Original Article

4-Methylthio-3-butenyl isothiocyanate (raphasatin) exerts chemopreventive effects against esophageal carcinogenesis in rats

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Abstract: To examine the effects of 4-methylthio-3-butenyl isothiocyanate on esophageal carcinogenesis, male 6-week-old F344 rats were subcutaneously injected with 0.5 mg/kg body weight *N*-nitrosomethylbenzylamine three times per week for 5 weeks and fed a diet supplemented with 80 ppm 4-methylthio-3-butenyl isothiocyanate, equivalent to 6.05 mg/kg body weight/day for the initiation stage, 4.03 mg/kg body weight/day for the promotion stage, or 4.79 mg/kg body weight/day for all stages. Although the incidence of lesions was not affected by 4-methylthio-3-butenyl isothiocyanate treatment, the multiplicity of squamous cell papilloma in the esophagus was significantly decreased in rats in the 4-methylthio-3-butenyl isothiocyanate initiation stage group (1.13 ± 0.74), 4-methylthio-3-butenyl isothiocyanate promotion stage group (1.47 ± 0.99), and 4-methylthio-3-butenyl isothiocyanate all stage group (1.47 ± 1.13) as compared with rats treated with *N*-nitrosomethylbenzylamine alone (3.00 ± 1.46). Immunohistochemical analysis revealed that 4-methylthio-3-butenyl isothiocyanate induced apoptosis, suppressed cell proliferation, and increased p21 expression when administered in the promotion phase. These modifying effects were not observed in the rats treated with 4-methylthio-3-butenyl isothiocyanate alone. Our results indicated that 4-methylthio-3-butenyl isothiocyanate may exert chemopreventive effects against *N*-nitrosomethylbenzylamine-induced esophageal carcinogenesis in rats. (DOI: 10.1293/tox.2016-0037; J Toxicol Pathol 2016; 29: 237–246)

Key words: 4-methylthio-3-butenyl isothiocyanate, raphasatin, esophageal cancer, *N*-nitrosomethylbenzylamine, chemoprevention

Introduction

Esophageal cancer is the tenth most common cancer and the sixth most common cause of cancer-related deaths worldwide (about 400,000 deaths/year)[1]. In addition, it is one of the most lethal malignancies, with a 5-year survival rate of only 17% in the USA from 1996 to 2004[2, 3]. In Japan,

the 10-year relative survival rate of men with esophageal cancer was reported to be 24.0%; this was lower than those of other digestive tract cancers (gastric cancer, 61.3%; colon cancer, 68.9%) from 2002 to 2006[4]. Therefore, effective chemopreventive approaches against this disease are urgently required.

Esophageal squamous cell carcinoma (ESCC) is one of the most predominant histological types of esophageal cancer. Epidemiological studies have shown that tobacco smoking, excessive alcohol consumption, and eating pickled vegetables are associated with increased risk of ESCC; these habits can lead to the endogenous generation of nitrosamines, a common type of carcinogen[5]. Furthermore, high concentrations of nitrate nitrogen, the precursor of nitrosamine, in drinking water can increase the risk of ESCC[6]. In contrast, the consumption of fruits and vegetables has been

shown to be associated with a reduced risk of ESCC[7, 8]. In these population-based prospective cohort studies, researchers found a dose-dependent decrease in the risk of ESCC associated with a higher intake of total fruits and vegetables. Of the five fruit and vegetable subgroups investigated in the study of Yamaji *et al.*, only individuals consuming cruciferous vegetables had a significantly reduced risk of ESCC on further adjustment for smoking and alcohol consumption status[8]. Among the different types of fruits and vegetables, cruciferous vegetables may provide a good source of effective cancer-preventing compounds[9]. In particular, isothiocyanates (ITCs), one of the constituents in cruciferous vegetables, have been extensively investigated and have attracted considerable attention owing to their promising chemopreventive effects[9].

ITCs have been shown to suppress tumor development in rodent carcinogenesis models of various organs[9–13]. Moreover, phenethyl isothiocyanate (PEITC) has been reported to suppress *N*-nitrosomethylbenzylamine (NMBA)-induced rat esophageal carcinogenesis when rats are treated throughout the experiment[14–16]. 4-Methylthio-3-butenyl isothiocyanate (MTBITC), a component of the daikon (Japanese white radish), was also found to have antimutagenic effects in an *in vitro* study[17] and was shown to suppress pancreatic carcinogenesis in hamsters treated with *N*-nitrosobis(2-oxopropyl)amine (BOP) when given during the initiation stage[18]. MTBITC is a pungent component of the daikon; therefore, heirloom types of daikon (e.g., Karami, Momoyama, and Sabaga), which are more pungent than the conventional type of daikon (Aokubi), are a rich source of MTBITC[19]. Although several *in vitro* studies have shown that MTBITC has chemopreventive properties, such as antiproliferative and pro-apoptotic effects[20–22], few reports have assessed the chemopreventive effects of this compound *in vivo*[18, 23], and it is unclear whether MTBITC exerts chemopreventive effects in esophageal carcinogenesis.

