Induction of SIRT1 and SIRT3 confers resistance to genotoxic apoptosis in thyroid cancers

Ki Hwan Kwon

Department of Medicine

The Graduate School, Yonsei University

Induction of SIRT1 and SIRT3 confers resistance to genotoxic apoptosis in thyroid cancers

Directed by Professor Woong Youn Chung

Doctoral Dissertation
submitted to the Department of Medicine
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Ki Hwan Kwon

June 2014

This certifies that the Doctoral Dissertation of Ki Hwan Kwon is approved.

Thesis Supervisor : Woong Youn Chung MD,PhD

Thesis Committee Member#1: Eun Jig Lee MD,PhD

Thesis Committee Member#2: Jeon Han Park MD, PhD

Thesis Committee Member#3: Chang Geol Lee MD, PhD

Thesis Committee Member#4: Euy-Young Soh MD, PhD

The Graduate School
Yonsei University

June 2014

ACKNOWLEDGEMENTS

When I enter Department of Medicine, the Graduate School, I have the source of trouble what to do and how to do. On this course, I met professor Woong Youn Chung and Professor Chung kindly take the responsibility for course of my study. At first, I begin to try a doctor's dissertation with my hart flutter and fear. And now, I am fininshing all dissertation courses and look back on last course. On my surgeon life, I have never learn about sirtuins and tried to review many paper to understand sirtuins. And I feel not to end the depth of learning basic study and have my little knowledge and learned modest for my looking shabby knowledge. I understand the way of study and the time of cultivation of the mind for collecting various many efforts and the course of completing thesis. 7 years the going over school, it came and went on study and job life, it was not easy in parallel. I gives a deep appreciation to professor Woong Youn Chung for encouraging and taking me to now and this dissertation reaching to a completion initially and until to catch a study it give and being kind to hold the teaching which is minute. And I thank professor Jan Dee Lee and Cho Rok Lee for helping me to collect laboratory results and assist. And I appreciated professor Eun Jig Lee, Jeon Han Park, Chang Geol Lee, university of Younsei and Euy-Young Soh, university of Ajou for kindly recommending this paper with the greatest care, too. I thank both parents for encouraging and supporting all ways. I thank my wife for not complaining and supporting silently. I love my sons, Seoung Chan and Yu Chan, treasure of my life. I pray to be a lamp for my sons as name of father. Last, I thank the God for keeping and taking care of me until now.

<TABLE OF CONTENTS>

ABSTRACT ····· iii

I. INTRODUCTION ····· 1
II. MATERIALS AND METHODS ····· 3
1. Cell lines and materials ····· 3
2. Immunoblot analysis ····· 3
3. Apoptosis detection ····· 3
4. MMT assay ····· 4
5. RNA isolation and real-time PCR ····· 4
6. Public data and stastical analysis ····· 5
III. RESULTS ····· 6
1. Analysis for cell viability by MTT assay with dose and time dependent manner ····· 6
2. Western blot expression of SIRT1 and SIRT3 in thyroid cancer cell lines by etoposides ····· 8
3. Analysis of apoptosis in cancer cell lines by FACS with Anexin V-FITC ····· 10
4. Analysis for expression of apoptosis-related genes by real time-PCR ··· 13
5. Correlation of Bax and p21 with SIRT1 activation by using gene network ····· 16
IV. DISCUSSION ····· 18
V. CONCLUSION ····· 22

REFERENCES ····· 23
ABSTRACT(IN KOREAN) ····· 27

LIST OF FIGURES

Figure1. Cell death along with dose and time dependent manner by etoposide in thyroid cancer cell lines. ·····6
Figure 2 Expression of SIRT1 and SIRT3 by etoposide is regulated by cell-type Specific manner in human thyroid cancer cell lines ·····8
Figure 3 SIRT1 and SIRT3 inducibility is related to etoposide-induced cell death ·····10
Figure 4 Differentially expression of apoptosis-related genes by etoposide induced gentoxic stress in thyroid cancer cell lines ····13
Figure 5 Correlation of Bax and p21 with SIRT1 activation in cDNA microarray data using BXD mice lines ·····16

ABSTRACT

Induction of SIRT1 and SIRT3 confers resistance to genotoxic apoptosis in thyroid cancers

Ki Hwan Kwon

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Woong Youn Chung)

Introduction: Sirtuin is NAD^+ dependent deacetylase, which plays great roles on gene silencing, DNA repair and apoptosis. The aim of study is to investigate whether the genotoxic stress such as etoposide treatment is able to induce SIRT1 and SIRT3 in thyroid cancer cell lines and to determine the effect of SIRT1 and SIRT3 induction on programmed cell death provoked by etoposide treatment and to determine the change of the apoptosis related genes (*p53*, *p21*, *Bcl-xL and Bax* **).**

Materials and Methods: Protein expression, apoptosis rate, cell viability and mRNA expression of SIRT1, SIRT3 and apoptosis related genes (*Bax, Bcl-xL*, *p53*, *p21*) were assessed in three thyroid cancer cell lines (TPC-1, FTC-133 and FRO) treated with 200 μM etoposide.

Results: In western blot, protein induction of SIRT1 and SIRT3 was increased in TPC-1 and decreased in FTC-133 and FRO cell lines. FTC-133 and FRO cells showed significantly increased levels of apoptosis compared with TPC-1 cell via FACS analysis. In RT-PCR, only *p21* expression was elevated in TPC-1 and Bax was highly elevated in FTC-133. In FRO, expression of all apoptosis related genes were decreased.

Conclusion: The SIRT1 and SIRT3 inducibility by etoposide was also different in thyroid cancer cell lines. The ability to survive under the genotoxic

stress was observed accordingly with SIRT1 and SIRT3 inducibility. In addition, SIRT1-Foxp3 signal might be involved in generation of resistance against genotoxic stress. Finally, the emerging role of *p21* under genotoxic stress should be addressed in future studies to develop new drug target of thyroid cancer. SIRT1 and SIRT3 might confer the prerequisite resistance to genotoxic drug induced apoptosis.

Key words: SIRT1, SIRT3, apoptosis related genes, thyroid cancer, apoptosis

Induction of SIRT1 and SIRT3 confers resistance to genotoxic apoptosis in thyroid cancers

Ki Hwan Kwon

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Woong Youn Chung)

I. INTRODUCTION

Thyroid hormone is oxygen consumptive hormone and plays important roles in human energic and metabolic homeostasis.[1] Thyroid cancer is frequent endocrine malignancy and well-differentiated thyroid cancers are efficiently treated by surgery and radiotherapy.[2] Anaplastic thyroid cancer is one of the most aggressive and fatal malignancies. Anaplastic thyroid cancer has a very poor prognosis. The mean survival rate is about 6 months from the time of diagnosis. In anaplastic thyroid cancer, the role of surgery and radiotherapy is controversial and chemotherapy is important but has not been effective treatment. The novel treatments are being tried as new strategies such as new chemotherapy agents, bovine serum ribonuclease, bovine morphogenic protein and p53 gene therapy.[3]

