Molecular mechanisms of anti-cancer activity of Wasabi 6-MSITC in human colorectal cancer cells

（ワサビ機能性成分のヒト大腸がん細胞における抗がん活性の分子機構に関する研究）

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Abbreviations

BAD = BCL2 associated agonist of cell death

BAK = BCL2 antagonist/killer 1

BAX = BCL2-associated X protein

BCL-2 = B-cell lymphoma 2

BCL-XL = B-cell lymphoma-extra large

BID = BH3 interacting domain death agonist

BIM = BCL2 like 11

BITC = benzyl isothiocyanate

CAD = caspase-activated DNase

CDK = cyclin-dependent kinase

CHOP = CCAAT/enhancer-binding protein homologous protein

cIAP1 = cellular inhibitor of apoptosis protein 1

cIAP2 = cellular inhibitor of apoptosis protein 2

COX-IV = cytochrome *c* oxidase subunit 4 isoform 1

$DiOC_6(3)$ = 3,3′-dihexyloxacarbocyanine iodide

DR4 = death receptor 4

DR5 = death receptor 5

ELK1 = ETS transcription factor

ERK1/2 = extracellular signal-regulated kinase 1/2

FADD = Fas associated via death domain

FAS = Fas cell surface death receptor

GAPDH = glyceraldehyde-3-phosphate dehydrogenase

GSH = glutathione

GST = glutathione S-transferase

HIF1α = hypoxia inducible factor 1 subunit alpha

HO-1 = heme oxygenase 1

HO-2 = heme oxygenase 2

HSP = heat shock protein

HTRA2 = HtrA serine peptidase 2

IAP = inhibitor of apoptosis protein

ICAD = inhibitor of caspase-activated DNase

ITC = isothiocyanate

JNK = c-Jun-*N*-terminal kinase

MAPK = mitogen-activated protein kinase

MCL-1 = myeloid cell leukemia 1

MEK1/2 = MAP/ERK kinase 1/2

$\Delta\Psi_m$ = mitochondrial membrane potential

6-MSITC = 6-(methylsulfinyl)hexyl isothiocyanate

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PARP = poly(ADP-ribose) polymerase

PEITC = phenethyl isothiocyanate

PON = paraoxonase

RAD17 = RAD17 checkpoint clamp loader component

RAF = Raf proto-oncogene, serine/threonine

RAS = RAS proto-oncogene, GTPase

SMAC = second mitochondria-derived activator of caspases

TERT = telomerase reverse transcriptase

TNF = tumor necrosis factor

TNFR = TNF receptor

XIAP = X-linked inhibitor of apoptosis

Abstract

Recently, cancer is the first leading cause of death, and the increase of its incidence and mortality as colorectal cancer has been observed in Japan. Lifestyles are associated with cancer risk, and consumption of fruit and vegetables on daily basis has provided preventive effects against colorectal cancer. Apoptosis, programmed cell death, plays an important role in the removal of seriously damaged cells or tumor cells. The tumor suppressor gene, *p53* plays a central role in apoptosis induction. Many chemotherapeutic drugs revealed their anti-cancer effects by activating P53, however, the *p53* gene is one of the most frequently mutated genes in many cancers.

6-(Methylsulfinyl)hexyl isothiocyanate (6-MSITC), a major bioactive compound in Wasabi, is a very popular pungent spice in Japan. Wasabi 6-MSITC has been reported to have cancer chemopreventive activities against colorectal carcinogenesis in rat model, however, the underlying mechanism is unclear. In this study, I investigated the anticancer activity and molecular mechanisms, using two types of human colorectal cancer cells (HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$).

First, 6-MSITC caused cell viability inhibition, cell cycle arrest in G_2/M phase and apoptotic cell death in both types of cells. The increase levels of P21, death receptor 5 (DR5) and pro-apoptotic BCL-2-associated X protein (BAX), and the decrease levels of anti-apoptotic B-cell lymphoma 2 (BCL-2) and B-cell lymphoma-extra large (BCL-XL) and inhibitor of apoptosis protein (IAP) family were observed in both types of cells treated with 6-MSITC. These data indicated that 6-MSITC inhibited cell viability of human colorectal cancer cells through cell cycle arrest in G_2/M phase and apoptosis induction by *p53*-independent molecular events.

Moreover, investigation of molecular mechanisms revealed that the activation of

extracellular signal-regulated kinase 1/2 (ERK1/2), rather than *p53*, is recruited for 6-MSITC-induced apoptosis. 6-MSITC stimulated ERK1/2 phosphorylation, and then activated ERK1/2 signaling including ELK1 phosphorylation, and upregulation of C/EBP homologous protein (CHOP) and death receptor 5 (DR5). This mechanism was confirmed as the MAP/ERK kinase 1/2 (MEK1/2) inhibitor U0126 blocked all of these molecular events induced by 6-MSITC, and enhanced the cell viability in both types of cells. These results indicated that 6-MSITC induced apoptosis in colorectal cancer cells via *p53*-independent, ERK1/2-mediated ELK1/CHOP/DR5 pathway

Further, 6-MSITC enhanced the ratio of pro-apoptotic BAX/anti-apoptotic myeloid cell leukemia 1 (MCL-1), and sequentially caused mitochondrial membrane potential ($\Delta\Psi_m$) loss, cytochrome *c* release, and caspase-3 activation in both types of cells. 6-MSITC-activated ERK1/2 has been involved in extrinsic apoptotic cell death via ELK1/CHOP/DR5, however, the MEK1/2 inhibitor U0126 had no effect on mitochondria dysfunction caused by 6-MSITC. Taken together, Wasabi 6-MSITC induced apoptosis of human colorectal cancer cells in *p53*-independent mitochondrial dysfunction pathway differently from ERK1/2-mediated ELK1/CHOP/DR5 pathway.

In conclusion, these findings will help in understanding the chemoprevention mechanisms of Wasabi 6-MSITC on colon carcinogenesis previously reported in animal model, and offer on opportunity that Wasabi 6-MSITC might be a potential compound for colorectal cancer chemoprevention even with *p53* mutation.

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Chapter 1 Introduction

1.1 Overview of Wasabi 6-MSITC

1.1.1 Isothiocyanates

Cruciferous vegetable (*Brassicaceae*), are commonly consumed, including broccoli, cabbage, watercress and mustard, are rich sources of isothiocyanates (ITCs) [1]. The ITCs are characterized by the presence of electrophilic sulfur group –N=C=S [2], derived from enzymic hydrolysis of glucosinolates by myrosinase present in cruciferous vegetables [3]. The chemopreventive effects of ITCs are attributed to the direct reaction of the carbon atom of the –N=C=S with many cellular proteins having a nucleophilic groups, such as cytochrome P450s, tubulins and proteasome [4, 5]. The lipophilicity of the side chains of ITCs might have an impact on protein binding and chemopreventive activity [6]. Additionally, ITCs have not shown to be capable of binding to DNA or RNA in cells [7].

Consumption of fruits and vegetables are known to be associated with reducing the risk of various cancers [8]. Epidemiological meta-analysis research has provided evidence that high intake of cruciferous vegetables has a potency to reduce approximately 18% of relative risk of human colon cancer [9]. Moreover, other studies have also indicated that cruciferous vegetables intake was inversely associated with the risk of gastric cancer [10], pancreatic cancer [11], prostate cancer [12] and breast cancer [13]. Isothiocyanates has appeared to be most attributable components of cruciferous vegetable in cancer chemoprevention. In fact, accumulated evidence from animal experiments and molecular data have reported that naturally occurring ITCs in cruciferous vegetables, such as sulforaphane, benzyl isothiocyanate (BITC) and

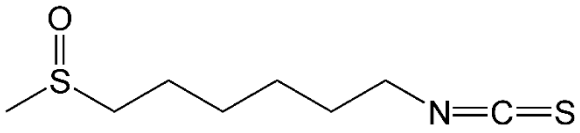
phenethyl isothiocyanate (PEITC) have chemopreventive properties of various cancers [14, 15].

1.1.2 Wasabi

Wasabi [*Wasabia japonica* (Miq.) Matsum.], also known as Japanese horseradish, belongs to family of cruciferous vegetable, is a very popular pungent spice in Japan. Wasabi is usually used as flavor for Sushi and Sashimi. The taste and flavor of Wasabi is similar to horseradish (*Armoracia rusticana*), also called as Western wasabi. The bioactive compounds of Wasabi have been identified as a series of isothiocyanate analogues [16, 17]. Several reports have indicated that the content of total isothiocyanates in Wasabi was 2.1 mg/g, whereas that of horseradish was 1.9 mg/g [18, 19]. The major bioactive component in Wasabi was identified as 6-(methylsulfinyl)hexyl isothiocyanate (6-MSITC) (Figure 1.1), was especially abundant in rhizome [16, 20]. The content of 6-MSITC in Wasabi rhizome has been estimated at approximately 400 μg/g, over 10 times more than that in horseradish [21, 22]

Several studies have shown that Wasabi has multiple physiological functions, such as appetite enhancement [23], anti-bacterial activity [24], platelet aggregation inhibitory activity [20] and anti-carcinogenic activity in N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced rat gastric carcinogenesis [25]. Oral administration of Wasabi 6-MSITC revealed the suppressive effects on rat colon carcinogenesis induced by 1,2-dimethylhydrazine [26]. The results suggest that 6-MSITC is a potential chemopreventive compound for colon carcinogenesis. Previous studies demonstrated the pharmacological properties of 6-MSITC, such as NRF2-mediated anti-oxidative activity [27–29] and anti-inflammatory effect in lipopolysaccharide-activated murine

macrophages [30–33], However, molecular mechanisms of cell death action by 6-MSITC in human colorectal cancer cells are unclear.



6-(Methylsulfinyl)hexyl isothiocyanate (6-MSITC)

Figure 1.1. Chemical structure of 6-(methylsulfinyl)hexyl isothiocyanate (6-MSITC)

1.1.3 Bioavailability and metabolism

The bioavailability and metabolism of ITCs in vegetables are key for considering the potency of these compounds on human health. ITCs and its metabolites have shown to be highly bioavailable in human body, whereas glucosinolates has been limited [34]. Several lines of studies have reported that ITCs are rapidly accumulated in human and animal cells after 0.5–3 h of exposure, with the peak of its intracellular concentration reached up to "mM" levels, over 100–200 times more than its extracellular concentration [35–38]. In particular, mouse hepatoma cells (Hepa-1c1c7 cells) treated with low concentrations (1–5 μM) of ITCs for 0.5 h exhibited very high intracellular concentrations of ITCs/dithiocarbamate (800–900 μM) [36]. In fact, published data have indicated that humans given 200 μmol of ITCs in broccoli sprout, mainly sulforaphane, exhibited the peak with its concentration of 1.62–2.27 μM in plasma at 1 h [39]. Moreover, clinical trial research have reported that ITCs had no significant toxicity to human health when humans were given 25 or 100 μmol of glucosinolates, or 25 μmol of ITCs, which were equivalent to about 10 g of dried broccoli or 1 g of dried broccoli sprouts, for 7 days [40].

After absorption, ITCs are primarily metabolized via the mercapturic acid pathway, the amount of mercapturic acid excretion in urine is a correspondence of the amount of ITCs consumption [41]. ITCs might penetrate cells by diffusion, and then they are rapidly conjugated to intracellular glutathione (GSH) with spontaneous or enzymatic glutathione S-transferase (GST) reactions via their –N=C=S, which are processed to N-acetyl cysteine ITCs [41, 42]. Accumulated data in human and rats have shown that approximately 70% of sulforaphane dose was recovered in 12–24 h of the urine as NAC conjugates after oral administration [43, 44]. When myrosinase in cruciferous vegetables is inactivated by boil and saute, glucosinolates transit to the colon, owing to their hydrophilicity, which are metabolized by intestinal microbacteria [45]. Several studies have reported that approximately 20% of glucosinolates were hydrolyzed and converted into ITCs by human microflora from feces [46, 47]. Indeed, publish research have indicated that high levels of ITCs in urine were inversely associated with the risk of colorectal cancer after five years, whereas a nonsignificant increase in the risk of colorectal cancer was observed for cases within five years [48].

1.2 Human cancer

1.2.1 Colorectal cancer

According to reports of World Health Organization (WHO) in 2015, 91 of 172 countries indicated that cancer is first or second leading cause of death at ages below 70 years [49]. A recent publication on the global burden of cancer worldwide has provided data that the numbers of new cancer cases and mortality with cancer might be estimated at 18.1 million and 9.6 million, respectively, in 2018 [49]. Colorectal cancer is the third most commonly occurring cancer (10.2% of the total cases) and the second most

common cause of mortality (9.2% of the total mortality) [49] There were over 1.8 million new colorectal cancer cases in 2018. In Japan, cancer is the first leading cause of death (approximately 28% of total mortality), and is estimated 380 thousand for mortality, especially, colorectal cancer is the first most commonly death in women, and it is estimated that 28.7 thousand of men (12.9% of the total mortality) and 24.8 thousand of women (15.8%) might be died with colorectal cancer in 2018 [50]. Recently, the increase of incidence and mortality rates in colorectal cancer has been observed in Japan [51]. Accumulated studies have noted that westernized lifestyles, including eating red meat, drinking alcohol and obesity, are associated with colorectal cancer risk [52].

1.2.2 Tumor suppressor gene *p53*

The tumor suppressor transcription factor, P53 is involved in multiples central cellular events, including DNA repair, maintenance genomic stability, senescence, cell cycle control and apoptosis [53, 54]. The P53 consists of 393 amino acid residues, and that gene is located on chromosome 17p and consists of 11 exons and 10 introns [55]. First, the P53 has been discovered as a cellular SV40 large T antigen-binding protein, which led to impact on cancer research and clinic [56]. According to recent survey, over 3600 genes, including, *p21*, *bax*, *noxa* and *mdm2*, have been identified as direct target of P53, which appears to tumor suppressor function [57]. However, the *p53* gene is one of the most frequently mutated genes in many different types of cancers [58, 59], and 74% of this mutations are missense mutations [60]. Several reports have indicated that mutation of *p53* gene occurs in approximately 40–50% of colorectal cancers, is closely related to the progression [61]. It has been well documented that many chemotherapeutic drugs revealed their anti-cancer effects by activating P53 with

genetoxic stress [62]. In fact, clinical research has shown that patients with mutant *p53* gene are frequently resistant to drug therapies, conferring poor prognosis [55]. On the other hand, many of the cancers retain wild-type *p53*, suggesting that *p53* role in carcinogenesis is complicated [63].

1.3 Cancer chemoprevention

1.3.1 Natural compounds

Fruit and vegetables are essential for human health. The World Health Organization (WHO) recommends to intake 400–600 g of fruit and vegetables in daily for reducing risks of various diseases including of cancer [64]. Naturally occurring compounds from dietary sources, called as phytochemical, such as polyphenol, isothiocyanate, terpenoid and lactone, have provided preventive effects against cancer, lacking of toxicity [65, 66]. Therefore, half of all drugs in clinical use are derived from natural compounds, their derivatives or their analogs [67]. Additionally, compounds derived from more than 3,000 plant species are used for medicines in cancer chemotherapy [67]. In fact, a number of publications have indicated that phytochemicals have a potency of anticancer activities against colorectal cancer though modulation of various signaling transduction and cell processes [68].