In this study, we aimed to clarify the potential chemopreventive effects of MTBITC in an *in vivo* model of esophageal carcinogenesis. For this purpose, we employed an NMBA-induced rat esophageal carcinogenesis model to allow the initiated cells to develop into preneoplastic/neoplastic lesions from squamous cells rapidly. NMBA is a carcinogenic nitrosamine that is found in the diet in regions where individuals are at high risk of ESCC[24]. The NMBA-induced rat esophageal carcinogenesis model has been extensively used because the relatively short-term (5 weeks) NMBA-treatment protocol results in a 100% tumor incidence within 23 weeks after the first administration of NMBA and allows for evaluation of the effects of compounds administered before, during, and after NMBA treatment[14, 16]. Using this model, we were able to assess the potential influence of MTBITC on the induction and subsequent progression of esophageal neoplasms. In addition, we performed immunohistochemical analysis to assess the mechanisms involved in MTBITC-dependent chemoprevention.

Materials and Methods

Chemicals

MTBITC was extracted and purified from the roots of heirloom varieties of daikon (*Raphanus sativus*) in Kyoto (Momoyama and Sabaga) as previously described[19] and then stored at −80°C until use. The purity of the MTBITC was 98.0%, as estimated by high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan). NMBA (Nard, Osaka, Japan) was dissolved in 20% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) in saline and was administered at a volume of 5 mL/kg body weight (BW).

Experimental animals and housing conditions

A total of 95 four-week-old male F344/DuCrlCrlj rats were obtained from Charles River Laboratories Japan (Yokohama, Japan) and acclimated for 1 week prior to testing. The rats were housed in polycarbonate cages (3–4 rats per cage) with softwood chips for bedding in a specific pathogen-free animal facility and maintained under controlled conditions (temperature, 23 ± 2°C; relative humidity, 55 ± 5%; air changes, 12 times/h; and lighting, 12-h light-dark cycle) with free access to the basal diet or test diet and tap water.

Experimental protocol

MTBITC was mixed into a powdered basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) at 80 ppm based on its ability to suppress pancreatic tumors in the hamsters[18]. The stability of MTBITC in the mixed diet was analyzed by HPLC, and the concentrations of MTBITC in the mixed diet preserved at 4°C for 4 days and 1 week were 96.6% and 90.7%, respectively, whereas those in mixed diets stored at room temperature for 4 days and 1 week were 75.3% and 75.1%, respectively. The MTBITC/diet admixture was prepared once per week and kept at 4°C in a refrigerator, and the contents of the feeding jar were exchanged twice per week.

As shown in Fig. 1, groups 1 (untreated) and 2 (MTBITC-treated) included 10 rats (5 weeks old) each that were fed the basal diet or a diet containing 80 ppm MTBITC without NMBA treatment or DMSO (vehicle) for analysis of the effects of MTBITC alone. Rats in groups 3 (DMSO-treated; n = 15) and 4 (NMBA-treated; n = 15) were fed the basal diet throughout the experiment and treated with DMSO or NMBA, respectively. NMBA was administered subcutaneously 3 times per week for 5 weeks at a dose of 0.5 mg/kg BW as previously described[16]. Groups 5–7 (n = 15 animals each) were given both NMBA and MTBITC as follows: for the initiation stage of treatment, from 1 week before NMBA treatment, rats were simultaneously given a diet mixed with 80 ppm MTBITC for 7 weeks and then the basal diet (group 5; NMBA+MTBITC/basal diet); for the promotion stage of treatment, starting 1 week after the end of NMBA treatment, the animals were continuously fed the MTBITC diet for 19
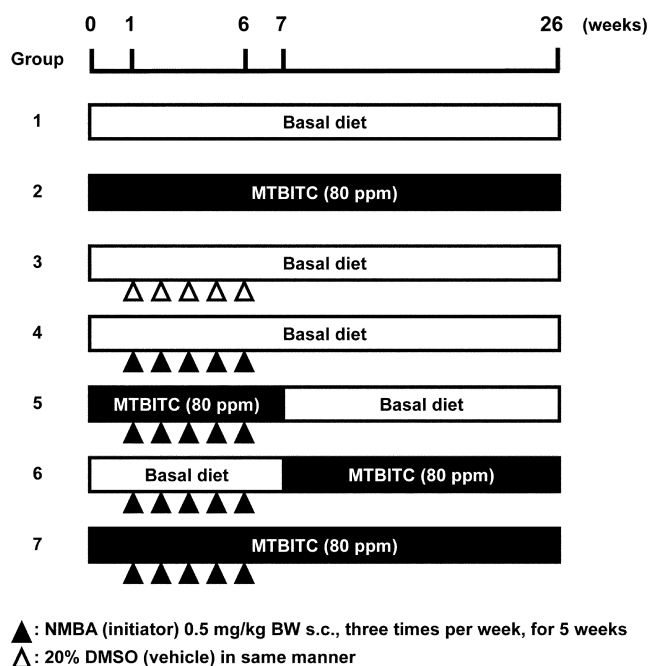
▲: NMBA (initiator) 0.5 mg/kg BW s.c., three times per week, for 5 weeks
△: 20% DMSO (vehicle) in same manner

Fig. 1. Experimental design.

weeks (group 6; NMBA/MTBITC); finally, for all stages of treatment, the MTBITC diet was given throughout the experimental period (group 7; NMBA+MTBITC/MTBITC).

