carcinogenesis are still controversial. Hirose et al. reported the enhancing effects of GTP on colon carcinogenesis in rats [17,18], although there were no proposed underlying mechanisms. Also, there has been no consistent association between the reduction of risk of CRC [19–21] or gastric cancer [22] and green tea consumption in previous epidemiologic studies, whereas green tea consumption has contributed to significantly reduced risk of breast, esophagus, kidney, liver, lung, pancreas cancers [23].

Cytokines play a key role in the pathogenesis of IBD, some of which may lead to colon carcinogenesis [24,25]. Ohkawara et al. reported that the macrophage-migration inhibitory factor (MIF) expression was increased in dextran sulfate sodium (DSS)-induced colitis in mice [26]. Interleukin (IL)-1β also contributes to the increased severity of DSS-induced colitis [27]. Colonic mucosa contains endogenous antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and the enzymes that scavenge or decompose ROS, which generate in response to certain inflammatory stimuli [28]. In the colonic mucosa of UC patients, nitrative and oxidative DNA lesion products, 8-oxo-7,8-dihydro-2′-deoxyguanosine were increased when compared with normal tissues [15]. EGCG, the main component of GTP, had previously been shown to be the pro-oxidant *in vitro* [8].

To determine the modifying effects (enhancement or inhibition) of GTP in colitis and inflammation-associated colon carcinogenesis, we first examined the effect of GTP on DSS-induced acute colitis in ICR male mice, since this model is useful to investigate roles of oxidative stress in acute colitis. Subsequently, we determined the influence of dietary GTP on inflammation-associated colon carcinogenesis using a mouse model initiated with 1,2-dimethylhydrazine (DMH) and promoted by DSS [29]. In this study, high doses of GTP were found to enhance acute colitis induced by DSS through modification of expression of IL-1β and MIF in the colon, whereas 0.1% dose of GTP decreased these cytokines production. Furthermore, high doses of GTP failed to prevent inflammation-associated colon carcinogenesis by DMH and DSS. We thus propose the hypothesis that GTP at excess dosage does not exert inhibitory effects on acute colitis and carcinogenesis in the inflamed colon.

2. Materials and methods

2.1. Animals

Male specific pathogen-free ICR mice (4 weeks of age) were purchased from Japan SLC (Shizuoka, Japan). They were housed three or five per cage and given fresh tap water *ad libitum* and commercial rodent MF pelleted diet (Oriental Yeast, Co., Kyoto, Japan), which were freshly changed every day, and handled according to the Guidelines for the Regulation of Animals, as provided by the Experimentation Committee of Kyoto University. The mice were maintained in a room controlled at 24°C ± 2°C with a relative humidity of 60% ± 5% and a 12-h light/dark cycle (06:00–18:00). All mice were quarantined for 1 week before starting the experiments.

2.2. Chemicals

GTP containing 70% total catechins and half of them is EGCG, and 3% caffeine was obtained from LKT Laboratories, (W. St. Paul, MN). DMH was purchased from SIGMA-Aldrich (Tokyo, Japan). DSS with a molecular weight of 36,000–50,000 was from MP Biomedicals, LLC (Aurora, OH). A rat/mouse MIF enzyme-linked immunosorbent assay (ELISA) kit was purchased from Sapporo Immunodiagnostic Laboratory, Co. (Sapporo, Japan). Mouse IL-1β, IL-6, and TNF-α ELISA kit were obtained from Endogen (Cambridge, MA). Catalase assay kit was purchased from Calbiochem, a brand of EMD Biosciences (San Diego). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan) unless specified otherwise.

2.3. Effects of GTP on acute colitis induced by DSS

Following quarantine for 1 week, a total of 85 male ICR mice were divided into an untreated control and six experimental groups (n = 12 or 13 for each group), as shown in Fig. 1. In the control group (group 1, n = 12), animals were given tap water and basal diet *ad libitum*. The GTP alone group (group 2, n = 12) was fed with the diet containing 1% GTP and did not receive DSS. In the DSS alone group (group 3, n = 12), animals were given tap water containing 2% DSS (w/v) for 6 days and fed with a basal diet to induce acute colitis. In the GTP groups, mice were fed with diets mixed with four different concentrations of GTP (0.1% for group 4, n = 12; 0.25% for group 5, n = 13; 0.5% for group 6, n = 12; and 1% for group 7, n = 12), starting at the DSS exposure. Body weight and intakes of food and drinking water were recorded every day during the experiment. At day 6, all mice were killed by deep anesthesia with diethyl ether for determining the effects of dietary GTP on DSS-induced acute colitis.

2.4. Isolation of colonic mucosa

At sacrifice, complete necropsy was done on all mice. All organs including colon were macroscopically inspected for the presence of lesions. As for colons, they were removed, washed in phosphate-buffered saline (PBS), and placed on filter papers. After measuring the length, they were cut opened longitudinally along the main axis with surgical scissors and the contents were removed. Colons of three mice randomly selected from each group were fixed in Mildform®10 N (Wako Pure Chemical Industries) and used for histopathology. Histopathological examination was performed on hematoxylin and eosin (H&E)-stained sections made from paraffin-embedded blocks. Colitis was recorded and scored according to the following morphological criteria described by Cooper et al. [30]: grade 0, normal colonic mucosa; grade 1, shortening and loss of the basal one-third of the actual crypts with mild inflammation and edema in the

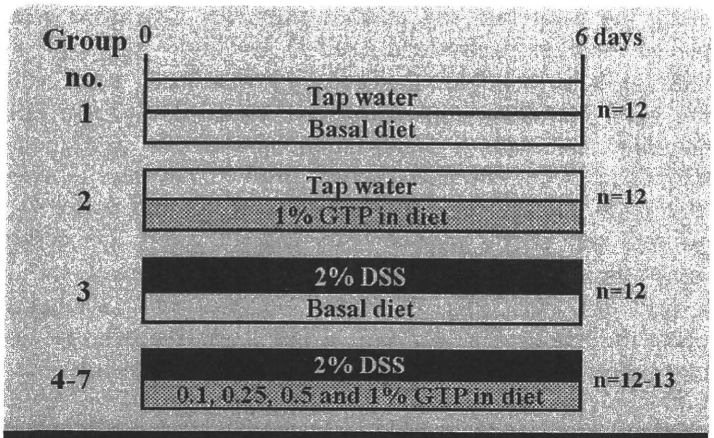

Fig. 1. Experimental protocol for determining the effects of GTP on acute colitis induced by DSS. In the untreated control group, male ICR mice were given tap water and basal diet *ad libitum*, freshly changed every day, for 6 days. In the DSS group, the mice were fed with a basal diet and 2% DSS (w/v) in tap water for 6 days to induce colitis. In the DSS + GTP groups, mice were fed with diets containing GTP (0.1, 0.25, 0.5, and 1%) for 6 days, stating the DSS exposure. The GTP alone group was fed with the diet mixed with 1% GTP and did not receive DSS (n = 12–13).

mucosa; grade 2, loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa; grade 3, loss of all crypts with severe inflammation in the mucosa, but with the surface epithelium still remaining; and grade 4, loss of all crypts and the surface epithelium with severe inflammation in the mucosa, muscularis propria, and submucosa. Their mucosal ulcers were counted on H&E-stained sections. Colonic mucosa of the remaining mice was scraped off using a razor before the specimens were frozen using in liquid nitrogen, until later use, according to a method previously reported by Perdue et al. [31] with slight modification.