In eukaryotes, genomic DNA is enwrapped by histone octamers and histone is modified by acetylation and deacetylation. The acetylated and deacetylated balance of chromatin is controlled by the histone acetyltransferase and histone deacetylase. Mammlian histone deacetylases are classified into four types. For example, Class I contains histone deacetylases 1, 2, 3 and 8. Class II contains histone deacetylases 4, 5, 6, 7, 9 and 10. Class III is Sir2 (silencing information regulator, sirtuins). Class IV is histone deacetylase 11.[4] Class III is NAD^+-dependent and influences enzymatic activity on histones and numerous transcriptional regulators.[5] Humans have seven sirtuins. These enzymes function as regulators for gene silencing, cell cycle regulation, metabolism, life longevity

and apoptosis. SIRT1 is closely connected to the regulation of transcription factors like *p*53, NF*κ*B, FOXO, Mef2 and PCC-1α.[6] SIRT1 functions in proliferation, senescence and apoptosis of cell.[7] SIRT2 is kwon as tubulin-deacetylase protein and plays an important role in the control of mitosis in the cell cycle and acts as an important protein that controls chromosomal instability.[5,8] SIRT3 is localized in mitochondria and connected to apoptosis because mitochondria functions in cell death and apoptosis. Anti-apoptotic proteins (Bcl-2 and Bcl-xL) are localized in the outer mitochondrial membrane and suppress apoptosis and promote cell survival. Pro-apoptotic proteins (Bad and Bax) interact with the outer mitochondrial membrane and promote apoptosis. *p*53 is localized in mitochondria and known as a tumor suppressor gene and responses to genotoxic stress and is connected to the expression of apoptosis related genes.[9] SIRT5 is little known about its activity but recently reported as NAD+-dependent protein lysine demalonylase and desuccinylase.[10] SIRT6 controls genomic DNA stability and DNA repair and organ integrity. SIRT7 is localized in the nucleus and and interacts with RNA polymerase I (Pol I) and histone. Absence of SIRT7 inhibites cell proliferation and raises apoptosis.[5]

As mentioned above, Sirtuins keep energy homeostasis and associated with cell cycle. It has been known that they could be associated with various diseases. Because thyroid plays an important role in homeostasis of energic metabolite of body, we think that sirtuins may be closely related to thyroid. We are more interesting in SIRT1 and SIRT3. SIRT1 has various biological roles in growth regulation, stress response, tumorigenesis, endocrine signaling and life longevity.[11] SIRT3 has been known as a significant mitochondria NAD+-dependent deascetylase and have functions as either oncogene or tumor suppressor in cancer.[12] SIRT3 is kwon as the significant molecule of cancer and potential drug target for cancer treatment.[13] In this study, we have investigated the cytoprotective effects of SIRT1 and SIRT3 in thyroid cancer cells under genotoxic stress by etoposide treatment which can induce DNA damage by preventing re-ligation of the DNA strands and causing DNA strands to break.[14] Etoposide induced the differential SIRT1 and SIRT3 activation in thyroid cancer

cell lines and this differential inducibility was related to resistance to etoposide-induced cell death. In addition, Bax, Bcl-xL, *p*53 and *p*21 might be molecules associated with cytoprotective effects of SIRT1 and SIRT3.

II. MATERIALS AND METHODS

1. Cell lines and materials

TPC-1 (papillary thyroid cancer cell line) and FTC-133 (follicular thyroid cancer cell line) cells were cultured in DMEM (Sigma, St Louis, MO, USA) with 10% fetal bovine serum (FBS)(Invitrogne, Grand Island, NY, USA). FRO (undifferentiated/anaplastic thyroid cancer cell line) cells were cultured in RPMI1640 (Sigma) with 10% FBS. Etoposide (Sigma) was treated at 200 μM for indicated times.

2. Immunoblot analysis

Cells were lysed in lysis buffer, and the cell lysates were separated using SDS-polyacrylamide gel electrophoresis. After the proteins were transferred to a nitrocellulose (NS) membrane (Amersham Biosciences, Freiburg, Germany), the membranes were blocked with 5% skim milk and incubated with the indicated primary antibodies overnight at 4□°C, and after washing, the membranes incubated with the secondary antibodies for 1□h at room temperature(RT). The immunoreactive bands were developed using peroxidase-conjugated secondary antibodies (Phototope-HRP Western Blot Detection Kit, New England Biolabs, Beverly, MA, USA). The primary antibodies used in this study were anti-SIRT1 (sc-15404, Santa Cruz Biotech, Santa Cruz, CA, USA), anti-SIRT3 (sc-365175, Santa Cruz Biotech), anti-actin (sc-1616, Santa Cruz Biotech) antibodies.

3. Apoptosis detection

The assay was performed using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences, Warsaw, Poland) according to the manufacturer's protocol.

Briefly, cells were washed twice with cold PBS and re-suspended in 1X binding buffer at a concentration of 1 x 10^6 cells/ml. One hundred microliter of the cell suspension (1 x 10^5 cells) was transfered to a 5 ml culture tube. The solution was added with 5 μL of FITC Annexin V and 5 μL Propidium Iodide (PI), and incubated for 15 min at RT (25°C) in the dark. After adding 400 μL of 1X Binding Buffer to each tube, flow cytometry analysis was performed. Data was acquired using a BD FACSVerse system and BD FACSuite software (BD Biosciences).

4. MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay

Cell viability was assessed by the MTT dye conversion assay. After treatment with etoposide, MTT (25 μL of 5 mg/ml MTT in sterile PBS) was added to 100 μL of a cell suspension and allowed to incubate for 2 h at 37 °C. After the reaction was stopped, the cells were lysed by the addition of 100 μL lysis buffer. Cell lysates were placed at 37 °C overnight to allow cell lysis and dye solubilization. The OD was read at 595 nm using a THERMOmax microplate reader (Molecular Devices, Menlo Park, CA, USA). Data are expressed as a percent of vehicle-treated (DMSO) control values. The assays were carried out on three independent experiments, each performed in triplicate.

5. RNA isolation and real-time PCR

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA), and complementary DNA (cDNA) was prepared from total RNA using M-MLV reverse transcriptase (Invitrogen) and oligo-dT primers (Promega, Madison, WI, USA). Quantative RT-PCR (qRT-PCR) was performed using cDNA, QuantiTect SYBR® Green RT-PCR Kits (Qiagen, Valencia, CA, USA) with following primers : Bax primers, 5'-CCC GAG AGG TCT TTT TCC GAG-3' and 5'-CCA GCC CAT GAT GGT TCT GAT-3'; Bcl-xL primers, 5'-GAG CTG GTG GTT GAC TTT CTC-3' and 5'-TCC ATC TCC GAT TCA GTC CCT-3'; p53 primers,

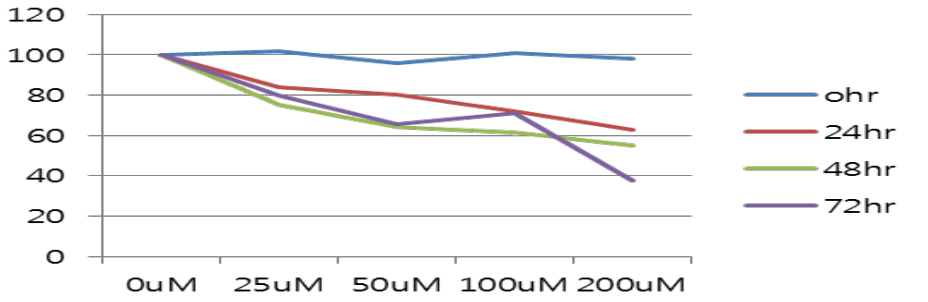
5’-CAG CAC ATG ACG GAG GTT GT-3’ and 5’-TCA TCC AAA TAC TCC ACA CGC-3’; p21 primers 5’-TGT CCG TCA GAA CCC ATG C-3’ and 5’-AAA GTC GAA GTT CCA TCG CTC-3’; GAPDH primers, 5’-GGA GCG AGA TCC CTC CAA AAT-3’ and 5’-GGC TGT TGT CAT ACT TCT CAT GG-3’. The relative expression was calculated using the Applied Biosystems® StepOne™ Real-Time PCR Systems (Foster City, CA, USA). qRT PCR experiments were performed in triplicate and were repeated three times.

