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Sulforaphane decreases telomerase activity and enhances TRAIL-mediated apoptosis by increasing ROS production

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GRADUATE SCHOOL

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Chapter I

SULFORAPHANE DECEASES VIABILITY AND TELOMERASE ACTIVITY IN HEPATOCELLULAR CARCINOMA HEB3B CELLS THROUGH THE REACTIVE OXYGEN SPECIES-DEPENDENT PATHWAY

Abstract

Sulforaphane (SFN), a dietary isothiocyanate, is a well-known natural product that possesses anti-cancer and chemopreventive activities. However, the molecular mechanism of the anti-telomerase activity of SFN is not well understood. In this study, we investigated the hypothesis that SFN inhibits cell viability and telomerase activity via downregulation of telomerase reverse transcriptase (hTERT) expression. We suggest that elevated intracellular reactive oxygen species (ROS) levels, due to exposure to SFN, has a critical role in abolishing since pretreatment with NAC, an antioxidant, resulted in the recovery of hTERT expression. SFN also suppressed the phosphorylation of Akt (Ser-473), thereby inhibiting hTERT phosphorylation and this effect was reversed by pretreatment with NAC. Taken together, these data suggest that ROS are essential for the suppression of SFN-mediated telomerase activity via transcriptional and posttranslational regulation of hTERT.

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1. Introduction

Sulforaphane (SFN), an isothiocyanate found in broccoli and cauliflower, is known to possess anti-cancer and anti-inflammatory activities [1]. It is well known that SFN inhibits cell proliferation and increases apoptosis in various cancer cell lines including human leukemia, colon, and prostate cancer cells, in vitro [2–4]. In our previous findings, we have shown that SFN induces apoptosis through the activation of reactive oxygen species (ROS)-dependent caspase-3 [2]. Others studies also support the notion that SFN dramatically induces ROS generation in cancer cells through a mitochondrial pathway [5]. Generally, ROS generation influences various physiological activities in cancer cells. ROS may play an especially important role in signal transduction through direct or indirect regulation of signal molecules [6]. Therefore, we focused on whether SFN-induced ROS generation regulates telomerase activity in cancer cells since these effects are still undocumented.

Telomerase is a specialized ribonucleoprotein reverse transcriptase that functions in telomere maintenance to promote neoplastic growth [7]. Activation of telomerase is necessary for the continued growth of several human tumors and plays a critical role in tumorigenesis [8,9]. Functional telomerase consists of several essential components, telomerase reverse transcriptase (hTERT), telomerase-associated protein-1 (TEP-1), hsp90, p23, and template RNA (hTR) [10]. Of these, we are primarily interested in the SFN-induced downregulation of telomerase activity via suppression of hTERT expression, because the hTERT is a core telomerase protein and its expression limited to germinal and cancer cells [8,11]. It is well known that the expression of dominant-

negative hTERT or its siRNA reduces the lifespan of human cancer cells by completely inactivating telomerase [6–8]. Therefore, it is thought that suppression of hTERT expression is an ideal strategy for the development of anti-cancer chemotherapeutics.

In this study, we evaluated the inhibitory effect of SFN on the viability of Hep3B cells. We also confirmed that treatment with SFN inhibits telomerase activity via downregulation of hTERT expression at the transcriptional and translational levels, with special emphasis on the role of SFN-induced ROS on the regulation of telomerase.

2. Materials and methods

2.1. Reagents and antibodies

Human hepatocellular carcinoma Hep3B cells, breast cancer MCF-7 cells, and prostate cancer PC-3 cells were purchased from American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified eagle medium (DMEM; Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum (FBS, Gibco BRL) and 1% penicillin-streptomycin (Sigma, St. Louis, MO) in an incubator maintained at 37°C with 5% CO_2. SFN, *N*-acetylcysteine (NAC), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide (MTT) were obtained from Sigma. Antibodies against Akt, phospho (p)-Akt, p-Akt-substrate, and β-actin were purchased from Cell Signaling (Beverly, MA). Antibodies against poly (ADP-ribose) polymerase (PARP) and hTERT were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell viability

Hep3B cells were grown to 70% confluence and treated with the indicated concentrations or for the indicated times with SFN. In a parallel experiment, we pretreated the cells with 5 mM NAC for 2 h at 37°C and then added 20 μM SFN for the indicated times. Control cells were supplemented with complete media containing 0.01% dimethyl sulfoxide (DMSO, vehicle control). Following treatment, viability was determined by MTT assays.

2.3. Flow cytometric analysis

In order to analyze the percentage of apoptotic cells, cells were washed twice with cold PBS. The collected cells were resuspended with annexin-V binding Ca^{2+} buffer in annexin-V-FITC staining solution (1.0 μg/ml) and incubated for 15 min at room temperature in the dark. Flow cytometric analysis was performed using a FACSCalibur (Becton Dickinson, San Jose, CA).

2.4. In vitro caspase-3 activity assay

The activity of caspase-3-like protease was measured using a caspase activation kit (R&D System, Minneapolis, MN) according to the manufacturer's protocol. This assay is based on spectrophotometric detection of the color reporter molecule *p*-nitroanaline (*p*NA), which is linked to the end of the caspase-specific substrate. The cleavage of the peptide by the caspase releases the chromophore *p*NA, which can be quantified spectrophotometrically at a wavelength of 405 nm.

2.5. Measurement of ROS

Intracellular ROS generation was measured by flow cytometry following staining with 10 μM of 2',7'-dichlorofluorescein diacetate (DCFDA; Sigma), which has been shown to be somewhat specific for detection of H_2O_2. After cells were collected, the fluorescence was analyzed using FACSCalibur and a Leica DC300F fluorescence microscopy.

2.6. Telomeric repeat amplification protocol (TRAP) assay

The TRAP assay was performed with a Telo TTAGGG Telomerase PCR-ELISA kit (Roche) according to the manufacturer's instructions. Amplified telomeric DNA products were separated by 10% nondenaturing polyacrylamide in 0.5× Tris-borate-EDTA buffer (pH 8.3) at 100 V for 1.5 h. Gels were stained with ethidium bromide and the image was captured with a Chemi-Smart 2000 WL (Vilber Lourmat, Deutsch, EU).

2.7. Western blot analysis

Total cell extracts were prepared using PRO-PREP protein extraction solution (iNtRON Biotechnology, Sungnam, Republic of Korea). Total cell extracts were separated on 10% polyacrylamide gels and then transferred to nitrocellulose membranes using standard procedures. The membranes were developed using an ECL reagent (Amersham, Arlington Heights, IL). The blots were visualized with the Chemi-Smart 2000 WL. Images were captured using Chemi-Capt and transported into Photoshop.

2.8. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the Trizol reagent (Gibco BRL) according to the manufacturer's recommendations. Genes of interest were amplified from cDNA that had been reverse-transcribed from 1 μg of total RNA using the One-Step RT-PCR Premix (iNtRON Biotechnology). Primers and conditions for the amplification of hTERT and GAPDH have been described previously [12]. PCR reaction was initiated at 94ºC for 2 minutes followed by 28 cycles of 94ºC for 1 min, 1-minute annealing temperature, 72ºC for 1 min followed by final extension at 72ºC for 5 minutes. Annealing temperatures for hTERT and GAPDH were 58ºC and 60ºC, respectively. After amplification, PCR products were separated on 1.5% garose gels and visualized by ethidium bromide fluorescence.

2.9. Immunoprecipitation (IP)

The total cell extracts were immunoprecipitated by incubating with antibody against hTERT overnight at 4°C. Immune complexes were collected using protein A/G-Sepharose beads (Santa Cruz Biotechnology), washed, and eluted in sample buffer. Samples were run on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to nitrocellulose membranes, and probed with specific antibodies against Akt-substrate or hTERT. Blots were developed using the ECL reagent.

2.10. Electrophoretic mobility shift assay (EMSA)

DNA-protein binding assays were carried out with nuclear extract. Synthetic complementary c-Myc (5′-GGA AGC AGA CCA CGT GGT CTG CTT CC-3') binding oligonucleotides (Santa Cruz Biotechnology) were 3'-biotinylated using the biotin 3'-end DNA labeling kit (Pierce) according to the manufacturer's instructions and annealed for 1 h at room temperature. Binding reactions were carried out for 20 min at room temperature in the presence of 50 ng/μl poly(dI-dC), 0.05% Nonidet P-40, 5 mM $MgCl_2$, 10 mM EDTA, and 2.5% glycerol in 1× binding buffer (LightShift^{TM} chemiluminescent EMSA kit) using 20 fmol of biotin-end-labeled target DNA and 10 μg of nuclear extract. Assays were loaded onto native 4% polyacrylamide gels pre-electrophoresed for 60 min in 0.5× Tris borate/EDTA and before being transferred onto a positively charged nylon membrane (Hybond^{TM}-N^+) in 0.5× Tris borate/EDTA at 100 V for 30 min. Transferred DNAs were cross-linked to the membrane at 120 mJ/cm^2 and detected using horseradish peroxidase-conjugated streptavidin according to the manufacturer's instructions.

2.11. Statistical analysis

Statistical analyses were conducted using SigmaPlot software (version 6.0). Values were presented as means ± SD. Significant differences between the groups were determined using unpaired student's *t*-test. Statistical significance was regarded at $P < 0.05$.

3. Results

3.1. SFN decreases cell viability in Hep3B cells

To determine the effect of SFN on cell viability, Hep3B cells were treated with increasing concentrations of SFN for 24 or 48 h and the cell viability was determined by the MTT assay. As shown in Fig. 1A, 24- or 48-h exposure times with 20 μM SFN decreased the cell viability by about 40 and 55% of total cell confluence, respectively. Treatment with SFN also increased the percentage of annexin-V^+ stained cells, indicating the presence of cells in early apoptotic stage (Fig. 1B). In this process, SFN exposure increased caspase-3 activity, and this increase was 2-fold higher as compared to the activity in untreated control cells (Fig. 1C). Additionally, Western blot analysis revealed that treatment with 20 μM of SFN for 48 h significantly increased the proteolytic cleavage of PARP (Fig. 1D). DMSO (0.01%), used as a vehicle control, did not affect cell viability, annexin-V staining, caspase-3 activity, and cleavage of PARP in parallel experiments. Collectively, these data indicate that treatment with SFN inhibits cell viability in Hep3B cells via triggering the activation of caspase-3.

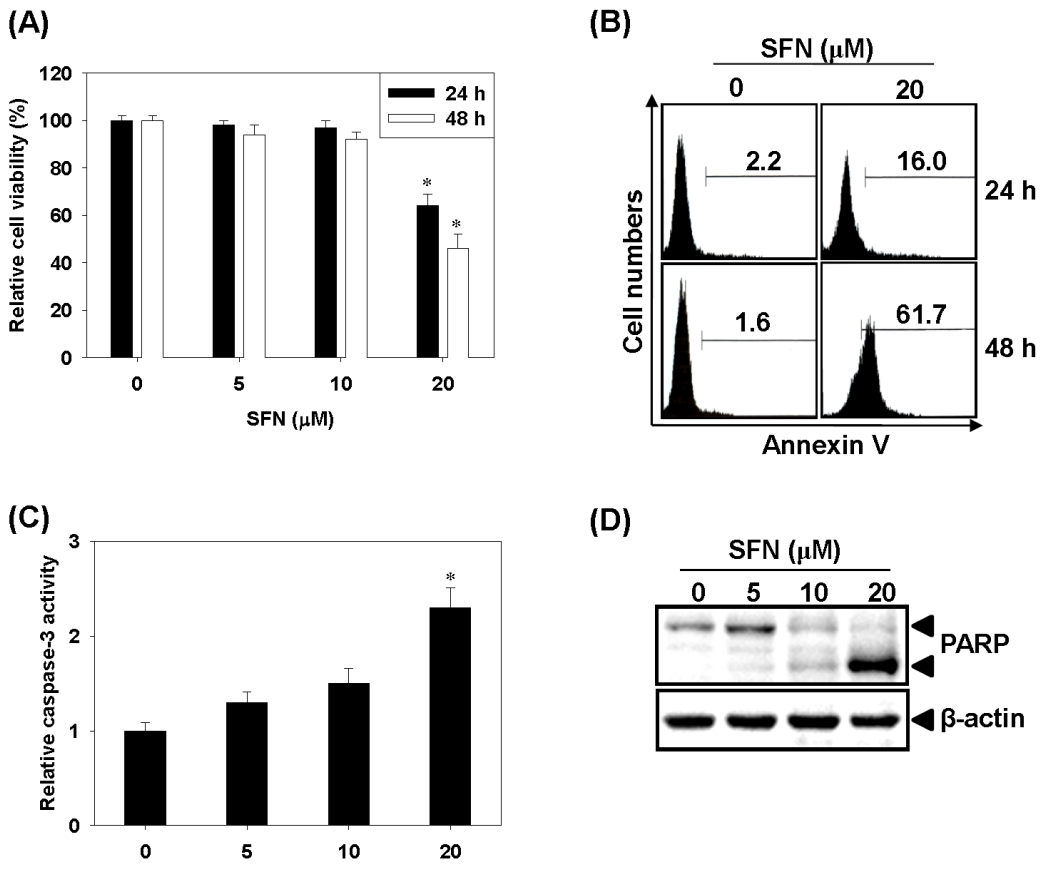

Fig. 1. SFN induces apoptosis in Hep3B cells. Cells were seeded at $1x10^5$ cells/ml and treated with the indicated concentrations of SFN for 24 or 48 h. (A) Cell viability was determined by the MTT assay. (B) Annexin V^+ cells are represented on the x-axis, while the number of cells counted is represented on the y-axis. (C) Caspase-3 activity was determined. (D) PARP Western blot analysis was performed by standard procedure. Data are expressed as overall means ± SD from three independent experiments. Statistical significance was determined by Student's *t*-test (* $P < 0.05$ vs. vehicle control).

3.2. SFN induces ROS-dependent apoptosis

To establish whether treatment with SFN induces ROS-dependent apoptosis, we first investigated whether SFN increases ROS generation in Hep3B cells by measuring the intracellular levels of H_2O_2 using DCFDA staining. As shown in Fig. 2A, flow cytometry (upper panel) and fluorescent microscopy (lower panel) analyses revealed that treatment with 20 μM SFN for 48 h increased ROS production by over 25% as compared to the control cells. Next, to investigate the effect of SFN-induced ROS generation on cell viability, Hep3B cells were pretreated with the antioxidant NAC for 2 h prior to treatment with SFN. Interestingly, SFN-induced cell death was completely neutralized to almost normal levels in the presence of NAC (Fig. 2B). In order to quantify the effects of NAC on SFN-induced cell death, we next investigated the extent of cell death and ROS generation using flow cytometry. As expected, we observed that SFN-induced ROS generation (upper panel) and the population of annexin-V^+ cells (lower panel) were completely reduced by pretreatment with NAC (Fig. 2C). Collectively, these data suggest that SFN induces apoptosis via ROS-dependent mechanisms in Hep3B cells and that NAC converts SFN-induced H_2O_2 to the level of untreated control.

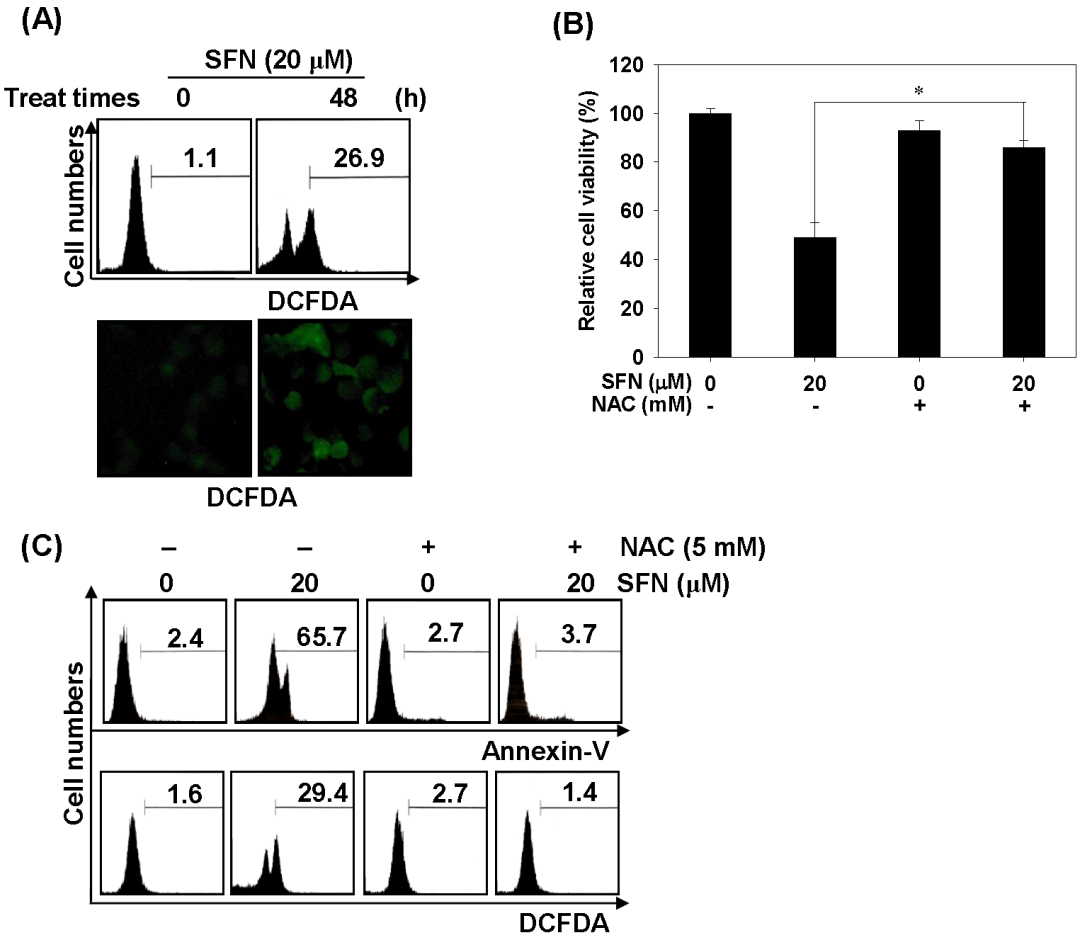

Fig. 2. SFN induces ROS-dependent apoptosis. Hep3B cells were incubated with the indicated concentration of SFN for 48 h after pretreatment with 5 mM NAC. (A) Intracellular ROS accumulation was assessed by DCFDA staining and then measured using flow cytometry and fluorescence microscopy. (B) Cell viability was determined by the MTT assay. (C) Apoptotic annexin-V^+ population and intracellular ROS level were determined in SFN-treated cells after pretreatment with NAC for 2 h. Data are expressed as overall means ± SD from three independent experiments. Statistical significance was determined by Student's *t*-test (* $P < 0.05$ vs. vehicle control).