Cancer chemoprevention strategies in colorectal cancer can be divided into three steps: 1) prevention of tumor initiation, the reduction of exposure of agents such as carcinogens to normal cells; 2) prevention of tumor promotion, the interference of carcinogenic processes; 3) prevention of tumor progression, suppression of further progression after an initial diagnosis of malignancy [69–71]. There are general hypothesis that disruption of apoptosis results in contributing to the development of

cancer, such as tumor initiation and progression, and treatment resistance [72, 73]. Thus, it is essential to consider that apoptotic cell death in tumorigenesis have a capable to prevent or control of cancer [72, 74].

1.3.2 Apoptosis

Cellular processes play a critical role in survival and apoptosis, which are essential for homeostasis, maintenance of growth and development of organisms [75]. Apoptosis is a highly regulated process of programmed cell death, which acts as a defense mechanism in response to cellular damage [76]. It is characterised by distinct cell morphological changes, including cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation [77]. Apoptosis has been known to be occurred by various stimuli, lead to cell death without severe inflammation [78, 79]. Several studies have highlighted that apoptosis occurring with high frequency in tumors treated with cytotoxic chemotherapeutic agents has potency of preventing tumor growth [80, 81]. In fact, many cancer chemopreventive compounds and cancer chemotherapeutic agents have been developed to induce apoptosis of serious damaged cells or tumor cells for cancer chemoprevention [82] and cancer therapy [83].

According to accumulated studies, there are two main apoptotic pathways, extrinsic and intrinsic pathway [76]. The extrinsic signaling pathway involves transmembrane death receptors which are member of tumor necrosis factor (TNF) receptor superfamily [84]. Once ligand binds to the receptors on cellular membrane, caspase-8 is activated, which lead to trigger apoptotic cell death [85, 86]. On the other hand, mitochondrial events are a key for the intrinsic signaling pathway [87]. Upon exposing of cells to intracellular stimuli, mitochondria dysfunction, such as mitochondria permeability transition, loss of mitochondrial membrane potential and release of cytochrome *c* and

second mitochondria-derived activator of caspases (SMAC) from mitochondria, is occurred, which triggers apoptotic cell death [87]. Additionally, the inhibitor of apoptosis protein (IAP) family is involved in suppression of apoptosis via directly inhibition of caspases activity [88]. These apoptotic events can eliminate damaged cells from the body, which have a potency of protection against tumor formation [89].

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Chapter 2 The *p53*-independent cell survival inhibition and apoptosis induction by Wasabi 6-MSITC in human colorectal cancer cells

2.1 Introduction

Wasabi [*Wasabia japonica* (Miq.) Matsum.] is a very popular pungent spice in Japan, usually used as flavor for Sashimi. In previously, oral administration of 6-MSITC in colon carcinogenesis model of rat revealed that 6-MSITC could suppress the number of colonic aberrant crypt foci induced by 1, 2-dimethylhydrazine [1]. These results suggest that 6-MSITC has a chemopreventive potency for colon carcinogenesis, however, the underlying mechanisms are not clear.

Cancer chemopreventive compounds have been considered to play an important role in the removal of serious damaged cells or tumor cells by apoptosis [2]. Apoptosis is highly regulated processes including a series of molecular events, such as chromatin condensation, DNA fragmentation, activation of caspase cascades, and cell death [3]. Accumulated studies have shown that activation of caspase-activated DNase (CAD) through cleavage of inhibitor of CAD (ICAD) by caspase-3 caused double-stranded DNA breaks and resulted in DNA fragmentation [4]. Moreover, the translocation of phosphatidylserine from the inner to the outer of plasma membrane is caused in apoptotic cell [5]. Apoptotic cells are rapidly recognized by macrophages and removed without severe inflammation [6]. In recent studies, caspase-3-mediated phosphatidylserine exposure has given in engulfed signals to phagocytes, and which led to trigger anti-inflammatory gene response in phagocytes [7].

The tumor suppressor protein, P53, has been reported to regulate cell cycle arrest,

apoptosis and DNA repair in a variety of cells [8]. It has been well documented that many chemotherapeutic drugs revealed their anti-cancer effects by activating P53 with genetoxic stress [9]. The importance of P53 in tumor suppression is highlighted by the observation that a number of human cancers show evidence for the loss of normal P53 function due to mutation within the *p53* gene [10]. On the other hand, many of the cancers retain wild-type *p53*, suggesting that *p53* role in carcinogenesis is complicated [11].

Based on the bioactive properties of Wasabi 6-MSITC and the roles of *p53* on apoptosis induction in cancer cells, I used two types of human colorectal cancer cells (HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$) to investigate the anticancer activity and molecular events of Wasabi 6-MSITC in this study.

2.2 Materials and methods

2.2.1 Materials

6-MSITC (purity >99%) (Figure 1.1) was kindly provided by Kinjirushi Co., Ltd. (Nagoya, Japan). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and β-actin antibody were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). The antibody against phospho-P53 (Ser15) was from Cell Signaling Technology, Inc. (Danvers, MA, USA), while against P53 was from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

2.2.2 Cell culture

Human colorectal cancer cell lines HCT116 $p53^{+/+}$ were obtained from the American Type Culture Collection (Manassas, VA, USA) and HCT116 $p53^{-/-}$ cells were kindly

supplied by Dr. Bert Vogelstein (Johns Hopkins Medical Institute, Baltimore, MD, USA). The cells were cultured at 37 ℃ in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin glutamine (PSG) for 24 h, and then treated by 6-MSITC in indicated times and doses.

2.2.3 Western blotting analysis

Western blotting was performed as described previously [12]. Briefly, the cells were lysed with modified RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and proteinase inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). The lysates were homogenized in an ultrasonicator for 10 s three times and incubated on ice for 30 min. The homogenates were centrifuged at 14,000 × *g* for 30 min and the supernatants were collected. The protein concentration was determined by Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts of lysate protein were run on SDS-PAGE and electrophoretically transferred to PVDF membrane (GE Healthcare UK Ltd., Amersham, England). The membrane was first blocked with TBST buffer [500 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 0.1% Tween 20] containing 5% nonfat dry milk, and then incubated with specific antibodies overnight at 4 ℃ and HRP-conjugated secondary antibodies for another 1 h. Bound antibodies were detected using the ECL system, and relative amounts of proteins associated with specific antibody were quantified using Lumi Vision Imager soft ware (TAITEC Co., Ltd., Saitama, Japan).

2.2.4 Cell viability assay

The cell survival rate was measured by an MTT assay as described previously [13]. In brief, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ (7.0×10^3/well) cells were plated into each well of 96-well plates. The cells were treated with various concentrations of 6-MSITC for 48 h. MTT solution was then added to each well and incubated for another 4 h. The resulting MTT-formazan product was dissolved by the addition of 0.04 N HCl-isopropanol solutions. The amount of formazan was determined by measuring the absorbance at 595 nm with Multiskan™ FC (Thermo Scientific™, Waltham, MA, USA). The cell viability was expressed as the optical density ratio of the treatment to control.

2.2.5 Cell cycle analysis

The cell cycle phases were determined by a flow cytometric analysis [14]. After treatment with 20 μM 6-MSITC for 24 h, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were fixed in 70% ethanol at −20 °C overnight and then resuspended in PBS. The cells were stained in 1 mL PBS containing 20 μg/mL RNase and 50 μg/mL propidium iodide (PI) for 30 min at room temperature. Fluorescence emitted from the propidium-DNA complex was analyzed with the flow cytometry (CyFlow®, Sysmex Partec GmbH, Görlitz, Germany).

2.2.6 Determination of apoptotic cells by flow cytometry

Quantitation of apoptotic cell death was assessed by FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA) according to the manufacturer's manual. In brief, after treatment with 20 μM 6-MSITC for 48 h, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were suspended in 100 μL of binding buffer and then incubated

with FITC Annexin V and propidium iodide staining solution for 15 min. The cells were analyzed at FL1 (530 nm) and FL3 (630 nm) with the flow cytometry (CyFlow®, Sysmex Partec GmbH, Görlitz, Germany).

2.2.7 ELISA detection of DNA fragmentation

DNA fragmentation was assessed with Cell Death Detection ELISA^{PLUS} (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's manual. In brief, HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ (2.1×10^4/well) cells were plated into each well of 12-well plates. After treatment with each sample, the cells were lysed with lysis buffer and then centrifuged at 200 × *g* for 10 min. The supernatant was added to each well of ELISA plate and incubated with immunoreagent buffer containing anti-histone biotin and anti-DNA POD for 2 h. The plate was washed with incubation buffer and then reacted with 100 μL of ABTS substrate buffer. Absorbance was measured at 405 nm and 490 nm with Multiskan™ FC (Thermo Scientific™, Waltham, MA, USA). The enrichment factor of apoptosis induction was calculated as the optical density ratio of the treatment to control.

2.2.8 Membrane array of apoptotic protein

Detection of apoptotic protein was assessed with Proteome Profiler™ Array Human Apoptosis Array Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's manual. In brief, HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells (1.19×10^6/100 mm-dish) were treated with each sample for 48 h. The harvested cells were lysed with Lysis Buffer and incubated on ice for 30 min. The lysates were centrifuged at 14,000 × *g* for 5 min and the supernatants were collected. The protein concentration was determined by Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories,

Inc., Hercules, CA, USA). The membrane of Human Apoptosis Array was first blocked with Array Buffer 1, and then incubated with 1.5 mL of the protein lysates (1.25mL of Array Buffer 1 and 0.25 mL of the supernatants) overnight at 4 °C, and 1.5 mL of Detection Antibody Cocktail in Array Buffer 2/3 for another 1 h. The membrane was incubated with Streptavidin-HRP in Array Buffer 2/3 for 30 min, and were detected using Chemi Reagent Mix. The relative amounts of proteins on membrane were quantified using Lumi Vision Imager soft ware (TAITEC Co., Ltd., Saitama, Japan).

2.2.9 Statistical analysis

The data represent the mean ± SD. All data were statistically analyzed by two-way analysis of variance (ANOVA). The results of two-way ANOVA were indicated as a table in the upper side of graphs. A probability of $p < 0.01$ was considered as significant.

2.3 Results

2.3.1 The effects of 6-MSITC on the P53 protein level

It has been known that many cancer chemotherapeutic drugs revealed their anti-cancer effects by activating P53 with genetoxic stress [9]. The phosphorylation of P53 on Ser15 is involved partially in apoptosis induction [15]. First, to confirm whether 6-MSITC induces the activation of P53, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were treated with 0-20 μM 6-MSITC for 48 h. As shown in Figure 2.1A, an increased P53 protein expression and phosphorylation of P53 on Ser15 were observed at 20 μM for 48 h in HCT116 $p53^{+/+}$ cells, but not in HCT116 $p53^{-/-}$ cells due to loss of *p53* gene. Moreover, a time-dependent P53 protein expression and phosphorylation induction was

observed at 20 μM from 24–48 h in HCT116 *p53*$^{+/+}$ cells, but not in HCT116 *p53*$^{-/-}$ cells (Figure 2.1B). These data indicated that 6-MSITC activated P53 in HCT116 *p53*$^{+/+}$ cells.

2.3.2 The inhibitory effects of 6-MSITC on the cell viability of HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells

Next, to examine the effects of 6-MSITC on the survival of HCT116 *p53*$^{+/+}$ and *p53*$^{-/-}$ cells, I performed an MTT assay. As shown in Figure 2.2A, 6-MSITC inhibited the proliferation of both HCT116 *p53*$^{+/+}$ and *p53*$^{-/-}$ cells at the concentration ranges of 5–20 μM in a dose dependent manner, with the IC_{50} values of 10.01 μM and 9.92 μM, respectively. Two-way ANOVA results indicated that *p53* had no significant effect on the inhibition of the survival. Cell nuclear condensation and cell adhesion loss were also observed in both types of cells treated with 20 μM 6-MSITC for 48 h (Figure 2.2B).

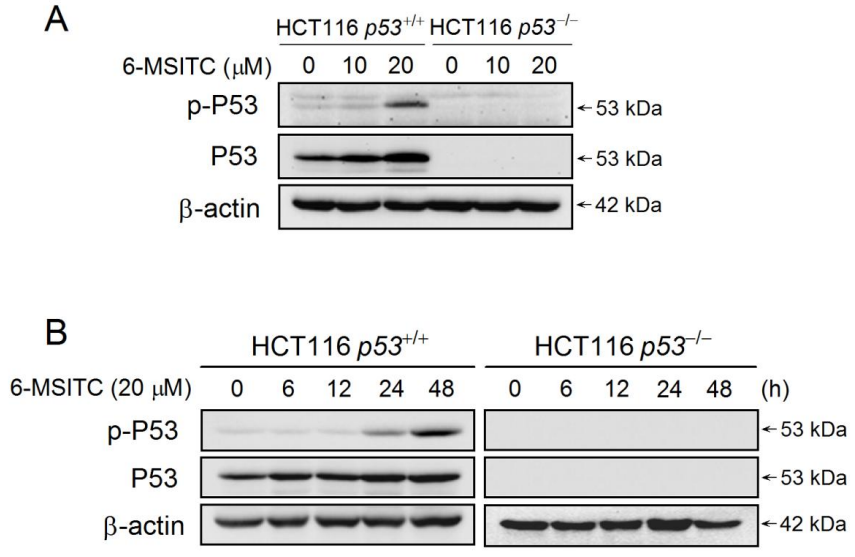


Figure 2.1. Phosphorylation and accumulation of P53 protein. A) A dose-response. **B)** A time-response. Both types of cells were treated with 6-MSITC in indicated concentrations and times. Whole cell lysate was used for Western blotting analysis with the indicated specific antibodies.