6. Public data and stastical analysis

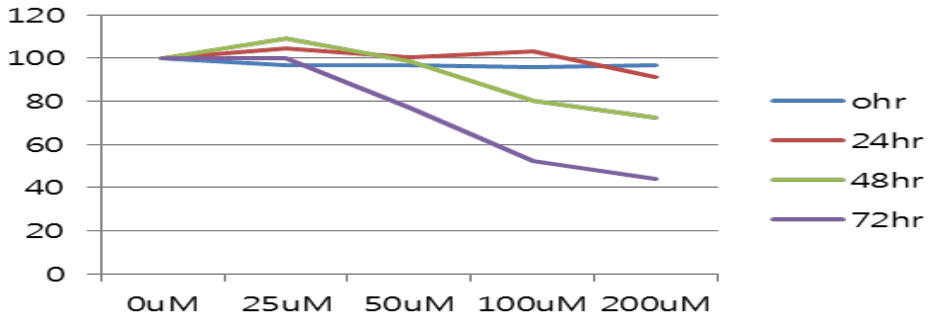
Analysis for gene expression using public repository data was performed using The Human Protein Atlas (http://www.proteinatlas.org/), BioGPS (http://biogps.org/#goto=welcome), NCBI Gene Expression Omnibus (GEO) profiles (http://www.ncbi.nlm.nih.gov/geoprofiles) and GeneNetwork (a free scientific web resourse, http://www.genenetwork.org/). Statistical analysis was carried out by means of GraphPad Prism (GraphPad Software Inc., CA, USA). Comparisons of average means were performed with Mann-Whitney U-test. Data are mean ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All reported P-values are two sided.

III. RESULTS

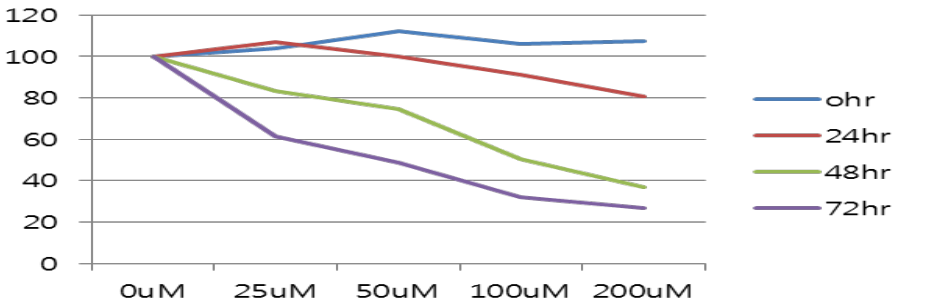
1. Analysis for cell viability by MTT assay with dose and time dependent manner



TPC1



FTC133



FRO

Figure 1. Cell death along with dose and time dependent manner by etoposide in thyroid cancer cell lines.

To investigate the effects of SIRT1 and SIRT3 induction in thyroid cancer cell lines, we first assessed the proper dosage of etoposide and time for inducing apoptosis in three human thyroid cancer cell lines. We gave increasing dose of etoposide to each cell line and check cell viability on indicated time and analysed cell viability by MTT assay and get representative data after triplicated 3 independent experiments were tried. We tried to investigate apoptosis rate on 6 hr, 24 hr, 48 hr and 72 hr for looking proper time and with 25 μM, 50 μM, 100 μM and 200 μM for looking for proper dosage. Cells were dead along with dose and time dependent manner. High dosage excessively increased sensivity to cytocixity and could not check apoptosis. For the next experiments, cells were treated with 200 μM etoposide to investigate the induction of SIRT1 and SIRT3 and expression of apoptosis related genes on western blot and analysis of RT-PCR

2. Western blot expression of SIRT1 and SIRT3 in thyroid cancer cell lines by etoposide

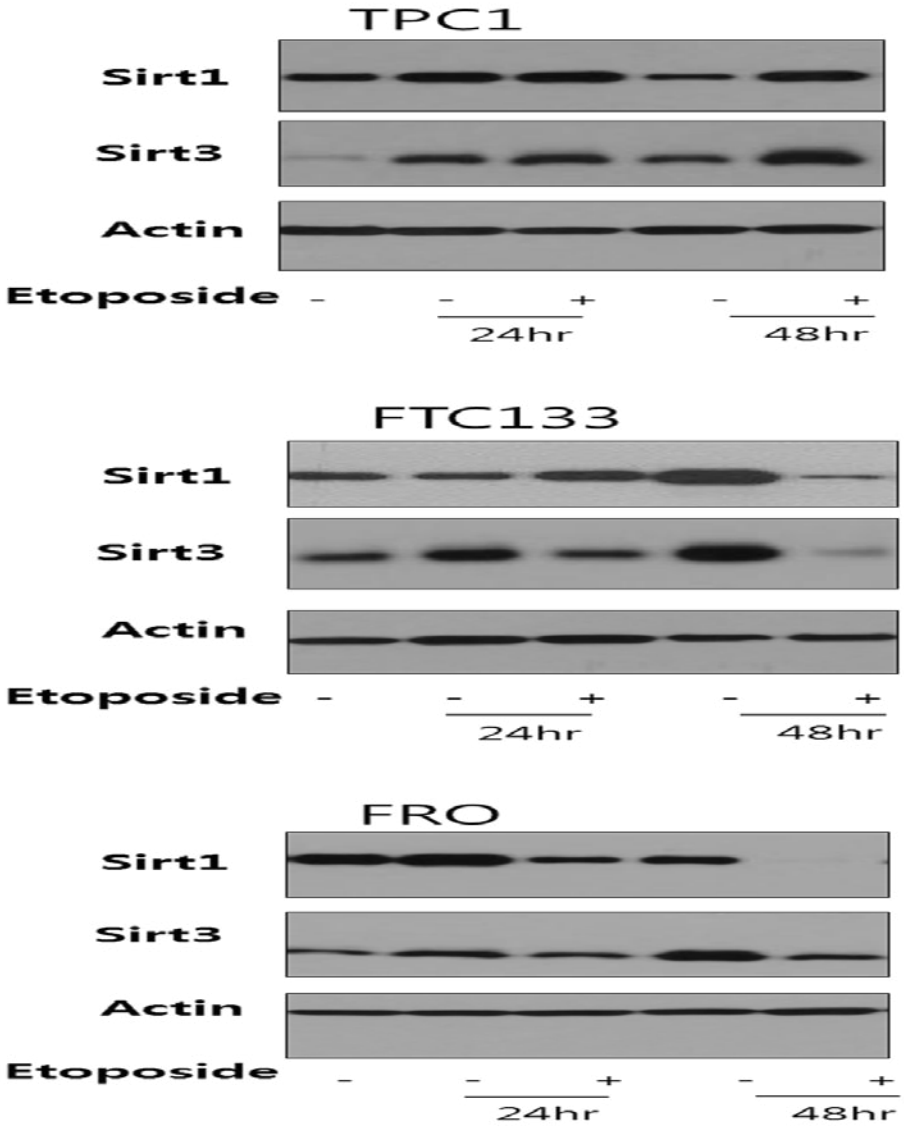
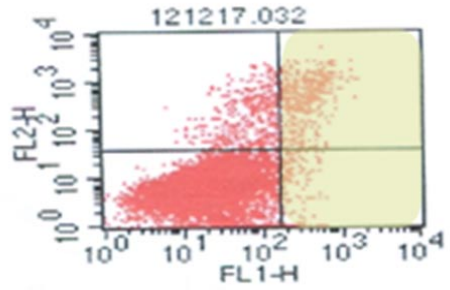


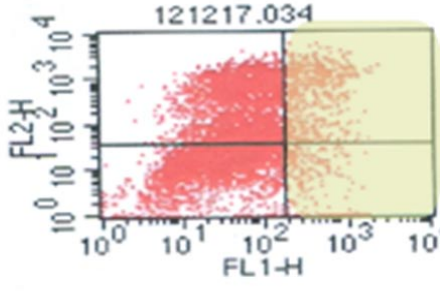
Figure 2. Expression of SIRT1 and SIRT3 by etoposide is regulated by cell-type Specific manner in human thyroid cancer cell lines.

we decided to perform western blot analysis to test whether genotoxic stress by etoposide can induce SIRT1 and SIRT3 differentially in cell type dependent manner. We assessed expression of SIRT1 and SIRT3 by Western blot after 200 μM etoposide treatment in each thyroid cancer cell lines and used β-actin for loading control. As shown in Fig 2. , TPC1 cells treated with etoposide (200 μM) for 24 or 48 hrs showed increased expression of SIRT1 and SIRT3. However, FTC133 and FRO showed reduced SIRT1 and SIRT3 expression under the same experimental conditions.