3.3. SFN represses telomerase activity via downregulation of hTERT

Because SFN has an anti-proliferative effect on a variety of cancer cells and the majority of cancer cells exhibit high telomerase activity that enables the uncontrolled growth of cancer cells [7–9], we examined whether SFN represses telomerase activity in Hep3B cells. In order to elucidate the regulatory effect on telomerase activity by treatment with SFN, we cultured Hep3B cells in the absence or presence of SFN and measured telomerase activity using a TRAP assay kit. As shown in Fig. 3A, we observed that SFN represses the telomerase activity in Hep3B cells in a dose-dependent manner (Fig. 3A). In addition, the expression of hTERT is closely related to telomerase activity in a variety of cancer cells [11,12]. Therefore, it is important to examine whether the inhibitory effect of SFN on telomerase activity is due to the downregulation of hTERT expression in Hep3B cells. Treatment with SFN decreased hTERT expression at both the transcriptional and translational levels in Hep3B cells (Fig. 3B). In addition to these results, pretreatment with NAC recovered telomerase activities that were decreased by treatment with SFN (Fig. 3C). Moreover, RT-PCR and Western blot analysis revealed that SFN-mediated downregulation of hTERT expression was significantly blocked by treatment with NAC (Fig. 3D). Additionally, because it is well known that The hTERT promoter site (-181 bp) has two c-myc binding regions and c-myc has previously been shown to directly regulate telomerase activity [13,14], we performed that SFN regulate the activity of c-Myc in the presence of NAC. DNA binding activity of c-Myc was significantly downregulated by SFN alone, but NAC restored SFN-induced c-Myc activity (Fig. 3E). SFN also significantly decrease telomerase activity in MCF-7 and PC-3 cells (Fig. 3F). These results indicate that the downregulation of telomerase activity and hTERT expression by SFN treatment were

significantly correlated with SFN-induced ROS generation and c-Myc activity in Hep3B cells.

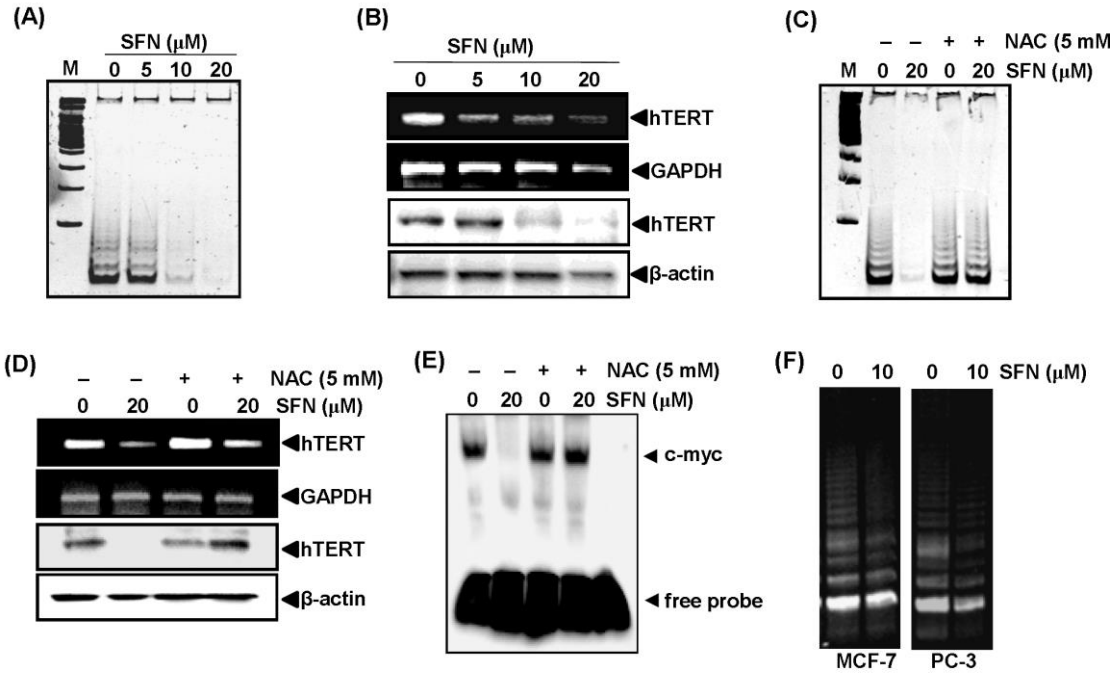

Fig. 3. SFN inhibits telomerase activity and hTERT expression. (A) After 48 h incubation with SFN, telomerase activity of Hep3B cells was measured using a TRAP assay according to the manufacturer's protocol. (B) Total RNA was isolated using Trizol reagent. One microgram of RNA was reverse-transcribed. The resulting cDNA was subjected to PCR with hTERT primers and visualized by EtBr staining (upper panel). GAPDH was used as an internal control. Equal amounts of cell lysate were subjected to Western blot analysis with antibody against hTERT (lower panel). β-Actin was used as an internal loading control. Telomerase activity and hTERT expression were determined in SFN-treated cells after pretreatment with NAC for 2 h. (C) TRAP assay was performed according to the manufacturer's protocol. (D) RT-PCR and Western blot analysis were performed to detect the expression of hTERT. (E) After treatment with NAC (5 mM), SFN was treated in the absence or presence of NAC (5

mM) and assayed for c-Myc activity. (F) Telomerase activity of MCF-7 and PC-3 cells was measured using a TRAP assay according to the manufacturer's protocol.

3.4. SFN deactivates Akt and thereby prevents phosphorylation of hTERT

Because previous studies have shown that Akt enhances human telomerase activity through the phosphorylation of hTERT [15,16], we assessed whether the repression of telomerase activity by treatment with SFN is due to the dephosphorylation of Akt. As shown in Fig. 4A, the total Akt level remained unchanged in all experiments, while the phosphorylation level of Akt was decreased after treatment with SFN for 48 h. However, deactivation of Akt by treatment with SFN was restored by pretreatment with NAC for 2 h. Since activated Akt is known to directly phosphorylate hTERT, which could subsequently translocate into the nucleus and participate in telomerase complexes, we also investigated whether SFN exposure reduces phosphorylation of hTERT as a result of activated Akt. Accordingly, the cells were treated under the indicated conditions and then their lysates were immunoprecipitated with antibody against hTERT. These isolates were subjected to Western blot analysis with a specific antibody against Akt-substrate (p-hTERT) or hTERT. As shown in Fig. 4B, treatment with SFN decreased hTERT phosphorylation at putative phosphorylation sites of Akt. Furthermore, pretreatment with NAC restored it. These results suggest that SFN suppresses the phosphorylation of hTERT via the ROS-dependent dephosphorylation of Akt.

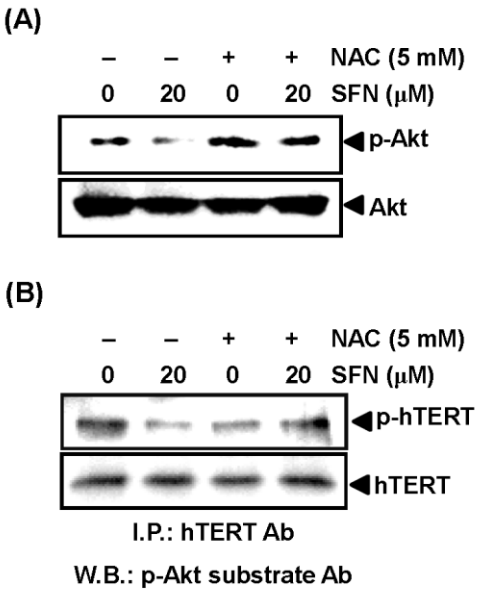

Fig. 4. SFN suppresses Akt-dependent hTERT phosphorylation. Hep3B cells were incubated with 20 μM of SFN for 48 h in the absence or presence of pretreatment with NAC for 2 h. (A) Western blot was performed by standard procedure with specific antibodies against Akt and p-Akt (Ser-473). (B) The lysates were subjected to immunoprecipitation with a specific antibody against hTERT and then blotted with antibody against Akt substrate or hTERT.

4. Discussion

Because telomerase is considered to be an ideal target for cancer therapy, many strategies have been developed to inhibit telomerase activity, such as use of small synthetic molecules and antisense nucleotides [16,17]. Therefore, because large numbers of published reports have suggested that the expression of hTERT is closely correlated with the telomerase activity [11,12], we focused on whether treatment with SFN downregulates telomerase activity via the transcriptional and posttranslational regulation of hTERT. This cooperation of transcriptional and posttranslational modification makes telomerase activity weaker at low dose of SFN compared to expression of hTERT. In this study, we showed that SFN treatment decreases cell viability and telomerase activity via the ROS-dependent pathway in Hep3B cells.

The expression of hTERT is possibly regulated by various transcriptional factors in its promoter region. Of these transcriptional factors, c-Myc has been found to bind with E-box sites (CACGTG) of the hTERT promoter region to regulate its gene transcription [18,19]. We also found that treatment with SFN decreases the DNA binding activity of c-Myc in Hep3B cells. Nevertheless, we cannot rule out the possibility that other transcriptional factors regulate the transcriptional expression of hTERT, since there are many transcriptional factors that bind its promoter regions. Kim et al. [20] reported that the physical interaction of hsp90 with the hTERT promoter is necessary for hTERT expression and regulation. They also found that the activity of the N-terminal–truncated–hTERT promoter, lacking the c-Myc recognition region but containing multiple Sp1/Sp3 sites, regulates telomerase activity, indicating that the involvement of

Sp1/Sp3 [21]. Therefore, further study is needed to elucidate the transcriptional factors that regulate hTERT expression and its SFN-induced suppression.

Mounting evidence suggests that ROS play an important role as signaling molecules that regulate many genes, including Akt. It has been reported that activation of Akt plays a critical role in triggering telomerase activation through hTERT phosphorylation before nuclear localization as well as in controlling the survival and apoptosis of mammalian cells. Specifically, phosphorylation at the Thr-308 and Ser-473 residues in Akt stimulates its activity, while the unphosphorylated form of Akt is practically inactive [22]. It is known that Akt forms a complex with hsp90 in vivo, and this complex protects Akt from protein phosphatase 2A-mediated dephosphorylation [23,24]. Several studies have suggested that Akt is dephosphorylated through enhanced binding to PP2A when cells are treated with H_2O_2 [25,26]. In the present study, we showed that SFN-induced ROS generation triggers p-Akt (Ser-473) downregulation and is then followed by inhibition of Akt-mediated hTERT phosphorylation. Although we could not rule out further processes such as hTERT nuclear translocation shortly after hTERT inactivation, our data consequently showed that telomerase activity was significantly decreased after exposure to SFN. It has also been reported that NF-κB plays a pivotal role in regulating telomerase by modulating nuclear translocation of hTERT [27]. Because we showed in a previous study that SFN significantly inhibits TNF-α-induced NF-κB activation [2], it is possible that these inhibitory effects are also correlated with the suppression of telomerase activity.

In summary, the present study demonstrated that SFN suppresses cell viability and telomerase activity in Hep3B cells. Moreover, the downregulation of hTERT transcription as well as the posttranslational modification of hTERT protein is involved

in SFN-induced repression of telomerase activity in Hep3B cells via the ROS-dependent pathway. However, we could not elucidate whether inhibition of telomerase activity was due to reduced hTERT protein expression.

5. 국문요약

Sulforaphane(SFN)은 식이성 isothiocyanate로서 항암 및 chemopreventive 활성을 지닌 것으로 잘 알려져 있다. 그러나 SFN의 anti-telomerase 활성의 분자적 기전은 잘 알려져 있지 않다. 이 연구에서는 SFN이 telomerase reverse transcriptase (hTERT) 발현의 downregulation을 통해 세포 생존력과 telomerase의 활성을 억제함을 확인하였다. 또한 SFN처리를 통해 증가되는 세포 내 reactive oxygen species (ROS)을 NAC(antioxidant)의 전처리 시 hTERT 발현의 복구시키는 것을 확인하였다. SFN은 또한 Akt (Ser-473)의 인산화를 억제함으로써 hTERT 인산화를 억제하였으며, NAC의 전처리 시 효과가 바뀌는 것을 확인하였다. 종합적으로, 이 결과는 ROS가 hTERT의 전사와 posttranslational 조절을 통해 SFN을 매개로 한 telomerase 활성의 억제에 필수적임을 확인하였다.

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Chapter II

TRAIL ATTENUATES SULFORAPHANE-MEDIATED NRF2 AND SUSTAINS ROS GENERATION, LEADING TO APOPTOSIS OF TRAIL-RESISTANT HUMAN BLADDER CANCER CELLS

Abstract

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) can preferentially initiate apoptosis in malignant cells with minimal toxicity to normal cells. Unfortunately, many human cancer cells are refractory to TRAIL-induced apoptosis through many unknown mechanisms. Here, we report that TRAIL resistance can be reversed in human bladder cancer cell lines by treatment with sulforaphane (SFN), a well-known chemopreventive isothiocyanate in various cruciferous vegetables. Combined treatment with SFN and TRAIL (SFN/TRAIL) significantly induced apoptosis concomitant with activation of caspases, loss of mitochondrial membrane potential (MMP), Bid truncation, and induction of death receptor 5. Transient knockdown of *Bid* prevented collapse of MMP induced by SFN/TRAIL, consequently reducing apoptotic effects. Furthermore, SFN increased both the generation of reactive oxygen species (ROS) and the activation of nuclear factor erythroid 2-related factor 2 (Nrf2), which is an anti-oxidant enzyme. Interestingly, TRAIL effectively suppressed SFN-mediated nuclear translocation of Nrf2, and the period of ROS generation was more extended compared to that of treatment with SFN alone. In addition, silencing of *Nrf2* increased apoptosis in cells treated with SFN/TRAIL; however, blockade of ROS generation inhibited apoptotic activity. These data suggest that SFN-induced ROS generation promotes TRAIL sensitivity and SFN can be used for the management of TRAIL-resistant cancer.

List of Figures

1. Introduction

Bladder cancer is the most common malignancy of the urinary tract and displays a high rate of recurrence; however, over 70% of recent new cases of bladder cancer are high risk non-muscle-invasive (Alfred Witjes *et al.*, 2017; Siegel *et al.*, 2017). Despite effective chemotherapy and radiotherapy, bladder cancer significantly recurs with resistance to treatments. Therefore, new therapeutics highlight to the use of tumor necrosis factor related apoptosis inducing ligand (TRAIL) that can promote apoptosis in a variety of cancer cells, but not in normal cells, when systemically delivered to animals and humans (Lemke *et al.*, 2014). TRAIL binds to two surface death receptors (DR4 and DR5), which transmit an apoptotic signal via their intracellular death domains (Pan *et al.*, 1997; Kelley and Ashkenazi, 2004). The death domains promote formation of the death-inducing signaling complex (DISC) and activate procaspase-8, which undergoes autocatalytic activation (Walczak, 2013). Active caspase-8 subsequently stimulates executioner caspases through a mitochondria-dependent and/or independent pathway (Crowder and El-Deiry, 2012). In the mitochondria-dependent apoptotic pathway, caspase-8 stimulates a mitochondrial amplification loop by cleaving Bid, a BH3-only member of the Bcl-2 family (Valley *et al.*, 2012), leading to apoptotic death. Researches has shown that systemic therapy with TRAIL is safe in animal models, and phase I clinical trials have been particularly designed to evaluate TRAIL toxicity and antitumor efficacy (Shah *et al.*, 2005; de Miguel *et al.*, 2016). However, *in vitro* data show that up to 50% of cancers acquire resistance against TRAIL by activating cell survival signaling pathways, such as Akt-mediated NF-κB and ERK (Lee *et al.*, 2006; Zhang

and Zhang, 2008). Therefore, understanding the molecular mechanism underlying TRAIL resistance and identifying strategies to reverse the resistance are high priorities for ongoing research on cancer treatment.