2.5. Assay for cytokine production

The colonic mucosa obtained was minced with surgical scissors and homogenized in ice-cold PBS using a homogenizer (Hielscher-UP 50H, Hielscher, Stahnsdorf, Germany). Tissue homogenates were then centrifuged at 1900*g* at 4°C for 10 min to obtain the supernatants. Total protein concentrations in the tissue supernatants were determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA), as specified by the manufacturer's protocol (dilution factor = 50), with γ-globulin according to the standard. The IL-1β, MIF, IL-6, and TNF-α concentrations were determined using each ELISA kit, according to the protocol of the manufacturer (dilution factor = 5–8), respectively. The amount of cytokines was calculated as ng of MIF and pg of IL-1β, IL-6, and TNF-α per mg of protein. Each assay was performed at least three times.

2.6. Measurement of SOD and catalase activities

The colonic mucosa was homogenized in ice-cold PBS using a homogenizer (Hielscher-UP 50H). The supernatants of the tissue homogenates were prepared by using the same process for the cytokine production experiment, as described earlier. Total SOD activity in the colonic mucosa was measured using a kit (Wako). The assay was carried out in 50 mM PBS, pH 7.8, in an incubator at 36°C. The kinetics of reduction of NBT to blue formazane was monitored through the absorbance changes with time at 560 nm. Solutions of NBT (0.24 mM) and xanthine (0.4 mM) were mixed in the plate and the reaction was started through the addition of a concentrated xanthine oxidase solution. The rate of NBT reduction was measured at 560 nm and then calculated as a percentage of inhibition of this reaction per mg protein of colonic mucosa. The SOD activity was expressed relative to control, which was standardized to 100. The assay was performed three times.

The samples for measuring catalase activity were prepared by the same method described for the SOD activity assay, and the activity was measured by using a kit (Calbiochem) following the manufacturer's instructions. Each of the test samples was assayed at 500 nm and measured on a Multiskan JX (Thermo Labsystems, Vantaa, Finland). The catalase activity was expressed relative to control, which was standardized to 100. The assay was performed three times.

2.7. Effects of GTP on colon carcinogenesis induced by DMH and DSS

Following quarantine for 1 week, a total of 111 male ICR mice were divided into an untreated control and nine experimental groups. DMH was dissolved in PBS and pH was adjusted to 6.5 by using 0.1 N NaOH. Group 1 (n = 12) served as an untreated control. Group 2 (the GTP alone group, n = 6) was fed the diet containing 1% GTP for 18 weeks (from week 2 to week 20) and received no further treatment. Mice of group 3 (the DSS alone group, n = 6) received 1-week treatment of 1% DSS (w/v) in drinking water (from week 2 to week 3). Group 4 (the DMH alone group, n = 6) was given two weekly intraperitoneal (i.p.) injection of DMH (20 mg/kg body weight). Group 5 (n = 12) was given DMH, as did for group 4 and fed with the diet containing 1% GTP for 18 weeks (from week 2 to week 20). Group 6 (n = 20) was treated with DMH and followed by 1-week treatment of 1% DSS (w/v) in drinking water (from week 2 to week 3). Mice in groups 7–10 (n = 12 each for groups 7–9 and n = 13 for group 10) were treated with DMH and DSS and fed the diets mixed with 0.01, 0.1, 0.5, and 1% GTP for 18 weeks, starting 1 week after the last DMH. Body weight and intake of food and drinking water were recorded once every week during the study. At week 20, all animals were sacrificed by deep anesthesia with diethyl ether to determine the effects of dietary GTP on colon carcinogenesis induced by DMH and DSS.

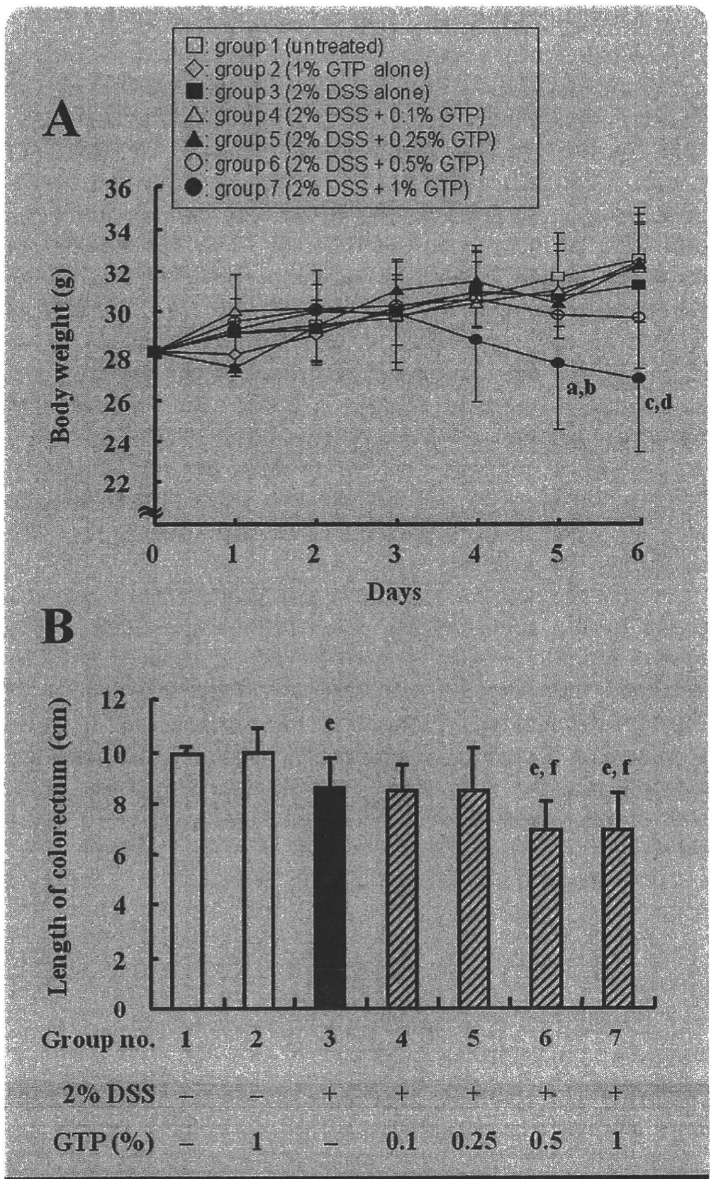

Fig. 2. Effects of dietary GTP on the body weight gain (A) and colon length (B) in the DSS-induced colitis experiment. Body weights of all mice were recorded every day. Colon length was measured at sacrifice (day 6) (Values are the mean ± SD). (a) Significantly different from group 1 of 5 days ($P < 0.01$). (b) Significantly different from group 3 of 5 days ($P < 0.001$). (c) Significantly different from group 1 of 6 days ($P < 0.001$). (d) Significantly different from group 3 of 6 days ($P < 0.001$). (e) Significantly different from group 1 ($P < 0.05$). (f) Significantly different from group 3 ($P < 0.05$) ($n = 12$–13).