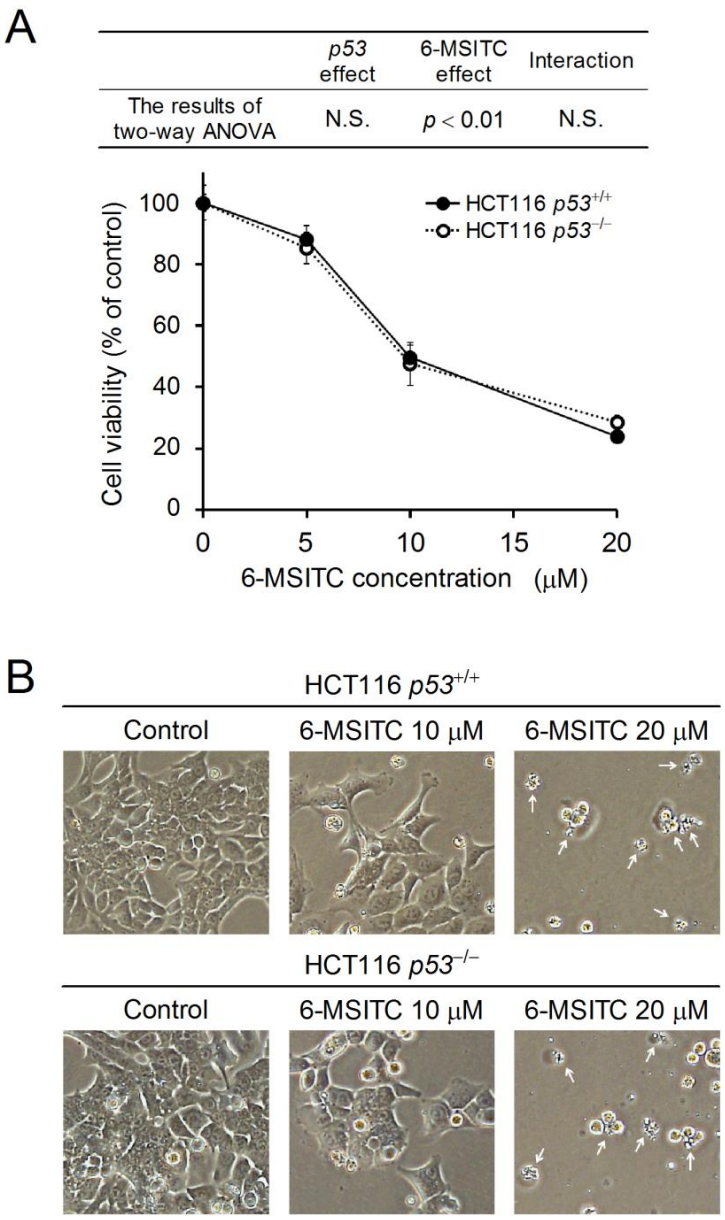


Figure 2.2. The inhibitory effects of 6-MSITC on the viability of HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells. A) Cell survival inhibition. Both types of cells were treated with 0–20 μM of 6-MSITC for 48 h. The cell survival rate was assessed colorimetrically after staining with MTT, and was expressed as the optical density ratio of the treatment to control. The data represent the mean ± SD of combined three independent experiments with quadruplicate. The results of two-way ANOVA were shown as a table in the upper side of graph (N.S.: not significant). **B)** Typical cell morphological changes. Both types of cells were exposed to 10–20 μM 6-MSITC for 48 h, and 0.2% DMSO was used as control. The cell morphological changes were observed under phase-contrast microscopy. White arrows indicated apoptotic bodies.

Collectively, there were no significant difference in the inhibition of cell survival and cell morphological changes between two cell lines, suggesting that 6-MSITC inhibited the viability of HCT116 cells in a *p53*-independent manner.

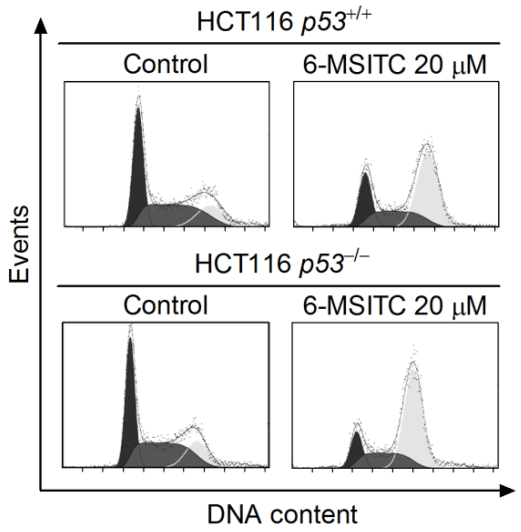
2.3.3 6-MSITC induces cell cycle arrest in G_2/M phase in a *p53*-independent manner

Moreover, to elucidate whether 6-MSITC-induced phosphorylation of P53 in HCT116 $p53^{+/+}$ cells influences the cell cycle distribution, I examined the cell distribution in different cell cycles with flow cytometric analysis. As shown in Figure 2.3A and B, a significant increase of G_2/M phase cells were observed in both types of cells after treatment with 6-MSITC for 24 h. Two-way ANOVA results indicated that *p53* had no significant effect on cell cycle distribution induced by 6-MSITC. These results indicated that 6-MSITC caused cell cycle arrest in G_2/M phase in both HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells by a *p53*-independent manner.

2.3.4 6-MSITC induces apoptotic cell death in a *p53*-independent manner

To elucidate whether 6-MSITC-induced inhibition of cell survival was due to apoptosis induction, I characterized the cells by several approaches. First, I used flow cytometric analysis with FITC-Annexin V/PI double staining to detect the apoptotic cells [16]. As shown in Figure 2.4A and B, the similar effect of 6-MSITC on apoptosis induction was observed in both types of the cells after exposure to 20 μM 6-MSITC for 48 h, with 4.89 fold and 4.72 fold of apoptotic cells in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells, respectively, comparing to its no treatment control. Moreover, the similar effect of 6-MSITC on DNA fragmentations were also observed in the treatments with 20 μM for 48 h (Figure 2.5). As shown in tables of Figure 2.4B and 2.5, two-way ANOVA results

A



B

		p53 effect	6-MSITC effect	Interaction
The results of two-way ANOVA	G_1 phase	N.S.	$p < 0.01$	N.S.
	S phase	N.S.	$p < 0.01$	N.S.
	G_2/M phase	N.S.	$p < 0.01$	N.S.

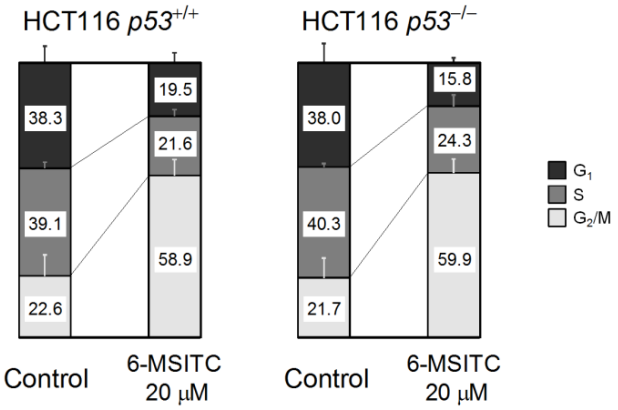


Figure 2.3. The effects of 6-MSITC on cell cycle distribution in HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells. A) Cell cycle analysis. Both types of cells were treated with 0.2% DMSO as control or 20 μM of 6-MSITC for 24 h. **B)** Quantitation of cell cycle distribution in (A). The data represent the mean ± SD of combined three independent experiments with duplicate. The results of two-way ANOVA were shown as a table in the upper side of graph (N.S.: not significant).

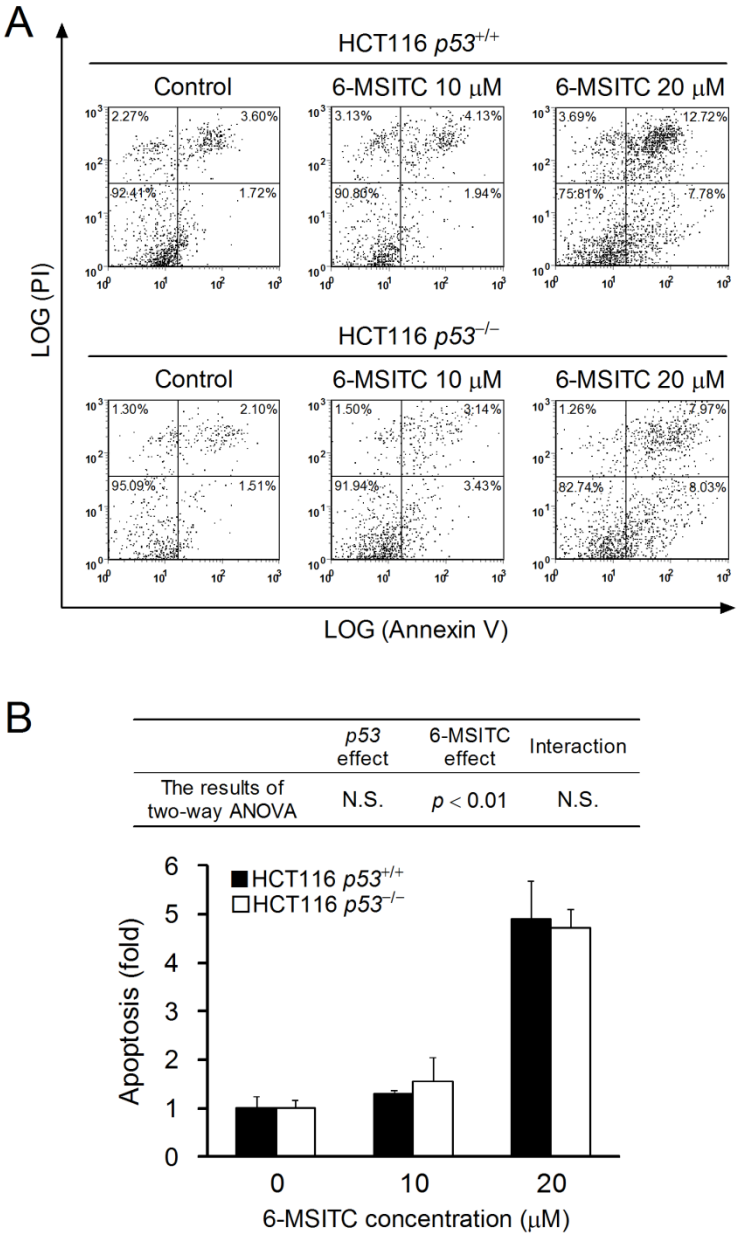


Figure 2.4. Apoptotic cell death induction by 6-MSITC in HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells. A) Flow cytometric analysis of apoptosis induction. Both types of cells were treated with 0.2% DMSO as control or 20 μM of 6-MSITC for 48 h. The harvested cells were stained with FITC Annexin V and propidium iodide (PI), followed by flow cytometric analysis. **B)** Quantitation of apoptotic cell death in (A). Both FITC Annexin V-positive/PI-negative cells and FITC Annexin V-positive/PI-positive cells were counted as apoptosis, and the fold change of apoptosis was expressed as ratio of the treatment to control. The data represent the mean ± SD of combined three independent experiments with duplicate or triplicate. The results of two-way ANOVA were shown as a table in the upper side of graph (N.S.: not significant).

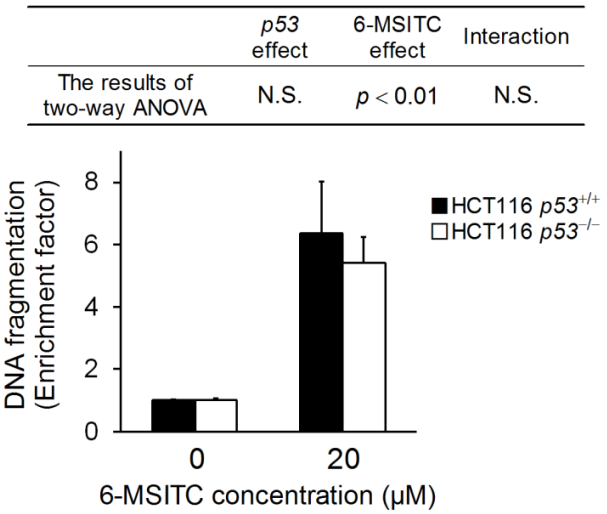


Figure 2.5. Detection of DNA fragmentation by 6-MSITC in HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells. Both types of cells were treated with 0 or 20 μM of 6-MSITC for 48 h, followed by Cell Death Detection ELISA^{PLUS} Kit as described in Materials and methods. The data represent the mean ± SD of combined three independent experiments with triplicate. The results of two-way ANOVA were shown as a table in the upper side of the graphs (N.S.: not significant).

indicated that *p53* had no significant effect on the apoptosis induction. These results suggested that 6-MSITC induced apoptotic cell death in both HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells in a *p53*-independent manner.

2.3.5 6-MSITC has anti-cancer potency of human colon cancer cells even though *p53* deficient

To clarify the molecular events of 6-MSITC-induced apoptotic cell death, I performed the membrane array of apoptotis-related protein. As shown in Figure 2.6B and C, the increased levels of P21, B-cell lymphoma 2-associated X protein (BAX) and death receptor 5 (DR5), but not other death receptors, such as Fas cell surface death

receptor (FAS), death receptor 4 (DR4) and tumor necrosis factor receptor (TNFR), were observed in both types of cells after treatment with 6-MSITC for 48 h. In contrast, the decreased levels of B-cell lymphoma 2 (BCL-2), B-cell lymphoma-extra large (BCL-XL), heat shock protein (HSP) family, such as HSP27, HSP60 and HSP70, and inhibitor of apoptosis protein (IAP) family, such as X-linked inhibitor of apoptosis (XIAP), cellular inhibitor of apoptosis protein 1 (cIAP1), cellular inhibitor of apoptosis protein 2 (cIAP2) and survivin, were observed in a same manner. These results have suggested that 6-MSITC-caused cell survival inhibition and apoptotic cell death might be associated with cell cycle arrest via P21, extrinsic apoptosis pathway via DR5 and intrinsic apoptosis pathway via mitochondria dysfunction.

2.4 Discussion

In the present study, I found a novel molecular mechanism of 6-MSITC on the inhibition of human colorectal cancer cells (HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$).

First, P53 activation was observed in HCT116 $p53^{+/+}$ cells exposed to 6-MSITC (Figure 2.1 A and B). However, 6-MSITC treatment caused the similar inhibitory effects on the cell survival both HCT116 $p53^{+/+}$ cells and HCT116 $p53^{-/-}$ cells (Figure 2.2). These results suggest that 6-MSITC might suppress the survival of colon cancer cells independent of *p53* status. Next, I characterized the 6-MSITC-treated cells by several approaches (Figure 2.3-2.5), and observed that 6-MSITC caused the similar effects on cell cycle arrest in G_2/M phase, translocation of plasma membrane and the induction of DNA fragmentation in both types of cells.