Sulforaphane (SFN, 1-isothiocyanato-4-(methylsulfinyl)-butane), a naturally occurring member of the isothiocyanate family from broccoli, has received intense attention for its cancer chemopreventive potential, because it can potently induce phase 2 detoxifying enzymes by activating nuclear factor erythroid-derived 2 related factor 2 (Nrf2) (Boddupalli *et al.*, 2012). Under normal conditions, Nrf2 is suppressed by binding to Kelch-like ECH-associated protein 1 (Keap1) anchored to the actin cytoskeleton in the cytoplasm (Kensler *et al.*, 2007). Under numerous oxidative stimuli, Nrf2 is released from Keap 1 and translocates from the cytoplasm to the nucleus, which sequentially binds to antioxidant responsive elements (AREs) to activate ARE-containing genes, such as heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase-1, and glutathione *S*-transferase-1, resulting in a net antioxidative effect and leading to cancer prevention (Kensler *et al.*, 2007; Jaramillo and Zhang, 2013; Kensler *et al.*, 2013). Additionally, many research confirmed that reactive oxygen species (ROS) are directly involved in mitochondrial dysfunction resulting to cancer apoptosis by downregulating Nrf2 (Choi *et al.*, 2008; Geng *et al.*, 2017). Previous studies have shown that SFN enhances TRAIL-mediated apoptosis in osteosarcoma (Ferreira de Oliveira *et al.*, 2014), prostate cancer (Shankar *et al.*, 2008), lung cancer (Jin *et al.*, 2007) both *in vitro* and *in vivo* by upregulating ROS generation; however, the role of Nrf2 on SFN-mediated TRAIL sensitization has not yet been studied. Thus, it is known that Nrf2 downregulates ROS generation in the cellular systems. Therefore, we need to assess the role of Nrf2 and ROS in stimulation of TRAIL-mediated apoptosis by

SFN, in order to overcome TRAIL resistance.

In the current study, we found that SFN is a potent enhancer of TRAIL-mediated apoptosis in TRAIL-resistant human bladder carcinoma cells. As a result, synergistic induction of apoptosis in cells treated with both SFN and TRAIL (SFN/TRAIL) was associated with increased generation of ROS, despite Nrf2 activation. Thus, our result raises the possibility that SFN/TRAIL might be promising for molecular-targeted chemotherapy in order to overcome TRAIL-resistance in bladder cancers.

2. Materials and methods

2.1. Reagents and antibodies

SFN, *N*-acetyl-cysteine (NAC), propidium iodide (PI) and z-VAD-fmk were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). SFN was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. DMSO (0.05%) was used as a vehicle control. 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine lodide (JC-1) and Enhanced Chemiluminescence (ECL) kit were obtained from Calbiochem (San Diego, CA) and Amersham Biosciences Corp. (Arlington Heights, IL), respectively. RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA) and GIBCO-BRL (Gaithersburg, MD). All other chemicals were purchased from Sigma-Aldrich. Antibodies against caspase-3, caspase-8, caspase-9, poly(ADP-ribose) polymerase (PARP), Bid, DR4, DR5, β-actin and nucleolin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against pNrf2 and HO-1 were purchased from Epitomics (Burlingame, CA) and Calbiochem (San Diego, CA), respectively. Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin were purchased from Amersham.

2.2. Cell culture

Human bladder cancer cell lines (253J, EJ, 5637, T24, J82, and HT1376) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured at 37°C in a 5% CO_2 humidified incubator and maintained in RPMI1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.3. PI and annexin-V FITC staining

Cells were seeded in 6-well plates at a density of 1×10^5 cells/ml and then treated with various concentrations of SFN in the presence or absence of TRAIL for 24 h. After harvesting, the cells were washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol at 4°C for 30 min. The DNA content of the cells was measured using a DNA staining kit (CycleTEST™ PLUS Kit, Becton Dickinson, SanJose, CA) according to the manufacturer´s instructions. For annexin-V staining, live cells were incubated with annexin-V staining kit (R&D Systems, Minneapolis, MN) according manufacturer´s instructions. A FACSCalibur flow cytometer (Becton Dickenson, San Jose, CA) was used to analyze the level of apoptotic cells containing sub-G_1 DNA content and annexin-V^+ population (Wang *et al.*, 2017).

2.4. Protein extraction and western blot analysis

Cells were harvested and lysed with lysis buffer [40 mM Tris (pH 8.0), 120 mM, NaCl, 0.5% NP-40, 0.1 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 100 μg/ml phenymethylsulfonyl fluoride]. After cleaning lysates by centrifugation, protein was quantified by the bicinchoninic acid (BCA) protein assay reagents (Pierce Biotechnology, Rockford, IL). In a parallel experiment, the cells were washed with ice-cold PBS, and cytoplasmic and nuclear proteins were extracted using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology). An equal amount of protein was separated by SDS-polyacrylamide gel, transferred onto polyvinylidene difluoride membrane (Schleicher & Schuell, Keene, NH) and then immunoblotted with the indicated antibodies. The protein content was visualized using an ECL kit according to the manufacturer´s instructions.

2.5. Mitochondrial membrane potential (MMP) assay

To measure MMP, a dual-emission fluorescent dye, JC-1, was used. JC-1 is internalized and concentrated by respiring mitochondria, which reflect change of MMP in living cells. Two excitation wavelengths, 527 nm (green) for the monomer form and 590 nm (red) for the JC-1 aggregate are formed. Briefly, cells were collected and incubated with 10 μM JC-1 for 30 min at 37℃. The cells were washed twice with cold PBS and analyzed using a flow cytometer (Becton Dickinson).

2.6. Transfection of small interference RNA (siRNA)

Cells were transfected with *Bid* and *Nrf2* siRNA, or an equal amount of nonspecific control RNA as a control (Dharmacon, Chicago, IL) using LipofectAMINE 2000 (Invitrogen) according to the manufacturer´s protocol. Following siRNA transfection, the cells were incubated for 24 h, followed by incubation under the indicated conditions.

2.7. Assay of Nrf2 activity

The DNA binding activity of Nrf2 was determined with the TransAM^{TM}Assaykits (Active Motif, Carlsbad, CA) according to the manufacturer´s instructions. An ELISA-based assay consisting of an immobilized oligonucleotide containing the ARE consensus binding site (5′-GTC ACA GTG ACT CAG CAG AAT CTG-3′) was used to measure Nrf2-DNA binding activity. Briefly, nuclear extracts isolated from cells were allowed to bind to the ARE on the oligonucleotide-coated 96-well plates for 1 h and washed three times with PBS containing 0.1% Tween-20. The Nrf2-bound complexes were detected with antibody against Nrf2 for 1 h and then incubated with horseradish peroxidase-conjugated secondary antibodies for another 1 h. For colorimetric detection, the developing solution was added and incubated at room temperature for 10 min before addition of the stop solution, followed by the measurement of absorbance at 450 nm.

2.8. Measurement of intracellular ROS generation

The generation of ROS was determined in cells treated with agent in the presence and absence of NAC, and evaluated with 2′,7′-dichlorofluorescein diacetate (DCF-DA; Molecular Probes, Leiden, Netherlands). In brief, cells were incubated with 10 μM DCF-DA at 37ºC for 30 min. The cells were then washed with PBS and FL-1 fluorescence was measured with a flow cytometer (Jang *et al*., 2017).

2.9. Statistical analysis

All data are presented as mean ± SE. Significant differences between groups were determined using an unpaired one-way ANOVA and Student´s *t*-test. A $p < 0.05$ was considered to indicate statistical significance. The results shown in each of the figures in this article are representative of at least three independent experiments.

3. Results

3.1. Effects of SFN/TRAIL on the apoptotic activity of human bladder cancer cells

In order to address the effects of TRAIL on apoptotic activity, six human bladder cancer cell lines (253J, EJ, 5637, T24, J82, and HT1376) were treated with the various concentrations of TRAIL for 24 h and then flow cytometry analyses were performed. Treatment with TRAIL (0-500 ng/ml) significantly increased apoptosis in 253J, EJ, and 5637 cells in a concentration-dependent manner (Fig. 1). However, TRAIL did not induced apoptosis in T24, J82 and HT1376 cells under the same conditions, suggesting that these bladder cancer cells were resistant to the cytotoxic activity of TRAIL. Next, the apoptotic activity of SFN in TRAIL-resistant bladder cancer cells was investigated. When the cells were treated with SFN at concentrations of 0-20 μM, no significant change appeared in the apoptotic rates, whereas a moderate increase in apoptotic rates appeared when the cells were treated with 40 μM SFN (Fig. 2A), which induces significant cell cycle arrest at G_2/M phase (Fig. 2B).

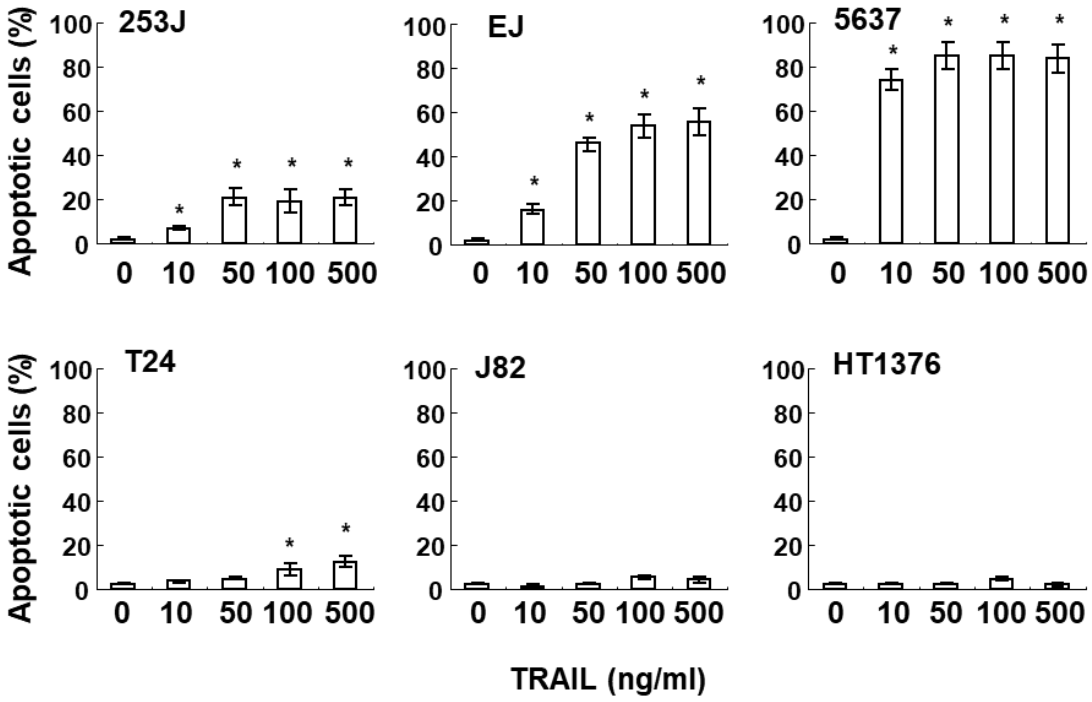

Fig. 1. TRAIL-mediated apoptosis in human bladder cancer cell lines. Six human bladder cancer cell lines (253J, EJ, 5637, T24, J82, and HT1376) were treated with the indicated concentrations (0-500 ng/ml) of TRAIL for 24 h. The apoptotic cells were determined by calculating the percentage of annexin-V^+ and PI^+ cells. Data from three independent experiments are expressed as the overall mean ± standard error (SE). Statistical significance was determined by one-way ANOVA (*, $p < 0.05$ vs. untreated control).

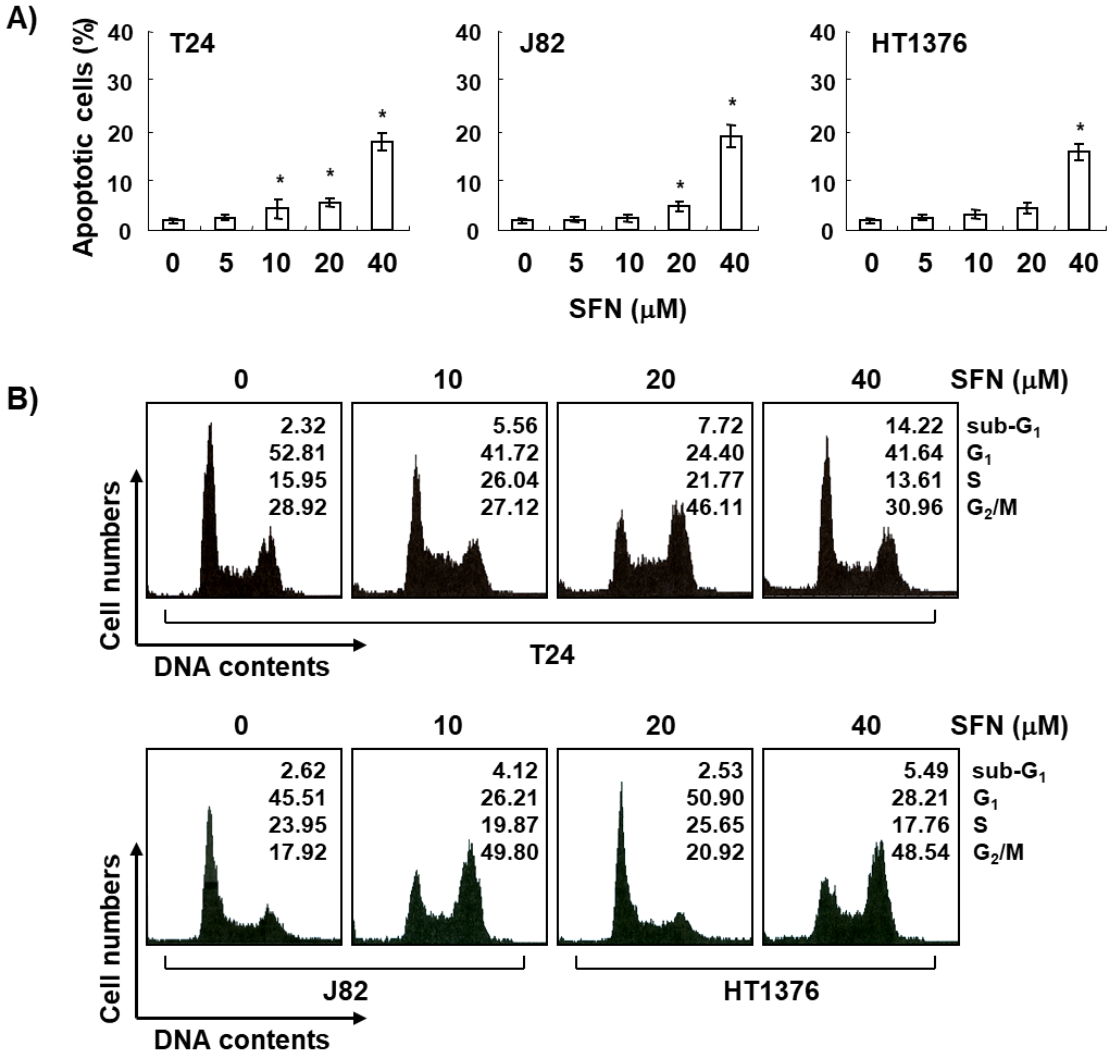

Fig. 2. Induction of apoptosis and cell cycle arrest at G_2/M phase by sulforaphane (SFN) in TRAIL-resistant bladder cancer cells. (A) Cells (T24, J82 and HT1376) were treated with the indicated concentrations of SFN (0-40 μM) for 24 h. The apoptotic cells were determined by counting the percentage of annexin-V^+ and PI^+ cells. The percentage of apoptotic cells is expressed as the mean ± standard error (SE) of three independent experiments. Significance was determined using one-way ANOVA (*, $p < 0.05$ *vs.* untreated control). (B and C) In a parallel experiment, T24 cells were cultured under the same conditions. After the 24-h culture, the cells were collected, fixed and stained with

PI for cell cycle distribution. The percentage of cells in each phase is presented. The data represent the average of two independent experiments.

3.2. Sub-toxic doses of SFN sensitizes TRAIL-mediated apoptosis via activation of caspase in TRAIL-resistant bladder cancer cells

In order to investigate whether SFN sensitizes TRAIL-mediated apoptosis, apoptotic activity was analyzed in three TRAIL-resistant bladder cancer cells: T24, J82, and HT1376. T24 cells were either treated with SFN (2.5-40 μM) alone, TRAIL (50 ng/ml) alone, or SFN/TRAIL for 24 h. SFN/TRAIL treatment resulted in a significant increase in the number of apoptotic cells compared to treatment with SFN or TRAIL alone (Fig. 3A). We further analyzed whether SFN/TRAIL affected activation of caspases and cleavage of PARP, a substrate protein of caspases. Western blot analysis showed that treatment with TRAIL alone causes only a slight decrease in the levels of pro-caspase-3, -8, and -9 with mild cleavage of PARP, whereas no changes occurred in those proteins after treatment of T24 cells with sub-toxic doses of SFN (5-20 μM) alone for 24 h. However, SFN/TRAIL treatment stimulated a significant reduction in the protein levels of pro-caspase-3, -8, and -9, concomitant with a significant cleavage of PARP (Fig. 3B). We also found that SFN/TRAIL resulted in a remarkable increase in the number of apoptotic cells in TRAIL-resistant J82 as well as HT1376 cells (Fig. 3C). To address the significance of caspase activation in SFN-mediated sensitization of apoptosis, we pretreated TRAIL-resistant bladder cancer cells with a general inhibitor of caspases, z-VAD-fmk. Apoptosis was completely prevented by treatment with z-VAD-fmk in the presence of SFN/TRAIL (Fig. 3C). These data indicate that SFN/TRAIL-induced apoptosis requires caspase activation.