The animals were observed daily and weighed once per week during the experimental period. The amounts of supplied and residual diet were weighed twice per week, when the contents of the feeding jars were exchanged, in order to calculate the average daily food consumption and MTBITC intake throughout the treatment period. At experimental week 26, all animals were anesthetized with isoflurane (Mylan Inc., Tokyo, Japan), and blood samples were collected from the abdominal aorta for hematology and serum biochemistry in groups 1 and 2. Other animals were then euthanized by exsanguination from the abdominal aorta. The protocol was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences (Tokyo).

Hematology and serum biochemistry

To examine whether the dose of MTBITC used in this study itself exerts any effects in rats, hematology and serum biochemistry were analyzed in groups 1 and 2. Hematological examination was performed using a K-4500 automatic hematology analyzer (Sysmex Corp., Kobe, Japan). Aliquots of whole blood samples were mixed with 4 volumes of the supplier's buffer containing 0.5% ethylenediaminetetraacetic acid (EDTA)·2K and applied to the analyzer for the following parameters: white blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet count (PLT). To

measure the differential leukocyte and reticulocyte counts, aliquots of whole blood samples were mixed with a one-fourth volume of 5.0% EDTA·2K solution. Blood smears were then processed for May-Grunwald Giemsa staining and analyzed with a Microx HEG-50S (Sysmex). Serum biochemistry was analyzed at SRL, Inc. (Tokyo, Japan), using sera frozen after centrifugation of whole blood (1,000 × *g* for 10 min). The following parameters were analyzed: total protein (TP), albumin (Alb), albumin/globulin ratio (A/G), total bilirubin (Bil), glucose, triglyceride (TG), total cholesterol (T-Cho), urea nitrogen (BUN), creatinine (Cre), sodium (Na), chlorine (Cl), potassium (K), calcium (Ca), inorganic phosphorus (IP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transpeptidase (γ GTP).

Organ weights and histopathological assessment

After necropsy, the esophagus, tongue, and stomach were removed from all rats. In groups 1 and 2, the brain, thymus, lungs, heart, spleen, liver, adrenal glands, kidneys, and testes were weighed, and the skin, mammary glands, femur with marrow, sternum with marrow, mesenteric lymph nodes, salivary glands (sublingual and submandibular), aorta, trachea, small intestine (duodenum, jejunum, and ileum), large intestine (cecum, colon, and rectum), pancreas, urinary bladder, epididymides, seminal vesicles, prostate gland, pituitary gland, thyroid glands, parathyroid glands, spinal cord with vertebrae (cervical, thoracic, and lumbar portions), trigeminal and sciatic nerves, eyes, Harderian glands, femoral skeletal muscle, and nasal cavity were removed to examine whether the dose of MTBITC used in this study itself exerts any effects in rats. To evaluate the chemopreventive effects of MTBITC, the esophagus of each rat was opened longitudinally, and surface nodules greater than 0.5 mm in a single dimension were mapped, counted, and measured. This size is used to ensure detection of proliferative lesions, and it was also applied in previous NMBA-induced esophageal carcinogenesis studies[14, 15]. The esophagus was unbent and fixed on a filter paper to make it flat. The organs were then fixed in 10% buffered formalin for 3 days. Testes and eyes were fixed in Bouin's solution and Davidson's solution, respectively. The nasal cavity, vertebrae, sternum, and femur were treated with a mixture of 10% formic acid and 10% buffered formalin for up to 3 weeks for decalcification. After fixation, the entire length of the esophagus was cut into 2 or 3 sections including all nodules greater than 0.5 mm in diameter. Tissue slices of all organs were routinely processed for paraffin embedding, and sections were prepared and stained with hematoxylin and eosin (HE) for histopathological evaluation. Esophageal lesions were classified into hyperplasia, atypical hyperplasia (i.e., dysplasia), papilloma, and carcinoma of squamous cells based on histopathological features in rats, in accordance with the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND)[25]. Diagnostic features are as follows. Atypical hyperplasia reveals the thickening of squamous epithelium with the presence of abnormal cell shape, size,

or nuclear morphology, and keratinization of cells within deeper layers of mucosa is seen. Papilloma is characterized by branching central fibrovascular stalks that are covered by a variably thick and differentiated squamous epithelium that is often heavily keratinized. Cells comprising a carcinoma show features such as loss of polarity (for example, basal-like tumor cells suddenly forming keratin pearls), an abundance of mitotic figures, cellular and nuclear pleomorphism, and foci of necrosis.

Immunohistochemistry

Esophagus tissues were embedded in paraffin, sectioned to at a thickness of 4-μm, and subjected to immunohistochemistry using a Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA) or Histofine Simple Stain Kit (Nichirei Corp, Tokyo, Japan) with 3, 3′-diaminobenzidine/H_2O_2 as a chromogen. The following primary antibodies were used: anti-cleaved caspase-3 rabbit monoclonal antibodies (clone 5A1E, 1:500; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-Ki-67 rabbit monoclonal antibodies (clone SP6, 1:1,000; Abcam, Cambridge, CA, USA), anti-p21 rabbit monoclonal antibodies (clone EPR3993, 1:2,000; Abcam), and anti-p53 mouse monoclonal antibodies (clone PAb 240, 1:2,500; Abcam). Antigen retrieval was performed in an autoclave for 10 min at 121°C in 10 mM citrate buffer (pH 6.0) for Ki-67, p21, and p53 or in a microwave for 10 min at 90°C in Target Retrieval Solution, pH 9 (Dako, Tokyo, Japan), for cleaved caspase-3.