2.8. Tissue harvest

At sacrifice, all animals were sacrificed by anesthesia with diethyl ether, and complete necropsy was done on all mice. All organs were macroscopically inspected for the presence of lesions, and colon was then removed. After the length of colons was measured, they were cut open longitudinally along the main axis and washed with PBS. The colon was placed on filter papers and fixed in Mildform® 10 N for at least 24 h for counting aberrant crypt foci (ACF), since ACF are early biomarkers for colon carcinogenesis [32,33]. For counting ACF with a stereoscopic microscope (SMZ1000, NikonInstech Co. Kawasaki, Japan), fixed colons were dipped in a 0.5% solution of methylene blue in distilled water for 30 sec, and briefly washed with distilled water. The large intestines were macroscopically inspected and the volume of tumors was measured. The tumor volumes was calculated using the equation $V = 4/3\pi r^3$, where r was the average tumor radius obtained from the three diameter measures. Histopathological examination was performed on H&E-stained sections made from paraffin-embedded blocks. Histological evaluation was done in a blind fashion by a pathologist (T. T.). Colonic neoplasms were diagnosed according to the description by Ward [34].

2.9. Statistical analysis

All measurements except for the incidences of the lesions and survivals are expressed as mean ± standard deviation (SD) of the mean. Statistically significant differences of the measures between the groups were determined using a Student's t-test (two-sided), Fisher's exact probability test, or Chi-square test. All statistical analyses were performed with the GraphPad InStat software (version 3.05, GraphPad Software, San Diego, CA). P values less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of GTP on DSS-induced acute colitis

The DSS-treated mice (groups 3–7) exhibited bloody feces and loss of body weight gain during the study (Fig. 2A). The mean body weights of groups 6 (DSS + 0.5% GTP) and 7 (DSS + 1% GTP, $P < 0.001$) were lower than group 3 (2% DSS alone group) at days 5 and 6, whereas the values of groups 4 (DSS + 0.1% GTP) and 5 (DSS + 0.25% GTP) were comparable to that of groups 1–3. At sacrifice, shortening of the colon was observed in the mice that received DSS (groups 3–7), as shown in Fig. 2B. The colon length of group 3 was shortened by 16% ($P < 0.05$) when compared with that of group 1 (untreated control). The shortening was evident in groups 6 and 7: shortened by 35% ($P < 0.05$) in group 6 and by 33% ($P < 0.05$) in group 7 when compared with that of group 3. The values of groups 4 and 5 did not significantly differ from group 3.

3.2. Cytokine production in colonic mucosa

The effects of GTP on DSS-induced cytokines production in colonic mucosa using ELISA were estimated. The levels of IL-1β and MIF of group 3 (DSS alone) were significantly larger than the values of group 1 (untreated) ($P < 0.01$ for each, Figs. 3A and 3B). This DSS-induced increase was attenuated by 79% and 85% in the 0.1% GTP treated group, respectively ($P < 0.01$ for each, Figs. 3A and 3B), when

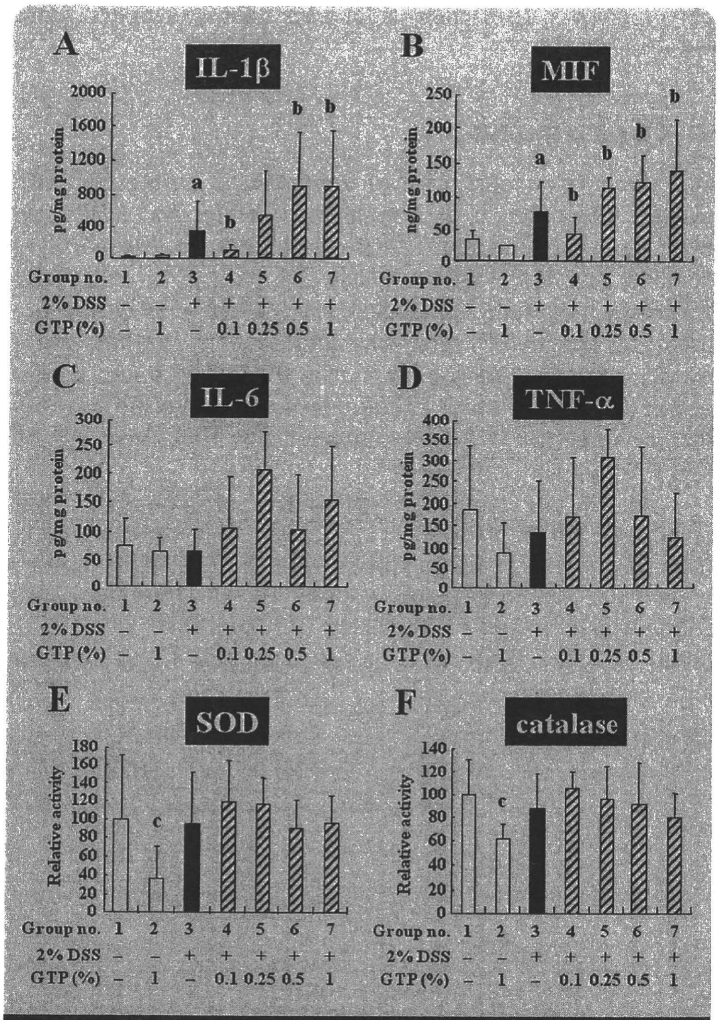

Fig. 3. Expression levels of IL-1β (A), MIF (B), IL-6 (C), TNF-α (D), and activities of SOD (E) and catalase (F) in colonic mucosa (Values are the mean ± SD).
(a) Significantly different from group 1 ($P < 0.01$).
(b) Significantly different from group 3 ($P < 0.01$).
(c) Significantly different from group 1 ($P < 0.05$) ($n = 9$).

compared with untreated group. However, the levels of IL-1β in groups 6 (DSS + 0.5% GTP) and 7 (DSS + 1% GTP) were increased by 2.6-fold when compared with the DSS alone group ($P < 0.01$ for each, Fig. 3A). The MIF levels were also elevated in these groups by 1.6- (group 6) and 1.8-fold (group 7) when compared with that of the DSS group ($P < 0.01$ for each group), whereas the 0.25% GTP (group 5) did not show significant effects (Figs. 3A and 3B). The IL-6 and TNF-α were variable among the groups without statistical significance (Figs. 3C and 3D).