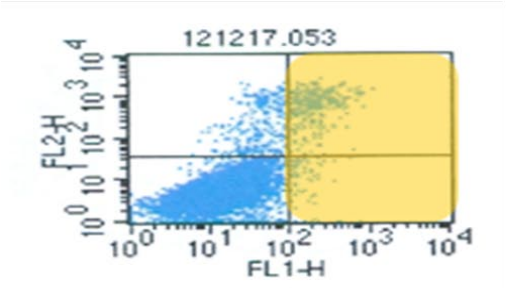
3. Analysis of apoptosis in cancer cell lines by FACS with Anexin V-FITC

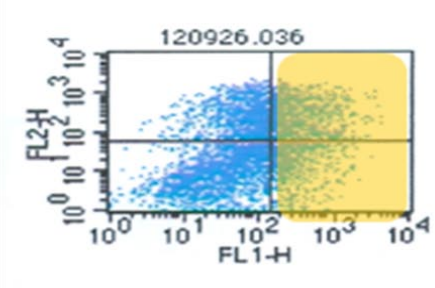
Untreated Treated



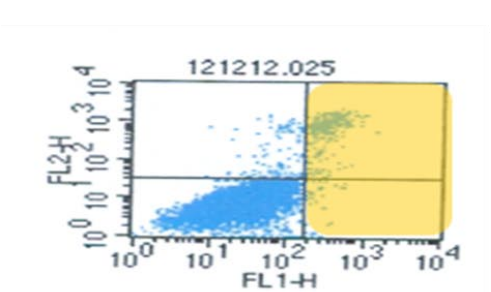


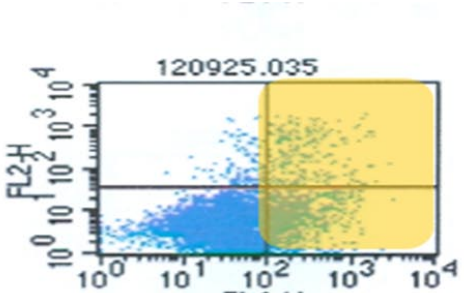
TPC1





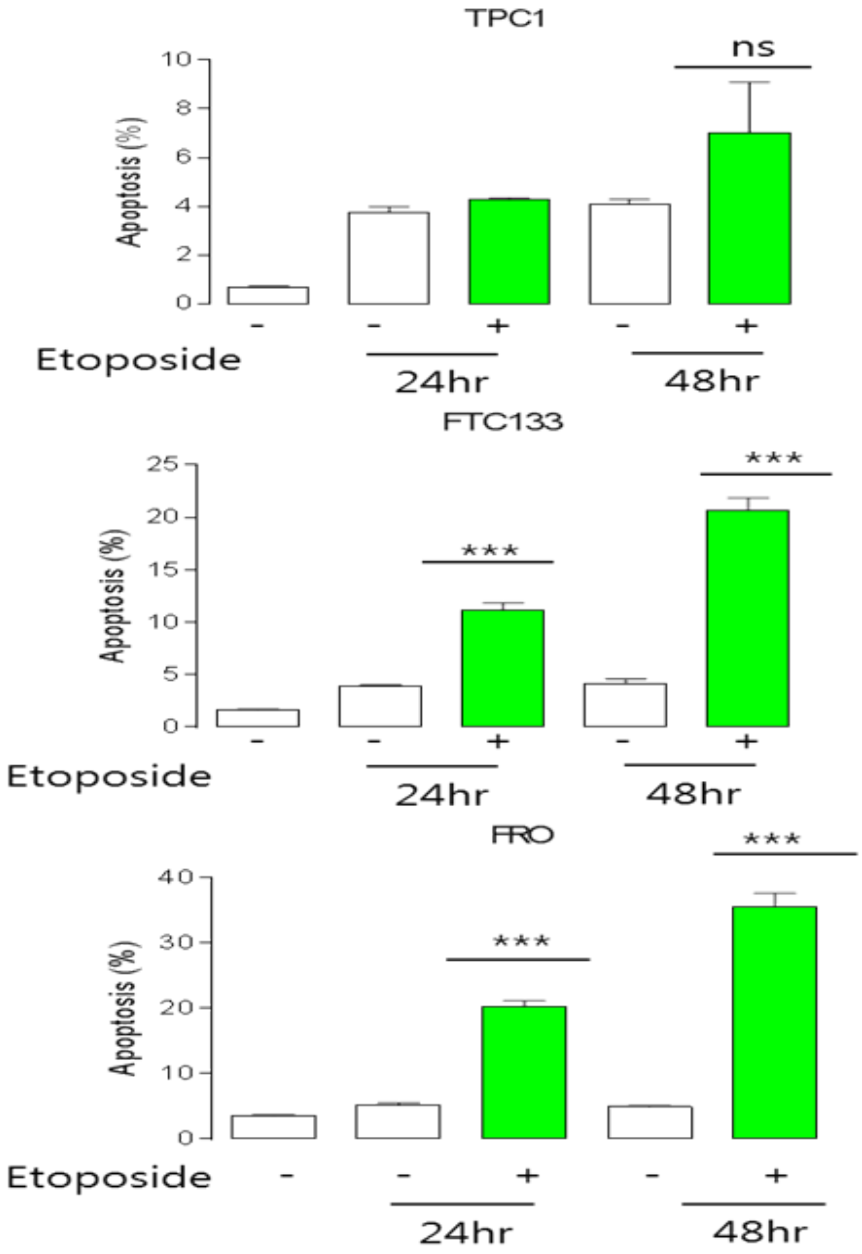
FTC133





FRO

(A)