P53, the tumor suppressor protein, play a central role in apoptosis [8]. Accumulated studies have reported that the loss of normal P53 function due to mutation of *p53* gene

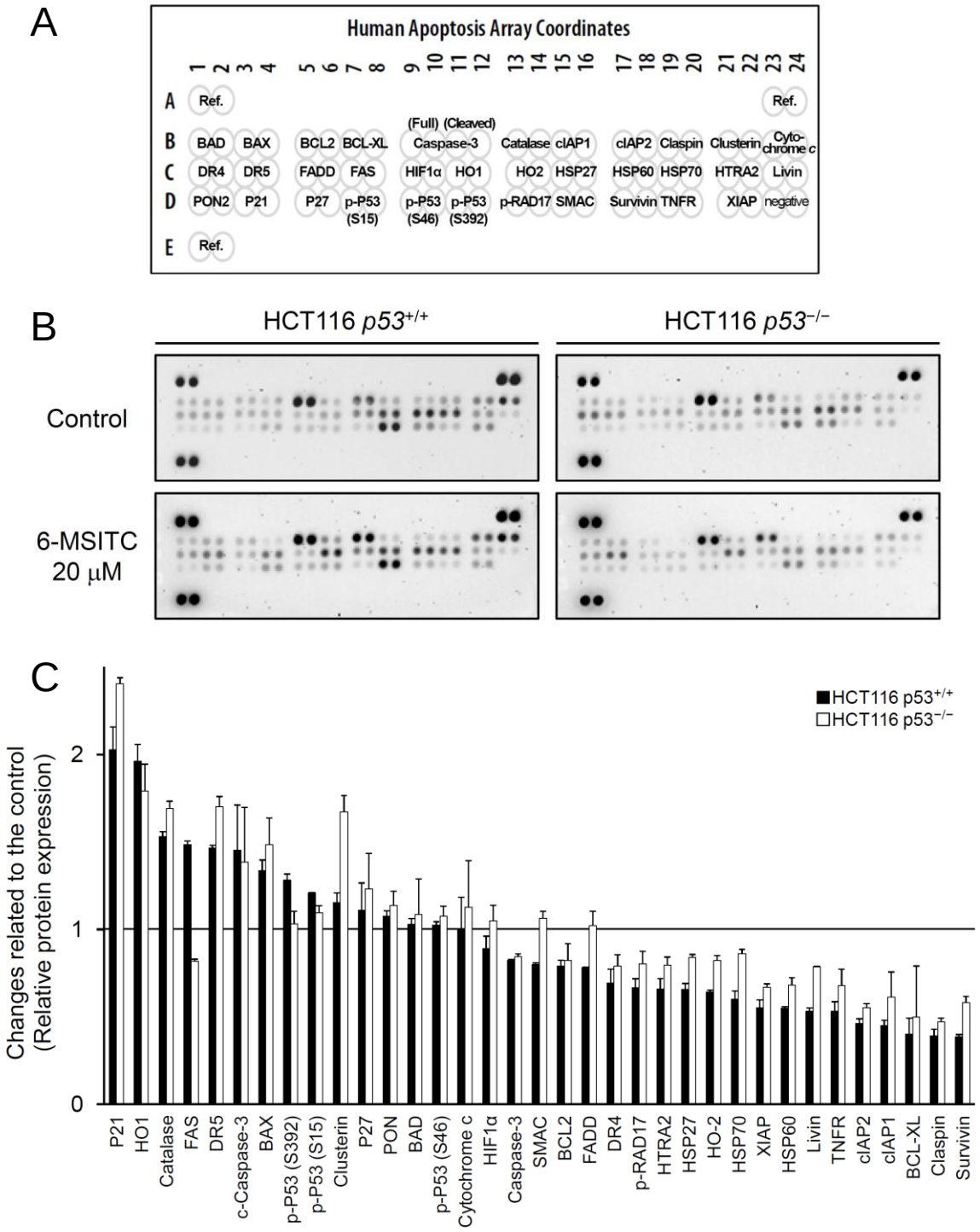


Figure 2.6. The effects of 6-MSITC on apoptotic protein expression. A) Coordinate reference. **B)** Spots data of the array. **C)** Determination of relative change in proteins level to control. Both types of cells were treated with 0 or 20 μM of 6-MSITC for 48 h, followed by Proteome Profiler™ Array Human Apoptosis Array Kit as described in Materials and methods. The data represent the mean ± SD of duplicate.

is observed in a number of human cancers [10]. Thus, *p53* mutations attenuate the effects on apoptosis induction by genotoxic chemotherapeutic drugs [9, 17]. In fact, exposure with cisplatin, oxaliplatin and 5-fluorouracil, apoptosis induction was observed with much higher level in HCT116 $p53^{+/+}$ cells than that in HCT116 $p53^{-/-}$ cells [17, 18]. On the other hand, anticancer antibiotic such as doxorubicin has the ability to induce *p53*-independent apoptosis in cancer cells [19]. However, doxorubicin also caused necrotic cell death and serious NF-κB activation [20, 21]. NF-κB activation by chemotherapeutic drugs contributes to chemoresistance and inflammation, which leads to some side effects [22]. In this study, I found that 6-MSITC induced the apoptosis in both HCT116 $p53^{+/+}$ cells and HCT116 $p53^{-/-}$ cells in a *p53*-independent manner. I also observed that 6-MSITC treatment did not cause NF-κB activation in both types of cells (data not shown).

An increase in the phosphorylation and total protein level of P53 was observed in 6-MSITC-treated HCT116 $p53^{+/+}$ cells (Figure 2.1). This result is different from that in leukemia cells [23], in which there was no significant change in total protein level of P53 from 24–48 h in 6-MSITC-treated cells. On the other hand, treatment with sulforaphane, an analog of 6-MSITC, also increased expression level of P53 in prostate cancer cells [24] and melanoma cells [25], but not in leukemia cells [26]. Therefore, the effects of 6-MSITC on the protein level of P53 might depend on the cancer cell lines.

The universal cell-cycle inhibitor, P21 has been shown to be directly upregulated by transcription factor P53 [27]. P21 play a crucial role in promoting cell cycle arrest via inhibition of cyclin-dependent kinases (CDKs) activity in response to various stimuli [28, 29]. In this study, 6-MSITC treatment caused the similar effects on the cell cycle distribution (Figure 2.3) and the increase of P21 level (Figure 2.6) in both HCT116 $p53^{+/+}$ cells and HCT116 $p53^{-/-}$ cells. These data suggest that 6-MSITC might cause cell

cycle arrest in G_2/M phase via P21 in a *p53*-independent manner.

Death receptors are members of the TNF receptor superfamily, which activated caspase-8 upon binding with ligands, eventually induce apoptotic cell death [30]. Interestingly, 6-MSITC increased DR5 levels, but not FAS, DR4 and TNFR in both types of cells (Figure 2.6). Previously, the increased of death receptors mRNA expression, especially DR5 were observed in other cancer cell lines treated with 6-MSITC (data not shown). Other studies have reported that interferon γ increased FAS level, but not DR4 and DR5 levels in human neural crest-derived cancer cells [31], and chemotherapeutic drugs, melphalan increased DR5 level with *p53*-dependenlty, whereas never increased DR4 level in myeloma cells [32], and doxorubicin increased different death receptors depending on types of breast cancer cells [33]. Thus, to clarify the effects of 6-MSITC on the increase of DR5 level, further investigation of molecular mechanism is required.

On the other hand, the increase of BAX level and the decrease of BCL-2 and BCL-XL levels were observed in both types of cells treated with 6-MSITC (Figure 2.6). This data suggests that 6-MSITC might cause mitochondria dysfunction via change levels of BCL-2 family in a *p53*-independent manner. Accumulated studies have reported that gene expression of BAX and BCL-2 is regulated by P53 [34]. Moreover, P53 directly binds to BAX, BCL-2 and BCL-XL, which induces mitochondrial membrane permeabilization [35, 36]. In fact, other ITCs, such as sulforaphane and benzyl isothiocyanate (BITC), have shown to induce apoptosis through mitochondria dysfunction in human colorectal cancer cells [37, 38].

Furthermore, the decrease of IAP family is observed in both types of cells treated with 6-MSITC. IAP family is involved in suppression of apoptosis via directly inhibition of caspases activity [39]. The expressions of IAP family genes have reported

to be frequently evaluated in human cancers [40–42]. These data suggests that 6-MSITC might promote caspases-mediated apoptosis through downregulation of IAPs levels.

In this study, I demonstrated that 6-MSITC had a potency to induce both intrinsic apoptosis and extrinsic apoptosis, independent of *p53* status. These findings suggest that 6-MSITC might be a potential compound for colon cancer chemoprevention even though with *p53* mutation. However, further investigation is required for understanding molecular mechanism of 6-MSITC-induced apoptosis in human colorectal cancer cells.

2.5 Summary

6-(Methylsulfinyl)hexyl isothiocyanate (6-MSITC) is a major bioactive compound of a popular pungent spice, Wasabi, in Japan. Although 6-MSITC has been reported to have cancer chemopreventive activities against colorectal carcinogenesis in rat model, the underlying mechanism is unclear. In this study, I investigate the anticancer activity using two types of human colorectal cancer cells (HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$). 6-MSITC inhibited the cell viability in both types of cells with similar IC_{50} value. Moreover, 6-MSITC caused cell cycle arrest in G_2/M phase and induced apoptosis in both types of cells in the same fashion. The results of apoptotic protein array showed that the increase levels of P21, death receptor 5 (DR5) and pro-apoptotic BCL-2-associated X protein (BAX), and the decrease levels of anti-apoptotic B-cell lymphoma 2 (BCL-2) and B-cell lymphoma-extra large (BCL-XL) and inhibitor of apoptosis protein (IAP) family were observed in both types of cells treated with 6-MSITC. Taken together, Wasabi 6-MSITC induced cell cycle arrest in G_2/M phase and apoptosis of human colorectal cancer cells by *p53*-independent molecular events, including extrinsic apoptosis pathway via DR5 and intrinsic apoptosis pathway via

mitochondria dysfunction. These findings suggest that 6-MSITC might be a potential agent for colon cancer chemoprevention even though with *p53* mutation.

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Chapter 3 Involvement of ERK1/2-mediated ELK1/CHOP/DR5 pathway in Wasabi 6-MSITC-induced apoptosis of human colorectal cancer cells

3.1. Introduction

A tumor suppressor protein, P53, has been reported to play a central role in cell cycle arrest and apoptosis, although there are many cell signaling pathways to regulate cell viability and death [1]. On the other hand, mitogen-activated protein kinase (MAPK) family plays critical roles in cellular maintenance, including cell growth, differentiation and apoptosis in response to various stimuli [2]. Three subfamilies of MAPK have been identified as c-Jun *N*-terminal kinase (JNK), p38 MAPK (p38) and extracellular signal-regulated kinase (ERK) [2]. The JNK and p38 are activated by various stresses, such as genotoxicity, and implicated in apoptosis induction, including death receptor-initiated extrinsic apoptotic pathways [3, 4]. Publication data has shown that activation of p38 is required for apoptosis induced by chemotherapeutic drugs, such as cisplatin and fluorouracil in human colorectal cancer cells [5]. In contrast, the ERK are also activated by numerous extracellular stresses, which generally represents a major signaling pathway to promote cell viability via the inhibition of apoptosis [6, 7]. RAS/RAF/MEK/ERK pathway is usually initiated by activation of RAS, small GTP-binding proteins, which transmits the signal by recruiting RAF kinases, to cellular membrane, which activates MEK, eventually activates ERK by phosphorylation and then phosphorylates cellular proteins, including transcription factors [8]. Accumulated evidence has provided that constitutive ERK1/2 activation is observed in human colorectal cancer, which may promote tumor progression [9, 10]. However, a

proapoptotic role of ERK1/2 signaling has also been reported in some studies [11–13].

A well documented case is that ERK1/2 signaling positively regulates the expression of death receptor 5 (DR5) to induce apoptosis [14–16]. ERK1/2 activation causes the phosphorylation of ELK1 and upregulation of C/EBP homologous protein (CHOP) through ERK/RSK-mediated ATF4 activation [15]. The phosphorylated ELK1 and increased CHOP bind the promoter (-323/-308, -276/-264) of *dr5* gene to co-operate its transactivation [14, 15]. DR5 is a member of tumor necrosis factor receptor (TNFR) superfamily and cytoplasmic death domain-containing cell surface protein [17]. Increase of DR5 expression by various stimuli, such as small molecule anti-cancer drugs and bioactive compounds, triggers extrinsic apoptotic pathway, ultimately leading to apoptotic cell death [18].

Based on the chemopreventive effect of Wasabi 6-MSITC on the roles of *p53* on apoptosis induction in cancer cells, I used two types of human colorectal cancer cells (HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$) to investigate the molecular mechanisms of Wasabi 6-MSITC in this study.

3.2. Materials and methods

3.2.1 Materials

6-MSITC was kindly provided by Kinjirushi Co., Ltd. (Nagoya, Japan). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the antibody against β-actin were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). SP600125, SB202190, U0126 and the antibodies against caspase-3, caspase-8, PARP, phospho-JNK, phospho-p38, phospho-ERK1/2, JNK, p38, ERK1/2, phospho-ELK1, CHOP and DR5 were from Cell Signaling Technology, Inc. (Danvers, MA, USA).

3.2.2 Cell culture

Human colorectal cancer cell lines HCT116 $p53^{+/+}$ were obtained from the American Type Culture Collection (Manassas, VA, USA) and HCT116 $p53^{-/-}$ cells were kindly supplied by Dr. Bert Vogelstein (Johns Hopkins Medical Institute, Baltimore, MD, USA). The cells were cultured at 37 °C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin glutamine (PSG) for 24 h, and then treated by 6-MSITC in indicated times and doses.

3.2.3 Western blotting analysis

Western blotting was performed as described previously [19]. Briefly, the cells were lysed with modified RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and proteinase inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). The lysates were homogenized in an ultrasonicator for 10 s three times and incubated on ice for 30 min. The homogenates were centrifuged at 14,000 × *g* for 30 min and the supernatants were collected. The protein concentration was determined by Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts of lysate protein were run on SDS-PAGE and electrophoretically transferred to PVDF membrane (GE Healthcare UK Ltd., Amersham, England). The membrane was first blocked with TBST buffer [500 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 0.1% Tween 20] containing 5% nonfat dry milk, and then incubated with specific antibodies overnight at 4 °C and HRP-conjugated secondary antibodies for another 1 h. Bound antibodies were

detected using the ECL system, and relative amounts of proteins associated with specific antibody were quantified using Lumi Vision Imager soft ware (TAITEC Co., Ltd., Saitama, Japan).

3.2.4 Cell viability assay

The cell survival rate was measured by an MTT assay as described previously [20]. In brief, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ (7.0×10^3/well) cells were plated into each well of 96-well plates. The cells were treated with various concentrations of 6-MSITC for 48 h. MTT solution was then added to each well and incubated for another 4 h. The resulting MTT-formazan product was dissolved by the addition of 0.04 N HCl-isopropanol solutions. The amount of formazan was determined by measuring the absorbance at 595 nm with Multiskan™ FC (Thermo Scientific™, Waltham, MA, USA). The cell viability was expressed as the optical density ratio of the treatment to control.

3.2.5 Determination of apoptotic cells by flow cytometry

Quantitation of apoptotic cell death was assessed by FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA) according to the manufacturer's manual. In brief, after treatment with 20 μM 6-MSITC for 48 h, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were suspended in 100 μL of binding buffer and then incubated with FITC Annexin V and propidium iodide staining solution for 15 min. The cells were analyzed at FL1 (530 nm) and FL3 (630 nm) with the flow cytometry (CyFlow®, Sysmex Partec GmbH, Görlitz, Germany).

3.2.6 Statistical analysis

The data represent the mean ± SD. All data were statistically analyzed by two-way or three-way analysis of variance (ANOVA), and then Tukey–Kramer test was used as a post hoc comparison in Figure 3.3 and Figure 3.5. The results of two-way and three-way ANOVA were indicated as a table in the upper side of graphs. A probability of $p < 0.01$ was considered as significant.