3.3. Activity of antioxidant enzymes

As shown in Figs. 3E and 3F, the activities of SOD and catalase of group 2 (1% GTP alone) were significantly lower than group 1 (untreated) ($P < 0.05$ for each value). These measures of group 3 (DSS alone) were comparable to the group 1. The treatment with GTP (0.1–1% in diet) together with DSS (2% in drinking water) did not affect these enzyme activities when compared with the DSS-treated mice.

3.4. Histopathology of colonic mucosa

Mucosal ulcerations were not observed in groups 1 (Fig. 4A) and 2 (Fig. 4B). Mucosal ulceration with marked inflammation and edema in the mucosa and submucosa was evident in groups 3 (Fig. 4C), 6 (Fig. 4F), and 7 (Fig. 4G). When compared these groups, regenerative changes of the crypt cells occurred in groups 4 (Fig. 4D) and 5 (Fig. 4E). The mean numbers of mucosal ulcer per mouse in all groups are illustrated in Fig. 5A. However, the numbers of groups 4 and 5 tend to be smaller than group 3 without statistical significance. When compared with group 3, the values of groups 6 and 7 showed similar levels of group 3. As for inflammation score, the score of each group did not reach to the significant differences.

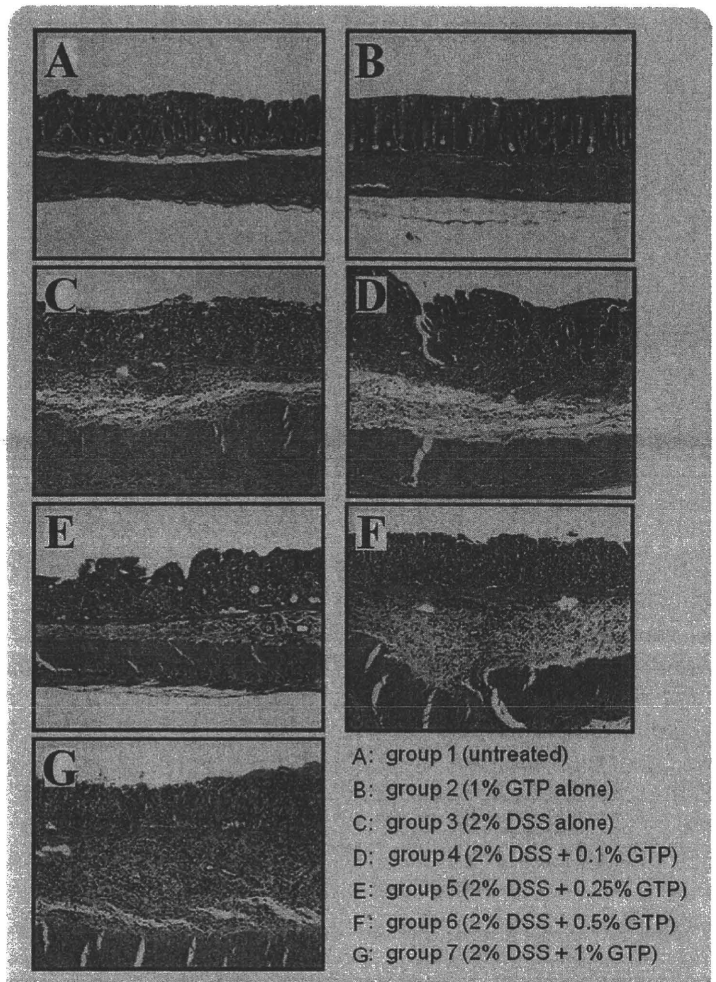

Fig. 4. Representative histopathology of the colon of mice belonging to groups 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F), and 7 (G). H&E-stain. Original magnification, A–G; ×40. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

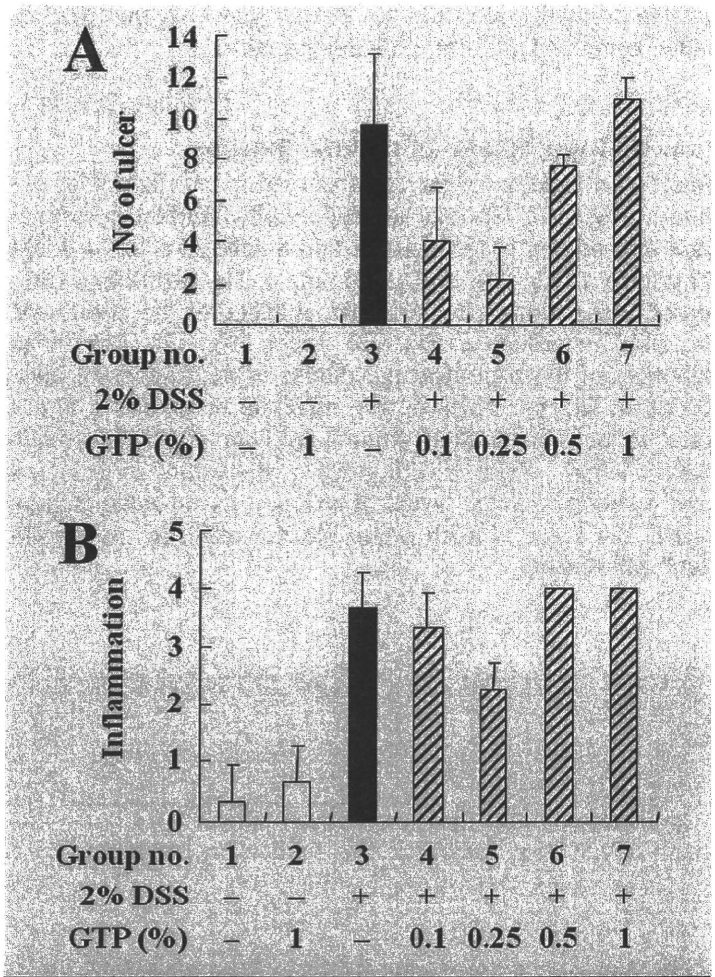

Fig. 5. Mean number of mucosal ulcers (A) and inflammation scores (B) of colon. (Values are the mean ± SD) (n = 3–4).