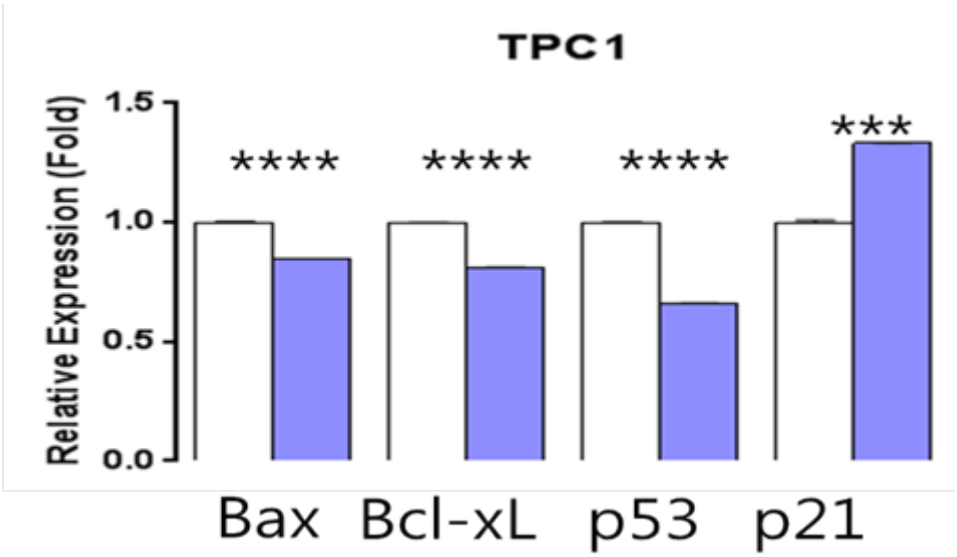


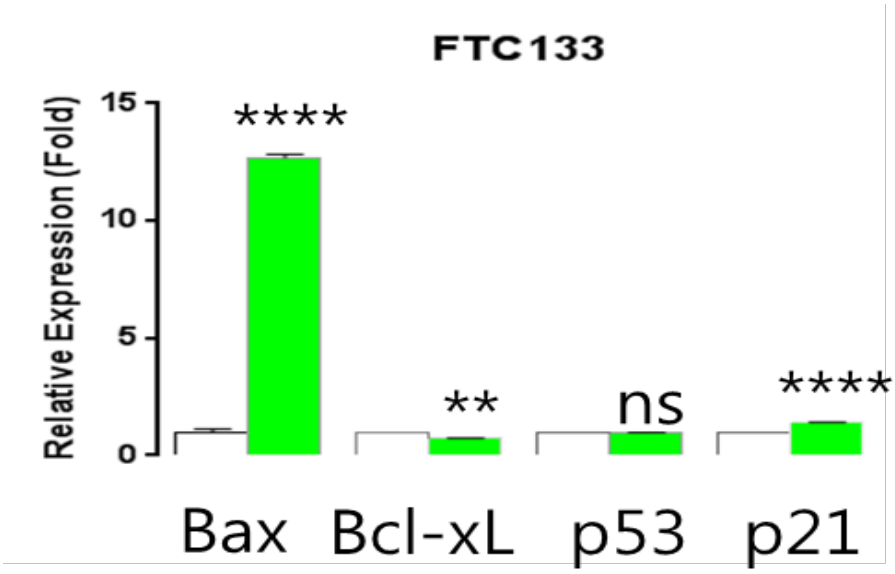
(B)

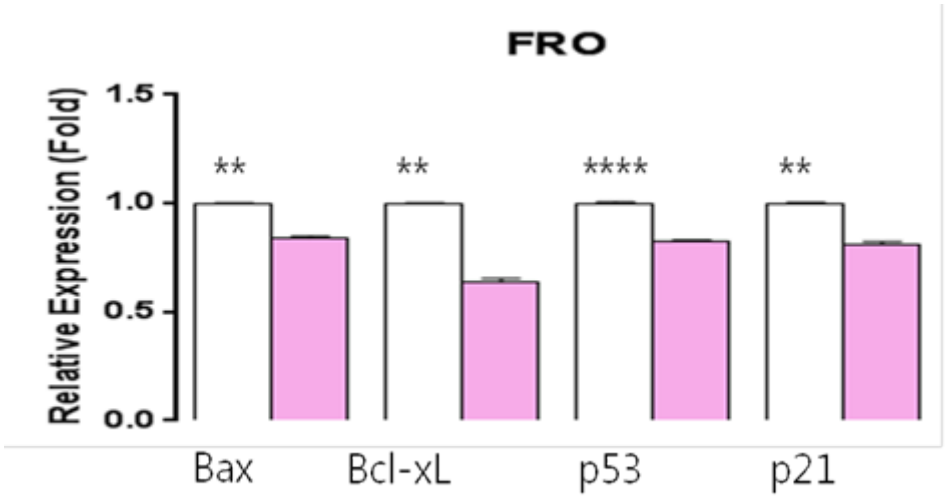
Figure 3. SIRT1 and SIRT3 inducibility is related to etoposide-induced cell death.

To support our hypothesis of cell type- specific activity of SIRT1 and SIRT3, each cell line was treated with 200 μM etoposide and we analyzed apoptosis by FACS with Annexin V-FITC. Data was representative for triplicated 3 independent experiments. After each cell line was treated with 200 μM etoposide for 24 hr and 48 hr, apoptosis induced experiment was implemented with triplicated, 3 independent and was expressed with mean±SEM. NS meant not significant and **** meant significant and p-value was less than 0.1. As shown in Figure 3. , TPC1 cells showed minimal increase of apoptosis (8.06%) with treatment with etoposide (200 μM) during 48 hr compared to nontreated control (5.34%). But, the etoposide treatment for 24 hr indicated statistically no significant increase of apoptosis. On the contrary, FTC133 cells presented a dramatic increase of apoptotic cell death (22.73%) compared to nontreated control (5.1%). This increase of apoptotic cell death was already observed after 24 hr treatment with statistical significance. In the case of FRO, a dramatic increase of apoptotic cell death (5.04% *vs. 32*.79%) at 48 hr was also observed and the apoptotic cell death was increased after 24 hr treatment with statistical significance. TPC1 cell showed less reduction of enzymatic activity of NAD(P)H-dependent cellular oxidoreductase compared to FTC133 and FRO cell. Taken together, TPC1 cell showing higher inducibility of SIRT1 and SIRT3 can be able to resist apoptosis and increase cell viability against etoposide-induced genotoxic stress.

4. Analysis for expression of apoptosis-related genes by real time-PCR







	TPC1	FTC133	FRO
Bax	↓	↑	↓
BCl-xL	↓	↓	↓
p53	↓	ns	↓
p21	↑	↑	↓

Figure4. Differentially expression of apoptosis-related genes by etoposide induced gentoxic stress in thyroid cancer cell lines.

To understand molecular expression of apoptosis-related genes in each thyroid cancer cell lines by etoposide treatment, we treated each cell lines with 200 μM etoposide for 48 hr and investigated mRNA expression of apotosis-related genes such as Bax, Bcl-xL, p53 and p21 by real time-PCR. In addition, we also quantified *p53* and cyclin-dependent kinase inhibitor 1 (*p21* / WAF1) which have been reported to be target proteins of SIRT1 and SIRT3. The mRNA expression of Bax was significantly reduced by etoposide treatment in TPC1 cells, whereas Bax expression was remarkably increased in FRO cells by etoposide treatment. Bcl-xL and *p53* showed decreased mRNA expression in all three kinds of cells after etoposide treatment. Interestingly, *p21* was significantly increased in TPC1 cells but decreased in FRO cells denoting that *p21* might have a cytoprotective effect of TPC1 cells. Taken together, we could postulate that decreased Bax and increased *p21* might be related to conferring resistance to etoposide-induced genotoxic apoptosis in TPC1 cells which have higher inducibility of SIRT1 and SIRT3.

