrug resistance of prostate cancer cells", *Molekuliarnaia biologiia,* vol. 42, no. 3, pp. 487-493.

Shin, S., Sung, B.J., Cho, Y.S., Kim, H.J., Ha, N.C., Hwang, J.I., Chung, C.W., Jung, Y.K. & Oh, B.H. 2001, "An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7", *Biochemistry,* vol. 40, no. 4, pp. 1117-1123.

Son, Y.O., Wang, L., Poyil, P., Budhraja, A., Hitron, J.A., Zhang, Z., Lee, J.C. & Shi, X. 2012, "Cadmium induces carcinogenesis in BEAS-2B cells through ROS-dependent activation of PI3K/AKT/GSK-3beta/beta-catenin signaling", *Toxicology and applied pharmacology,* vol. 264, no. 2, pp. 153-160.

Sparks, A.B., Morin, P.J., Vogelstein, B. & Kinzler, K.W. 1998, "Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer", *Cancer research,* vol. 58, no. 6, pp. 1130-1134.

Tamura, M., Gu, J., Danen, E.H., Takino, T., Miyamoto, S. & Yamada, K.M. 1999, "PTEN interactions with focal adhesion kinase and suppression of the extracellular matrix-dependent phosphatidylinositol 3-kinase/Akt cell survival pathway", *The Journal of biological chemistry,* vol. 274, no. 29, pp. 20693-20703.

Van der Wilt, C.L., Pinedo, H.M., Smid, K. & Peters, G.J. 1992, "Elevation of thymidylate synthase following 5-fluorouracil treatment is prevented by the addition of leucovorin in murine colon tumors", *Cancer research,* vol. 52, no. 18, pp. 4922-4928.

Wang, S., Liu, Z., Wang, L. & Zhang, X. 2009, "NF-kappaB signaling pathway, inflammation and colorectal cancer", *Cellular & molecular immunology,* vol. 6, no. 5, pp. 327-334.

Woodward, W.A., Chen, M.S., Behbod, F., Alfaro, M.P., Buchholz, T.A. & Rosen, J.M. 2007, "WNT/beta-catenin mediates radiation resistance of mouse mammary progenitor cells", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 104, no. 2, pp. 618-623.

Yang, P., Guo, L., Duan, Z.J., Tepper, C.G., Xue, L., Chen, X., Kung, H.J., Gao, A.C., Zou, J.X. & Chen, H.W. 2012, "Histone methyltransferase NSD2/MMSET mediates constitutive NF-kappaB signaling for cancer cell proliferation, survival, and tumor growth via a feed-forward loop", *Molecular and cellular biology,* vol. 32, no. 15, pp. 3121-3131.

Yeung, J., Esposito, M.T., Gandillet, A., Zeisig, B.B., Griessinger, E., Bonnet, D. & So, C.W. 2010, "beta-Catenin mediates the establishment and drug resistance of MLL leukemic stem cells", *Cancer cell,* vol. 18, no. 6, pp. 606-618.

Zhou, Q., Yan, B., Hu, X., Li, X.B., Zhang, J. & Fang, J. 2009, "Luteolin inhibits invasion of prostate cancer PC3 cells through E-cadherin", *Molecular cancer therapeutics,* vol. 8, no. 6, pp. 1684-1691.

감사의 글

한 사람이 무엇을 해내기 위해서는 그 일이 작은 일이라 할지라도 온전히 혼자만의 힘으로는 이루지 못한다는 것을 절실히 깨달은 2년이었습니다. 학위과정 동안 얼마나 많은 분들의 가르침과 조언으로 성장해왔는지를 생각해 보면, 저는 참 복이 많은 사람 같습니다. 지난 2년을 돌아보면 아쉬움이 많지만 앞으로가 더욱 기대되는 것은 많은 분들의 도움으로 성장할 수 있었기 때문입니다.