3.5. The effects of GTP on inflammation-associated colon carcinogenesis

At week 20, the mean of body weight and colon length did not significantly differ among the groups (data not shown) (Fig. 6). Data on enumeration of ACF (insert in Fig. 7A) is given in Fig. 7A. The multiplicity of ACF formation of group 5 was the greatest, and the value was followed by group 4 and groups 6–10. ACF was not observed in groups 1–3. Colonic tumors developed in groups 6–10, but not in groups 1–5. The multiplicities of adenoma (insert in Fig. 7B) and adenocarcinoma (insert in Fig. 7C) showed a slight tendency to increase among the groups 7–10, respectively (Figs. 7B and 7C). The values of groups 9 and 10 tend to be larger than that of group 6 ($P < 0.16$ for each lesion), these 0.5% and 1% doses of GTP were failed to decrease colonic tumor induced by DMH/DSS. As to tumors volumes, the values of groups 7–10 have a slight tendency to be greater than that of group 6 without statistical significance (Fig. 7D). 1% GTP + DMH + DSS increased tumor incidence (78%) when compared with DMH + DSS (56%), whereas in the 0.01–0.5% GTP + DMH + DSS showed 30–44% tumor incidence (data not shown).

4. Discussion

EGCG, the main constituent of green tea, is a potential antioxidant, which serves as a metal ion chelater and ROS scavenger [35]. GTP has many favorable properties, including an effective chemopreventive effect. The properties were supported by numerous epidemiological studies in several countries [23] suggesting promising anticancer properties and a variety of action mechanisms. On the other hand, a few cohort and case-control studies have suggested no association between green tea consumption and decreased risk of CRC development [36–38].

In the carcinogenesis experiment of this study, oral administration of 1% GTP in diet failed to suppress colonic tumor formation when compared with the DMH/DSS-treated mice (Figs. 7B and 7C). Furthermore, 1% GTP treatment after DMH exposure tend to increased ACF formation when compared with the DMH alone group (Fig. 7A). These findings

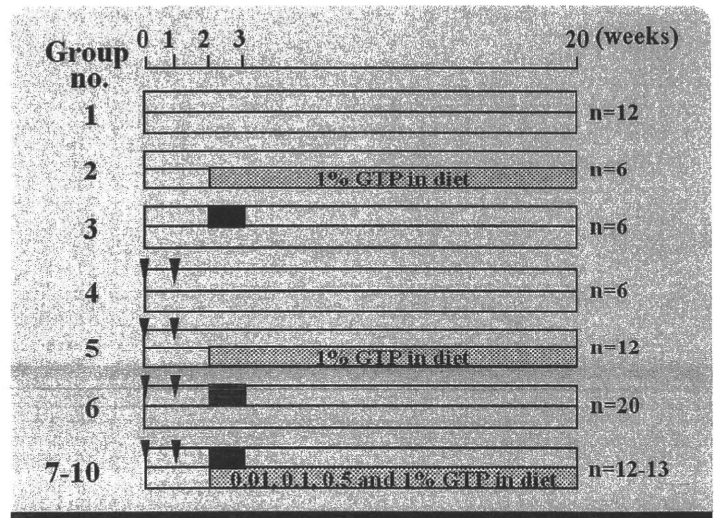

Fig. 6. Experimental protocol for examining the modifying effects of GTP on DMH/DSS-induced mouse colon carcinogenesis. Group 1 was an untreated control. Male ICR mice in groups 4–10 were initiated with two weekly i.p. injection of DMH (20 mg/kg body weight). Seven days after the last DMH injection, mice in groups 3 and 6–10 were given 1% DSS (w/v) in drinking water for 1 week. In groups 7–10, 0.01, 0.1, 0.5, and 1% GTP-containing diets were given, respectively, starting at the beginning of DSS treatment, and then continued on these experimental diets for 18 weeks. To examine whether GTP promotes DMH-induced colon carcinogenesis, 1% GTP-added diet was fed after DMH treatment without DSS exposure (group 5). Group 2 was fed with 1% GTP-added diet without any further treatment for the same period. All animals were sacrificed by anesthesia with diethyl ether at 20 weeks (n = 6 or 12–13 or 20).

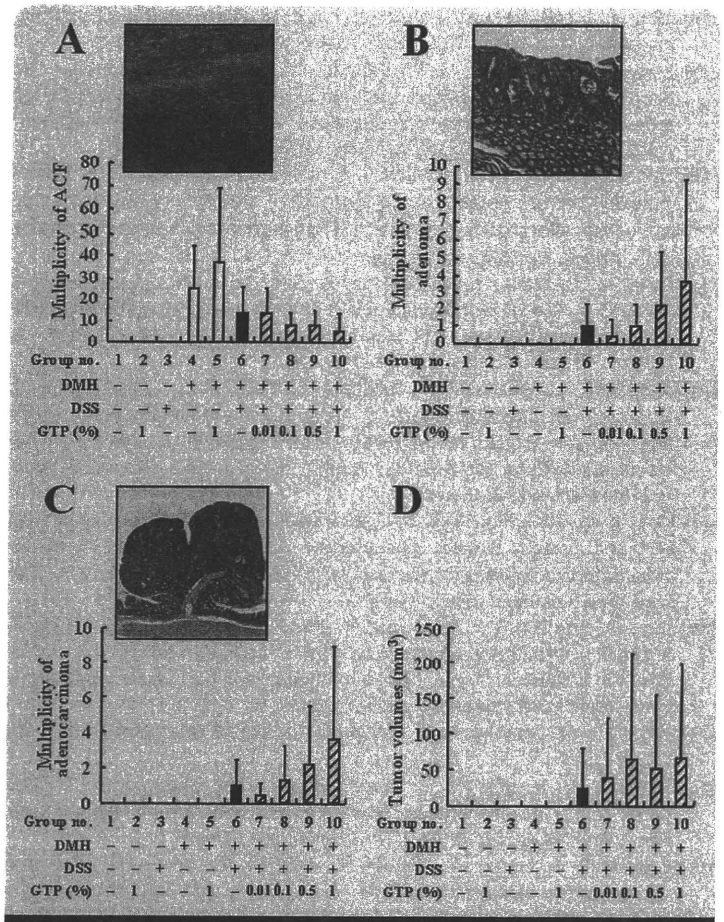

Fig. 7. The multiplicity of ACF (A), adenoma (B), adenocarcinoma (C), and volumes of tumors (D) (n = 6 or 12–13 or 20). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

are in line with those reported by Hirose et al. [17] who demonstrated that dietary GTP enhanced colon carcinogenesis in rats initiated with DMH. Moreover, feeding with 3,600 ppm of GTP in the diet resulted in significant increase in multiplicity of colonic tumors, when compared with the carcinogen, azoxymethane (AOM) alone group [39]. In fact, it has recently been reported that the levels of EGCG found in the small intestine and colon plateaued after administration by gavage between 500 and 2,000 mg/kg [40]. Furthermore, Lambert et al. [41] reported that after oral administration of EGCG at 163.8 μmol/kg, high concentrations of EGCG were observed in the small intestine (46.2 ± 13.5 nmol/g) and colon (7.9 ± 2.4 nmol/g). Assuming that 1 g of tissue is equivalent to 1 mL, these high concentrations are in the range of those used in cell culture experiments. The results of this study, together with previous findings, may thus suggest that oral intake of high dose of GTP may adversely affect colon carcinogenesis of certain individuals who have inflamed colon. To support this notion, green tea at a high dose (6 g/day) caused toxic symptoms that included nausea, emesis, insomnia, fatigue, diarrhea, abdominal pain, and confusion in patients with androgen independent prostate cancer [42]. Also, adverse events, for example, excess gas, upset stomach, nausea, heartburn, stomach ache, abdominal pain, dizziness, headache, and muscle pain, were reported in healthy individuals who took EGCG or Polyphenon E, a preparation that contains EGCG (400 mg twice/day or 800 mg once/day) for 4 weeks [43]. However, all of the reported events were rated as being mild.