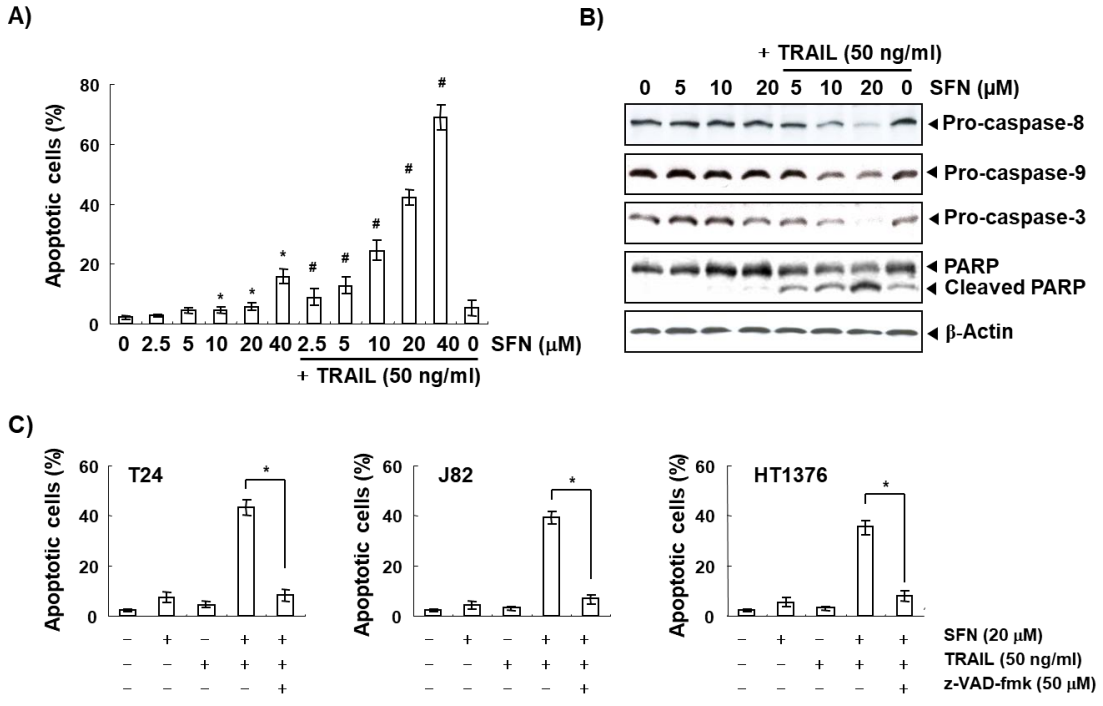

Fig. 3. Caspase-dependent apoptosis by combined treatment with sulforaphane (SFN) and TRAIL (SFN/TRAIL) in TRAIL-resistant bladder cancer cells. (A) T24 cells were incubated with SFN (2.5-40 μM) in the presence and absence of 50 ng/ml TRAIL for 24 h. The apoptotic cells were determined by counting the percentage of annexin-V^+ and PI^+ cells. The percentage of apoptotic cells is expressed as the mean ± standard error (SE) of three independent experiments. Significance was determined using one-way ANOVA (*, $p < 0.05$ *vs*. untreated control and $^\#$, $p < 0.05$ *vs*. TRAIL alone-treated cells). (B) T24 cells were treated with the indicated concentrations of SFN/TRAIL for 24 h. The cells were collected and cell lysates were prepared. Equal amounts of cell lysates (30 μg) were resolved by SDS-polyacrylamide gels, transferred to nitrocellulose membranes and probed with the indicated antibodies. Actin was used as an internal control. (C) Cells (T24, J82 and HT1376) were treated with SFN/TRAIL 24 h after pretreatment with or without a pan-caspase inhibitor (z-VAD-fmk, 50 μM) for 1 h and

the apoptotic cells were determined by counting annexin-V^+ and PI^+ cells. Data are expressed as the mean ± standard error (SE) of three independent experiments. *, $p < 0.05$ indicates a significant difference from the value obtained for cells treated with SFN/TRAIL in the absence of z-VAD-fmk.

3.3. SFN-mediated TRAIL sensitization requires Bid cleavage, leading to mitochondria-mediated apoptosis

The pro-apoptotic and anti-apoptotic members of the Bcl-2 family play pivotal roles in regulating cell growth and death. These proteins induce diverse upstream survival and distress signals in order to decide the fate of the cells. Therefore, we investigated whether SFN/TRAIL induces apoptosis by modulating the expression of Bid. T24 cells treated with TRAIL and SFN alone resulted in a no cleavage of Bid; however, there was significant cleavage in Bid upon SFN/TRAIL treatment (Fig. 4A). Subsequently, the role of the mitochondria in SFN/TRAIL-induced apoptosis was further investigated by examining the values of MMP. Treatment of cells with SFN or TRAIL alone for 24 h had no effect on loss of MMP in J82 and HT1376 cells; however, SFN/TRAIL treatment, as expected, caused a significant increase in loss of MMP in both J82 and HT1376 cells (Fig. 4B). To further confirm the role of Bid in SFN/TRAIL-induced apoptosis, we examined whether transient knockdown of *Bid* using siRNA could provide protection against cell death and loss of MMP. Transient *Bid* knockdown significantly reduced the SFN/TRAIL-mediated loss of MMP (Fig. 4C) and induction of apoptosis (Fig. 4D). These data indicate that SFN enhances TRAIL-induced Bid cleavage, leading to MMP loss and consequent apoptosis.

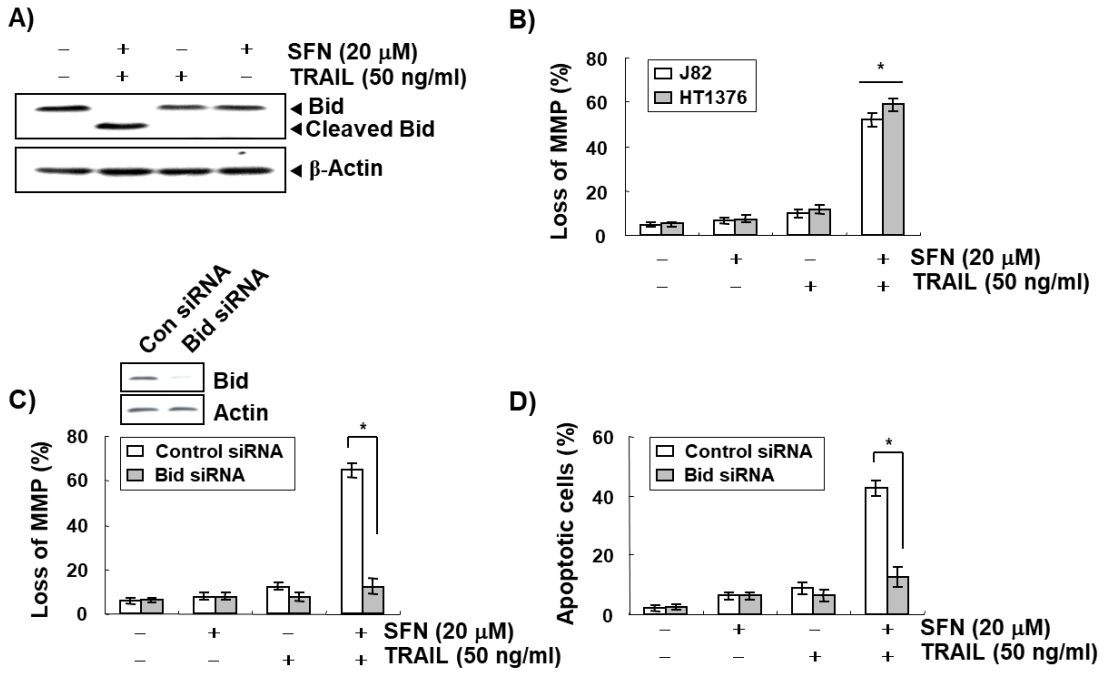

Fig. 4. Effects of sulforaphane (SFN) and TRAIL on Bid expression and MMP values in TRAIL-resistant bladder cancer cells. (A) T24 cells were treated with 20 μM SFN or 50 ng/ml TRAIL alone or with a combination of SFN and TRAIL (SFN/TRAIL) for 24 h. Equal amounts of cell lysate (30 μg) were resolved by SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with anti-Bid antibody. Actin was used as an internal loading control. (B) J82 and HT1376 cells were treated with 20 μM SFN in the presence or absence of 50 ng/ml TRAIL for 24 h and then loss of MMP was evaluated by flow cytometric analysis. Significance was determined using Student´s *t*-test ANOVA (*, $p < 0.05$ *vs.* untreated control). (C and D) T24 cells were transiently transfected with siRNA construct specific for *Bid* or a control construct for 24 h. The cells were then treated with 20 μM SFN in presence or absence of 50 ng/ml TRAIL for 24 h, followed by the analysis of the loss of MMP (C) as well as annexin-V^+ and PI^+ staining (D) using a flow cytometer. The significance was determined by a Student's *t*-

test (*, $p < 0.05$ *vs*. control siRNA in the presence of SFN/TRAIL)

3.4. SFN enhances DR5 expression, resulting in cell death

Because our previous study showed that SFN increases DR5 expression by deactivating Akt in TRAIL-resistant lung adenocarcinoma (Jin *et al.*, 2007), we, in the current study, confirmed whether SFN can also downregulate Akt phosphorylation in TRAIL-resistant bladder cancers, leading to DR5 upregulation. Western blot analysis showed that DR5 protein levels increased in a time- and a concentration-dependent manner, whereas the DR4 expression was weakly sustained by SFN exposure in TRAIL-resistant bladder cancer cells (Fig. 5A and 5B). We next determined the functional role of DR5 expression in response to SFN/TRAIL. As shown in Fig. 5C, the levels of DR5 significantly decreased in response to SFN alone and SFN/TRAIL. We also investigated whether DR5 expression is required for apoptosis induced by SFN/TRAIL. As expected, SFN/TRAIL significantly decreased cell viability and increased the population of apoptotic cell (Fig. 5D and Fig. 5E). However, the transient knockdown of DR5 resulted in a significant decrease of SFN/TRAIL-mediated apoptosis. Thus, these results suggest that SFN-induced DR5 upregulation enhances TRAIL-mediated apoptosis in TRAIL-resistant bladder cancer cells.

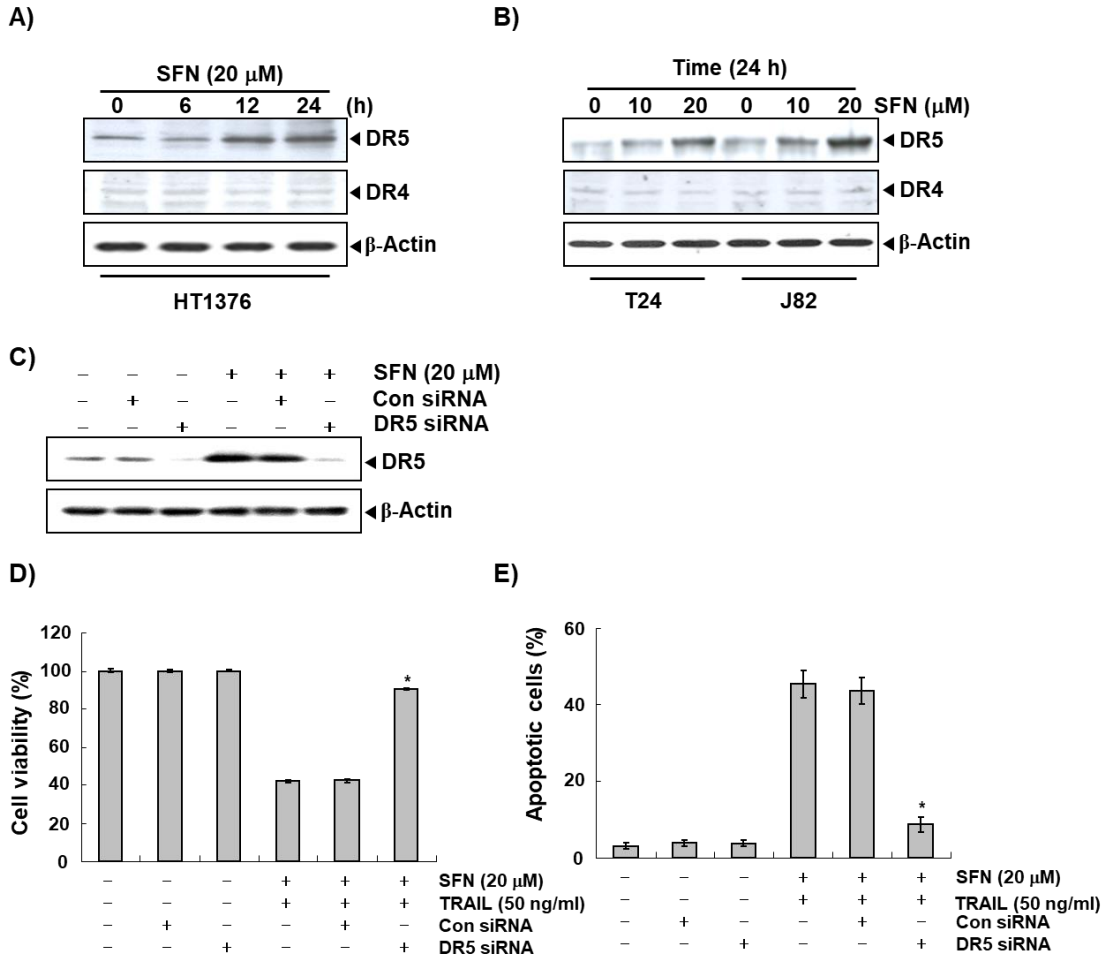

Fig. 5. Effects of DR5 expression by sulforaphane (SFN) in TRAIL-resistant bladder cancer cells. (A and B) Cells (HT1376, T24 and J82) were treated with 20 μM SFN for the indicated times (A) or with the indicated concentrations of SFN for 24 h (B). (C) T 24 cells were treated with SFN in the presence and absence of siRNA targeting DR5. Western blot analysis was performed with antibody against DR5. β-Actin was used an internal control. (D and E) In a parallel experiment, T24 cells were transfected for 48 h and then treated with SFN (20 μM) and TRAIL (50 ng/ml) for an additional 24 h. The degree of cell viability (D) and apoptosis (E) was determined by an MTT assay and annexin-V^+ and PI^+ staining. Results are expressed as percentage of the control ± SE of

three independent experiments. A Student's *t*-test (*, $p < 0.05$ *vs*. SFN/TRAIL) was used for determination of significance.

3.5. SFN/TRAIL increases ROS generation in spite of Nrf2 activation

We next investigated whether SFN/TRAIL treatment upregulates ROS generation accompanied by inactivation of Nrf2-mediated antioxidant mechanisms, leading to apoptosis. Unexpectedly, the levels of total phosphorylated Nrf2 (pNrf2) rapidly increased in response to SFN in J82 and T24 cells, following the levels of HO-1 proteins that increased in a time-dependent manner (Fig. 6A). In addition, we found that ROS generation was also rapidly initiated within 30 min after SFN treatment, peaked at 2 h and then gradually declined (Fig. 6B). To confirm that SFN-induced Nrf2 activation is indeed due to its induction of ROS generation, we used NAC, a well-known ROS scavenger, to antagonize the effects of ROS. Consistent with results of pNrf2 expression, levels of Nrf2 activation significantly increased 2 h after treatment with SFN, which remained elevated at 24 h in J82 cells; however, in the presence of NAC, SFN could not induce Nrf2 activation (Fig. 6C), suggesting that transient ROS production decreased SFN-induced activation of Nrf2. Moreover, examination of cytosolic and nuclear fractions showed that SFN increased the levels of pNrf2 expression in the nucleus in a time-dependent manner; however, NAC inhibited SFN-induced nuclear translocation of pNrf2 reduced in the presence of (Fig. 6D). In addition, SFN/TRAIL did not significantly affect the levels of pNrf2 in the nucleus and markedly reduced Nrf2 proteins in the cytosol, suggesting that TRAIL promotes degradation of SFN-induced Nrf2 (Fig.6E). These results indicate that SFN/TRAIL effectively suppressed the activation of Nrf2 through degradation of Nrf2 proteins.