5. Correlation of Bax and p21 with SIRT1 activation by using gene network

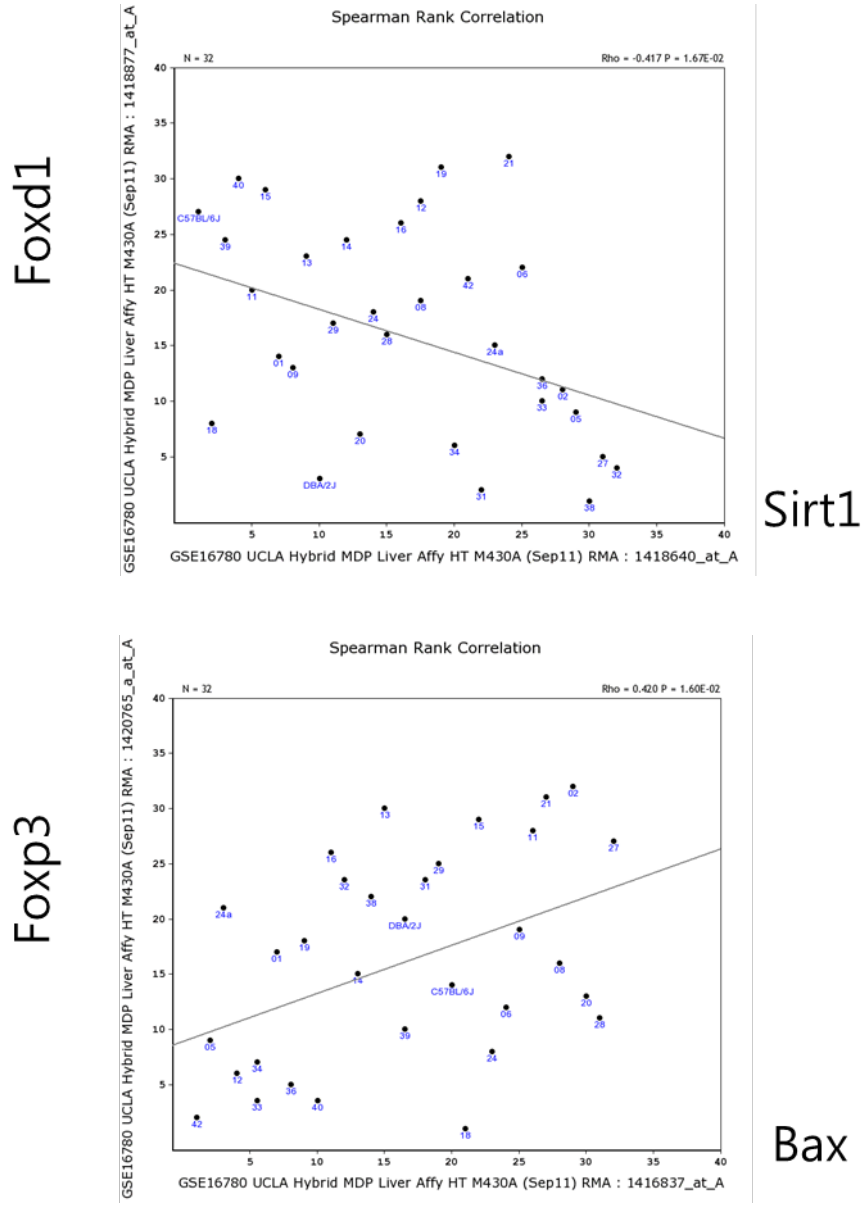


Figure 5. Correlation of Bax and p21 with SIRT1 activation in cDNA microarray data using BXD mice.

As our qRT-PCR data indicated that mRNA expresison of Bax and p21 might be related to Sirt1 or Sirt3 inducibility, we analyzed the correlation of Bax/p21 with Sirt1 activation using GeneNetwork. Recently, Sirt1 has been regarded as a negative regulator of Forkhead Box Protein P3 (Foxp3) transcription factor by deacetylation of Foxp3.[15] In addition, Ex-527, a Sirt1 inhibitor, enhanced Foxp3 expression during ex vivo Treg expansion.[15] Accordingly, mRNA expression of Foxp3 showed positive relationship with Bax in GSE16780 UCLA Hybrid MDP Liver Affy HT M430A (Sep11) RMA Database (Spearman Rank Correlation : Rho=0.420, p=1.60^{E}-02 ; Pearson Correlation : Rho=0.453, p=8.49^{E}-03, Supplementary Fig. 5), suggesting that Sirt1-Foxp3-Bax signaling pathway might be operatinal in TPC1 cells to generate the resistance against etoposide induced genotoxic stress. Interestingly, we also found the statistically significant negative correlation between Foxp3 and p21 (cyclin-dependent kinase inhibitor 1A (CDKN1A), Cip1) in EPFL/LISP BXD CD Brown Adipose Affy Mouse Gene 2.0 ST Exon Level (Oct13) RMA (Spearman Rank Correlation : Rho=-0.471, p=2.89^{E}-03 ; Pearson Correlation : Rho=-0.489, p=1.82^{E}-03, Fig. 5). Taken together, we postulated again that Sirt1-Foxp3 signal may have a role in TPC1 cells to survive under etoposide treatment.

IV. DISCUSSION

Etoposide is known as the topoisomerase II inhibitor and anticancer drug used to induce apoptosis in response to DNA damage.[16] Karpinich et al have reported that topoisomerase II inhibitor etoposide killed 10% of the cell (L929 mouse fibroblast) within 24 hr and 30% after 48hr and almost 50% within 72 hr.[17]

Our study showed that apoptosis was gradually increased to about 30% after 48 hr treatment of 200 μM etoposide except a papillary thyroid cancer cell line TPC1.

On our results of FACS, apoptosis rate in a papillary thyroid cancer cell line TPC1 was not significantly increased state by etoposide compared to untreated cell after 48 hr. Apoptosis rate of both follicular and an anaplastic thyroid cancer line was significantly increased state compared to untreated tissue. Expression of SIRT1 and SIRT3 increased in TPC1 cell line by etoposide and decreased in FTC133 and FRO cell lines. These results showed that induced expression of SIRT1 and SIRT3 might be correlated to apoptosis by etoposide.

SIRT1 enhances cell survival under stressful condition by regulating *p53* dependent apoptosis and is overexpressed in several cancers, such as acute myleloid leukemia, primary colon cancer, prostate cancer and non-melanoma skin cancers.[18] It was also reported that there was reductive state of SIRT1 in some human cancers, such as glioblastoma, bladder carcinoma, prostate cancer and ovarian cancer.[18] SIRT1 knockout gives rise to growth arrest and reduces resistance to drug in cancer cell. Overexpression of SIRT1 inhibits tumor suppressor and DNA repair genes (FOXO family, *p73*, Rb, MLH1 and Ku70). SIRT1 may have dual functions as either oncogene or tumor suppressor gene.[18] Most of tumor had lost expression of *p53* and about half of tumor had little or no expression of SIRT1. When insufficiency of SIRT1 or *p53* alone is, compensatory signal pathways inhibit tumorigenesis. When function of both SIRT1 and *p53* was lost, cells lost a function in genomic integrity and enhance tumor development.[19] SIRT1 inhibits apoptosis and SIRT1 inhibitors (cambinol, sirtinol, salermide, JGBl741, suramin analogs, and the tenovins) increase *p53* acetylation under etoposide treatment and induce cancer cell apoptosis.[20] SIRT3

has pro-apoptotic or anti-apoptotic functions on different cell types. SIRT3 enhances Ku70-Bax interactions and prevent the release of Bax into the mitochondria and prevents apoptosis under stressful condition. In contrast, SIRT3 promotes growth arrest and apoptosis in several cancers, such as colon cancer and osterosarcoma. And SIRT1 and SIRT3 regulate JNK signaling pathway and continuously suppress apoptosis.[21, 22]

Our study showed that overexpression of SIRT1 and SIRT3 promote cell survival (such as papillary thyroid cancer line TPC1) and lower level of SIRT1 and SIRT3 increased apoptosis (such as both follicular and anaplastic thyroid cancer).

On our results of RT-PCR, *p21* was elevated and other apoptosis related genes (Bax, Bcl-xL and *p53*) were decreased in a papillary thyroid cancer cell line, Bax was markedly increase and *p21* was mild elevated and *p53* was not changed and Bcl-xL was decreased in a follicular thyroid cancer cell line. All apoptosis related genes were decreased state in an anaplastic thyroid cancer cancer cell line. Saltman et al have showed that *p53* (0%, 12%, 32%), Bcl-2 (68%, 42%, 0%) and *p21* (68%, 42%, 0%) were expressed in well-differentiated thyroid cancer, poorly differentiated thyroid cancer and anaplastic thyroid cancer, respectively that overexpression of *p53* has been closely connected to marker of aggressive behavior (large tumor size, tall cell variant of PTC and extra-thyroid extension). *p21* is well-known as a cyclin-dependent kinase inhibitor induced by *p53*. *p21* overexpression enhances cell cycle arrest in the G1 phase of tumor cell proliferation.[23] Several studies have shown that *p21* was regulated by *p53*-dependent.[24, 25] Our study showed that *p53* was decreased in all thyroid cancer cell line and *p21* was increased in a well differentiated thyroid cancer cell line except an anaplastic thyroid cancer line. Mahyar-Roemer et al showed that physiological concentration of *p21* expression was stimulated by n-butyrate and induced apoptosis by independence of *p53* and deletion of *p21* increased apoptosis and *p21* Waf1/Cip1 could protect human colon carcinoma cells against *p53* dependent and *p53*-independent apoptosis induced by natural chemopreventive and therapeutic agents.[25] These results suggest that *p21* has

anti-apoptotic role against *p53* dependent and *p53* independent apoptosis. Our study showed that *p21* has protective role against cytotoxic stress in thyroid cancer cell lines.