In the experimental animal studies, green tea catechin (GTC) significantly inhibited AOM-induced rat colon carcinogenesis when GTC was administered during the postinitiation stage at doses of 0.01–0.1% [44], which were comparable to the average intake of green tea of Japanese people [44]. In this study, when feeding with 0.01% GTP-containing diet was started at the beginning of DSS exposure, tumors multiplicity tended to decrease, but 0.5 and 1% GTP in the diet failed to decrease tumor frequency (Figs. 7B and 7C). Thus, our findings suggest that modifying (beneficial or harmful) effects of GTP on colitis-related colon carcinogenesis depend on the dose in the diet.

We demonstrated for the first time that dietary GTP at dose levels of 0.5 and 1% profoundly enhances the DSS-induced acute colitis in mice presumably through increase in IL-1β and MIF expression. IL-1β and MIF, hallmarks of proinflammatory cytokines, were significantly enhanced by feeding with 0.5% and 1% GTP, whereas 0.1% GTP in diet suppressed the expression (Figs. 3A and 3B). Even though, 0.25% GTP in the diet had a tendency to improve both ulcer and inflammation (Figs. 5A and 5B) it did not show a decrease of IL-1β and MIF production (Figs. 3A and 3B). It is difficult to define what role 0.25% GTP in the diet plays on colitis. In patients with colitis-associated colon cancer, several inflammatory mediators and cytokines were increased in their serum [45–48]. IL-1β is a pivotal mediator in the inflammatory immune response that is characteristic of destructive inflammatory diseases [49]. The recent identification of the inflammasome, a multiprotein complex responsible for the activation of the IL-1β converting enzyme (ICE, caspase-1), has generated new possibilities for the elucidation of the etiology and pathophysiology of IBD as a new treatment target [50]. Evidence presented earlier suggests that certain proinflammatory cytokines and enzymes play a major role in mediating tumorigenesis. Thus, elevation of inflammatory cytokines such IL-1β and MIF may be the major factors for GTP-worsened colitis. Meanwhile, IL-6 and TNF-α levels in the present study were not signifcantly different in the colon of DSS-treated groups, or even GTP-, treated animals when compared with control mice. IL-6 [51] and TNF-α [52] also play important roles in the colitis induced by trinitrobenzenesulfonic acid and acetic acid. However, Kwon et al. [53] reported constitutive expression of TNF-α in DSS-treated colonic mucosa and present results of IL-6 and TNF-α levels may related to differences of strain and drugs.

Colonic oxidative stress is responsible for the pathogenesis of colitis through the release of inflammatory mediators and cytokines [24,54]. Therefore, ROS has the potential to promote proliferation of inflammatory and epithelial cells. Concomitantly, ROS induces resistance to apoptosis via activating redox-sensitive transcription factors, the most well-

known being nuclear factor-κB and activator protein-1 [55]. Previous studies have shown that EGCG not only has an antioxidative property but also pro-oxidative property [7,9]. We previously reported that EGCG activates oxidative stress signaling pathways *in vitro* [12]. A major cellular defense against ROS is provided by SOD and catalase, both of which detoxify superoxide radical to water and molecular oxygen. Therefore, the activities of antioxidant enzymes in the colonic mucosa of mice given GTP were investigated in this study. The activities of SOD and catalase were decreased in the 1% GTP alone group when compared with an untreated group, although dietary GTP did not significantly affect their activities in the DSS-administrated mice (Figs. 3E and 3F). These findings are inconsistent with those of cytokine production (Figs. 3A and 3B). At present, we are unable to explain the contradictory results, but a time-course experiment, which is underway in our laboratory, will provide detailed insights into the redox regulation of dietary GTP in the inflamed colon of mice.

In conclusion, our results described here, together with some previous reports by others, suggest that higher doses of dietary GTP deteriorate colitis and fail to prevent colon carcinogenesis in the inflamed colon. Our findings may also indicate that excess intake of GTP or derived supplements cannot be expected to provide chemopreventive effects in certain patients who are at particular risk for developing epithelial malignancies in the inflamed large bowel.

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Induction of HITS, a newly identified family with sequence similarity 107 protein (FAM107B), in cancer cells by heat shock stimulation

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Abstract. The Family with sequence similarity 107 (FAM107) possesses an N-terminal domain of unknown function (DUF1151) that is highly conserved beyond species. In human, FAM107A termed TU3A/DRR1 has been reported as a candidate tumor suppressor gene which expression is down-regulated in several types of cancer, however no studies have investigated the other family protein, FAM107B. In the present study, we designated FAM107B as heat shock-inducible tumor small protein (HITS) and studied its expression and functional properties in cancer. HITS is an 18-kDa nuclear protein expressed in a variety of tissues including stomach, colon, lung and lymphoid organs. In human gastric and colorectal cancers and a mouse model of colon cancer, its expression in tumor cells was much lower than normal epithelial cells, while expression pattern and intensity varied among different histological types of cancer. In functional analysis *in vitro*, forced expression of this protein suppresses the cellular responses to growth factors. Furthermore, HITS gene carries the promoter region providing heat shock transcription factor (HSF) binding sites and amplifying the transcription of HITS by heat shock or hyperthermia treatment both *in vitro* and *in vivo*. Thus HITS would be a potential tumor suppressor gene similar to TU3A containing heat responding elements, which contrasts with previously described oncogenic activities of other heat shock proteins such as HSP70 and HSP90.

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Key words: FAM107, TU3A, heat shock protein, tumor suppressor gene

Introduction

The Family with sequence similarity 107 (FAM107) possesses an N-terminal domain of unknown function (DUF1151) that is conserved beyond species including mammalian, Xenopus, fish and Drosophila, and shows no homology match to other functional conserved domains (http://www.ncbi.nlm.nih.gov/structure/cdd/cdd.shtml). This family includes several hypothetical eukaryotic proteins of unrevealed function. In mammals, FAM107 consists of two genes FAM107A and FAM107B, coding proteins of 144 amino acids (aa) and 131 aa respectively (http://www.uniprot.org/uniprot).