사람마다 배움의 속도가 다르다지만, 저는 유독 느렸던 것 같습니다. 그런 저 자신에 대해 조급함을 가지고 힘들어 했을 때, 누구보다도 믿어주시고 기다려 주신 강희경 교수님께 먼저 감사함을 전합니다. 아무것도 모르고 들어온 저를 기초부터 이끌어주시고, 실험을 하면서 깊이 생각하고, 나무가 아닌 숲을 볼 수 있게 가르쳐주셔서 너무 감사합니다. 아직 저는 모자라지만 "과학을 하는 사람은 생각하지 않는 것을 제일 두려워해야 한다." 고 하신 교수님 말씀 깊이 새기며 나아가겠습니다. 그리고 부족한 제 논문에 관심 가져주시고 심사해주신 유은숙 교수님과 박덕배 교수님께도 감사의 마음을 전합니다. 또한 학위과정 동안 늘 관심 가져주시고 가르침 주신 이영기 교수님, 조문제 교수님, 현진원 교수님, 고영상 교수님, 이대호 교수님, 은수용 교수님, 윤상필 교수님께도 감사의 마음을 전합니다.

학위과정 2년동안 약리학 교실에서 생활하면서, 참 많은 것을 배웠습니다. 부족한 후배 끝까지 믿어주시고 조언해주신 강정일 박사님께 감사의 마음을 전합니다. 또한 실험과 논문 그에 따른 아주 사사로운 고민까지 들어주시고 보다 큰 사고를 할 수 있게 도와주신 강경진 박사님께 감사와 사랑의 마음을 전합니다. 그리고 항상 열정적인 그 모습 자체가 배울 점이 많은 한상철 선배님, 여러 가지 일로 고민이 많을 때 감정적으로 어루만져주시고, 선배로써 조언을 아끼지 않으셨던 은아언니 정말 감사합니다. 학위과정 처음 1년 동안 실험실 생활 같이 하며 의지 많이 한 유나, 그리고 힘들었던 2012년을

함께 부딪친 동환이와 내 친구 나진이에게도 감사함을 전하며, 유나, 동환, 나진이도 남은 학위과정을 잘 마무리 할 수 있기를 희망합니다. 또한 짧은 기간이었지만 함께 한 수연이와 문정이에게도 고맙다는 말 전하고 싶습니다.

생화학교실에 김영미 박사님, 김기천 선생님, 김아름다슬 선생님, 미생물학 교실에 채두병 선생님, 생리학교실 최연희 선생님, 오금희 선생님, 강문석 선생님, 양윤실 선생님께도 감사함을 전합니다.

자주 연락 못해서 정말 미안하지만 그래도 먼저 연락해주고 걱정해주는 좌씨 미경, 미정이, 이영이 그리고 대구에서 씩씩하게 생활하는 곧 대박날 사장님 은혜, 지현이, 언제나 잊지 않고 안부 전해주시는 이슬언니, 가끔 먼저 연락해주는 행근이에게도 고맙단 말 전하고 싶습니다. 항상 먼저 연락해주고, 먼저 챙겨주는데 나는 그렇지 못해서 미안한 아름이 이제 교육대학원 들어가서 꼭 원하는 것을 이룰 수 있기를 바라고, 역시 고맙단 인사 하고 싶습니다. 만날 때 마다 유쾌한 지선언니, 영미, (강)지혜, (김)지혜, 수미 그리고 나진이에게도 감사의 마음 전하며 우리 다음에 꼭 날씨가 좋아서 만났으면 합니다.

가장 나에게 큰 힘이 되는 우리 가족들 은화언니 은희언니 은애언니와 큰형부 작은형부에게 감사의 마음 전합니다. 그리고 이제 의젓한 민혁이 민석이 민아와 천사 같은 쌍둥이들 혜원이 예원이 까지, 조카들에게 지면을 빌어 감사와 사랑을 전합니다. 그리고 대구에 있는 믿음직한 형권이 오빠에게도 감사의 마음을 전합니다. 포항에 있는 사랑하는 가족들에게도 고맙다는 말 전하고 싶고, 지면을 빌어 자주 연락 못해서 죄송하다는 말씀 꼭 드리고 싶습니다. 외할아버지와 하늘에서 지켜보고 계실 할머니 그리고 삼촌들, 숙모들에게도 감사의 마음을 전합니다. 지금 가장 보고싶고 항상 옆에 계실줄 믿고있는 우리 엄마 아빠, 막내딸 잘 해나가고 있으니까 걱정하지 마세요. 항상 언제나 늘 사랑합니다. 끝으로 오늘의 저를 있게 해준 사랑하는 부모님의 영전에 이 작은 결실을 바칩니다.