Immunohistochemical analysis was performed for rats in groups 2–7. All immunostained slides were counterstained with hematoxylin for microscopic examination. To compare the effects of MTBITC on early-stage esophageal carcinogenesis, we analyzed normal looking epithelia from rats in groups 2 and 3 or diffuse hyperplastic epithelia (i.e., the majority of the mucosa consisting of squamous cells in increased layers other than focal preneoplastic/neoplastic lesions in NMBA-treated animals) in groups 4–7. The numbers of positive cells were counted in 5 randomly selected areas per epithelium at a magnification of 200× from 10 randomly selected rats per group, and cell numbers were normalized to the unit length of the muscularis mucosae in accordance with Taniai *et al.*[26].

Statistical analysis

All data were expressed as means ± SDs per group, and the data of groups 3 and 4 were compared with those of the other groups to evaluate the effects of MTBITC on NMBA-induced rat esophageal carcinogenesis. For comparison of numerical data between multiple groups, Bartlett's test for equal variance was used to determine whether variances were homogenous between the groups. If a significant difference in variance was not observed, one-way analysis of variance (ANOVA) was performed. If significant differences were found, Dunnett's multiple comparison tests (for multiplicity data) or Tukey-Kramer's multiple range tests (for other data) were performed for comparisons between the groups. If a significant difference was found using Bartlett's test, Kruskal-Wallis tests were performed. If significant differences were found, Steel tests (for multiplicity data) or Steel-Dwass tests (for other data) were applied.

The significance of differences in data for hematology, serum biochemistry, and organ weights (both absolute and relative weights) between groups 1 and 2 was analyzed with Student-Welch tests. For histopathological findings, Fisher's exact probability tests were performed. Differences with *P* values of less than 0.05 were considered statistically significant.

Results

One rat in group 3 became emaciated due to malocclusion and was excluded from the study. No treatment-related clinical signs or deaths were detected during the 26-week study period. The treatments did not affect body weight. Analysis of food consumption revealed that rats in groups 2, 5, 6, and 7 consumed 4.64, 6.05, 4.03, and 4.79 mg MTBITC/kg BW/day, respectively (Table 1).

No MTBITC-related changes were observed in hematological parameters, serum biochemistry, organ weights, and histopathological features in groups 1 and 2, except for incidental and spontaneous changes (data not shown). A significant low value for lung weight was observed in group 2, but it was thought to be incidental, since the difference was minimal and no significant histopathological change was observed.

The incidence and multiplicity data for macroscopic esophageal nodules greater than 0.5 mm in diameter in each group are summarized in Table 2. The nodules were diagnosed as squamous cell hyperplasia, atypical hyperplasia, papilloma, or carcinoma, as described later. No statistically significant differences in the incidence of nodules were detected among groups 4–7. In contrast, the multiplicities of nodules in groups 5–7 were significantly lower than that in group 4 (*P*<0.01).

The incidence and multiplicity data of esophageal preneoplastic/neoplastic lesions, as identified by histological assessment, are shown in Table 3, and the representative appearance of each lesion is shown in Fig. 2. There were no significant differences in the incidences of lesions among groups 4–7. However, the multiplicity data for atypical hyperplasia (*P*<0.05 in groups 5 and 7) and for papilloma (*P*<0.01 in groups 5–7) were significantly decreased compared with those in group 4. As for carcinoma, the animals in groups 6 and 7 revealed a tendency to exhibit slightly lower incidence and multiplicity data than those in group 4, without statistical significance. Moreover, focal hyperplasia and papilloma of squamous cells in the tongue was sporadically detected in rats in groups 4–7; however, there were no significant differences in the incidence and multiplicity data (data not shown). Additionally, no changes were observed in the stomach in all groups of rats.

The results of the immunohistochemical analysis are graphically summarized in Fig. 3. The numbers of epithelial cells positive for cleaved caspase-3, the activated form of

Table 1. Body Weight, Food Consumption, and Intake of MTBITC

Group	No. of animals	Body weight (g)		Food consumption (g/rat/day)	Intake of MTBITC (mg/kg BW/day)
		Initial	Final		
1. Untreated	10	84.1 ± 6.7	364 ± 14.6	15.4	0
2. MTBITC-treated	10	84.2 ± 6.5	369 ± 28.0	16.3	4.64
3. DMSO-treated	14	84.5 ± 5.4	378 ± 14.3	16.5	0
4. NMBA-treated	15	84.0 ± 5.4	361 ± 21.9	15.8	0
5. NMBA+MTBITC/basal diet	15	84.0 ± 5.3	360 ± 23.3	15.7	6.05
6. NMBA/MTBITC	15	83.9 ± 5.3	364 ± 21.4	16.1	4.03
7. NMBA+MTBITC/MTBITC	15	83.9 ± 5.2	364 ± 27.5	16.7	4.79

Body weight values represent the mean ± SD.

Table 2. Incidence and Multiplicity Data for Macroscopic Esophageal Nodules

Group	No. of animals	Incidence (%)	Multiplicity (no./rat)
1. Untreated	10	0	
2. MTBITC-treated	10	0	
3. DMSO-treated	14	0	
4. NMBA-treated	15	15 (100)	4.53 ± 1.77
5. NMBA+MTBITC/basal diet	15	13 (87)	1.67 ± 0.98**
6. NMBA/MTBITC	15	12 (80)	1.73 ± 1.10**
7. NMBA+MTBITC/MTBITC	15	12 (80)	1.87 ± 1.19**

Multiplicity values represent the mean ± SD. ** $P<0.01$ versus the NMBA-treated group.