3.3. Results

3.3.1 The effects of 6-MSITC on caspase-3 activation and PARP cleavage in HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells

Caspase-3 is an effector caspase, which has been implicated in the execution phase of apoptosis. Caspase-3 has been shown to cleave over 100 substrates including poly(ADP-ribose)polymerase (PARP), and eventually leads to apoptosis [21]. First, in both types of cells treated with 6-MSITC, caspase-3 activation and PARP cleavage were detected (Figure 3.1). These results indicated that 6-MSITC induced apoptosis via caspase-3 in HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells.

3.3.2 The effects of 6-MSITC on MAPKs, ERK1/2, JNK and p38

Accumulated studies reported that MAPKs activated by various stimuli can participate in cell death [7, 22]. Thus, I investigated the effects of 6-MSITC on the activation of MAPKs. Interestingly, a time-dependent activation of ERK1/2, but not JNK and p38, was observed in HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells after treatment with 20 μM 6-MSITC for 6–48 h (Figure 3.2).

3.3.3 The suppressive effects of MAPKs inhibitors on 6-MSITC-caused cell viability inhibition

Furthermore, MTT assay of the cells treated with the MAPK inhibitors revealed that

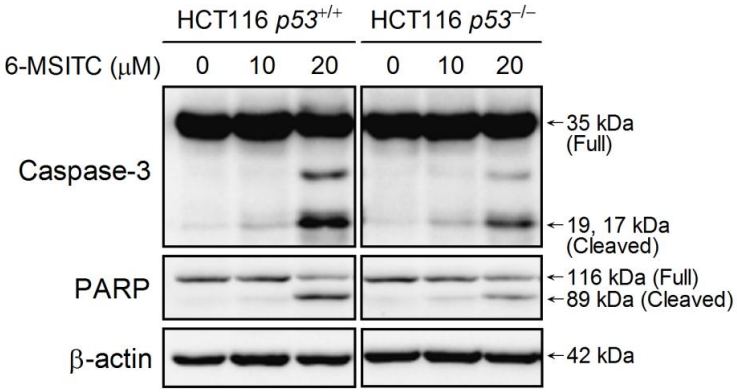

Figure 3.1. Caspase-3 activation and PARP cleavage in HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells. Both types of cells were treated with 0–20 μM 6-MSITC for 48 h, whole cell lysates were used for Western blotting analysis with the indicated specific antibodies.

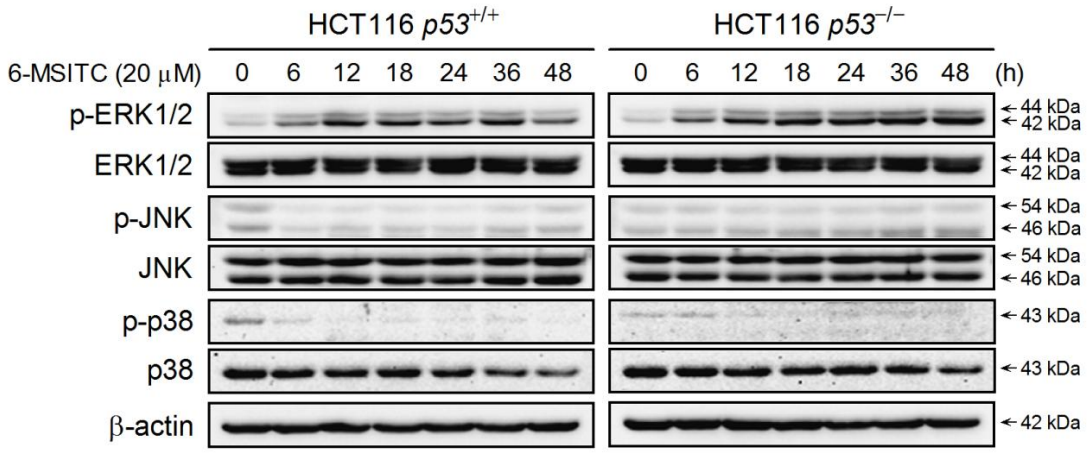

Figure 3.2. Phosphorylation of MAPKs, ERK1/2, JNK and p38 in HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells. Both types of cells were treated with 20 μM 6-MSITC for the indicated times, and the whole cell lysate was used for Western blotting analysis with the indicated specific antibodies.

MEK1/2 inhibitor (U0126), but not JNK inhibitor (SP600125) and p38 inhibitor

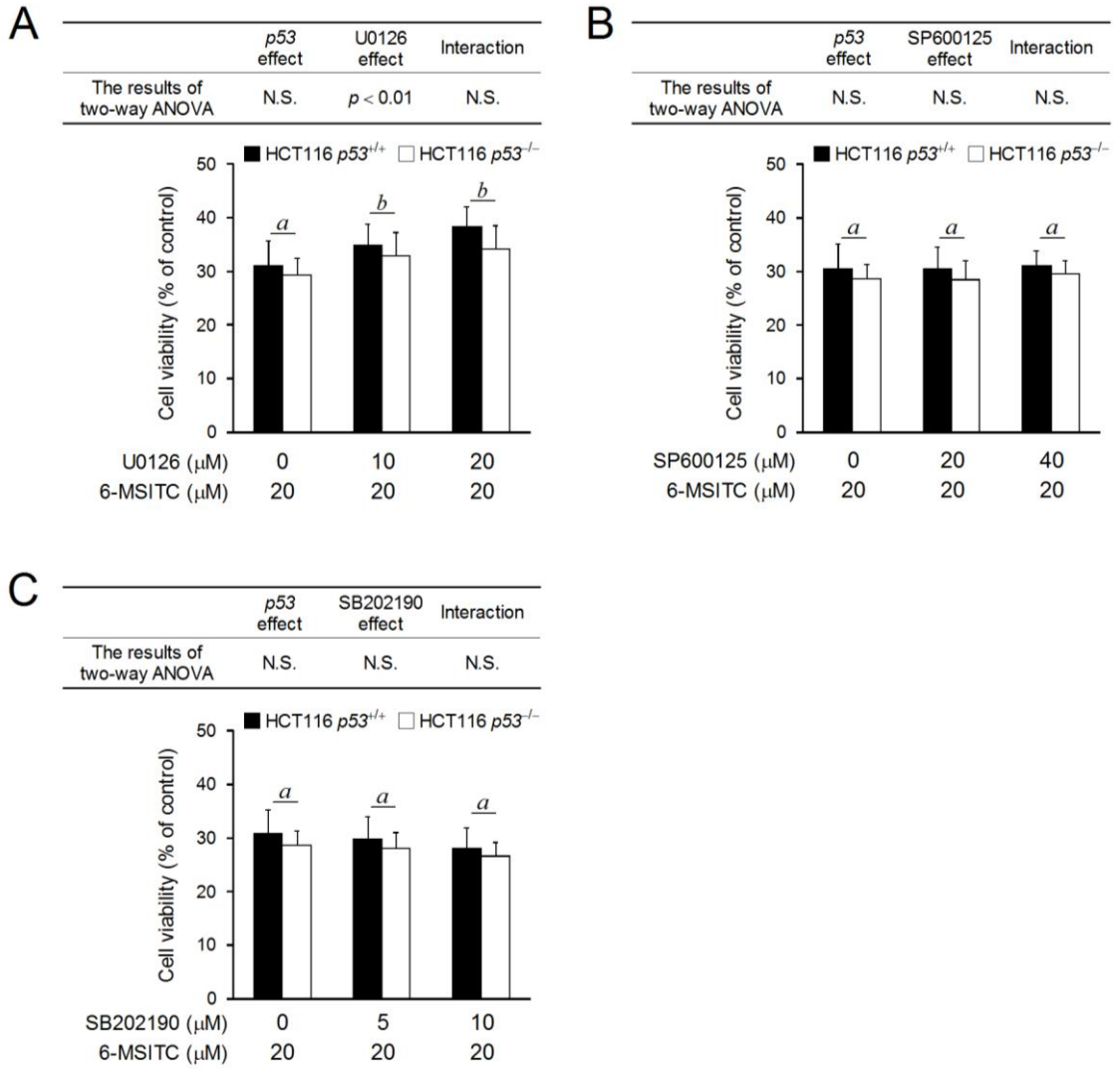

Figure 3.3. The cell viability of HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells treated with 6-MSITC and MAPKs inhibitors. Both types of cells were pretreated with the indicated concentrations of **A)** MEK1/2 inhibitor (U0126), **B)** JNK inhibitor (SP600125) and **C)** p38 inhibitor (SB202190) for 2 h and then exposed to 20 μM 6-MSITC for 48 h. The cell viability rate was assessed colorimetrically after staining with MTT, and was expressed as the optical density ratio of the treatment to control. The data represent the mean ± SD of combined three independent experiments with quadruplicate. The results of two-way ANOVA were shown as a table in the upper side of graphs (N.S.: not significant). Different superscript letters indicate significant difference analyzed with Tukey–Kramer test among the groups after two-way ANOVA.

(SB202190), enhanced significantly the cell viability that was suppressed by 6-MSITC (Figure 3.3). As shown in tables of Figure 3.3, two-way ANOVA results indicated that U0126 had significant effect on the inhibition of cell viability in HCT116 cells treated with 6-MSITC while SP600125, SB202190 and *p53* had not such effect.

3.3.4 MEK1/2 inhibitor, U0126 suppresses 6-MSITC-caused cell apoptotic cell death

To further elucidate whether the ERK1/2 activation is associated with 6-MSITC-induced apoptosis, I next examined apoptotic events in both types of cells treated with 6-MSITC in the presence or absence of U0126. As shown Figure 3.4, U0126 blocked 6-MSITC-induced caspase-3 activation and PARP cleavage in both types of cells. U0126 also blocked 6-MSITC-induced apoptotic cell death in both types

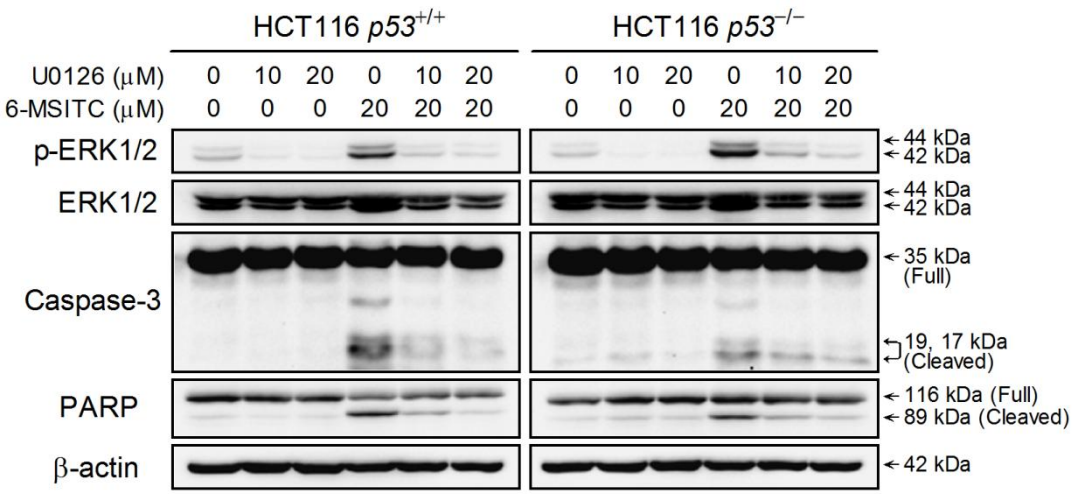

Figure 3.4. The inhibitory effects of U0126 on 6-MSITC-induced caspase-3 activation and PARP cleavage in HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells. Both types of cells were pretreated with the indicated concentrations of MEK1/2 inhibitor (U0126) for 2 h and then exposed to 20 μM 6-MSITC for 48 h. The whole cell lysate was used for Western blotting analysis with the indicated specific antibodies.

of cells (Figure 3.5) in the same fashion. Three-way ANOVA results indicated that U0126, but not *p53*, had significant effect on the apoptosis induction. These results indicated that ERK1/2 activation is required for 6-MSITC-induced apoptotic cell death in both HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells.

	p53 effect	6-MSITC effect	U0126 effect	Interaction
The results of three-way ANOVA	N.S.	$p < 0.01$	$p < 0.01$	N.S.

	p53 × 6-MSITC	*p53* × U0126	6-MSITC × U0126
The results of two-way interaction	N.S.	N.S.	$p < 0.01$

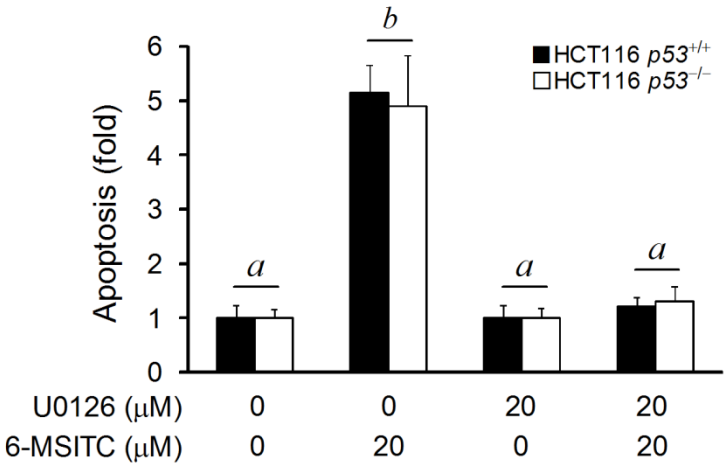

Figure 3.5. The inhibitory effects of U0126 on 6-MSITC-induced apoptotic cell death in HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells. Both types of cells were pretreated with the indicated concentrations of MEK1/2 inhibitor (U0126) for 2 h and then exposed to 20 μM 6-MSITC for 48 h. The harvested cells were stained with FITC Annexin V and propidium iodide (PI), followed by flow cytometric analysis. Both FITC Annexin V-positive/PI-negative cells and FITC Annexin V-positive/PI-positive cells were counted as apoptosis, and the fold change of apoptosis was expressed as ratio of the treatment to control. The data represent the mean ± SD of combined four independent experiments with duplicate. The results of three-way ANOVA were shown as a table in the upper side of graph (N.S.: not significant). Different superscript letters indicate significant difference analyzed with Tukey–Kramer test among the groups after two-way ANOVA (6-MSITC × U0126).