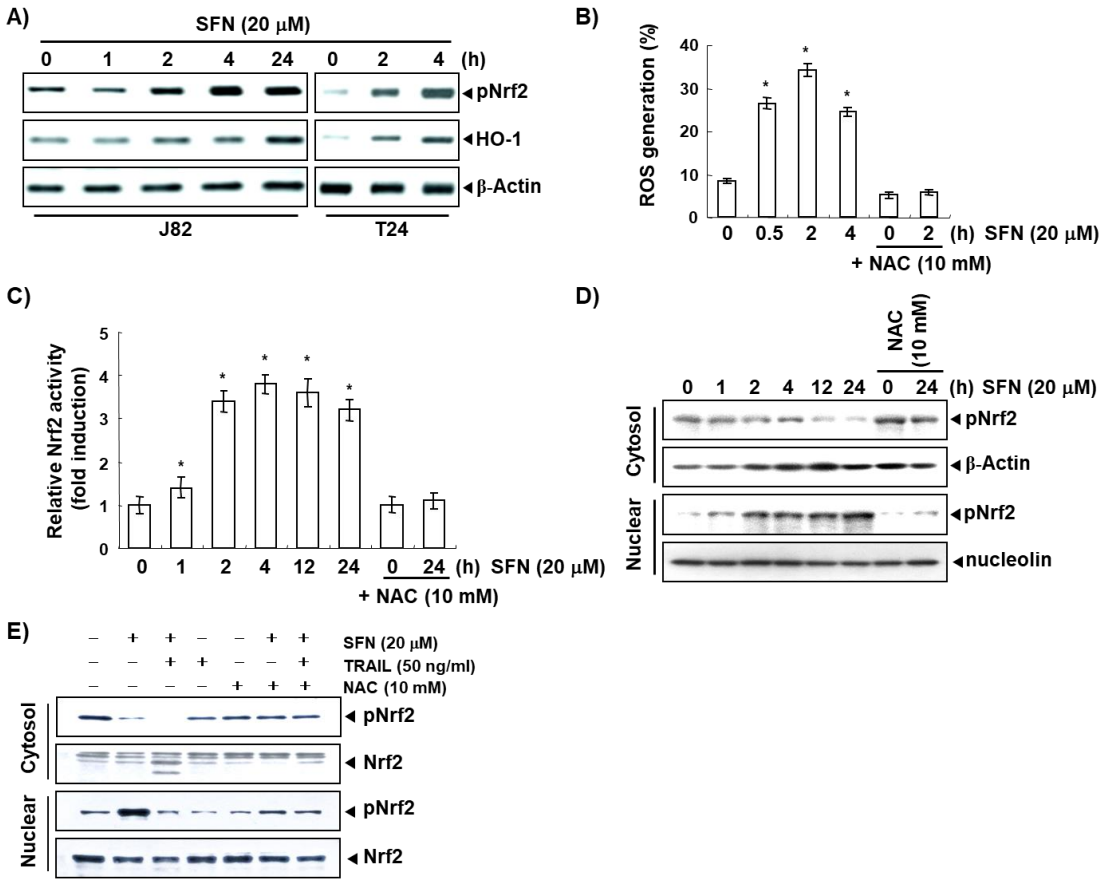

Fig. 6. Nrf2 activation by sulforaphane (SFN) in TRAIL-resistant bladder cancer cells. (A) J82 and T24 cells were treated with 20 μM SFN for the indicated times, and then nuclear (for pNrf2) and total cytosol (for HO-1) proteins were isolated. Equal amounts of cell lysates (30 μg) were resolved by SDS-polyacrylamide gels, transferred to nitrocellulose membranes and probed with anti-pNrf2 and anti-HO-1 antibodies. The amounts of proteins were visualized using an ECL detection system. Actin was used as an internal control. (B) J82 cells were treated with or without 10 mM NAC for 1 h before challenging with 20 μM SFN for the indicated times. For the measurement of ROS generation, the cells were stained with JC-1 for 20 min at 37˚C. The mean JC-1 fluorescence intensity was then detected using a flow cytometer. (C) Transcriptional

activity of Nrf2 was measured by using an immobilized oligonucleotide containing the ARE consensus binding site. In brief, J82 cells were pretreated with or without 10 mM NAC for 1 h and then incubated 20 μM SFN for the indicated times. Equal amounts of nuclear proteins (15 μg) were used for Nrf2/ARE transcription factor assay. The ARE-bound complexes were detected with a phospho-Nrf2 antibody followed by the addition of a secondary HRP-conjugated antibody. (D) In a parallel experiment, T24 cells were pretreated with or without 10 mM NAC for 1 h and then incubated with 20 μM SFN. Nuclear and cytosol proteins were isolated and used for western blot analysis at the indicated times. (E) T24 cells were pretreated with or without 10 mM NAC for 1 h and then incubated with 20 μM SFN and 50 ng/ml TRAIL alone or combination with SFN and TRAIL. After 24 h of incubation, nuclear and total cytosol proteins were isolated. Equal amounts of cell lysates (30 μg) were resolved by SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies against pNrf2 and Nrf2. The results are expressed as the mean ± SE of three independent experiments. Significance was determined using one-way ANOVA (*, $p < 0.05$ *vs*. untreated control).

3.6. TRAIL helps to sustain SFN-mediated ROS generation

Although treatment with TRAIL alone had a minimal effect on the ROS´ status; a more significant change in ROS´ status was observed in bladder cancer cells treated with SFN/TRAIL. Treatment with SFN alone induced maximum ROS levels at 2 h which then gradually declined by 24 h; however, a sustained generation of ROS was observed for 24 h in the cells treated with SFN/TRAIL (Fig. 7A). Next, we evaluated whether SFN/TRAIL-mediated apoptosis was dependent on ROS accumulation. Pretreatment of J82 cells with NAC effectively inhibited the cellular apoptosis induced by SFN/TRAIL treatment (Fig. 7B). Furthermore, transient knockdown of *Nrf2* upregulated SFN/TRAIL-induced apoptosis and also triggered TRAIL-resistant cells into apoptotic stages in the presence of TRAIL alone (Fig. 7C), indicating that intracellular ROS play a critical role in the synergistic induction of apoptosis by SFN/TRAIL. NAC-mediated anti-apoptotic effect was blocked in *Nrf2* knockdown cells treated with SFN/TRAIL. Taken together, these data indicate that SFN/TRAIL-induced apoptosis is mediated by excessive ROS generation, which overcomes Nrf2-mediated antioxidative activity, leading to apoptosis.

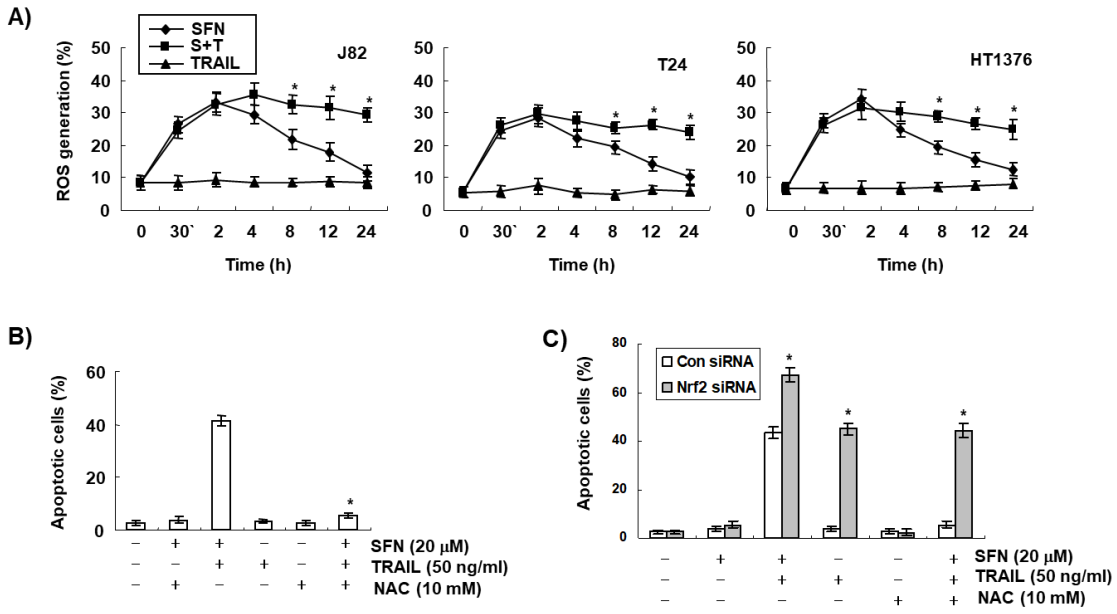

Fig. 7. Sensitization TRAIL-mediated apoptosis by sulforaphane (SFN) through ROS generation. (A) Cells (J82, T24 and HT1376) were treated with 20 μM (SFN) and 50 ng/ml TRAIL alone or combination with SFN and TRAIL (SFN/TRAIL) for the indicated times. The cells were incubated with 10 μM DCF-DA at 37ºC for 30 min and ROS generation was measured using a flow cytometer. Results are expressed as percentage of the control ± SE of three independent experiments. A one-way ANOVA (*, $p < 0.05$ *vs*. SFN alone-treated group) was used for determination of significance. (B) J82 cells were pretreated with or without 10 mM NAC for 1 h, then incubated with 20 μM SFN and 50 ng/ml TRAIL alone or SFN/TRAIL for 24 h. (C) *Nrf2*-targeted siRNA was transiently transfected into J82 cells for 48 h and then treatment with SFN was in the presence or absence of TRAIL for 24 h. Results are expressed as percentage of the control ± SE of three independent experiments. The degree of apoptosis was determined by a flow cytometer using annexin-V^+ and PI^+ staining. A Student´s *t*-test (*, $p < 0.05$ *vs*. each control siRNA-transfected group) was used for determination of significance.

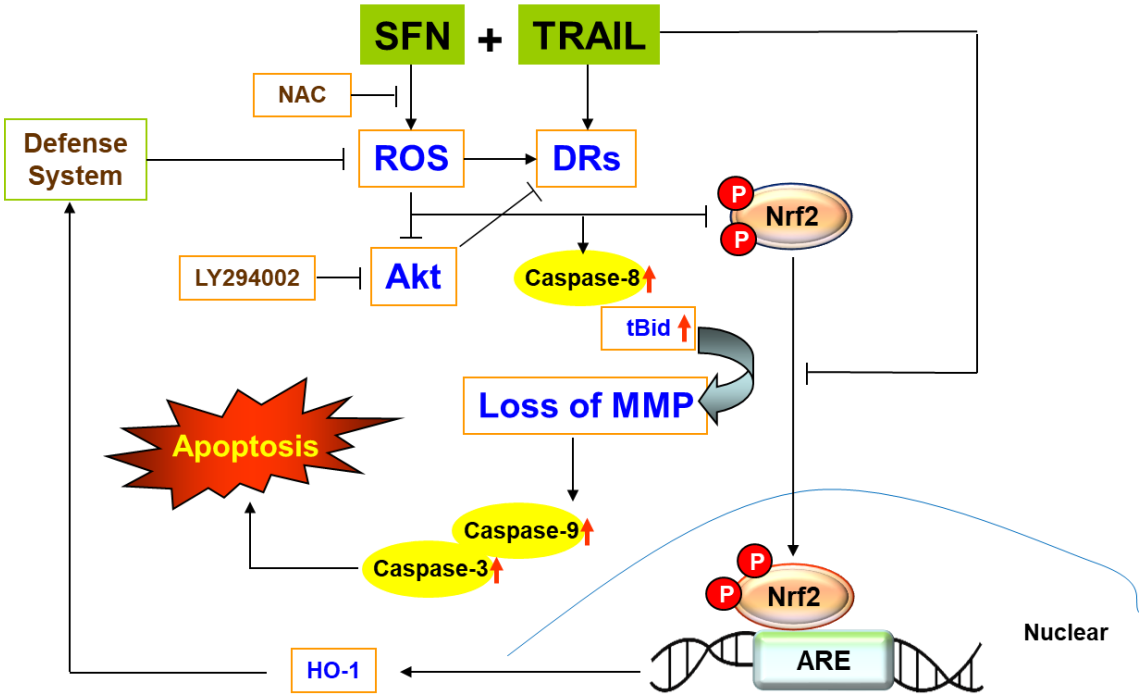

Fig. 8. Schematic model for sulforaphane (SFN) and TRAIL-mediated apoptosis. SFN sensitizes TRAIL-resistant bladder cancer cells to apoptosis by sustaining excess ROS generation, resulting from inhibition of Nrf2. However, Nrf2 activity and expression, intriguingly, increases in response to SFN at the early stage (by 4 h), which prevents cell damages from ROS stress; however, TRAIL helps to sustain ROS generation induced by SFN (up to 24 h), thereby overcoming TRAIL resistance in bladder cancer cells.

4. Discussion

Many studies on chemopreventive drugs have suggested that their beneficial effects on suppression of carcinogenesis and other chronic diseases are mediated through activation of Nrf2 (Shu *et al.*, 2010; Paredes-Gonzalez *et al.*, 2015). Conversely, genetic analyses of human tumors indicated that Nrf2 may be oncogenic and cause resistance to chemotherapy (Niture *et al.*, 2010; Chen *et al.*, 2012). Thus, Sporn and Liby (Sporn and Liby, 2012) rationalized controversial results that the activation, or alternatively inhibition of Nrf2 is a useful strategy for the prevention or treatment of cancer. SFN is a well-known chemopreventive phytochemical found in cruciferous vegetables such as broccoli and is also a known potent inducer of Nrf2/ARE, leading to upregulation of specific genes encoding detoxifying enzymes, such as HO-1 and glutathione (GSH) (Dinkova-Kostova *et al.*, 2017). Data from a previous study suggested that SFN inhibits 4-aminobiphenyl-induced DNA damage though activation of Nrf2 in bladder cells and tissues (Ding *et al.*, 2010). Some researchers also determined that SFN is a powerful sensitizer of TRAIL against many different cancer cells by increasing ROS generation (Jin *et al.*, 2007; Choi *et al.*, 2008; Shankar *et al.*, 2008; Ferreira de Oliveira *et al.*, 2014; Geng *et al.*, 2017). Nevertheless, it is not understood whether SFN sensitizes TRAIL-resistant bladder cancer cells to apoptosis by combining ROS generation and Nrf2 activation. In the current study, we revealed that SFN enhances TRAIL-mediated apoptosis in TRAIL-resistant bladder cancer cells by consistently upregulating ROS generation, resulting from inhibition of Nrf2 (Fig. 8). Surprisingly, SFN increased Nrf2 activity concomitant with ROS generation, but SFN-mediated Nrf2 acted to downregulates ROS generations, which does not diminish excessive ROS generation in

the presence of TRAIL.

TRAIL is known to trigger apoptosis by binding to DR4 and DR5, which contain cytoplasmic death domains responsible for recruitment of adaptor molecules involved in caspase activation (Pan *et al.*, 1997; Schneider *et al.*, 1997; Kelley and Ashkenazi, 2004). Moreover, a study using a phage display of DR-selective TRAIL variants showed that DR5 plays a more prominent role than DR4 in mediation of apoptotic signals emanating from TRAIL, in cells expressing both DRs (Kelley *et al.*, 2005). This indicates that SFN/TRAIL combination is capable of DR5 upregulation and may be a promising strategy for sensitization of tumors to TRAIL-induced apoptosis (Shankar *et al.*, 2008; Bergantin *et al.*, 2014). In addition, previous researches showed that inhibition of Akt-mediated X-linked inhibitor of apoptosis protein expression reverses TRAIL resistance in human bladder cancer cell lines (Shrader *et al.*, 2007) and phosphoinositide-3-kinase (PI3K) inhibitors, wortmannin or LY294002, also downregulates active Akt, causing reversal of cellular resistance to TRAIL (Kandasamy and Srivastava, 2002). In the current study, Akt phosphorylation significantly decreased upon SFN treatment accompanied by downregulation of DR5, suggesting that SFN sensitizes TRAIL-induced apoptosis by inhibiting Akt-mediated DR5 expression (Supplementary Fig 1). This finding is similar to previous demonstration of a linkage between PI3K/Akt and cell death in TRAIL-resistant A549 cells by SFN (Jin *et al.*, 2007). Therefore, SFN could be used to treat bladder-resistant cells when combined with TRAIL by suppressing Akt activity and DR5 expression.

Nrf2 was originally identified to be a critical regulator of intracellular antioxidants by upregulating ARE-containing genes (Kensler *et al.*, 2007). Nrf2 is negatively modulated by Keap1, a substrate adaptor for the Cul3-dependent E3 ubiquitin ligase

complex (Jaramillo and Zhang, 2013; Kensler *et al.*, 2013). Normally, Keap1 constantly targets Nrf2 for ubiquitin-dependent degradation and thus maintains low constitutive expression of Nrf2-downstream target genes. In response to oxidative stress or chemopreventive compounds, Keap1-dependent ubiquitin ligase activity is inhibited and Nrf2 subsequently translocates into the nucleus to activate transcription of ARE-containing genes (Jaramillo and Zhang, 2013; Kensler *et al.*, 2013). During the post-induction period, Keap1 travels into the nucleus to remove Nrf2 from the ARE regulatory sequences and to shuttle Nrf2 back into the cytoplasmic degradation machinery, thereby turning off the Nrf2-dependent responses (Sun *et al.*, 2007). However, there is a controversy whether Nrf2 is tumor suppressive or, conversely, oncogenic, leading to the question that Nrf2 should be targeted for anticancer therapeutic approaches (Kensler and Wakabayashi, 2010). It has been reported that enhancing Nrf2 activity is important for the prevention of cancer (Iida *et al.*, 2004), especially if low doses of drugs are used during the earliest stages of carcinogenesis (Yanaka, 2011). However, in fully malignant cells, enhancement of Nrf2 activity (caused by mutation) can protect tumors from the cytotoxic effects induced by ROS as a result of chemotherapy or that may be produced endogenously by oncogenic signaling in advanced tumors (DeNicola *et al.*, 2011). The effects of Nrf2 inducers are still largely unknown and need further investigation. Carcinogenesis is a continuum and there may be many different premalignant genotypes and phenotypes within a given susceptible organ *in vivo* (Sporn and Liby, 2012). In the current study, we observed that kinetics of ROS generation correlate with nuclear accumulation of Nrf2 treated with SFN. Recently, Rushworth *et al.*, (2011) reported that elevated nuclear Nrf2 reduced ROS levels in acute myeloid leukemia (AML) cells and permitted them to resist

anticancer drug treatment by activating Nrf2-regulated cytoprotective and detoxification genes. This suggests that activation of the Nrf2 reduces the amounts of cellular ROS created by SFN and thus protects bladder cancer cells against apoptosis. Interestingly, we observed that SFN alone enhanced ROS generation at the early times and gradually diminished; however, Nrf2 activity sustained up to 24 h. This indicates that SFN alone-induced Nrf2 downregulates ROS generation to decelerate ROS-mediated cell damages. However, in the presence of TRAIL, SFN-induced ROS generation sustained up to 24 h, suggesting that Nrf2 could not downregulate excessive ROS generation induced by SFN or TRAIL may induce the degradation of Nrf2. In conclusion, SFN upregulates both ROS generation and Nrf2 activity, and TRAIL potently boosts SFN-mediated ROS generation beyond the ability of Nrf2, and this consequently leads to apoptosis in TRAIL-resistant bladder cancer cells.