Table 3. Incidence and Multiplicity Data for Esophageal Preneoplastic/Neoplastic Lesions

Group	No. of animals	Incidence (%)			Multiplicity (no./rat)		
		Atypical hyperplasia	Papilloma	Carcinoma	Atypical hyperplasia	Papilloma	Carcinoma
1. Untreated	10	0	0	0			
2. MTBITC-treated	10	0	0	0			
3. DMSO-treated	14	0	0	0			
4. NMBA-treated	15	14 (93)	15 (100)	9 (60)	2.73 ± 1.58	3.00 ± 1.46	0.67 ± 0.62
5. NMBA+MTBITC/basal diet	15	14 (93)	12 (80)	8 (53)	1.67 ± 1.23*	1.13 ± 0.74**	0.60 ± 0.63
6. NMBA/MTBITC	15	14 (93)	12 (80)	6 (40)	1.80 ± 1.15	1.47 ± 0.99**	0.47 ± 0.64
7. NMBA+MTBITC/MTBITC	15	11 (73)	12 (80)	6 (40)	1.53 ± 1.36*	1.47 ± 1.13**	0.47 ± 0.64

Multiplicity values represent the mean ± SD. * $P<0.05$ versus the NMBA-treated group. ** $P<0.01$ versus the NMBA-treated group.

caspase-3 involved in apoptotic signaling, were significantly higher in groups 5–7 than in group 3 ($P<0.01$). The animals in group 4 also showed a tendency to exhibit a higher number of positive cells than the animals in group 3; however, this difference was not statistically significant. Moreover, the numbers of positive cells in groups 6 and 7 were significantly increased as compared with those in group 4 ($P<0.05$).

We next examined cell proliferation by analysis of Ki-67, an established cell proliferation marker. The numbers of Ki-67-positive cells in groups 4 and 5 were significantly increased as compared with that in group 3 ($P<0.01$). In contrast, groups 6 and 7 exhibited no obvious differences in the numbers of positive cells compared with that in group 3. Moreover, groups 6 and 7 showed significant decreases in the numbers of positive cells compared with that in group 4 ($P<0.01$).

There were no obvious differences in the number of cells positive for p21, a potent cyclin-dependent kinase (CDK) inhibitor, in groups 3–5. However, the numbers of p21-positive cells were significantly increased in groups 6 and 7 as compared with those in groups 3 ($P<0.01$) and 4 ($P<0.01$ versus group 6 and $P<0.05$ versus group 7).

The numbers of cells positive for p53, a well-studied tumor suppressor, were significantly increased in groups 4–7, i.e., all the NMBA-treated groups, compared with that in group 3 ($P<0.01$). However, there were no obvious immunoreactive differences between group 4 and groups 5–7.