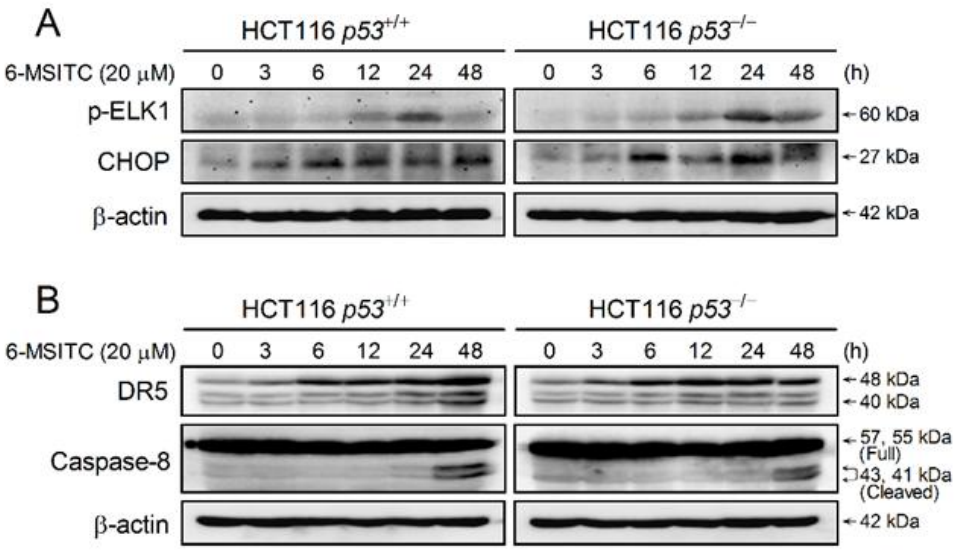

Figure 3.6. 6-MSITC-activated ERK1/2 positively regulated DR5 level in HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells. A) Time-course to detect the phosphorylation of ELK1 and level of CHOP. **B)** Time-course to detect the level of DR5 and activation of caspase-8. Both types of cells were treated with 20 μM 6-MSITC for the indicated times, and the whole cell lysate was used for Western blotting analysis with the indicated specific antibodies.

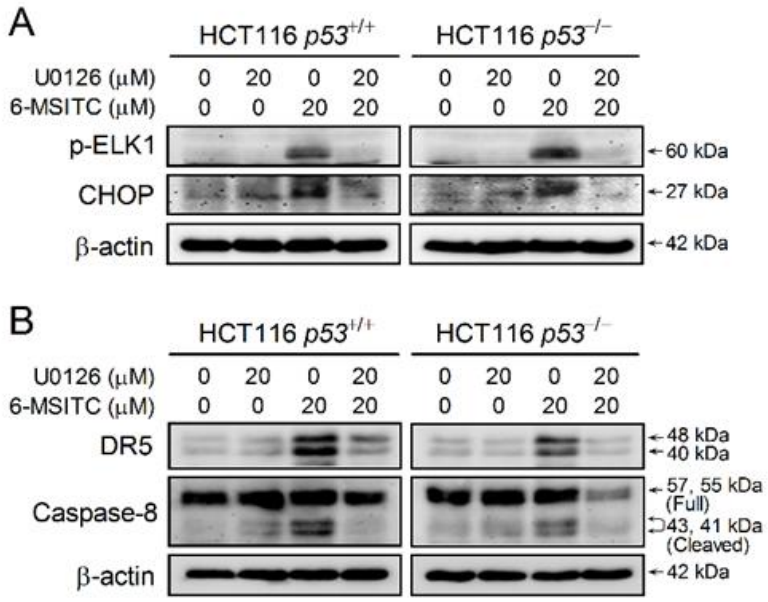

Figure 3.7. The suppressive effect of U0126 on 6-MSITC-induced activation of ELK1 and CHOP A), and activation of DR5 and caspase-8 B). Both types of cells were pretreated with the indicated concentrations of MEK1/2 inhibitor (U0126) for 2 h and then exposed to 20 μM 6-MSITC for 24 h (A) and 48 h (B). The whole cell lysate was used for Western blotting analysis with the indicated specific antibodies.

3.3.5 6-MSITC-activated ERK1/2 upregulates DR5 level via ELK1 and CHOP

ERK1/2 is recently reported to positively regulate DR5 expression by stimulating ELK1 phosphorylation as well as CHOP expression [14–16]. Thus, I investigated the ELK1 phosphorylation and CHOP level in both types of cells treated with 20 μM 6-MSITC from 3–48 h. As shown in Figure 3.6A, a time-dependent phosphorylation of ELK1 and increase of CHOP level from 6–24 h were observed in both types of cells. Coincident with this, a time-dependent increase of DR5 level and activation of caspase-8 were also observed in both types of cells (Figure 3.6B). Moreover, U0126 blocked phosphorylation of ELK1 and increase of CHOP level in both types of cells treated with 6-MSITC (Figure 3.7A). U0126 also further blocked increase of DR5 level and activation of caspase-8 in both types of cells treated with 6-MSITC (Figure 3.7B). These results demonstrated that ERK1/2-mediated activation of ELK1 and upregulation of CHOP by 6-MSITC positively regulated DR5 level.

3.4. Discussion

6-MSITC is a major bioactive compound in Wasabi, and has been reported to suppress the rat colon carcinogenesis *in vivo* [23] with unclear molecular mechanism. In the present study, I found a novel molecular mechanism for 6-MSITC on the inhibition of the viability of human colorectal cancer cells (HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$) in this study.

I found that 6-MSITC treatment caused the similar action on caspase-3 activation and PARP cleavage in both types of cells (Figure 3.1). To clarify the upstream signaling of 6-MSITC-induced apoptosis, I further investigated the effects of 6-MSITC on MAPKs signaling which is demonstrated to participate in multiple cellular events

including apoptosis induction [7, 22]. Interestingly, 6-MSITC induced the phosphorylation of ERK1/2, but not JNK and p38, in both types of cells (Figure 3.2). MEK1/2 inhibitor (U0126), but not JNK inhibitor (SP600125) and p38 inhibitor (SB202190), enhanced significantly the cell viability that was suppressed by 6-MSITC (Figure 3.3). U0126 further blocked 6-MSITC-induced apoptotic cell death, caspase-3 activation and PARP cleavage (Figure 3.4 and 3.5.) These data suggested that ERK1/2 activation is recruited for 6-MSITC-induced apoptosis. To future confirm the effect of this overexpression system on cell death-induced by 6-MSITC, the constructs of active ERK1/2 and/or inactive variants will be conducted in our next research. I also noted that 6-MSITC reduced the phosphorylation of JNK and p38 after 6 h late of treatment. It is reported that p38 activation is required for cancer cell survival in colon cancer [24], and activation of JNK promotes colon carcinogenesis [25]. Thus, it is possible that 6-MSITC also inhibited cell survival of both HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells by inhibiting p38 and JNK activation. Future study is required for this.

Recent studies have reported that activation of ELK1 and increase of CHOP through ERK1/2 positively regulated DR5 expression by directly binding to *dr5* gene as enhancer [14–16]. In the present study, I found that 6-MSITC enhanced ELK1 phosphorylation and CHOP level, and finally increased DR5 level in both types of cells (Figure 3.6). Furthermore, U0126 inhibited both phosphorylation of ELK1 and increase of CHOP level, consequently downregulated DR5 level in both types of cells treated with 6-MSITC (Figure 3.7). These results suggested that activation of ERK1/2 by 6-MSITC upregulated DR5 level without *p53*-dependence, leading to apoptotic cell death. Several lines of studies have reported that various cellular stress, such as DNA damage, oxidative stress and endoplasmic reticulum stress, induced CHOP expression in a *p53*-independent manner [26, 27]. Accumulated data have shown that CHOP

expression is mainly regulated at the transcriptional level [28]. In addition, expression of CHOP is also regulated at post-transcriptional level, such as mRNA stability [28]. In fact, MEK1/2 inhibitor such as U0126, but neither JNK inhibitor nor p38 MAPK inhibitor, has been reported to suppress mRNA expression level of CHOP in HCT116 cells [29]. On the other hand, recent studies have shown that DR5 protein might be degraded through deubiquitination [30, 31]. In previous study, 6-MSITC could upregulate the levels of NRF2 by inhibiting the ubiquitination and proteasomal turnover of NRF2 [32]. The effect of 6-MSITC on upregulatinging the levels of DR5, CHOP need to clarify possible proceed via transcriptional or post-transcriptional or post-translational level, which need to be clarified in our next work. Therefore, 6-MSITC seems to be a potential candidate of DR5 inducer for colon cancer chemoprevention and chemotherapy.

Several lines of studies have shown that some analogs of 6-MSITC also have similar actions. For example, sulforaphane up-regulated DR5 in human cancer cells [33, 34], and benzyl isothiocyanate (BITC) also increased DR5 expression and induced apoptosis through LKB1 activation in *p53*-mutated cells [35]. DR5 is a member of TNF receptor superfamily and cytoplasmic death domain-containing cell surface protein [17]. Increase of DR5 expression by various stimuli, such as small molecule anti-cancer drugs and bioactive compounds, triggers extrinsic apoptotic pathway, ultimately leading to apoptotic cell death [18]. Furthermore, *dr5* gene mutation was detected at very low frequency in various cancers [18, 36]. Hence, increase of DR5 expression by bioactive reagents has been recognized as effective cancer therapeutic approach to sensitize effect of combination with TRAIL [18]. Although P53 plays a key role in expression of DR5 [37], DR5 has been also reported to be regulated in a *p53*-independent manner [38]. For instance, celecoxib, a nonsteroidal anti-inflammatory drugs (NSAIDs), increased DR5

expression and sensitized the effect of TRAIL-induced apoptosis to colon cancer cells, with *p53* independence [39].

In conclusion, the activation of ERK1/2, rather than *p53*, is recruited for 6-MSITC-induced apoptotic cell death and suggests that ERK1/2-mediated ELK1/CHOP/DR5 pathway is involved in the molecular mechanisms. These findings will help to understand the chemoprevention mechanisms of 6-MSITC on colon carcinogenesis previously reported in animal model.

3.5. Summary

In this study, I investigate the molecular mechanisms using two types of human colorectal cancer cells (HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$). 6-(Methylsulfinyl)hexyl isothiocyanate (6-MSITC) has been found to have a potency to induce apoptosis in both types of cells in a *p53*-independent manner. As well as tumor suppressor protein P53, mitogen-activated protein kinase (MAPK) signaling are known to play crucial roles in apoptotic events. Signaling analysis data revealed that the activation of ERK1/2, rather than *p53*, is recruited for 6-MSITC-induced apoptosis. 6-MSITC stimulated ERK1/2 phosphorylation, and then activated ERK1/2 signaling including ELK1 phosphorylation, and upregulation of C/EBP homologous protein (CHOP) and death receptor 5 (DR5). The MEK1/2 inhibitor U0126 blocked all of these molecular events induced by 6-MSITC, and enhanced the cell viability in both types of cells in the same manner. These results indicated that 6-MSITC induced apoptosis in colorectal cancer cells via *p53*-independent, ERK1/2-mediated ELK1/CHOP/DR5 pathway, suggesting that 6-MSITC is a potential compound for colorectal cancer chemoprevention even though with *p53* mutation.

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Chapter 4 Apoptosis induction by Wasabi 6-MSITC in human colorectal cancer cells through *p53*-independent mitochondrial dysfunction pathway

4.1 Introduction

Mitochondria are cytosolic organelles that are engaged in energy metabolism and control of stress response [1]. Accumulated evidence have demonstrated that a mitochondria-initiated death pathway plays an important role in triggering apoptosis in response to various extracellular stimuli, which may contribute to tumor suppression with elimination of cells [2–4]. B-cell lymphoma 2 (BCL-2) family proteins [e.g. BCL-2-associated X protein (BAX) and myeloid cell leukemia 1 (MCL-1)] play critical roles in mitochondria-initiated death pathway [5, 6]. BAX is localized to the cytoplasm under normal condition. MCL-1 prevents pro-apoptotic function of BAX [6, 7]. Upon stimulation, activated BAX leads directly to mitochondrial membrane permeability transition [8], which promotes cytochorome *c* release from mitochondria to cytosol [9]. Released cytochrome *c* can activate caspase-9, and its downstream caspase-3 [9]. Caspase-3 is executioner for apoptosis, which cleaves specific substrates such as poly(ADP-ribose) polymerase 1 (PARP), and eventually leads to apoptotic cell death [10].

P53, tumor suppressor protein, has known to be participated in mitochondrial apoptosis [11]. The roles of P53 in mitochondria are reported to be control of BCL-2 family proteins activity through directly interaction and transcriptional regulation, which cause mitochondria dysfunction, including membrane permeabilization and membrane potential loss [12, 13]. The expression of BAX is upregulated by P53 via the

binding to its promoter regions, whereas the expression of MCL-1 is downregulated by P53 [14, 15]. Several studies have shown that genotoxic chemotherapeutic agents increased BAX level via *p53*, and induced *p53*-dependent apoptosis [16, 17].

4.2 Materials and methods

4.2.1 Materials

6-MSITC (purity > 99%) (Fig. 1A) was kindly provided by Kinjirushi Co., Ltd. (Nagoya, Japan). 3,3′-Dihexyloxacarbocyanine iodide ($DiOC_6(3)$) was from Molecular Probes® (Eugene, OR, USA). The antibodies against caspase-3, caspase-9, PARP, and GAPDH were from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibodies against BAX, cytochrome *c* and COX-IV were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). MCL-1 antibody was from BD Biosciences (San Diego, CA, USA).

4.2.2 Cell culture

Human colorectal cancer cell lines HCT116 $p53^{+/+}$ were obtained from the American Type Culture Collection (Manassas, VA, USA) and HCT116 $p53^{-/-}$ cells were kindly supplied by Dr. Bert Vogelstein (Johns Hopkins Medical Institute, Baltimore, MD, USA). The cells were cultured at 37 ℃ in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin glutamine (PSG) for 24 h, and then treated by 6-MSITC in indicated times and doses.

4.2.3 Western blotting analysis

Western blotting was performed as described previously [18]. Briefly, the cells were lysed with modified RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and proteinase inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). The lysates were homogenized in an ultrasonicator for 10 s three times and incubated on ice for 30 min. The homogenates were centrifuged at 14,000 × *g* for 30 min and the supernatants were collected. The protein concentration was determined by Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts of lysate protein were run on SDS-PAGE and electrophoretically transferred to PVDF membrane (GE Healthcare UK Ltd., Amersham, England). The membrane was first blocked with TBST buffer [500 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 0.1% Tween 20] containing 5% nonfat dry milk, and then incubated with specific antibodies overnight at 4 °C and HRP-conjugated secondary antibodies for another 1 h. Bound antibodies were detected using the ECL system, and relative amounts of proteins associated with specific antibody were quantified using Lumi Vision Imager software (TAITEC Co., Ltd., Saitama, Japan).

4.2.4 Flow cytometric detection of mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was assayed by detecting the retention of $DiOC_6(3)$ on mitochondria. The cationic lipophilic fluorochrome $DiOC_6(3)$ is a cell permeable marker that specifically accumulates into mitochondria depending on $\Delta\Psi_m$. HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells were treated by 20 μM 6-MSITC for various times and then incubated with 50 nM of $DiOC_6(3)$ for 30 min. The cells were washed with PBS, resuspended in 1 mL of PBS

and analyzed at FL1 (530 nm) with the flow cytometry (CyFlow®, Sysmex Partec GmbH, Görlitz, Germany).