Taken together, the current study suggests that SFN sensitizes cells to TRAIL-mediated apoptosis by generating ROS via overcoming Nrf2-mediated defense system. In conclusion, the use of TRAIL in combination with SFN might provide an effective therapeutic strategy for the safe treatment of some TRAIL-resistant human bladder cancer cells.

5. 국문요약

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL)는 정상 세포에 대한 독성을 최소화하면서 악성세포에서 세포 사멸을 일으킬 수 있다. 불행히도, 많은 인간 암세포는 알려지지 않은 많은 기전을 통해 TRAIL에 의해 유도되는 세포사멸사에 대해 내성을 지니고 있다. 다양한 십자화과 채소 유래의 chemopreventive isothiocyanate인 Sulforaphane(SFN)의 처리시 인간 방광암 세포주에서 TRAIL저항성이 역전될 수 있음을 확인하였다. SFN과 TRAIL의 병행 요법은 caspase의 활성화, mitochondrial membrane potential (MMP)의 감소, Bid truncation와 death receptor(DR) 5의 유도하여 세포사멸을 유도하였다. Bid의 일시적인 knockdown은 SFN에 의해 유도되는 MMP의 붕괴를 방지하여 SFN/TRAIL의 세포사멸 효과를 감소시켰다. 또한 SFN은 reactive oxygen species (ROS)의 생성과 항산화 효소인 nuclear factor erythroid 2-related factor 2 (Nrf2)의 활성을 증가시켰다. 흥미롭게도, SFN의 단독처리에 비해 TRAIL의 병해 요법은 SFN-mediated Nrf2의 nuclear translocation을 효과적으로 억제하였고, ROS의 생성기간은 연장되었다. 또한, SFN/TRAIL의 병행 요법시 Nrf2의 silencing은 세포사멸을 증가시켰고, ROS의 생성을 막았을 땐 세포사멸 활성을 억제하였다. 이 결과를 바탕으로 SFN에 의해 유도되는 ROS의 생성은 TRAIL 민감성을 향상시키고 SFN을 TRAIL 내성 암의 치료에 사용될 수 있음을 확인하였다.

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Chapter III

CHEMOPREVENTIVE AND CHEMOTHERAPEUTIC EFFECT OF SULFORAPHANE

Abstract

Numerous animal studies and epidemiological evidence have substantiated the chemoprevention effect of increasing cruciferous vegetable intake against cancer, which has been attributed to the activity of various isothiocyanates that are enzymatically hydrolyzed from glucosinolates. Glucoraphanin, a major glucosinolate in cruciferous vegetable including broccoli and broccoli sprouts could be converted by sulforaphane. The chemoprevention properties of sulforaphane against many types of cancers have been shown through different mechanisms as “blocking” and “suppressing” effects. The “blocking” function of sulforaphane is governed through inhibiting Phase 1 metabolism enzymes that convert procarcinogens to carcinogens and inducing Phase 2 metabolism enzymes that promote excretion of carcinogens. Multiple studies have demonstrated the “suppressing” effects of sulforaphane in modulating diverse cellular activities while inducing apoptosis, autophagy, anti-telomerase activity, anti-invasive and anti-metastatic effects and cell cycle arrest. It has been shown that these mechanisms work synergistically to gives better chemoprevention effect. There are several signaling cascades involving for those mechanisms such as the NF-KB signaling pathway, Wnt/beta catenin pathway, the Keap-1-Nrf2 pathway and MAPK/JNK signaling pathway and intracellular ROS production. However, to make any progress in developing safe and effective cancer chemopreventive and chemotherapeutic strategies, a better understanding of these molecular mechanism underling beneficial effects of sulforaphane is required.

1. Introduction

Numerous studies have shown the chemo preventive efficacy of high consumption of cruciferous vegetables against various types of cancers. The Sulforaphane, a major isothiocyanate derived from its glucosinolate precursor (glucoraphanin) which is abundant in cruciferous vegetables such as broccoli, broccoli sprouts and cabbage (D. Clarke et al., 2008). Nevertheless, previous studies show that Sulforaphane modulates multiple targets, which regulate many cellular activities including oxidative stress, apoptosis induction, cell cycle arrest, angiogenesis and metastasis suppression and detoxification of carcinogens.

Numerous studies have substantiated the chemoprevention effect of increasing cruciferous vegetable intake against cancer, which has been attributed to the activity of various isothiocyanates that are enzymatically hydrolyzed from glucosinolates (Li et al., 2010). Glucosinolates are a class of amino-acid-derived secondary metabolites, mainly found in broccoli sprouts. Fahey *et al* reported that glucoraphanin is present in crucifers including broccoli and cauliflower in concentrations of 10 times of that in mature plants (Fahey et al., 1997). When sulforaphane is absorbed, it is conjugated with glutathione and metabolized via the mercapturic acid pathway to be excreted predominantly as *N*-acetylcysteine conjugates (Gasper et al., 2005). This reaction catalyzed by glutathione-*S*-transferases (GST), a family of eukaryotic and prokaryotic phase II metabolic isozymes that inhibits phase I enzymes. There are 3 mammalian GST gene families exist as cytosolic, mitochondrial, and microsomal (Gasper et al., 2005). Cytosolic GSTs are divided into 7 classes based upon their structure: α (*GSTA1-A5*), μ

(*GSTM1-M5*), ω (*GSTO1* and *O2*), π (*GSTP1*), θ (*GSTT1, GSTT2 and GSTT4*), σ (*GSTS1*), and ζ (*GSTZ1*). The Mitochondrial GSTs are belongs to the class κ (*GSTK1*) (Niranjan Reddy et al., 2011). Even though the catalytic activity of relatively few GSTs in isothiocyanates and glutathione conjugation has been studied, some studies suggested, GSTM1-1 and GSTP1-1 have the greatest activity on sulforaphane in general. It is important the high expression of *GSTM1* in the liver combined with its activity may be effect in the metabolism of dietary isothiocyanates. Although Epidemiological studies conducted in the United States have been suggested that*GSTM1*-positive persons attain greater cancer protection from either broccoli consumption or total cruciferous vegetable consumption than do *GSTM1*-null persons (Higdon et al., 2007). However the possible role of other GST polymorphisms in the association between consumption of cruciferous vegetables, isothiocyanate excretion, and cancer risk remains to be explored.

2. Mechanism of chemoprevention

Given the substantial raise in cancers across the globe in developed and developing countries, associated morbidity and mortality, together with the dramatic incensement of costs of treatment, there is increasing interest in strategies for disease prevention. Cancer chemoprevention evokes the use of natural, synthetic or biological substances to reverse, retard or prevent any of the multiple stages of the carcinogenic process. The several recent clinical trials in preventing cancer in high-risk populations successfully show that chemoprevention is a rational and appealing strategy. Numerous epidemiological studies have been reported that Sulforaphane used as an effective therapeutic agent to reduction of various types of cancers in human. The chemoprevention mechanism of Sulforaphane exerts through different mechanisms, including modulation of drug metabolism enzyme, induction of apoptosis and induction of cell cycle arrest (Fimognari et al., 2002). It is suggested that these mechanisms seem to work synergistically to provide the observed health strategies for disease prevention. There are several signaling cascades involving for those mechanisms such as the NF-KB signaling pathway, Wnt/beta catenin pathway, the Keap-1-Nrf2 pathway and MAPK/JNK signaling pathway and intracellular ROS production. However, to make any progress in developing safe and effective cancer chemo preventive strategies, a better understanding of these molecular mechanism underling beneficial effects of Sulforaphane is required.

A. Induction of apoptosis

Apoptosis is the process of programmed cell death is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanism that occurs under physiological as well as pathological conditions. Series of biochemical events lead to characteristic changes in cell morphology and death including bebbling of the plasma membrane, chromatin condensation, cell shrinkage, nuclear fragmentation and chromosomal DNA fragmentation. The apoptosis process mainly controlled by a diverse range of cell signals, which may originated either as extracellularly (extrinsic inducers) or intracellularly (intrinsic inducers) (Korraah et al., 2012). Extracellular signals such as growth factors, nitric oxide or toxins can be either trigger or repress apoptosis process. On the other way, in response to stress conditions cell can be initiated apoptotic signals intracellularly, as a massage for death a cell by itself. As a response to binding of nuclear receptors by increase intracellular calcium concentration, nutrient deprivation, hypoxia conditions, radiation and viral infections cell can trigger the release of intracellular apoptotic signals. Although mitochondria are key regulator of intrinsic apoptotic pathway that affect to the apoptosis process in different ways. The release of mitochondrial proteins, SMACs (small mitochondria-derived activator of caspases) binds to IAPs (inhibitor of apoptosis proteins) and deactivates them by allowing to proceeding apoptosis process (Du et al., 2000). Moreover, IAPs can suppress the cysteine protease or caspases activity, which are involve for the degradation of the cell, therefore mitochondria are indirectly involved for the regulation of these proteins. Because of the formation of a mitochondrial apoptosis-induced channel (MAC), cytochrome c can be released from mitochondria in the outer mitochondrial membrane hence cytochrome c binds to Apoptotic protease

activating factor-1 (Apaf-1) and ATP, which then bind to pro-caspase-9 to create a protein complex known as an apoptosome (Zou et al., 1999). Then subsequently recruit multiple procaspase-9 molecules to the complex and facilitate their autoactivation. The caspase-9 recruited to the apoptosome is able to efficiently cleave and activate downstream target caspases such as caspase-3. These caspases subsequently cleave many important intracellular substrates, leading to characteristic morphological changes in apoptosis and form the apoptotic bodies.

Apoptosis is important for normal development and tissue homeostasis, but too much or too little apoptosis has been implicated in an extensive variety of diseases, such as ischemic damage, autoimmune diseases and various types of cancers (Fadeel et al., 2005). However, chemotherapy induced apoptosis on the way it has a possibility to decreases the severity of these diseases. Sulforaphane has been shown to the inhibitory effects of tumor cell growth *in vitro* and *in vivo* by inducing apoptotic mechanism, has emphasize attention. Exposure of human prostate cancer cells to growth suppressive concentrations of Sulforaphane resulted in ROS generation, which was accompanied by caspase dependent apoptosis (Singh et al., 2005). Incubation of human pancreatic cell lines MIA PaCa-2 and PANC-1 at higher Sulforaphane doses (> 10 μmol/L) resulted in induction of apoptosis (Pham et al., 2004). There is accumulating evidence that Sulforaphane can induce apoptosis, by the way it can inhibit the growth of tumor cells. The apoptotic mechanism induced by Sulforaphane have been demonstrated many *in vitro* and *in vivo* studies from human epithelial carcinoma (Devi, J.R. and Thangam, E.B., 2012), colon (Gamet-Payrastre, et al 2000), bladder (Abbaoul et al., 2012), salivary gland adenoid cystic carcinoma (Chu,et al 2009), pancreatic (Pham et al., 2004), lung (Liang et al., 2008), ovary (Chuang et al., 2010), cervical (Sharma et al., 2011),

and hepatocarcinoma (Park et al., 2007). Human colon cancer cell line HT29 cells treated Sulforaphane in concentration dependent manner induced cell death via an apoptosis process. Moreover this apoptosis process clearly confirmed by observing translocation of phosphatidylserine from inner layer to outer layer of plasma membrane, nuclear chromatin condensation and ultrastructural modifications related to the apoptotic cell death. Further determined this apoptosis process mediated through the proteolytic cleavage of poly (ADP-ribose) polymerase, release of cytochrome C from mitochondria to cytosol, increased the bax protein content that express after 24 hrs of the treatment of 5 and 15 μM Sulforaphane by confirming bax dependent apoptotic pathway (Gamet-Payrastre et al., 2000). Ovarian carcinoma cell line OVCAR-3 and SKOV-3 resulted in approximately 50% cell density decrease after 48 hrs of incubation with Sulforaphane 2 μM and flow cytometry analysis showed increase in the sub G1 cell population. Further ovarian carcinoma cell line OVCAR-3 showed 6, 8, and 17% apoptosis with the treatment of 2, 10, and 50 μM Sulforaphane respectively. Also, time dependently treatment as 2 days and 3 days of Sulforaphane in OVCAR-3 cells showed 18% and 42% of cell population underwent apoptosis, respectively (Chuang et al., 2010). Hence, apoptotic mechanism is an important target of Sulforaphane, thus prevention of cancer with increased efficacy.

B. Induction of cell cycle arrest

Cell cycle checkpoints are control important mechanisms that ensure the fidelity of cell division at each phase of cell cycle have been accurately completed before progression into the next phase. The regulation of four distinct phases of cell cycle; G1, S, G2 and M involves processes crucial to the survival of a cell, including the detection and repair of genetic damages as well as the prevention of uncontrolled cell division (Sa and Das., 2008). Cyclins and cyclin-dependent kinases (CDKs) are two key classes of regulatory molecules determine a cell's progress through the cell cycle (Tarailo-Graovac and Chen, 2012). Cyclins form the regulatory subunits and have no catalytic activity. CDKs the catalytic subunits of an activated heterodimer; and CDKs are inactive in the absence of a partner cyclin. When CDKs are activated by a bound cyclin, CDKs perform a common biochemical reaction called phosphorylation that activates or inactivates target proteins to orchestrate coordinated entry into the next phase of the cell cycle. CDKs are constitutively expressed in cells since cyclins are synthesised at specific stages of the cell cycle, in response to various molecular signals. There is accumulating evidences that Sulforaphane inhibits the proliferation of cancer cells both *in vitro* and *in vivo* by causing cell cycle arrest. Cell cycle arrest by Sulforaphane mediates through different mechanisms such as up-regulation of cyclins, tubulin depolimerization by disrupting mitotic progression. The exposure of KB and YD-10B human oral carcinoma cells to 20 and 40 μM of Sulforaphane for 12 h effectively inhibit the cell proliferation by arresting cell cycle at the G2/M phase (Kim et al., 2010). Using human colon cancer cell line HT29 was reported Sulforaphane induced cell cycle arrest correlate with an incensement of expression of cyclins A and B1 during their exponentially growing phase (Gamet-Payrastre et al., 2000). The cell cycle arrest by

Sulforaphane has been reported in G1 arrest in epithelial ovarian cancer cells (Bryant et al., 2010), G2/M phase arrest in salivary gland adenoid cystic carcinoma (Chu et al., 2009), acute lymphoblastic leukemia (Suppipat et al., 2012), bladder (Abbaoui et al., 2012), osteosarcoma (Kim et al, 2011), prostrate (Xiao et al., 2009), and lung (Liang et al., 2008). Moreover, several recent studies shows that Sulforaphane induce cell cycle arrest through the tubulin depolymerization. It is known that microtubules play a pivotal role for the formation of mitotic spindle, used by most eukaryotic cells to segregate their chromosomes correctly during cell division. Exposure to concentrations of up to 15 μM Sulforaphane of Bovine aortic endothelial (BAE) cells for 24 hrs induced G2/M accumulation and pre-metaphase arrest. Furthermore this study shows the same concentration of Sulforaphane disrupting mitotic progression and has a possibility to disturb normal polymerization of mitotic and cytoplasmic microtubules (Xiao et al., 2009). The synchronized BALB/c mouse mammary carcinoma cell line F3II was treated at concentrations up to 15 mM Sulforaphane resulted in elevated numbers of prophase/ prometaphase mitotic morphology. Moreover, this study indicates cells treated with 15 mM Sulforaphane displayed aberrant mitotic spindles, and higher doses of Sulforaphane inhibited tubulin polymerization in vitro (Jackson and Singletary, 2004). By the mechanism inhibition of tubulin polymerization has been much attention recently using Sulforaphane based angeogenisis studies also. Therefore Sulforaphane mediated cell cycle arrest governs through more than one possible mechanism.