As for all examined markers, the animals in the group 2 exhibited no obvious differences in the immunoreactive cell populations compared with those in group 3.
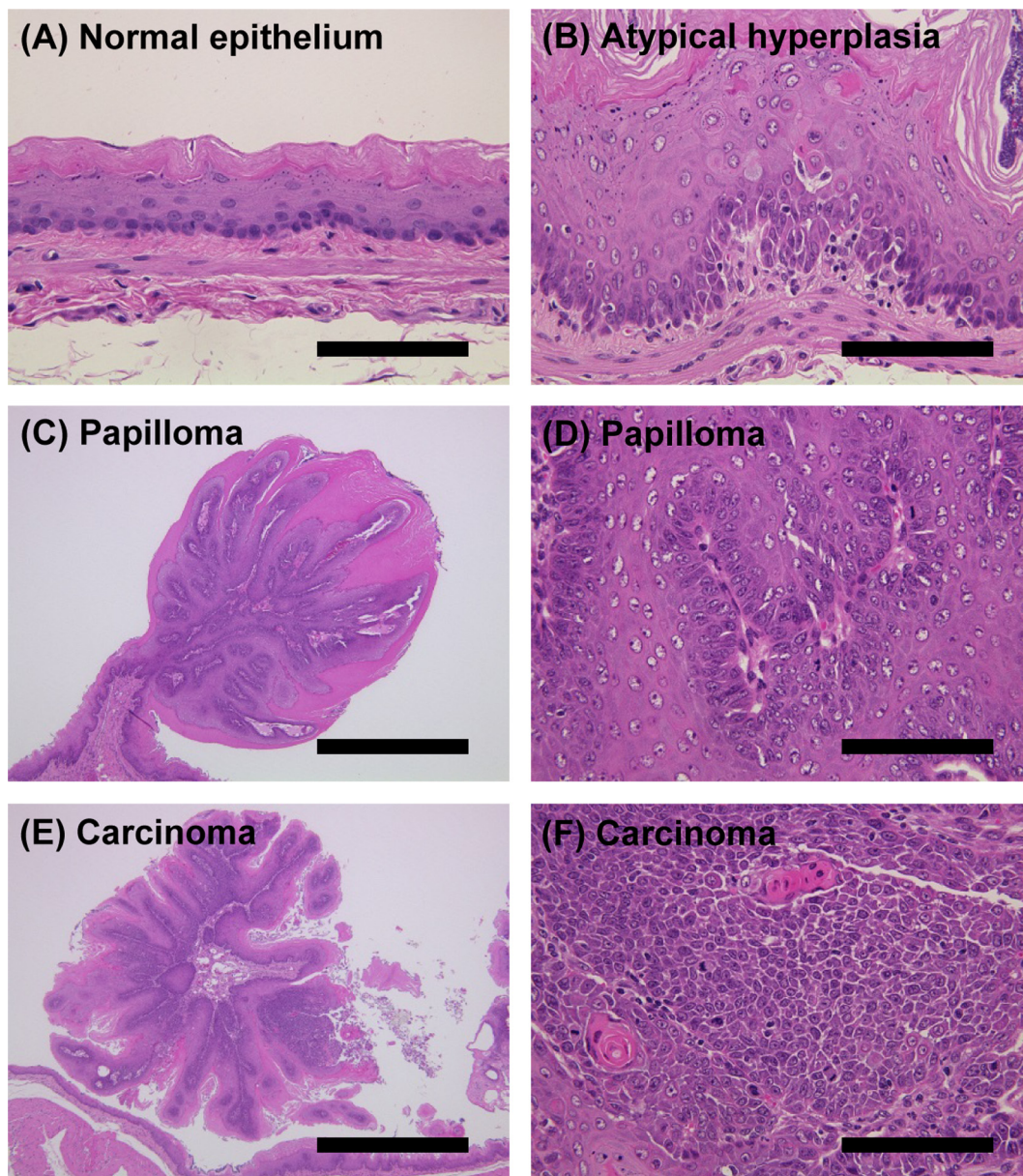
Fig. 2. Representative appearances of esophageal lesions. Normal epithelium (A) in rats in the DMSO-treated group, and squamous cell atypical hyperplasia (B), papilloma (C, D), and carcinoma (E, F) in rats in the NMBA-treated group (HE staining). Atypical hyperplasia exhibits thickening of the squamous epithelium along with the presence of an abnormal cell shape, size, or nuclear morphology, and keratinization of cells within deeper layers of the mucosa is seen. Papilloma is characterized by branching central fibrovascular stalks that are covered by a variably thick and differentiated squamous epithelium that is often heavily keratinized. Cells comprising a carcinoma show features such as loss of polarity (for example, basal-like tumor cells suddenly form keratin pearls), an abundance of mitotic figures, cellular and nuclear pleomorphism, and foci of necrosis. Bars = 100 μm (A, B, D, F) or 1,000 μm (C, E).

Discussion

In the present study, we showed that 26 weeks of dietary administration of 80 ppm MTBITC (equivalent to 4.64 mg/kg BW/day) alone did not have any effects in male F344 rats. Importantly, MTBITC treatment clearly inhibited NMBA-induced esophageal carcinogenesis when administered during the initiation and/or promotion phase, causing induction of apoptosis and inhibition of cell proliferation in the promotion phase.