FAM107A designated TU3A (Tohoku University cDNA clone A on chromosome 3), also called DRR1 (down-regulated in renal cell carcinoma gene 1) is a candidate tumor suppressor gene located on chromosome 3p21.1 (1,2). Recently several studies have reported that TU3A expression is down-regulated in a variety of cancers by epigenetic silencing such as promoter hypermethylation (3-5). In addition, the reexpression of TU3A suppresses cancer cell growth and induces apoptosis (2,6).

In this study, we examined expression, tissue localization and functional properties of FAM107B, and designated FAM107B as heat shock-inducible tumor small protein (HITS) because of its unique expression pattern in cancer cells and relatively small molecular weight (18 kDa) among heat shock proteins (HSPs). In human, HITS protein is encoded by the gene on chromosome 10p13 and consists of 131 aa, the sequence of which is almost 98% identical with mouse and rat homologues. FAM107A (TU3A) and FAM107B (HITS) proteins share 65% sequence similarity in their DUF1151 regions. At present, no functional information is available for HITS despite recent accumulating data on TU3A. Herein, we demonstrate heat shock-inducible unique expression pattern and biological properties of HITS in cancer cells, that is distinct from other HSPs equipped with oncogenic activities (7,8).

Materials and methods

Cells and tissue specimens. HEK293 and HeLa cells were cultured in DMEM with 10% fetal calf serum (FCS), and THP-1 and Jurkat cells in RPMI-1640 with 10% FCS. HITS cDNA was subcloned into pcDNA3.1 D/V5-His-TOPO (Invitrogen, CA) and transfected into 293 cells using Lipofectamine 2000 reagent (Invitrogen) to express HITS-V5-His fusion protein. Stable transfectants were obtained through selection by neomycin. Inducible expression of HITS was generated by Tet-On advanced inducible gene expression system (Clontech, CA). Full-length cDNA of HITS was introduced into a retrovirus vector, pRetroX-Tight Pur, with a Tet-inducible promoter. Virus stock was prepared by transfecting vector plasmid into GP2-293 packaging cells (Clontech) and then infected to the HeLa Tet-On advanced cell line (Clontech). For selecting stable transfectants (HeLa-Tet-HITS or control HeLa-Tet-MOCK), infected cells were cultured in the medium containing puromycin.

Matched pairs of fresh non-neoplastic (normal) and tumor tissues were obtained from the surgical specimens of stomach and colorectal cancer patients (9). Each patient signed an informed consent form. These tissue samples were stored in -80˚C till use for extraction of total RNA. Surgical specimens were then fixed in neutral-buffered 10% formalin, embedded in paraffin and processed for histopathological diagnosis and immunohistochemical examinations. Histological types of stomach and colorectal cancers were classified according to the WHO Classification (10,11). Paraffin sections of normal brain, thyroid, thymus, lung, esophagus, liver, pancreas, uterus, prostate and lymph node were prepared from autopsy cases. This study was approved by the Institutional Review Boards of Kanazawa Medical University and Kanazawa University.

Colon carcinogenesis mouse model. Crj:CD-1 (ICR) male mice at 5-week-old (Charles River Japan) received a single intraperitoneal injection of azoxymethane (AOM) at a dose of 10 mg/kg body weight. Starting 1 week after the AOM injection, mice were exposed to 2% dextran sodium sulfate (DSS) in the drinking water for 7 days (12). They were autopsied at the week 12. The histopathological findings of the large bowel lesions were examined by hematoxylin and eosin (HE)-staining and immunohistochemistry. The animal study was conducted according to the Guidelines for Experimental Animals at Kanazawa Medical University and the National Guidelines for Animal Usage in Japan (http://www.lifescience.mext.go.jp/policies/pdf/an_material011.pdf).

Antibodies. Anti-V5 (Invitrogen), anti-HSP90 (Stressgen, MI), anti-Lamin A/C and anti-ß-actin (Santa Cruz Biotech, CA) antibodies (Abs) were purchased. To generate anti-HITS anti-serum, rabbits were immunized with KLH-conjugated polypeptides MAEPDYIEDDNPE. The anti-serum was purified by affinity chromatography against the polypeptides cross-linked to agarose beads.

Immunostaining. Cultured cells were fixed with 2% paraformaldehyde and permealized by 0.5% Triton-X. After blocking with 2% FCS, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-V5 Ab or anti-HITS rabbit serum plus FITC-conjugated secondary Ab, and then subjected to FACS or fluorescence microscope analysis.

Expression and localization of HITS and HSP90 in paraffin sections of tissues were observed by immunohistochemical examinations using the avidin-biotin-peroxidase complex (ABC) method. Microwaved tissue sections were incubated with the respective antibodies (1:500 dilution each) overnight at 4˚C, followed by the standard procedure (13).

Complementary DNA synthesis and semi-quantitative RT-PCR. Total RNA was isolated from cultured cells and fresh surgical tissues with RNeasy mini kits (Qiagen, Germany) according to the manufacturer's instruction, and a 10 μg aliquot of total RNA was subjected to cDNA synthesis using First-Strand cDNA synthesis kit (Amersham, UK). Mixtures containing 1 μl of synthesized cDNA were amplified by PCR for 25 cycles (94˚C for 30 sec, 50˚C for 30 sec, 72˚C for 30 sec) after denaturation for 2 min at 94˚C. The primer pairs for RT-PCR are listed below: HITS forward: 5'-AGCACAGAAGAAGA AATCTGA-3'; HITS reverse: 5'-CTTGGGCGACTTCTTGG CCTG-3'. ß-actin forward: 5'-ACACTGTGCCCATCTACG AGGGG-3'; ß-actin reverse: 5'-ATGATGGAGTTGAAGGT AGTTTCGTGGAT-3'.

DNA microarray analysis. Total RNAs from heat shock-treated and non-treated Jurkat cells were subjected to cRNA preparation using a MessageAmp II®-Biotin Enhanced Kit (Ambion, TX). Target hybridizations were performed using a Human Genome U133 plus 2.0 Gene-Chip microarray system (Affymetrix, UK). The hybridized cRNAs were detected using a GeneChip® Scanner 3000. The digital data were processed using the GeneChip Operating Software (GCOS) version 1.4.

Western blot analysis. To obtain nuclear and cytoplasmic extracts, cultured cells were treated with NucBuster Protein Extraction kit (Novagen, Germany) according to the manufacturer's instruction, and extracts from $3x10^5$ cells were separated by standard sodium dodecylsufate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (Amersham). Membranes were probed with the respective primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody and developed using chemiluminescence (Amersham).