C. Induction of autophagy

Autophagy is a highly conserved pathway from yeast to mammals, which involve cell degradation of unnecessary or dysfunctional cellular components through the actions of lysosomes. It is considered to be important for cellular homeostatic quality control, specially under nutrient-deficient or stress conditions, by breakdown of cellular components in order to either supply the nutrients required for alternate energy metabolism pathways or remove toxic components for cell survival. There are three different forms of autophagy that are commonly described; macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Kaushik et al., 2008). Macroautophagy requires the formation of a double-membrane vesicle, known as autophagosome, which upon formation sequesters cytosolic components for delivery to the lysosome. Although autophagy is mostly a non-specific degradative process, there are some selective forms of autophagy in terms of cargo selectivity (e.g. mitophagy for mitochondria, nucleophagy for nucleus, peroxophagy for peroxisomes, reticulophagy for endoplasmic reticulum, xenophagy for pathogens, and granulophagy for stress granules). In microautophagy, cytosolic materials are directly engulfed by invagination of the lysosomal membrane while CMA involves the recognition by the hsc70-containing complex. CMA, is a very complex and specific pathway, utilizing heat shock chaperone of 70 kDa, hsc70, which recognizes the pentapeptide KFERQ motif upon misfolding of protein complexes (Badadani, 2012). Then this complex moves to the lysosomal membrane-bound protein that will recognize and bind with the CMA receptor, allowing it to enter the cell. While recognition, the substrate protein get unfold and it is translocated across the lysosome membrane with the assistance of the lysosomal hsc70 chaperone. The process of initiation, nucleation and elongation/maturation of the

autophagy vesicle requires several different AuTophaGy (ATG) proteins such as Atg-13, Atg-17 and Atg-7 together ULK1 kinase complex which consists of ULK1, and receives stress signals from mTOR complex 1 .When inhibited the activity of the mTORC1 kinase, autophagosome formation occurs. The ULK1-ATG13-FIP200 complex controlled the initiation process while Beclin-1-Class III PI3K requires for the nucleation process. The elongation and maturation process require associated protein complex, and two conjugation systems. Autophagosome formation also requires Atg12 and LC3B, protein conjugation systems that resemble ubiquitin. LC3B is normally present in the cytoplasm (LC3B I) and it is only upon its post-translational modification, where a phosphatidylethanolamine lipid group is added (LC3B II) that it associates with the membrane of the autophagosome (Hancock et al., 2012). Then autophagosome move through the cytoplasm of the cell to a lysosome, and the two organelles fuse; intersection with endosomal pathways also occurs. Within the lysosome, the contents of the autophagosome are degraded via acidic lysosomal hydrolases. Emerging evidence indicates that autophagy plays a role in human diseases including neurodegenerative diseases and various types of cancers (Zhu et al., 2009). Although autophagy exerts a cell protective role, against cancers by isolating damaged organelles, allowing cancer cells to survive against cytotoxic agents; on the other hand, autophagy results in the death of cancerous cells through autophagic cell death or type II cell death.

Recently, sulforaphane was shown to induce autophagy that functions as a cell protective mechanism in various cancer types (Kanematsu et al., 2010). Therefore, inhibition of autophagy potentiates anti-angiogenic effects of Sulforaphane by inducing apoptosis. As an example as the in vitro model of angiogenic endothelial cells Human umbilical vein endothelial cells (HUVECs) were treated low concentration of

Sulforaphane for 16 hrs resulted in the formation of acidic vesicular organelles (AVOs) and the recruitment of LC3 to autophagosomes, the characteristic marker of autophagy. Then the specific autophagy inhibitor (3-methyladenine) were treated these cells potentiated the proapoptotic effect of Sulforaphane (Nishikawa et al., 2010). Although, Sulforaphane exerts its angiogenic effects by inhibiting the formation of capillary like structures these cells by inhibition of autophagy (Nishikawa et al., 2010). Further, using human breast cancer cells, MCF-7 and MDA-MB-231 were shown appearance of autophagic vacuoles, punctuates pattern of microtubule-associated protein 1 light chain 3 (LC3) and the accumulation of acidic vesicular organelles as a typical features of autophagy in the treatment of Sulforaphane (Kanematsu et al., 2010). Nevertheless, the treatment of Sulforaphane was induced autophagy in human colon cancer cells (Nishikawa et al., 2010), prostate cancer cells (Herman-Antosiewicz et al., 2006) also were reported. The induction of autophagy in response to Sulforaphane was reported in human prostate cancer cell lines LNCaP and PC-3 by showing typical morphological features of autophagy including formation of acidic vesicular organelles and appearance of membranous vacuoles in the cytoplasm. This induction of autophagy by Sulforaphane was related with induction of LC-3 protein and this study also indicates that induction of autophagy represents a cytoprotective mechanism against Sulforaphane-induced apoptosis in human prostate cancer cells (Herman-Antosiewicz et al., 2006). The recent study has implicated ROS generation and autophagic response in human prostate cancer cells treated with Sulforaphane. The treatment of Sulforaphane resulted in minimal effect on catalase protein level in wild-type LNCaP and PC-3 cells and their Rho-0 variants. Further, Sulforaphane treatment resulted in cleavage of LC-3 in both wild-type LNCaP and PC-3 cells while this LC-3 cleavage partially suppressed

in Rho-0 variants of both cell lines. The wild-type LNCaP and PC-3 cells treated for 9 h with 40 μmol/L sulforaphane were shown punctuate pattern of LC-3 immunostaining. Therefore this study indicated that Sulforaphane-induced autophagy was partially depending on the ROS production (Xiao et al., 2009). Therefore, those studies indicate a cytoprotective role of autophagy against Sulforaphane -induced apoptosis and that the combination of sulforaphane treatment with autophagy inhibition may be a promising strategy for cancer chemoprevention.

D. Anti-telomerase activity

It is well known that cancer may be induced by a plethora of both external and internal factors, including genetic mutations. Among those internal factors, telomerase activity, generally not detectable in most somatic cells in contrast, during carcinogenesis it is activated as frequently as 85-95%, confers survival of tumor cells (Ahmed and Tollefsbol, 2003). Telomerase activity is one of the most important factors that have been linked to multiple process that the ends of linear chromosomes from DNA repair and degradation, and their maintenance is critical for long-term cell proliferation and survival.

Mammalian telomeres consist of tandem DNA sequence TTAGGG repeats to the 3' end of DNA strands in the telomere regions that are bound by a specialized six-protein complex known as shelter in and may be replenished by telomerase. Telomerase are the region of repeated nucleotide contains non coding DNA and hinders the loss of important DNA from chromosome ends. As a consequence, there are 100–200 nucleotides may be lost at each of the chromosome repetition. Human telomerase composed of two essential components, a catalytic subunit with reverse transcriptase activity, telomerase reverse transcriptase (*hTERT*) that is essential for the reconstitution of telomerase activity and a telomerase RNA component (*hTERC*) (Horikawa et al., 1999). Many studies have been defined *hTERT* expression as the rate limiting factor in regulating telomerase activity thus *hTERT* regulation occurs primarily at the transcriptional level. Therefore it has been shown that ectopic expression of *hTERT* in telomerase-negative cells is sufficient to restore telomerase activity.

Among the various strategies of molecular therapeutics, epigenetic alterations have gained much attention as govern fundamental role during carcinogenesis process. In fact

DNA methylation is a one of major form of epigenetic control of gene expression that involves a covalent modification at the fifth carbon position of cytosine residues within CpG dinucleotides, resulting in the transcriptional silencing of the affiliated gene (Ding et al., 2004). Recently it has been more attention for the promoter hypermethylation of tumor suppressor genes since it may involve for the silencing of genes in cancer cells, resulting in tumor initiation and progression (Sharma et al., 2009). In addition to DNA methylation, another important factor in the epigenetic control of gene expression is histone modification, including acetylation, methylation, ubiquitination and phosphorylation. The *hTERT* promoter contains two E-box regions and five GC boxes and CpG Island, indicating a role for methylation in the regulation of *hTERT* expression. The growing body of evidence indicates that *hTERT* contains an increased level of DNA methylation in its promoter region in various types of cancers (Kyo et al., 2008).

The knowledge of the molecular basis of carcinogenesis has more attention for the discovery of effective and less toxic chemopreventive agents; sulforaphane has considerable attention due to it exert anti-telomerase activity. Many studies revealed that Sulforaphane has been shown anti-telomerase activity in different types of cancers. The bioactive components in cruciferous vegetables like Sulforaphane could alter the histone modifications and DNA methylation necessary for the oncogene activation or silencing. This study suggests these bioactive components involve for the epigenetic modifications such as inhibition of expression of *hTERT* like oncogene during the carcinogenesis process and induce the expression of tumor suppressor genes such as $p21^{WAF1/CIP1}$ (Meeran et al., 2010). Sutapa *et al.* have been reported treatment with Sulforaphane significantly suppressed the telomerase activity in a concentration dependent manner in cervical cancer cell line HeLa as observed by decrease in the

telomeric repeat band pattern (Mukherjee et al., 2009). Moreover, Sulforaphane potentially causes epigenetic repression of *hTERT* expression in human hepatocellular carcinoma Hep3B cells. Hep3B cells treated with Sulforaphane inhibit telomerase activity via downregulation of telomerase reverse transcriptase (*hTERT*) expression. This study further suggests due to exposure to low dose of Sulforaphane may be elevated intracellular reactive oxygen species (ROS) levels and due to suppression of the phosphorylation of Akt (Ser-473), resulted in suppressed the telomerase activity via transcriptional and posttranslational regulation of *hTERT* expression (Moon et al., 2010). Using human breast cancer cell lines MCF-7, MCF-10A and MDAMB-231 Sulforaphane was found to potentially suppress the telomerase activity. The MCF-7 and MDAMB-23 cells treated with Sulforaphane in concentration dependent manner and time dependent manner resulted in inhibition of the catalytic regulatory subunit of telomerase, human telomerase reverse transcriptase (*hTERT*) in both cell lines. The intense hypermethylation at the translation site and CTCF binding site were detected in non Sulforaphane treated MCF-7 and MDAMB-23 cells. In this regards treatment of 5 μM and 10 μM sulforaphane for 6 days reduction in methylation status as 32% and 21% in MCF-7 cells where as 64% and 35% in MDA-MB-231 cells and 21% and 14% in MCF-10A cells respectively. Therefore MDA-MB-231 cells were shown dramatic demethylation status in the regions of the *hTERT* 5′-regulatory region with the concentration dependent exposure of Sulforaphane rather than the MCF-7 cells. However the demethylation effect of Sulforaphane treatment was negligible in MCF-10A cells. The Sulforaphane treatment also suppressed the DNA methyltransferases (*DNMTs*), especially *DNMT1* and *DNMT3a*, suggesting that Sulforaphane may repress *hTERT* by impacting epigenetic pathways. In Chromatin immunoprecipitation (ChIP)

analysis of the *hTERT* promoter revealed that Sulforaphane treatment cause to increase the level of active chromatin markers acetyl-H3, acetyl-H3K9 and acetyl-H4, whereas decreasing the trimethyl-H3K9 and trimethyl-H3K27 inactive chromatin markers in a dose-dependent manner. Further this study revealed suppression of hTERT expression facilitated the induction of apoptosis in human breast cancer cells (MMeeran et al., 2010). Nevertheless the combination treatment of sulforaphane with epigallocatechin gallate, EGCG the major components of green tea on the expression of *hTERT* was reported in ovarian carcinoma cells. In this study, both paclitaxel-sensitive (SKOV3TR-ip1) and paclitaxel-resistant (SKOV3TR-ip2) ovarian carcinoma cell lines alone or in combination exposure to Sulforaphane or EGCG. The combined treatment of 10 μM Sulforaphane or 20 μM EGCG treatment increases apoptosis significantly while reducing the expression of *hTERT*, the main regulatory subunit of telomerase in paclitaxel-resistant SKOV3TR-ip2 ovarian carcinoma cells after 6days of treatment (Chen et al., 2013). Therefore, due to suppression of *hTERT* expression by Sulforaphane it may use effectively for development of anti-cancer chemotherapeutics.

E. Anti-invasive and anti-metastasis effect

Invasion and metastasis are the most insidious and life-threatening aspects of cancer. Cancer metastasis is a complex process that involves the spread of cancer cells to distant organs of the body from its original site. To metastasize, cancer cells must penetrate the extracellular matrix component by proteolytic degradation. For this process many factors are involve; one group of enzymes known to play a key role is the Matrix metalloproteinases (MMPs) (Curran and Murray, 2000). The evidence that links MMPs with metastasis is also extensive and has been broadly reviewed. However, recent studies suggest that MMPs play a complex role in metastasis and thus they may make important contributions at other steps in the metastasis process. MMPs involve the breakdown of physical barriers to metastasis, therefore promoting invasion and entry into and out of blood or lymphatic vessels known as intravasation and extravasation respectively. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases belongs to a larger family of proteases known as the metzincin superfamily. MMPs are secreted as inactive zymogens and require activation for catalytic function. Currently, 24 MMP genes have been identified in humans, among them two gelatinase enzymes; MMP-2 and MMP-9 in particular have been implicated in cancer invasion and metastasis process. Due to the collagen degradation in the basement membrane these enzymes also known as type IV collagenases. Like all MMP family members, the activity of MMP-2 and MMP-9 is tightly regulated at multiple levels, including gene transcription, activation of the latent precursor, secretion, and inhibition by tissue inhibitory of MMPs (TIMPs). The TIMPs are the low molecular weight, locally synthesized proteins that bind to active form of the MMPs, inhibiting proteolytic activity (Gomez et al., 1997). The latent form of the MMP-2 and MMP-9 proteinases

can be formed complexes withTIMP-2 and TIMP-1, respectively. There are numerous studies have been shown imbalance between MMP/TIMP implicated in the progression of tumors to malignant phenotypes. There are numerous studies have reported that MMPs are key regulators of progression of tumors at both primary and metastatic sites. On the other point of view suggest that MMPs are involve for the creating and maintaining the micro-environment that suitable for the initiation and maintenance of growth of both primary and metastatic tumors. Therefore inhibition of MMPs might thus lead inhibition of progression of cancer and to inhibition of dissemination. Because of this potential, researchers have much focused on different approaches to block the activation of MMPs by using chemopreventive agents.

Several numbers of studies have shown anti-invasive and anti-metastasis effect of Sulforaphane. The human oral squamous carcinoma cell line YD10B down-regulated the expression of MMP-1 and MMP-2 with the treatment of Sulforaphane. The results of this study also showed that low concentration of 1μM Sulforaphane inhibits the migration and invasion of these oral carcinoma cell line (Jee et al., 2011). The anti-invasive effect of Sulforaphane was reported in melanoma cells in the *in vitro* invasion assay with the exposure of Sulforaphane. According to this invasion assay invasion of B16F-10 melanoma cells across the collagen matrix was inhibit considerably by Sulforaphane. Also, inhibition of the MMP activity was conducted via gelatin zymographic analysis, but the inhibition of the expression of MMP in this experiment was not shown (Thejass and Kuttan, 2005). Further, this study reported the anti-metastatic potential of Sulforaphane in metastasis melanoma cells. The metastatic melanoma cells were injected as three different modes as simultaneous injection with cancer cells, prophylactic injection before cancer cell injection and injection after the

development of tumor into tail vein of C57BL/6 mice and observed the effectiveness after the injection of Sulforaphane. All of these modes reduced the formation of nodule in the lung, while simultaneous mode of Sulforaphane application was shown most effectiveness (Thejass and Kuttan, 2005). The expression of metastasis markers such as MMP-9, MMP-7, MMP-2 and MT1-MMP in prostrate carcinoma cells were reduced, as indicated by immunohistochemistry in a mouse orthotopic prostate carcinoma model with the treatment of Sulforaphane. Further, this study indicated the combination effect of Sulforaphane and TRAIL was more effective in inhibiting these metastasis markers (Shankar et al., 2008). Therefore these results suggest Sulforaphane can inhibit prostate cancer progression by suppressing metastasis via down-regulation of MMPs.

F. Anti-inflammatory effects

Even though, inflammatory process is a beneficial host response to external challenge or cellular injury involving signals that both initiate and maintain inflammation, prolonged inflammation can be harmful and can contribute to the pathogenesis of many diseases (Lucas et al., 2006). In this regard, macrophages are major mononuclear phagocytic cells that release a broad spectrum of inflammatory mediators, including pro-inflammatory and cytotoxic cytokines, growth factors, bioactive lipids, hydrolytic enzymes, reactive oxygen intermediates, and nitric oxide (NO), with all of these mediators being implicated in the pathogenesis of tissue injury (Watkins et al., 1995). Therefore, regulating the expression levels of pro-inflammatory mediators is thought to be the most effective strategy for the treatment of inflammation. For this purpose anti-inflammatory substances used; it is refers to the property of a substance or treatment that reduces inflammation as opposed to opioids, which affect the central nervous system. Research shows Sulforaphane is a highly pleiotropic substance capable of interacting with numerous molecules and protein complex that controls the transcription of DNA of particular molecules targets involved in inflammation. The accumulating evidence from clinical and epidemiological studies indicate Sulforaphane mediates its anti-inflammatory process by regulating Nrf2 (Fragoulis et al., 2012) and (Lin et al., 2008), nuclear factor-kB (NF-kB) and AP-1(Kim et al., 2011).

Through regulation of these signaling cascades Sulforaphane may have beneficial effects to suppress various types of inflammatory diseases such as atherosclerosis (Kim et al., 2011), cardiovascular and neurodegenerative diseases (Jeffery and Araya., 2009). The epidemiological studies demonstrated that relationship between the high

consumption of cruciferous vegetables related with the reduction of incidence of above inflammatory diseases.

Sulforaphane possesses its anti-inflammatory activity by suppressing of LPS-stimulated pro-inflammatory mediators, iNOS, COX-2 and TNF-α in RAW macrophages. According to western blot analysis, this induction of iNOS and COX-2 were represses at transcriptional level in time and dose dependent manner with the treatment of sulforaphane (Heiss et al., 2001). An another study on anti-inflammatory effect of sulforaphane in primary rat microglia cells indicates the suppression the LPS-induced expression level of pro-inflammatory mediators such as TNF-α, IL-1β and IL-6 with the Sulforaphane treatment. Exposure of primary rat microglia cells to sulforaphane resulted in significantly decreases the LPS-induced NO expression in concentration dependent manner. In this regards sulforaphane may be an effective therapeutic strategy to prevent neurodegenerative diseases and inflammation in the brain (Brandenburg et al., 2010). Nevertheless the Synergistic anti-inflammatory effects of sulforaphane with nobiletin have been reported using lipopolysaccharide-stimulated RAW 264.7 cells. The combination treatment of sulforaphane and nNobiletin synergistically decreased iNOS and COX-2 protein expression levels which related to the expression of pro-inflammatory mediators NO and COX-2 respectively, and induced heme oxygenase-1 (HO-1) protein expression in lipopolysaccharide-stimulated RAW 264.7 cells (Guo et al., 2012). Further Synergistic anti-inflammatory effects of Sulforaphane with curcumin and phenethyl isothiocyanate have been reported using RAW 264.7 cells. The combination of Sulforaphane with curcumin or Sulforaphane with phenethyl isothiocyanate has been shown synergistic effect in down-regulating inflammatory mediators such as TNF, IL-1, NO and PGE_2 (Cheung et al., 2009).