Few *in vivo* studies have reported the effects of MT-BITC on cancer[18, 23], and the current study is the first report demonstrating the chemopreventive effects of MTBITC on esophageal tumorigenesis in rats. Several other ITCs have also been shown to inhibit carcinogen-induced lung, colonic, pancreatic, and esophageal tumorigenesis[10–16], and the characteristics of these ITCs differ[21, 27], which may contribute to the observed variations in carcinogenesis. For ex-
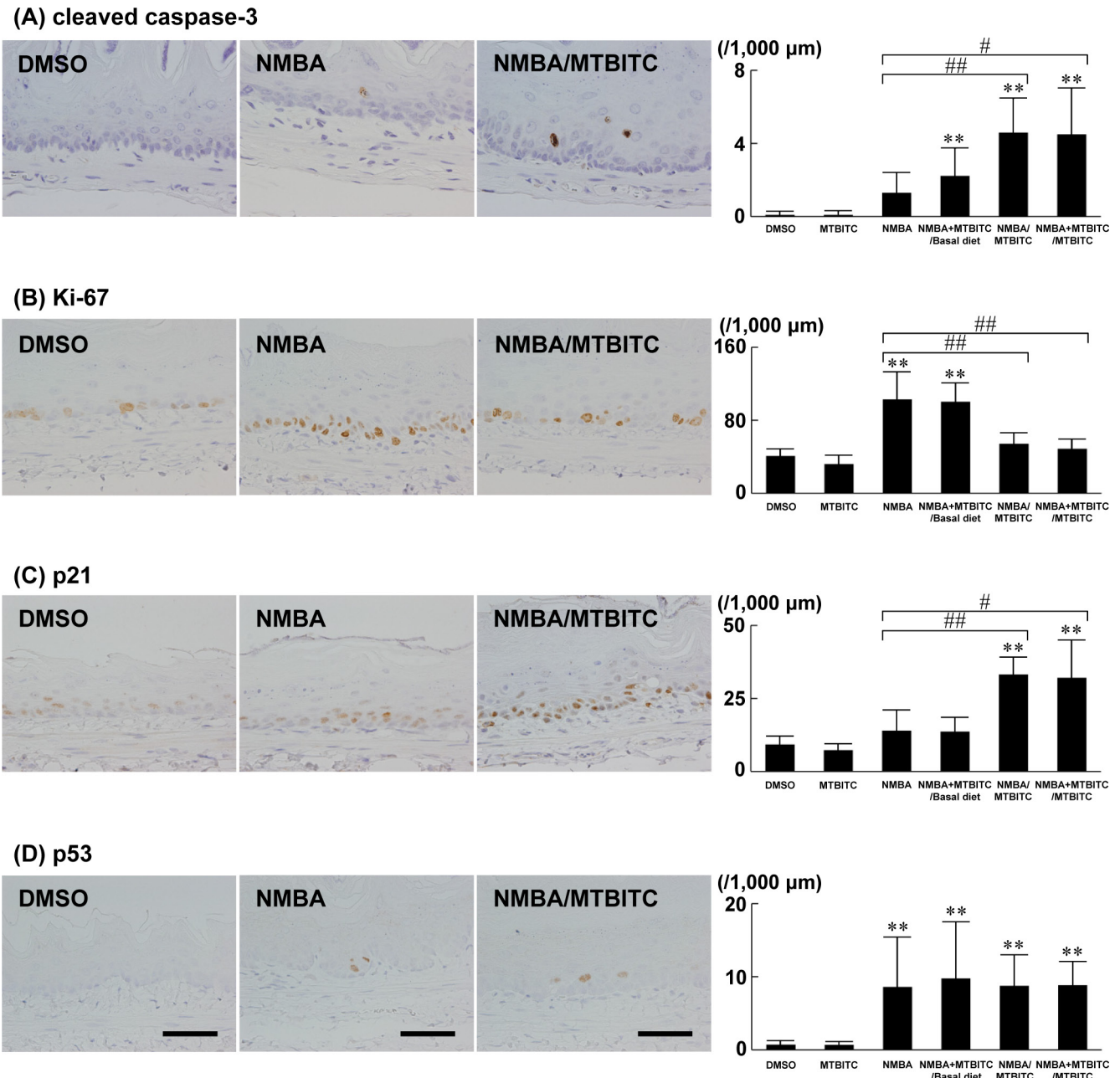
Fig. 3. Distribution of cleaved caspase-3-, Ki-67-, p21-, and p53-positive cells in the esophageal epithelium. Photomicrographs show the cellular distributions of markers in the DMSO-treated, NMBA-treated, and NMBA/MTBITC groups. The graphs show positive cells per unit muscularis mucosae length (1,000 µm) of the epithelium in the DMSO-treated, MTBITC-treated, NMBA-treated, NMBA+MTBITC/basal diet, NMBA/MTBITC, and NMBA+MTBITC/MTBITC groups. Values represent the mean + SD. (A) Cleaved caspase-3, (B) Ki-67, (C) p21, and (D) p53. Bars = 50 µm. ** $P<0.01$ versus the DMSO-treated group. # $P<0.05$ versus the NMBA-treated group. ## $P<0.01$ versus the NMBA-treated group.

ample, unlike dietary administration of 80 ppm MTBITC in the present experiment, 500 ppm of PEITC fails to exert chemopreventive effects on NMBA-induced tumorigenesis in the rat esophagus when administered during the promotion stage[14]. In contrast, 80 ppm MTBITC, benzyl isothiocyanate, or sulforaphane (SFN), a type of ITC, prevents BOP-induced pancreatic carcinogenesis in hamsters when administered during the initiation stage but not during the promotion stage[13, 18]. These reports, combined with our current findings, indicate that the chemopreventive effects of ITCs may differ depending on the target organ and/or the target molecules of the carcinogen.

In the present study, we also investigated the effects of MTBITC on cell proliferation and apoptosis in the esophageal epithelium of NMBA-treated rats. We found that cleaved caspase-3-positive cells were significantly in-

creased in animals treated with MTBITC during the promotion stage, as compared with that in animals treated with NMBA alone, indicating that MTBITC treatment increased apoptosis. MTBITC has been reported to induce apoptosis in HeLa (human cervical epithelial carcinoma), A549 (human alveolar basal epithelial carcinoma), MCF-7 (human mammary epithelial carcinoma), and PC-3 (human prostate epithelial carcinoma) cell lines[20]. Specifically, MTBITC was shown to upregulate the expression of Bax and caspase-3, two pro-apoptotic genes, and downregulate the expression of Bcl-2 and Bcl-xl, two anti-apoptotic genes. In terms of cell proliferation, we found that the numbers of Ki-67-positive cells were significantly increased in animals treated with NMBA alone or with MTBITC during the initiation stage as compared with that in animals treated with DMSO. Preneoplastic cells are generally known to have higher proliferative activity than normal cells[28, 29], and similar high proliferative activity was previously observed in a rat NMBA carcinogenesis model identical to the model used in this study[30]. However, the numbers of Ki-67-positive cells were significantly decreased in animals treated with MTBITC during the promotion stage as compared with that in animals treated with NMBA alone, indicating that MTBITC treatment decreased cell proliferation in the esophagus in rats treated with NMBA.