Our study showed that Bax was markedly elevated state in a follicular thyroid cancer cell line. Bax plays an important role in generating cytochrome c release from mitochondria and regulating the fate of a cell. Bax is present mainly in the cytosol during growth arrest and cannot induce cell death in cytosol and localizes to mitochondria during apoptosis.[26] FGF-2 signaling through MEK enhances the levels of the anti-apoptotic proteins (Bcl-xL and Bcl-s) and inhibits induction of the pro-apoptotic protein Bad by etopside genotoxic stress. These effects were selective due to unchanged expression of Bax. The upregulation of Bcl-xL and Bcl-2 was not by mRNA transcription but by increasing translation. The balance between two types of apoptosis related proteins regulate cell fate. Overexpression of Bcl-2 or Bcl-xL inhibits apoptosis. In contrast, upregulation of Bad or Bax induces cell death.[27] Etoposide alone triggered upregulation of Bax.[28] Bax can antagonize Bcl-xL under epotoside treatment.[29]

Our study showed that Bcl-xL was decreased in all thyroid cancer and Bcl-xL might be decreased due to marked elevation of Bax in a follicular thyroid cancer line.

Zhu et al had reported that *p21* and Bax was expressed independently of *p53* by triptolid induced apoptosis in anaplastic thyroid cancer. Basal level of Bcl-xL is a strong negative correlation with the sensitivity to cytotoxic agents and Bcl-xL has a specific role in regulating the survival of cancer cells exposed to chemotherapeutic agents.[30] The most remarkable relationship was a strong negative correlation between basal Bcl-x levels and sensitivity to drugs in all the mechanistic classes except antimetabolites. Bcl-x may play a special role in general resistance to cytotoxic drugs.[31] The downregulation of Bcl-2 and Bcl-xL effectively enhances cytotoxicity and tumor cell death through targeting NFκB.[32] We suggest that lower of Bcl-xL may be negative correlation with etoposide agents and increase apoptosis. Potent inhibition of Bcl-xL alone could sufficiently potentiate the significant activity of chemotherapeutic agents.[33]

Therefore, lower below basal level of Bcl-xL may result in apoptosis. Apoptosis could be closely connected to substantial decrease in the expression of Bcl-xL protein.[34] But it was unclear to decrease level of Bcl-xL by etoposide induced genotoxic stress.

Mutations of *p53* have appeared to be high frequency in many cancer types, such as lymphomas, leukemias and cancers of lung, esophagus, breast, liver, bone, bladder, ovary and brain. Mutations of *p53* appear to be frequent in undifferentiated thyroid carcinoma.[2, 35] *p53* has dual function as a protector of genome integrity and as a significant regulator of apoptosis. Deletion of *p53* enhances resistance to chemo- and radiotherapy and a more malignancy.[2]

Our study showed that *p53* was decreased in an anaplastic thyroid cancer line and apoptosis gradually increased for 48 hr. At the functional loss of *p53*, other *p53* family proteins (*p73* and *p63*) may function instead of *p53* for activating the tumor suppression pathway and induce cell cycle arrest and apoptosis in response to DNA damage.[2]

Inhibition of SIRT1 activity lead to decrease cell proliferation and induction of apoptosis by *p53* independent manner.[36] With regard to the apoptotic response of tumor cells to genotoxic therapy, our results show marked variation among different thyroid tumor types.

To the best of our knowledge, the direct relationship between SIRT1 and Bax or *p21* independently from *p53* pathway has never been investigated. However, Foxp3 which is deacetylated and degraded by SIRT1 activation showed statistically significant strong positive correlation with Bax and negative correlation with *p21* in our analysis using GeneNetwork, suggesting SIRT1-Foxp3 signal might be a signature molecular event in TPC1 cells to generate resistance against etoposide-induced genotoxic stress.[37]

V. CONCLUSION

The SIRT1 and SIRT3 inducibility was different in thyroid cancer cell lines. The ability to survive under the genotoxic stress was observed accordingly with SIRT1 and SIRT3 inducibility. In addition, SIRT1-Foxp3 signal might be involved in generation of resistance against genotoxic stress. Finally, the emerging role of *p21* under genotoxic stress should be addressed in future studies to develop new drug target of thyroid cancer.

SIRT1 and SIRT3 might confer the prerequisite resistance to genotoxic drug induced apoptosis. Bax, Bcl-xL and *p21* might be mediators for this resistance.

REFERENCES

1. Da-Silva WS, Harney JW, Kim BW, Li J, Bianco SD, Cresecenzi A, et al. The small polyphenolic molecule kaempferol increases cellular energy expenditure and thyroid hormone activation. Diabetes 2007;56(3):767-76.
2. Messina RL, Sanfilippo M, vella V, Pandini G, Vigneri P, Nocolosi ML, et al. Reactivation of p53 mutants by p53 reactivation and induction of massive apoptosis in thyroid cancer cell. Int J cancer 2012 May 15;130(10):2259-70.
3. Are C, Shaha AR. Anaplastic thyroid carcinoma: Biology, Pathogenesis, Prognostic Factors and Treatment Approaches. Ann Surg Oncol. 2006 Apr;13(4):453-64.
4. Borbone E, Berlingieri MT, De Bellis F, Nebbioso A, Chiappetta G, Mai A, et al. Histone deacetylase inhibitors induce thyroid cancer-specific apoptosis through proteasome-dependent inhibition of TRAIL degradation. Oncongene. 2010 Jan 7;29(1):105-16.
5. Dali-Yucef N, Lagouge M, Froelich S, Koehl C, Schoonjans K, Auwerx J. Sirtuins: the ‘magnificent seven’, function, metabolism and longevity. Ann Med. 2007;39(5):335-45. Review.
6. Hallows WC, Lee S, Denu JM. Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases. Proc Natl Acad Sci U S A 2006 Jul 5;103(27):10230-5.
7. Yang T, Fu M, Pestell R, Sauve AA. SIRT1 and endocrine signaling. Trends Endocrinol Metab. 2006 Jul;17(5):186-91.
8. Dryden SC, Nahhas FA, Nowak JE, Goustin AS, Tainsky MA. Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle. Mol Cell Biol. 2003 May;23(9):3173-85.
9. Allison SJ, Milner J. SIRT3 is pro-apoptotic and participated in distinct basal apoptotic pathways. Cell Cycle. 2007 Nov 1;6(21):2669-77
10. Du J, Zhou Y, Su X, Yu JJ, Khan S, Jiang H, et al. SIRT5 is a NAD-dependent protein lysine demalonylase and desuccinylase. Science. 2011:334(6057):806-9.
11. Kim EJ, Um SJ. SIRT1: roles in aging and cancer. BMB Rep. 2008 Nov 30;41(11):751-6. Review.
12. Chen Y, Fu LL, Wen X, Wang XY, Liu J, Cheng Y, et al.