3. Signaling cascade involving for the chemoprevention process

A. The NF-KB signaling pathway

Nuclear factor (NF)-κB, a transcription factor that mediates the transcription of various genes linked to inflammation, immunity, cell survival and cell proliferation (Mantovani et al., 2008). NF-κB is found in almost all animal cell types and important in regulating cellular responses because when it in an inactive state in the cell do not require new protein synthesis to be activated thus it belongs to the category of "rapid-acting" primary transcription factors. Therefore NF-κB to be a first responder to harmful cellular stimuli such as ultraviolet irradiation, stress, cytokines, free radicals, oxidized LDL, and bacterial or viral antigens. The one of central activator of NF-κB is receptor activator of NF-κB (RANK), which is a type of tumor necrosis factor receptor (TNFR) (C. Hofbauer and E. Heufelder., 2001). Osteoprotegerin (OPG, TNFRSF11B), a soluble homolog of RANK primarily secreted by bone marrow stromal cells and osteoblasts, acts as a decoy receptor of RANKL to block the binding of RANKL to RANK and, thus, osteoprotegerin is tightly involved in regulating NF-κB activation.

All proteins of the NF-κB family share a Rel homology domain which is a protein domain found in a family of eukaryotic transcription factors in their N-terminus. The subfamily of NF-κB proteins, including RelA, RelB, and c-Rel, have a transactivation domain in their C-termini. The longer precursor proteins, p105 and p100 involve generating the mature NF-κB subunits, p50 and p52, respectively.

Nevertheless, the NF-κB members p50 and p52 play critical roles in modulating the specificity of NF-κB function. Although the repressors of κB site transcription, p50 and p52 homodimers participate in target gene transactivation by forming hetero dimers with RelA, RelB, or c-Rel. The transcriptional activation of NF-κB-regulated genes, which include apoptosis (e.g., IAPs, XIAP, Bcl-2, and Bcl-xL) (Rayet and Gélinas., 1999), cell cycle arrest (e.g., c-Myc, COX-2, and cyclinD1) (Chefetz et al., 2011), anti-inflammatory (e.g., iNOS, COX-2 and TNF-α) (Lee et al., 2013) and metastasis (e.g., COX-2, MMP-9, and VEGF) (Klettner et al., 2013) genes. Thus, many attempts have been made to develop that chemopreventive agents capable of suppressing NFκB activity may be potentially useful in the prevention and management of various types of diseases including cancers. Sulforaphane was shown to suppress the 12-O-tetradecanoyl phorbol-13-acetate (TPA) induced MMP-9 expression and cell invasion by inhibiting phosporylation of IκB in MCF-7 breast cancer cells (Lee et al., 2013). Vascular endothelial growth factor (VEGF) which is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis suppress NF-κB transcriptional activation by Sulforaphane was reported in PC-3 human prostate cancer cells (Xu et al., 2005). The apoptosis induction by Sulforaphane regulating the NF-κB was detected using LNCAP (wild-type p53) and PC-3 (p53 deficient) human prostate cancer cells. Treatment of PC-3 cells with Sulforaphane resulted in a marked decrease in the levels of inhibitor of apoptosis (IAP) family proteins (cIAP1, cIAP2 and XIAP), which was accompanied by inhibition of nuclear translocation of p65-nuclear factor kappa B (NF-κB) (Choi et al., 2007). TNF-a-resistant leukemia cells (THP-1, HL60, U937, and K562), combination treatment of Tumor necrosis factor-α (TNF-α), with a sub-toxic dose of Sulforaphane, significantly triggered apoptosis which was associated with

caspase activity and poly (ADP-ribose)-polymerase cleavage related to inhibition of TNF-a-induced NF-κB activation through the inhibition of IκBa phosphorylation, IκBa degradation, and p65 nuclear translocation (Moon et al., 2008). Nevertheless Sulforaphane inhibit vascular smooth muscle cell (VSMC) proliferation which is cause to the vascular diseases through NF-κB dependent pathway (Kwon et al., 2012). In addition Sulforaphane exhibited its anti-inflammatory properties by down-regulating the COX-2 expression in lipopolysaccharide (LPS)-activated Raw 264.7 cells by inhibiting NF-κB (Woo et al 2007). Further Sulforaphane attenuates the LPS-induced expression of IL-1b, IL-6, NO and TNF-α expression in primary rat microglia through suppression of activation of NF-κB pathway (Brandenburg et al., 2010).

B. The Keap-1-Nrf2 pathway

The Keap1-Nrf2 pathway is the key regulator of cytoprotective responses to endogenous and exogenous oxidative stresses caused by reactive oxygen species (ROS) and electrophilic stress (Tanigawa et al., 2013). The transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) is a major signaling protein within the pathway that binds together with small Maf proteins to the antioxidant response element (ARE) in the regulatory regions of target genes, and Keap1 (Kelch ECH associating protein 1), a repressor protein that binds to Nrf2 and promotes its degradation by the ubiquitin proteasome pathway. Keap1 is a cysteine-rich protein, consist of cysteine residues like C151, C273 and C288, have been governed critical role by altering the conformation of Keap1 leading to nuclear translocation of Nrf2 which can bind small Maf1 protein and subsequent target gene expression such as heme oxygenase 1 (HMOX1), NAD(P)H quinone oxidoreductase 1 (NQO1), glutamate-cysteine ligase (GCL) and glutathione S transferases (GSTs). Extensive epidemiological evidence and animal experimental studies suggest that Sulforaphane has been shown that Nrf2 translocation to the nucleus is essential to its binding of ARE sequence and activation of its antiinflammatory (e.g., TNFα, IL-1β, Cox-2, and iNOS), anti-oxidant (e.g., heme oxygenase 1 (HMOX1), NAD(P)H quinone oxidoreductase 1 (NQO1), apoptosis (e.g., IAPs, XIAP, Bcl-2, and PARP) effects. Fuchs endothelial corneal dystrophy (FECD) and normal ex vivo corneas and human corneal endothelial cell lines were pretreated with Sulforaphane cause to significantly upregulated major ARE-dependent antioxidants and ameliorated oxidative stress–induced apoptosis in FECD (Ziaei et al., 2013). Further the exposure of neural crest cells (NCC) to sulforaphane alone significantly increased Nrf2 activation and the expression of Nrf2 downstream antioxidants as well as the activities of the

antioxidant enzymes. Moreover this study indicates treatment of NCCs with Sulforaphane along with ethanol significantly decreased ethanol-induced oxidative stress and apoptosis by Nrf2 pathway (Chen et al., 2012).

In addition experimental animal studies have shown strong anti-inflammatory effect of Sulforaphane on LPS-stimulated primary peritoneal macrophages derived from Nrf2 (+/+) and Nrf2 mice. Pretreatment of Sulforaphane in Nrf2 (+/+) primary peritoneal macrophages potently inhibited LPS stimulated mRNA expression, protein expression and production of TNFα, IL-1β, Cox-2, and iNOS by exerting its anti-inflammatory activity mainly via activation of Nrf2 pathway (Lin et al., 2008). Similarly, several clinical evaluation of sulforaphane has been largely conducted by utilizing preparations of broccoli or broccoli sprouts rich in either sulforaphane or its precursor form in plants, exposures to food- and air-borne carcinogens considerable region in Qidong, China. This study has been shown the preparations of broccoli sprouts may enhance the detoxification of aflatoxins and air-borne toxins, which may in turn attenuate their associated health risks, including cancer, in exposed individuals that related to the enhanced transcription of Nrf2 target genes. Furthermore, Ferguson and Schlothauer were reported high consumption of brassicaceous vegetables, including broccoli, has regularly associated with low cancer risk since expression of detoxifying enzymes, including hemoxygenase-1 (HO-1), various GST and glutathione reductase (GR) which regulate by Nrf2 (Ferguson and Schlothauer., 2012).

C. MAPK signaling pathway

Mitogen-activated protein kinases (MAPKs) are important signal transducing enzymes that mediate intracellular signaling associated with a variety of cellular activities including cell proliferation, differentiation, survival, program cell death, and transformation. MAPKs are evolutionary conserved enzymes connecting cell-surface receptors to critical regulatory targets within cells. MAPK signaling axis comprises at least three components: a MAPK, MAPK kinase (MAPKK, MKK or MEK) and a MAPKK kinase or MEK kinase (MAPKKK or MEKK). These modules may be activated by STE20 kinases or small GTP-binding proteins. MAPKKKs, phosphorylate and activate MAPKKs, which in turn phosphorylate and activate MAPKs. Mammals express at least four distinctly regulated groups of MAPKs, extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38α/β/γ/δ) and ERK5 that are activated by specific MAPKKs: MEK1/2 for ERK1/2, MKK3/6 for the p38, MKK4/7 (JNKK1/2) for the JNKs, and MEK5 for ERK5. Each MAPKK, however, can be activated by more than one MAPKKK, increasing the complexity and diversity of MAPK signaling. The JNK and p38 signaling cascades are activated by pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α and or in response to various cellular stresses such as hypoxic, oxidative stress or etc. The JNK pathway consists of JNK, a MAP2K such as SEK1 (or MKK4) or MKK7, and a MAP3K such as ASK1, MEKK1, mixed-lineage kinase (MLK), or transforming growth factor-β-activated kinase 1 (TAK1). In the p38 signaling pathway, distinct MAP2Ks such as MKK3 and MKK6 activate p38 and are themselves activated by the same MAP3Ks (such as ASK1 and TAK1) that function in the JNK pathway. In the ERK signaling pathway, ERK1 or ERK2 (ERK1/2) is activated by MEK1/2, which in

turn is activated by a Raf isoform such as A-Raf, B-Raf, or Raf-1 (or C-Raf). The kinase Raf-1 is activated by the small GTPase Ras, which activation is mediated by the receptor tyrosine kinase (RTK)-Grb2-SOS signaling axis. Members of the Ras family of proteins, including K-Ras, H-Ras, and N-Ras, play a key role in transmission of extracellular signals into cells. MAPK signaling pathways have been implicated in the pathogenesis of many human diseases especially cancer and neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Kim and Choi., 2010). Although Sulforaphane attributed to its ability to inhibit the activity of MAPK/JNK signaling, there are numerous examples where Sulforaphane may have beneficial effects to suppress pathogenesis of these diseases. As an example, Sulforaphane may represent a high therapeutic potential for treatment or prevention of cardiovascular diseases (Jayakumar et al., 2013), Parkinson’s disease (Morroni et al., 2013), nephropathy (Guerrero-Beltrán et al., 2012), sensorineural deafness/retinal dystrophic syndromes and other forms of inherited neurological disorders (Kong et al., 2007) by suppressing MAPK signaling pathway. Nevertheless by regulating MAPK signaling pathway, either up-regulating or down-regulating Sulforaphane also may act as a cancer suppressor agent. Using human colon carcinoma cell line, HT-29, Sulforaphane was found to potently induce the activity of MAPKs pathways, including ERK, JNK and p38 in a dose- and time-dependent manner. This study has been shown the role of MAPKs activation Sulforaphane -induced G1 phase cell cycle arrest in HT-29 cells (Shen et al., 2006). Treatment with TRAIL in combination with subtoxic concentrations of sulforaphane sensitizes TRAIL-resistant human lung adenocarcinoma A549 cells to TRAIL mediated apoptosis through downregulation of ERK (Jin et al., 2007). Nevertheless anti-tumor activity triggered by Sulforaphane was reported both *in*

vitro and *in vivo* on a panel of human myeloma cell lines as well as primary myeloma tumor cells and using a myeloma xenograft mouse model which related to the MAPK activation (Jakubikova et al., 2011).

D. Wnt/Beta catenin signaling pathway

The Wnt signaling pathways are a group of signal transduction pathways that consists of family of secreted lipid-modified signaling glycoprotiens mediate cell growth, differentiation, migration and polarity. Wnt proteins are capable of signaling through several pathways, characterized being the canonical Wnt pathway, the noncanonical planer cell polarity pathway, and the noncanonical Wnt/calcium pathway. The difference between the canonical and noncanonical pathways is that the β-catenin protein involve in the canonical pathway while noncanonical pathway functioning independent from that β-catenin protein. The canonical Wnt pathway results an accumulation of β-catenin protein in the cytoplasm and it translocates to the nucleus gradually to act as a transcriptional co-activator of transcription factors that belongs to the TCF/LEF family (McCarthy et al., 2011). In the absence of Wnt signaling, the β-catenin would not accumulate in the cytoplasm since it degrades with the involvement of destruction complex. This destruction complex regulated by a multiprotein complex encompassing kinases such as GSK3β (glycogen synthase kinase-3 β) and CK1α (casein kinase 1 α), and the scaffolding proteins APC (adenomatous polyposis coli), Axin 1, Axin 2, and PP2A (protein phosphatase 2A). This destruction complex binds and phosphorylates β-catenin at serine and threonine residues, by targeting it for ubiquitination, which subsequently undergo the proteolytic digestion. However in the presence of Wnt ligands, co-activation of the low-density lipoprotein receptor-related proteins (LRP-5/6) and Frizzled (Fz) leads to inhibition of the destruction complex. This inhibition occurs due to the translocation of both negative regulator of Axin and destruction complex by Wnt to the plasma membrane. This negative regulator of Axin begins to be localized to the cytoplasmic tail of LRP-5/6. The other proteins in the

destruction complex phosphorylate and gradually bind Axin and also to the cytoplasmic tail of LRP-5/6. Due to the de-phosphorylation of Axin its level and stability become decreased. Then Dishevelled (Dsh) protein phophorylate and become activated form and its Dix and PDZ domains inhibits the GSK3β activity of the destruction complex. This is facilitates the accumulate β-catenin and localize to the nucleus. In the nucleus, β-catenin binds to members of the TCF/LEF family transcription factors, therefore capable of modulating expression of target genes. Therefore, canonical Wnt pathway has implications in the genesis of the many human cancers (Surana et al., 2013). The canonical Wnt pathway itself offers ample targeting principle points for cancer chemoprevention.

There are several studies were shown that Sulforaphane was able to down regulate Wnt/ β-catenin signaling pathway in different types of cancers. Sulforaphane inhibits breast cancer stem cells and down regulates Wnt/β-catenin signaling pathway. The treatment of Sulforaphane concentrations as 1,5,10 μM for 4 days decreased the protein level of β-catenin by up to 85% in MCF7 and SUM159 cells. This downregulation of β-catenin protein level detected its transcriptional level for further confirmation by using a TCF/LEF TOP-dGFP lentiviral reporter system. Since known, β-catenin activates TCF/LEF in nucleus, involve the transcription of destabilized GFP (dGFP) gene and this dGFP gene expression was quantified by flow cytometry and analyzed by fluorescence microscopy. According to the flow cytometry analysis, approximately 30%-40% of dGFP-positive cells were reduced by 5μM Sulforaphane treatment out of the 3% of dGFP-positive transfected cells. The GSK3β inhibitor, LiCl, resulted in reversed the Sulforaphane induced β-catenin phosphorylation. As well as another GSK3β inhibitor, BIO increased the dGFP-positive cells population while 5 μM

Sulforaphane treatments decreased this cell population as 60% in the presence of BIO (Li et al., 2010). Park *et al* reported Sulforaphane resulted to induce down regulation of β-catenin in human cervical carcinoma HeLa and hepatocarcinoma HepG2 cells (Park et al., 2007).

5. 국문요약

수많은 동물 연구와 역학연구를 통해 십자화과 작물 섭취의 증가는 glucosinolates로부터 가수분해되는 다양한 isothiocyanate의 활성에 의한 항암 chemoprevention 효과를 입증하였다. 브로콜리와 브로콜리 새싹을 포함하는 십자화과 작물의 주요 glucosinolate인 Glucoraphanin은 Sulforaphane(SFN)으로 전환될 수 있다. 많은 종류의 암에 대해 SFN의 chemoprevention 특성은 “blocking”과 “suppressing”의 각기 다른 기전을 통해 확인되었다. SFN의 “blocking”기능은 전발암물질을 발암물질로 전환시키는 1상 대사 효소를 억제와 발암 물질의 배설을 촉진하는 2상 대사 효소를 유도시킨다. 여러 연구 결과에 따르면 apoptosis, autophagy, 항 telomerase 활성, 항 invasive 효과, 항 metastatic 효과와 cell cycle arrest을 유도하면서 SFN이 다양한 세포 활동을 조절하는 “suppressing” 효과를 입증하였다. 이러한 기전은 상승작용으로 작용하여 보다 나은 chemoprevention 효과를 나타낸다. 이러한 기전은 NF-κB 신호 전달 경로, Wnt/베타 카테닌 경로, Keap-1-Nrf2 경로 및 MAPK / JNK 신호 전달 경로 및 세포 내 ROS 생성과 같은 메커니즘에 관련된 몇 가지 신호 전달 체계가 있다. 그러나 안전하고 효과적인 chemopreventive과 chemotherapeutic 전략을 개발하기 위해서는 SFN의 영향을 받는 분자기전에 대한 이해가 필요하다.

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