We also investigated the expression of p21 in the rat esophageal epithelium to determine the effects of MTBITC on the cell cycle checkpoint in this model. p21 is a potent CDK inhibitor that blocks cell cycle progression at the G_1, G_2, and/or G_0 phase[31]. In the present study, we found that animals treated with MTBITC during the promotion stage showed higher numbers of p21-positive cells than animals treated with NMBA alone. Several studies have shown that ITCs upregulate the expression of p21 and suppress the expression of cyclins and CDKs, resulting in induction of cell cycle arrest by PEITC in MCF-7 and MDA-MB-231 cells (derived from human breast adenocarcinomas) and by SFN in LNCaP and PC-3 cells (derived from human prostate adenocarcinomas)[32, 33]. SNF has been known to induce apoptosis derived from reactive oxygen species[34] and to elevate the transcription of p21 through the inhibition of histone deacetylase (HDAC) activity[33] in human prostate adenocarcinoma cells. Furthermore, MTBITC shares a similar chemical structure with SFN; therefore, MTBITC might have similar activity regarding ROS induction and HDAC inhibition. Further investigation is needed to clarify this. Consequently, our results suggest that MTBITC treatment may suppress cell proliferation through increased expression of p21, at least in part, in the rat esophageal epithelium.

Interestingly, rats in all NMBA-treated groups showed increased numbers of p53-positive cells in the esophagus compared with that in the DMSO-treated group, regardless of the presence of MTBITC treatment. p53 is a short-lived protein in normal tissues; however, mutant p53 protein has an extended half-life and is often highly expressed in tumor cells[35]. Mutations in the *p53* gene have been observed in NMBA-induced esophageal tumors in rats[36] and in esoph-

ageal tumors in humans[37]. Thus, the increased number of p53-positive cells may be explained by NMBA treatment, and MTBITC may not have affected the expression of p53 in the esophagus of NMBA-treated rats. However, we did not investigate mutations in the *p53* gene directly in our study.

Compared with the esophagus in the vehicle control group, administration of MTBITC alone did not alter these factors on the esophagus epithelium, suggesting that MTBITC may have selectively affected the NMBA-treated esophageal cells, while the normal esophageal cells were left unaffected. Selective targeting and negligible toxicity in normal cells are important prerequisites for probable chemopreventive compounds. Similarly, ITCs, including MTBITC, have been reported to induce cell cycle arrest and apoptosis in preneoplastic/neoplastic cells but not in normal cells in *in vitro* studies[20, 32, 33]. This might be due to a fact that ITCs could target a particular molecular event present in hyperplastic and cancer cells but absent in normal cells, for example, higher cell proliferative activity[28, 29].

MTBITC has been shown to inhibit genotoxicity *in vitro* and *in vivo*[17, 23] and to induce several types of antioxidative enzymes, including quinone reductase, NAD(P) H:quinone oxidoreductase, heme oxygenase-1, thioredoxin reductase, and glutathione S-transferase in HepG2 (human hepatocellular carcinoma) cells or hepatocytes of rats and mice[21, 22]. Moreover, induction of antioxidative enzymes is transcriptionally regulated through the antioxidant response element (ARE), and activation of gene transcription through the ARE is mediated primarily by nuclear factor E2-related factor 2 (Nrf2)[38]. Indeed, several types of ITCs, including MTBITC, activate Nrf2 in primary cultures of rat hepatocytes[21]. Furthermore, several ITCs have been reported to inhibit the induction of cytochrome P450 (CYP) enzymes. CYP enzymes are generally known to activate certain carcinogens, and simultaneous administration of PEITC has been reported to inhibit NMBA-induced esophageal carcinogenesis in rats through its ability to reduce the metabolism of NMBA and to inhibit the DNA adduct formation induced by NMBA metabolites[15]. PEITC also inhibits CYP1A2, CYP2E1, and CYP3A in rat liver microsomes[39]. In addition, MTBITC inhibits CYP3A2 in primary cultures of rat hepatocytes[21]. Based on these reports, MTBITC treatment during the initiation phase may affect the Nrf2-ARE pathway and the inhibition of CYP enzymes in the rat esophagus. Further studies are needed to confirm this hypothesis.

In conclusion, we showed for the first time that treatment with approximately 4–6 mg MTBITC/kg BW/day suppressed NMBA-induced esophageal tumorigenesis when administered during the initiation and/or promotion stages. Converting this dose based on body surface area as described in the FDA guidance[40], the human equivalent dose would be 0.65–0.98 mg/kg BW/day. The above effects were related to the induction of apoptosis and the suppression of cell proliferation in NMBA-treated esophageal cells with elevated p21 expression. On the other hand, these changes were not observed in the animals treated with MTBITC alone, suggesting the specificity of MTBITC to NMBA-

initiated cells but not to the normal esophageal epithelium. Based on the previously reported concentration of MTBITC in heirloom daikon varieties such as Karami, Momoyama, and Sabaga (421, 276, 227 μmol/100 g, respectively)[19], the estimated daily dose of MTBITC in this study was equal to about 60–160 g heirloom daikon in humans (60 kg BW); therefore, it is feasible to consume a sufficient amount of heirloom daikon containing MTBITC to achieve chemoprevention for esophageal cancer. Further studies of the efficacy of MTBITC will provide additional insights into the health benefits of daikon.

Disclosure of Potential Conflicts of Interest: Isamu Suzuki is an employee of BoZo Research Center Inc., Shizuoka, Japan, and Tadashi Hirata is an employee of Japan Tobacco Inc., Kanagawa, Japan. The authors declare that they have no conflicts of interest.

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