4.2.5 Subcellular fractionation for cytochrome c detection

The subcellular fractions were prepared with Mitochondria/Cytosol Fractionation Kit (BioVision Inc., Milpitas, CA, USA) according to the manufacturer's manual. The harvested cells were suspended in Cytosol Extraction Buffer. After incubation on ice for 10 min, the cells were homogenized for 40 strokes and centrifuged at 700 × *g* for 10 min at 4 °C. The supernatant was further centrifuged at 10,000 × *g* for 30 min at 4 °C to isolate cytosol and mitochondria fractions. The supernatant was used as cytosolic fraction. The pellet was dissolved in Mitochondria Extraction Buffer and used as mitochondria fraction.

4.2.6 Statistical analysis

The data represent the mean ± SD. All data were statistically analyzed by two-way analysis of variance (ANOVA). The results of two-way ANOVA were indicated as a table in the upper side of graphs. A probability of $p < 0.01$ was considered as significant.

4.3 Results

4.3.1 6-MSITC activates caspase-9 with time dependently in both HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells

The activation of caspase-8 was also observed from 24–48 h in both types of cells (Figure 3.6). Moreover, activations of caspase-9 and caspase-3, and PARP cleavage, as other hallmarks of apoptosis [9, 10], were observed from 24–48 h in both types of cells

(Figure 4.1). This data showed that 6-MSITC-induced apoptosis in both types of cells might be involved in mitochondria dysfunction.

4.3.2 6-MSITC induces mitochondrial dysfunction in a *p53*-independent manner

Mitochondria dysfunction is a key event for apoptosis [2–4], including cytochrome

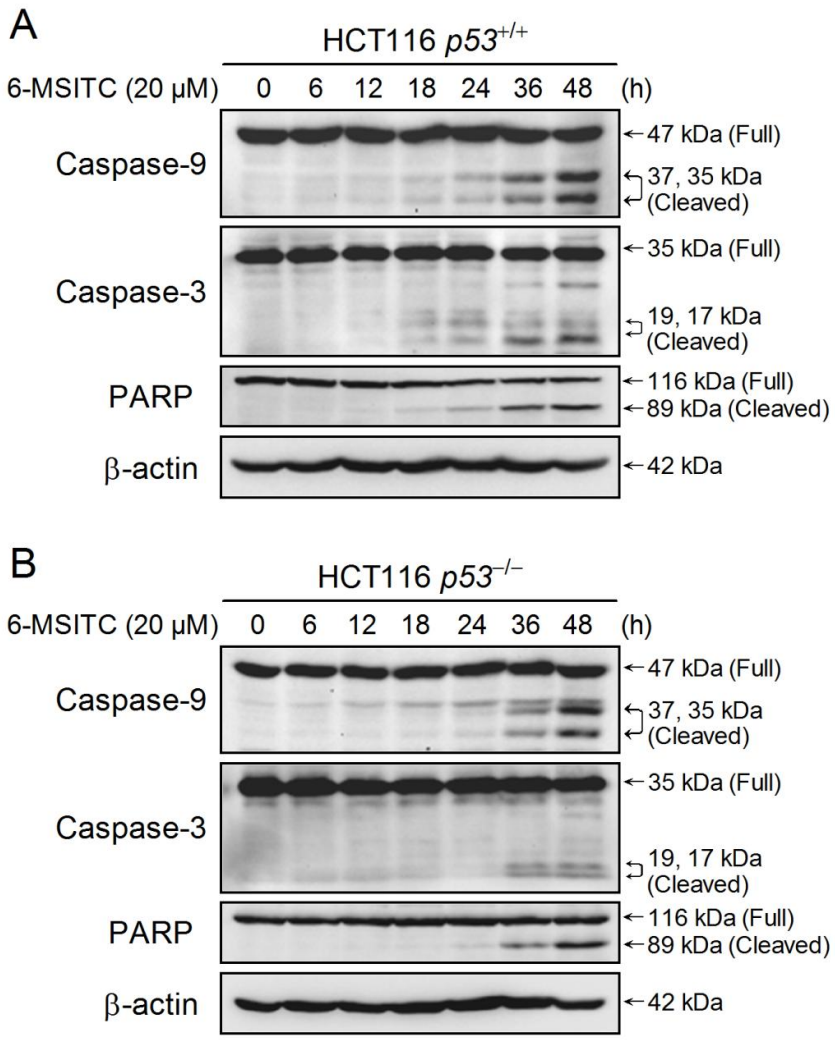

Figure 4.1. Activation of caspase-9 and caspase-3 and cleavage of PARP. A) HCT116 $p53^{+/+}$ and **B)** HCT116 $p53^{-/-}$ cells were treated with 20 μM 6-MSITC for the indicated times, and the whole cell lysate was used for Western blotting analysis with the indicated specific antibodies.

c release from mitochondria to cytosol following $\Delta\Psi_m$ loss, which causes caspase-9-dependent activation of caspase-3 [9]. The members of BCL-2 family play crucial roles in regulation of mitochondrial dysfunction [5, 6]. Thus, I investigated whether these events were involved in the apoptosis of HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells treated with 20 μM 6-MSITC. As shown in Figure 4.2, the ratio of BAX/MCL-1 was increased in a time-dependent manner in both types of cells, and *p53* had no significant effect on the ratio according to the results of two-way ANOVA

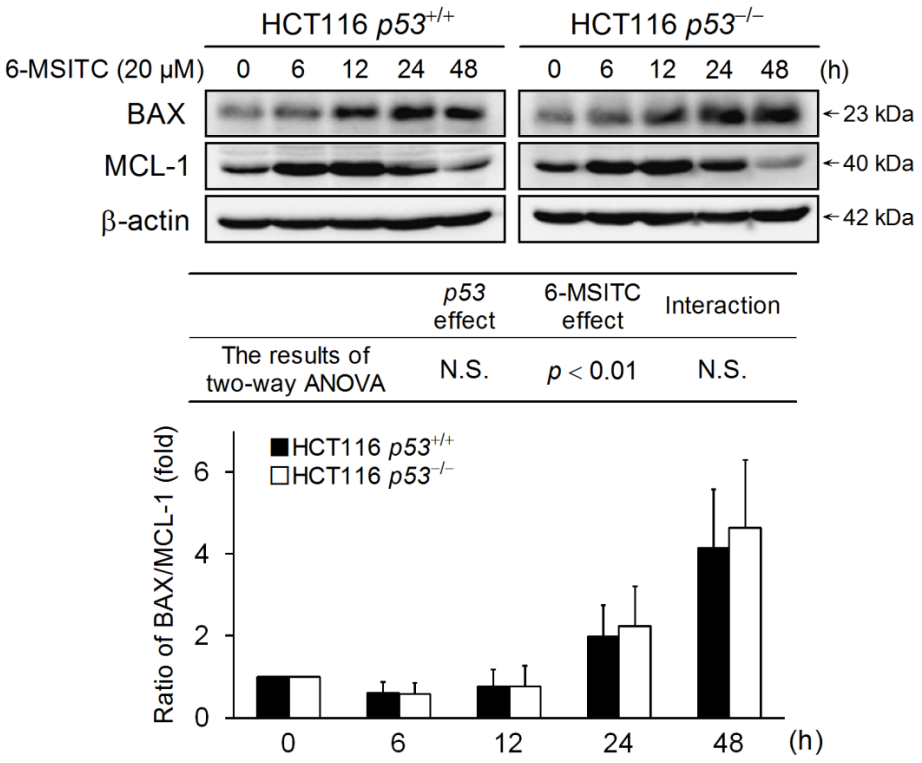

Figure 4.2. The change in the protein levels of pro-apoptotic BAX and anti-apoptotic MCL-1 in HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells. Both types of cells were treated with 20 μM 6-MSITC for the indicated times, and the whole cell lysate was used for Western blotting analysis with the indicated specific antibodies. Densitometry of the blots was performed using the Lumi Vision Imager software. Histograms show the densitometric analysis of BAX to MCL-1. The data represent the mean ± SD of combined three independent experiments with single replicate. The results of two-way ANOVA were shown as a table in the upper side of graph (N.S.: not significant).

analysis. A time-dependent loss of $\Delta\Psi_m$ from 6–48 h was also observed in the same treatment after staining with mitochondria-specific dye $DiOC_6(3)$ (Figure 4.3). Consequently, a time-dependent accumulation of cytochrome *c* in cytosol concomitantly with the efflux of cytochrome *c* from mitochondria occurred (Figure 4.4). These results revealed that 6-MSITC induced the apoptosis in both types of HCT116 cells via *p53*-independent mitochondria dysfunction.

4.3.3 MEK1/2 inhibitor, U0126, has no suppressive effect of 6-MSITC-induced mitochondrial apoptosis

The 6-MSITC-induced apoptosis via death receptor 5 (DR5) was associated with

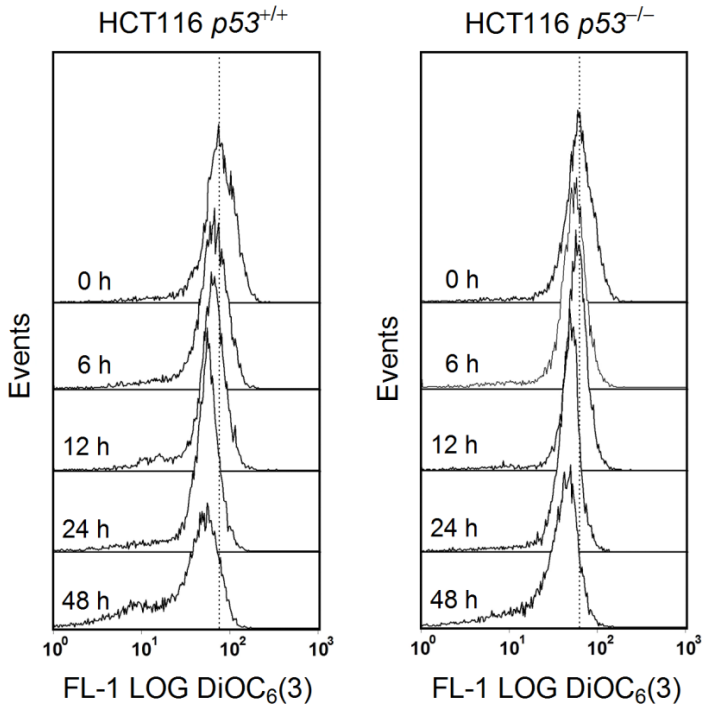

Figure 4.3. Loss of $\Delta\Psi_m$ in HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells treated with 6-MSITC. Both types of cells were treated with 20 μM 6-MSITC for the indicated times. The harvested cells were then incubated with 50 nM $DiOC_6(3)$ for 30 min, followed by flow cytometric analysis as described in Materials and methods.

MEK1/2-ERK1/2 pathway (Figure 3.4-3.7). As shown in Figure 4.5, MEK1/2 inhibitor, U0126 has no inhibitory effect on 6-MSITC-caused $\Delta\Psi_m$ loss and cytochrome *c* release. This data indicated that 6-MSITC-activated MEK1/2-ERK1/2 pathway was involved in extrinsic apoptosis, but not intrinsic apoptosis via mitochondrial dysfunction.

4.4 Discussion

In the present study, I found a novel molecular mechanism of 6-MSITC on the inhibition of human colorectal cancer cells (HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$).

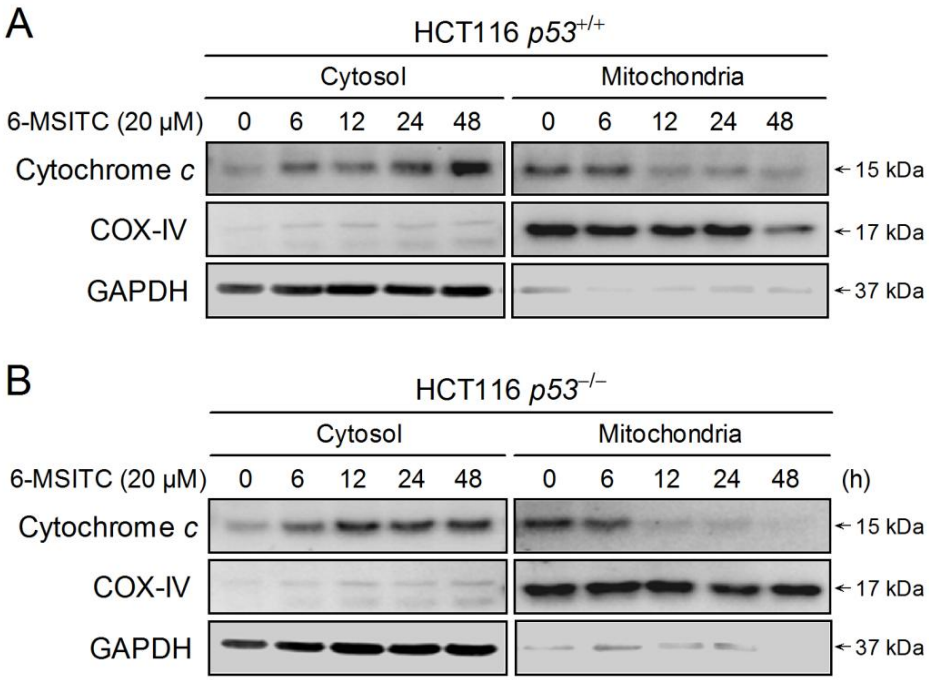

Figure 4.4. Release of cytochrome *c* to cytosol from mitochondria. A) HCT116 *p53*$^{+/+}$ cells and **B)** HCT116 *p53*$^{-/-}$ cells were treated with 20 μM 6-MSITC for the indicated times. The harvested cells were fractionated into cytosolic and mitochondrial fractions as described in Materials and methods. Cytochrome *c* and compartment-specific cytosolic GAPDH or mitochondria COX-IV proteins were detected by Western blotting analysis with corresponding antibodies.

First, I observed that 6-MSITC caused the similar effects on increased BAX/ MCL-1 ratio, mitochondria $\Delta\Psi_m$ loss and cytochrome *c* release in both types of cells.