Sirtuin-3(SIRT3), a therapeutic target with oncogenic and tumor-suppressive function in cancer. Cell Death Dis. 2014 Feb 6;5:e1047.
13. Alhazzazi TY, Kamarajan P, Verdin E, Kapila YL. Sirtuin-3(SIRT3) and the Hallmarks of Cancer. Genes Cancer. 2013 Mar;4(3-4):164-71.
14. Yuan J, Luo K, Liu T, Lou Z: Regulation of SIRT1 activity by genotoxic stress. Genes & development 2012;26:791-6.
15. Kwon HS, Lim HW, Wu J, et al Three novel acetylation sites in the Foxp3 transcription factor regulate the suppressive activity of regulatory T cells. Journal of immunology 2012;188:2712-21.
16. Mizumoto K, Rothman RJ, Farber JL. Programmed cell death(apoptosis) of mouse fibroblasts induced by the topoisomerase II inhibitor etoposide. Mol. Pharmacol. 1994 Nov;46(5):890-5.
17. Karpinich NO, Tafani M, Rothman RJ, Russo MA, Farber JL. The course of etoposide-induced apoptosis from damage to DNA and p53 activation to mitochondrial release of cytochrome c. J Biol Chem. 2002 May 10;277(19):16547-52.
18. Martinez-Pastor B, Mostoslavsky R. Sirtuins, metabolism, and cancer. Front Phamacol. 2012 Feb 21;3:22.
19. Yi, Luo J. SIRT1 and p53, effect on cancer, senescence and beyond. Biochim Biophys Acta. 2010 Aug;1804(8):1684-9.
20. Lin Z, Fang D. The Roles of SIRT1 in Cancer. Genes Cancer. 2013 Mar 4(3-4):97-104.
21. Park SH, Ozden O, Jiang H, Cha YI, Pennington JD, Aykin-burns N, et al. Sirt3, Mitochondrial ROS, Ageing, and Carcinogenesis. Int J Mol Sci. 2011;12(9):6226-39.
22. Alhazzazi TY, Kamarajan P, Verdin E, Kapila YL. SIRT3 and cancer: tumor promoter or suppressor? Biochim Biophys Acta. 2011 Aug;1816(1):80-8.
23. Saltman B, Singh B, Hedvat CV, Wreesmann VB, Ghossein R. Patterns of expression of cell cycle/apoptosis genes along the spectrum of thyroid carcinoma progression. Surgery. 2006 Dec;140(6):899-905.
24. Zirbes TK, Baldus SE, Moenig SP, Nolden, Kunze D, Shafizadeh ST, et al. Prognostic impact of p21/waf1/cip1 in colorectal cancer. Int J Cancer. 2000 Jan 20;89(1):1-8.
25. Mahyar-Roemer M, Roemer K. p21 Waf1/Cip1 can protect human

colon carcinoma cells against p53-dependent and p53-independent apoptosis induced by natural chemopreventive and therapeutic agents. Oncogene. 2001 Jun 7;20(26):3387-98.

26. Deng Y, Wu X. Peg3/Pw1 promoted p53-mediated apoptosis by inducing Bax translocation from cytosol to mitochondria. Proc Natl Acad Sci USA 2000 Oct 24;97(22):12050-5.
27. Pardo OE, Arcaro A, Salemo G, Raquz S, Downward J, Seckl MJ. Fibroblast growth factor-s induces translational regulation of Bcl-XL and Bcl-s via a MEK-dependent pathway:correlation with resistance to etoposide-induced apoptosis. J Biol Chem. 2002 Apr 5;277(14):12040-6.
28. Miao L, Yi P, Wu M. Etoposide upregulates Bax-enhancing tumor necrosis factor-related apoptosis inducing ligand-mediated apoptosis in the human hepatocellular carcinoma cell line QGY-7703. Eur J Biochem. 2003 Jul;270(13):2721-31.
29. Simonian PL, Grillot DA, Merino R, Nunez G. Bax can antagonize Bcl-XL during etoposide and cisplatin-induced cell death independently of its heterodimerization with Bcl-XL. J Biol chem. 1996 Sep 13;271(37):22764-72.
30. Zhu W, Hu H, Qiu P, Yan G. Triptolide induces apoptosis in human anaplastic thyroid carcinoma cells by a p53-independent but NF-kappaB-related mechanism. Oncol Rep. 2009 Dec;22(6):1397-401.
31. Amundson SA, myers TG, Scudiero D, Kitada S, Reed JC, Formace AJ Jr. An informatics approach identifying markers of chemosensitivity in human cancer cell lines. Cancer Res. 2000 Nov 1;60(21):6101-10.
32. Ahmad A, Banerjee S, Wang Z, kong D, Sarkar FH. Plumbagin-induced apoptosis of human breast cancer cells is mediated by inactivation of NF-kappaB and Bcl-s. J Cell Biochem. 2008 Dec 15;105(6):1461-71.
33. Shoemaker AR, Oleksijew A, Bauch J, Belli BA, Borre T, Bruncko M, et al. A small-molecule inhibitor of Bcl-XL potentiates the activity of cytotoxic drugs in vitro and vivo. Cancer Res. 2006 Sep 1;66(17):8731-9.
34. Lebedva I, Rando R, Ojwang J, Cossum P, Stein CA. Bcl-xL in prostate cancer cells: effects of overexpression and down-regulation of chemosensitivity. Cancer Res. 2000 Nov 1;60(21):6052-60.
35. Fagin JA, Matsuo K, Karmakar A, chen DL, Tang SH, Koeffler HP.

High prevalence of mutations of the p53 gene in poorly differentiated human thyroid carcinomas. J Clin invest. 1993 Jan;91(1):179-84.

36. Jung-Hynes B, Ahmad N. Role of p53 in the anti-proliferative effects of Sirt1 inhition in prostate cancer cells. Cell cycle. 2009 May 15;8(10):1478-83.

37. van Loosdregt J, Brunen D, Fleskens V, Pals CE, Lam EW, confer PJ. Rapid temporal control of Foxp3 protein degradation by sirtuin-1. PloS one 2011 Apr 20;6(4):e19047.

ABSTRACT(IN KOREAN)

서투인1과 서투인3은 갑상선암들에서 유전독성물질에 대한 저항성에 기여한다

<지도교수 정 웅윤>

연세대학교 대학원 의학과

권 기 환

Sirtuin은 NAD+ dependent deacetylase이며 이는 유전자 침묵화(gene silencing), 유전자수복(DNA repair) 그리고 세포사멸(apoptosis)에 중요한 역할을 한다. 본 연구에 목적은 갑상선암세포에서 etoposide와 같은 약으로 genotoxic stress를 주었을 때 SIRT1과 SIRT3가 유도되는지 여부를 조사하고자 하였고 etoposide에 의해 유발된 세포예정사(program cell death)에 대해 SIRT1과 SIRT3가 어떤 역할을 하는지 조사해보고자 하였으며 세포 사멸 관련 유전자(*p53*, *p21*, Bcl-xL, Bax)들이 어떻게 변하는지 조사하고자 하였다. 세가지 갑상선 세포주(cell line)에 대해 200 *u*M의 etoposide로 처치를 하였을 때 단백질발현(protein expression), 세포사멸율(apoptosis rate), 생존률(viability) 그리고 SIRT1과 SIRT3의 mRNA 표현(expression) 그리고 세포사멸 연관 유전자(apoptosis related gene; Bax, Bcl-xL, *p53*, *p21*)에 대해 조사를 하였다. Western blot상에서 SIRT1과 SIRT3의 단백질 유도는 TPC-1 세포 주에서 증가하였고 FTC-133과 FRO세포 주에서는 감소하였다. FACS분석으로 FTC-133과 FRO 세포는 TPC-1 세포와 비교하였을 때 의미있게 세포사멸 수치가 증가하였다. RT-PCR상 TPC-1에서 유일하게 p21만 증가하였고 FTC-133에서 Bax가 매우 증가하였다. FRO는 모든 세포사멸 연관 유전자가 감소하였다.

Etoposide에 의해 SIRT1과 SIRT3의 유도 정도는 갑상선 세포주에 있는 세포 특징에 따라 다르게 발현된다. SIRT1과 SIRT3는 genotoxic drug 유도하였을때 세포사멸에 대한 저항성에 기여하는 것으로 보이며 Bax, Bcl-xL과 *p21*은 이러한 저항성에 대한 신호 매개자일 것이다.

핵심되는 말: SIRT1, SIRT3, Thyroid cancer, apoptosis, apoptosis related genes