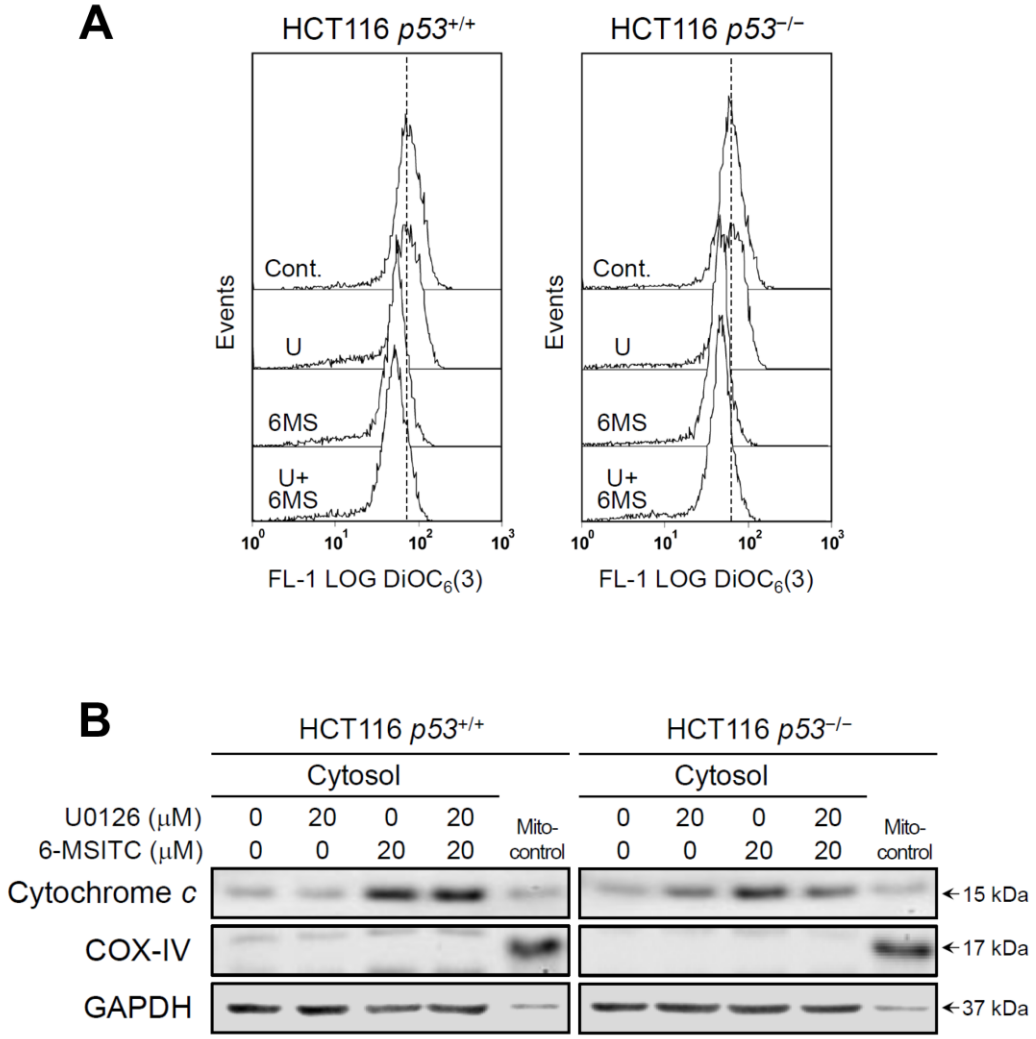

Figure 4.5. The effects of U0126 on 6-MSITC-caused $\Delta\Psi_m$ loss and cytochrome *c* release. A) Loss of $\Delta\Psi_m$. Both types of cells were pretreated with 20 μM of MEK1/2 inhibitor (U0126) for 2 h and then exposed to 20 μM 6-MSITC for 48 h. The harvested cells were then incubated with 50 nM $DiOC_6(3)$ for 30 min, followed by flow cytometric analysis as described in Materials and methods. **B**) Release of cytochrome *c* to cytosol from mitochondria. Cell treatment and culture were the same as (A). The harvested cells were fractionated into cytosolic and mitochondrial fractions as described in Materials and methods. Cytochrome *c* and compartment-specific cytosolic GAPDH or mitochondria COX-IV proteins were detected by Western blotting analysis with corresponding antibodies.

The ratio of BAX and BCL-2 is well used as critical for apoptosis. MCL-1 is one member of BCL family, recent study has indicated that suppression of anti-apoptotic MCL-1 and B-cell lymphoma-extra large (BCL-XL) caused activation of BAX and BCL2 antagonist/killer 1 (BAK), which led to apoptosis without BH3-only proteins such as BH3 interacting domain death agonist (BID) and P53 in HCT116 cells [19]. Furthermore, MCL-1 has been frequently observed as amplified expression in human cancers [20], and recently considered as a target for colon cancer therapy [21, 22]. These results of presented study suggested that 6-MSITC inhibited the viability of HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells through the induction of apoptotic cell death in mitochondrial dysfunction pathway**,** independent of *p53* status.

Furthermore, death receptors (e.g. DR4 and DR5) have been known as cell surface proteins that initiated extrinsic death pathway via caspase-8 activation upon interacting with death ligands (e.g. TRAIL), which linked to mitochondria dysfunction and activation of caspase cascades, led to induce apoptotic cell death [23]. Additionally, death receptors are expected as targets for cancer therapy, because they have capability of inducing apoptosis in cancer cells independent with P53 [24]. 6-MSITC has reported to induce extrinsic apoptosis via caspase-8 but not mitochondrial intrinsic apoptosis in leukemia cell lines [25]. In this study, I demonstrated that 6-MSITC had potency to induce both intrinsic and extrinsic apoptosis, independent of *p53* status.

The MEK1/2-ERK1/2 pathway is known to be involved in numerous molecular events, such as proliferation, cell cycle, drug resistance and cell death in response to various stimuli [26, 27]. In this study, 6-MSITC-activated ERK1/2 was not participated in mitochondria dysfunction (Figure 4.5), although 6-MSITC induced apoptotic cell death through increase of DR5 level via MEK1/2-ERK1/2 signaling (Figure 3.4–3.7). Several studies have shown that drug reagents and ion irradiation caused mitochondria

dysfunction mediated by ERK1/2 signaling in cancer cells [28–30]. In contrast, the protective effects of ERK1/2 signaling on mitochondria in cell death events are also reported in some studies [31–33]. Especially, anti-apoptotic MCL-1 is reported to be stabilized by ERK1/2-mediated phosphorylation at Ser163 [34]. Moreover, MCL-1 is also reported to be dissociated with BCL like 11 (BIM) through ERK1/2-dependent phosphorylation of BIM at Ser65, which promote to interact with pro-apoptotic BAX, eventually inhibit mitochondrial apoptotic cell death [35]. In fact, the temporary increase of MCL-1 level was observed at 6–12 h in both types of cells treated with 6-MSITC (Figure 4.2). The effect of 6-MSITC on mitochondria dysfunction is required to be more clarified in my next work.

In conclusion, 6-MSITC inhibited the cell viability and induced apoptosis in both HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells via *p53*-independent mitochondrial dysfunction pathway. These findings suggest that 6-MSITC might be a potential compound for colon cancer chemoprevention even though with *p53* mutation.

4.5 Summary

In this study, I used two types of human colorectal cancer cells (HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$) to investigate the molecular mechanisms of 6-(methylsulfinyl)hexyl isothiocyanate (6-MSITC) with a focus on mitochondria. The data from mitochondrial analysis revealed that 6-MSITC enhanced the ratio of pro-apoptotic BCL-2-associated X protein (BAX)/anti-apoptotic myeloid cell leukemia 1 (MCL-1), and sequentially caused mitochondrial membrane potential ($\Delta\Psi_m$) loss, cytochrome *c* release, and caspase-3 activation in both types of cells. 6-MSITC-activated ERK1/2 has been involved in extrinsic apoptotic cell death via ELK1/CHOP/DR5, however, the MEK1/2

inhibitor U0126 had no effect on mitochondria dysfunction caused by 6-MSITC. Taken together, Wasabi 6-MSITC induced apoptosis of human colorectal cancer cells in *p53*-independent mitochondrial dysfunction pathway differently from ERK1/2-mediated ELK1/CHOP/DR5 pathway. These findings suggest that 6-MSITC might be a potential agent for colon cancer chemoprevention even with the occurrence of *p53* mutation.

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Chapter 5 Discussion and conclusion

5.1. Discussion

5.1.1 Cancer preventive effects

Previously, oral administration of 6-MSITC in colon carcinogenesis model of rat has been reported that 6-MSITC could suppress the number of colonic aberrant crypt foci induced by 1, 2-dimethylhydrazine [6], suggesting that 6-MSITC has a chemopreventive potency for colon carcinogenesis, however, the underlying mechanisms are not clear. In the present study, 6-MSITC has the capable of cell survival inhibition and cell cycle arrest in G_2/M phase in human colorectal cancer cells even though with *p53* deficiency. Furthermore, 6-MSITC induced apoptotic cell death through ERK1/2-mediated ELK1/CHOP/DR5 pathway and mitochondria dysfunction pathway in human colorectal cancer cells with a *p53*-independent manner. In addition, 6-MSITC also has the potency to decrease levels of IAP family proteins, caspases inhibitor proteins. These findings will help us to understand the chemoprevention mechanisms of 6-MSITC on colon carcinogenesis previously reported in animal model.

According to epidemiological research, high intake of cruciferous vegetables, containing isothoicyanates (ITCs) has a potency to reduce the risk of human colon cancer [2]. Additionally, ITCs have not been appeared to be significant toxicity to human health [3]. In fact, the intraperitoneal injection and oral administration of Wasabi 6-MSITC have not shown the toxicity to mice [4, 5]. Thus, these evidence suggest that ITCs may be the potential compounds for cancer chemoprevention [6]. Accumulated data have shown that ITSs selectively inhibited cell survival and caused cell death in cancer cell [7–10], especially sulforaphane, an analogues of 6-MSITC, induced

apoptosis in HCT116 cells, but not normal colon mucosal epithelial cell [9]. The telomerase reverse transcriptase (TERT) has been known to be widely high expressed in more than 90% of human cancers, but not normal cells, and play a critical role in the maintenance of telomeres which might be associated with human carcinogenesis [11, 12]. Recently, sulforaphane has been reported to suppress the expression of *TERT* via epigenetic regulation, which selectively induce apoptotic cell death in human cancer cells, owing to high *TERT* expression [13–15]. The colorectal cancer chemopreventive properties of Wasabi 6-MSITC are need to further investigate molecular mechanisms related to genetics and epigenetics.

5.1.2 Chemical property

Wasabi 6-MSITC belongs to ITC family, characterized by the presence of electrophilic sulfur group –N=C=S. The lipophilicity in side chains of ITCs might have an impact on protein binding and chemopreventive activity [16]. Several studies has indicated that increased alkyl chain lengths enhance the inhibitory activity of arylalkyl ITCs, such as benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC), against tumorigenesis in mouse model [16–18], whereas too longer alkyl chain lengths (C_8–C_{10}) might decline the chemopreventive effects, owing to its poor solubility [19]. Additionally, alkyl ITCs, such as hexyl isothiocyanate, have been shown to enhance the inhibitory effect of tumorigenesis as alkyl chain lengths is longer (C_{12}) [17]. In fact, 6-MSITC (C_6), methyl sulfinyl group of alkyl ITCs, indicated the inhibitory effect on HCT116 cells survival stronger than sulforaphane (C_4) (data not shown).

Accumulated data have suggested that the chemopreventive effects of ITCs are associated with direct reaction of the carbon atom of the –N=C=S with many cellular proteins having a nucleophilic groups [20, 21]. The central carbon atom of the –N=C=S

group has a highly electrophilicity, which reacts rapidly with oxygen-, sulfur-, or nitrogencentered nucleophiles, eventually gives rise to carbamates, thiocarbamates, orthiourea derivatives, respectively [22]. Thus, the ITCs have a potency to react with α-amino groups of N-terminal residues, and cysteine and lysine [21]. Several evidence have shown that ITCs bind to both α- and β-tubulin, and then lead to misfolding and aggregation, which may trigger G_2/M phase arrest and apoptosis induction [23, 24]. High concentration of 6-MSITC (40 μM) presented degradation of α-tubulin in Western blotting, suggesting that 6-MSITC may cause G_2/M phase arrest via tubulin degradation. It is well known that cysteine is often located in the catalytic centers of enzymes, such as caspases. In fact, caspase-3 was degraded, not cleaved in both types of cells treated with 40 μM 6-MSITC which had no effect of inducing apoptosis (data not shown). This data suggests that 6-MSITC may bind to catalytic center of caspase-3, lead to degrade its protein. On the other hand, the methyl sulfinyl ITC group, 6-MSITC and sulforaphane have a potency to induce anti-oxidant enzymes with higher level than other ITCs, suggesting that this group may have another potency of anti-cancer [25, 26]. The chemical property and structure activity of Wasabi 6-MSITC are need to further investigate.

5.1.3 P53 and ERK1/2 in cancer

The tumor suppressor gene, *p53* is involved in multiples central cellular events, including DNA repair, maintenance genomic stability, senescence, cell cycle control and apoptosis [27, 28]. It has been well documented that many chemotherapeutic drugs revealed their anti-cancer effects by activating P53 with genetoxic stress [29]. However, the mutation of *p53* gene has been reported to occur in approximately 40–50% of colorectal cancers, is closely related to the progression [30]. In the present study,

6-MSITC has shown to induce apoptosis in a *p53* independent manner. Other ITCs, such as sulforaphane, BITC and PEITC, have been also reported to induce apoptotic cell death in *p53*-independent mechanisms [31–33]. Furthermore, mutated P53 has noted to gain other functions that help to contribute to tumor progression [34, 35], and reduce the effect of apoptosis induction [36]. Interestingly, the ITCs have a potency to rather decrease mutated P53 level, but not the wild-type P53 level via a transcription-independent mechanism in cancer cells [37]. Publish study has shown that 6-MSITC is capable of inducing apoptosis in *p53*-mutated cancer cell lines [38].

As well as *p53*, the RAS/RAF/MEK/ERK signaling is frequently dysregulated in colorectal cancer [39–41]. According to accumulated research, the gene mutation of *ras* and *raf* are observed with 32–40% and 15% in colorectal cancers, respectively, which contribute to activate ERK1/2 constitutively [41]. The level of ERK1/2 activation has reported to be associated with tumor progression [42]. In contrast, several studies have documented that certain compounds upregulated DR5 through ERK1/2 signaling, which led to induce apoptotic cell death [43, 44]. Actually, DR5 has been indicated to be higher expressed in colorectal cancer cells due to ERK1/2 activation than normal pairs [45], meanwhile, in colorectal advanced-stage cancers, DR5 levels rather decrease [46]. Moreover, *dr5* gene mutation was detected at very low frequency in various cancers [47, 48]. Thus, DR5 has been recognized as the target of colorectal cancer therapeutic approach, and bioactive reagents that have a potency to increase of its expression may be the candidates for cancer chemopreventive and chemotherapeutic reagents. In this study, 6-MSITC rather activated ERK1/2, which led to induce apoptosis via ELK1/CHOP/DR5 in a *p53*-independent manner, suggesting that 6-MSITC might be a potential agent to induce DR5 for colon cancer chemoprevention even though with p53 mutation.

5.2. Conclusion

This study characterized the effects of colorectal cancer chemoprevention of Wasabi 6-MSITC at cellular model through targeting the molecular mechanisms of apoptotic cell death.

1. Wasabi 6-MSITC had a potency to induce cell cycle arrest in G_2/M phase and apoptotic cell death in human colorectal cancer cells in a *p53*-independent manner.

2. Wasabi 6-MSITC induced extrinsic apoptosis in colorectal cancer cells via *p53*-independent, ERK1/2-mediated ELK1/CHOP/DR5 pathway.

3. Wasabi 6-MSITC induced apoptosis of human colorectal cancer cells in *p53*-independent mitochondrial dysfunction pathway.

These findings will help us to understand the chemoprevention mechanisms of Wasabi 6-MSITC on colon carcinogenesis previously reported in animal model, suggesting that Wasabi 6-MSITC might be a potential compound for colorectal cancer chemoprevention even though with *p53* mutation.

5.3. References

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