Drug resistance. HeLa-Tet-MOCK and -HITS transfected cells were suspended in DMEM containing 10% FCS with or without 1 μg/ml doxycycline, and 5000 cells/well were seeded and treated with paraquat (1-5 μM), etoposide (30, 100 μM), 5-FU (160, 320 μg/ml) or CDDP (10, 20 μM). After treatment for 2 days, relative number of viable cells was measured by MTT assay using Cell Counting Kit-8 (Dojin, Japan), and cell survival was calculated by the ratio against untreated cells.

Cell responses to growth factors. HeLa-Tet-MOCK and -HITS transfected cells were suspended in DMEM containing 0.2% FCS with or without doxycycline, and 5000 cells/well were seeded in 96-well plates. After serum depletion for 2 days, human recombinant fibroblast growth factor-2 (FGF-2),

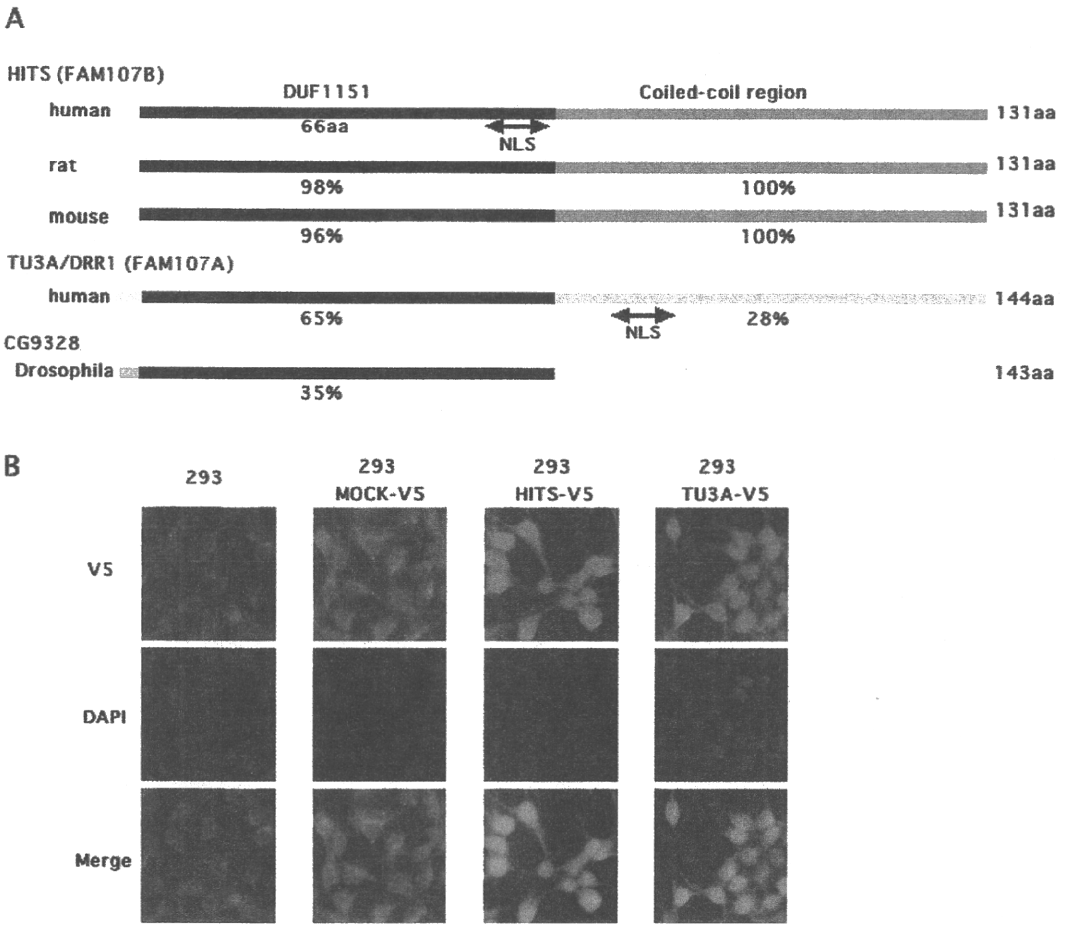

Figure 1. Molecular structures of FAM107 family proteins and subcellular localization of HITS (FAM107B) and TU3A (FAM107A) proteins. (A) Molecular structure of FAM107 family of proteins was compared among different species. In rat and mouse HITS, human TU3A/DRR1 and Drosophila CG9328, homology of each protein to human HITS protein sequence is shown by %. NLS, nuclear localization signal. (B) Fusion proteins of HITS and TU3A tagged with V5 (HITS-V5 and TU3A-V5, respectively) were expressed in 293 cells. As controls, untransfected 293 cells and those transfected with empty insert (MOCK-V5) were examined. These cells were immunostained with FITC-labeled anti-V5 antibody. Nuclei were counterstained with DAPI. A merged image is generated in each cell type.

epidermal growth factor (EGF) (both from Wako Pure Chemical, Japan) or increased amount of FCS were added to octuplicate wells at indicated concentrations. Proliferation of cells after 2 days of stimulation was measured by MTT assay as described above.

Luciferase assay. According to the published genomic sequences, 5'-flanking region of HITS (-316 to +189) was amplified and ligated into pGL3-Basic vector (Promega, WI) to generate a HITS promoter-firefly luciferase reporter construct (pGL3-HITS). As previously described, pGL3-Basic or pGL3-HITS and pRL-thymidine kinase control vector (pRL-TK; Promega) were transfected to 293 cells (14). Next day, the cells were harvested after heat shock treatments and assayed by the Dual-Luciferase Assay System (Promega), using a luminometer. The relative light units were calculated after normalization against Renilla luciferase activities of the pRL-TK internal control vector.

Hyperthermia and heat shock treatment. For heat shock stimulation, cultured cells were incubated at 42°C for 2 h, followed by recovery at 37°C for indicated periods. Then the cells were harvested and processed for luciferase assay, RT-PCR, Western blotting or DNA microarray analysis.

For hyperthermia treatment of rat tumor model, Rat Walker 256 sarcoma was subcutaneously inoculated at a flank of Sprague-Dawley Rats. When tumors grew to ~2 cm in diameter, hyperthermia treatment was performed on an air heating machine with a spray tube that can cover the tumor area of the rat (AOYUE 615, Tongyi Electronic Equipment Factory, Zhongshan City, China). Heated air was ejected from this tube and the flow was adjusted manually. The temperature at the center of tumor was monitored by thermocouples (Physitemp, NJ). The air flow of the heating machine was adjusted and kept at 43°C for 2 h. Tumor tissues were removed 6 h later and processed for histopathological and immunohistochemical examinations.

Statistical analysis. Between-group statistical significance was determined using Student's t-test. $P<0.05$ was considered as statistically significant.

Results

Expression and subcellular localization of HITS in normal tissues. The FAM107 family of proteins share homology in an N-terminal domain that is conserved among species (Fig. 1A). According to the protein data base, FAM107B protein

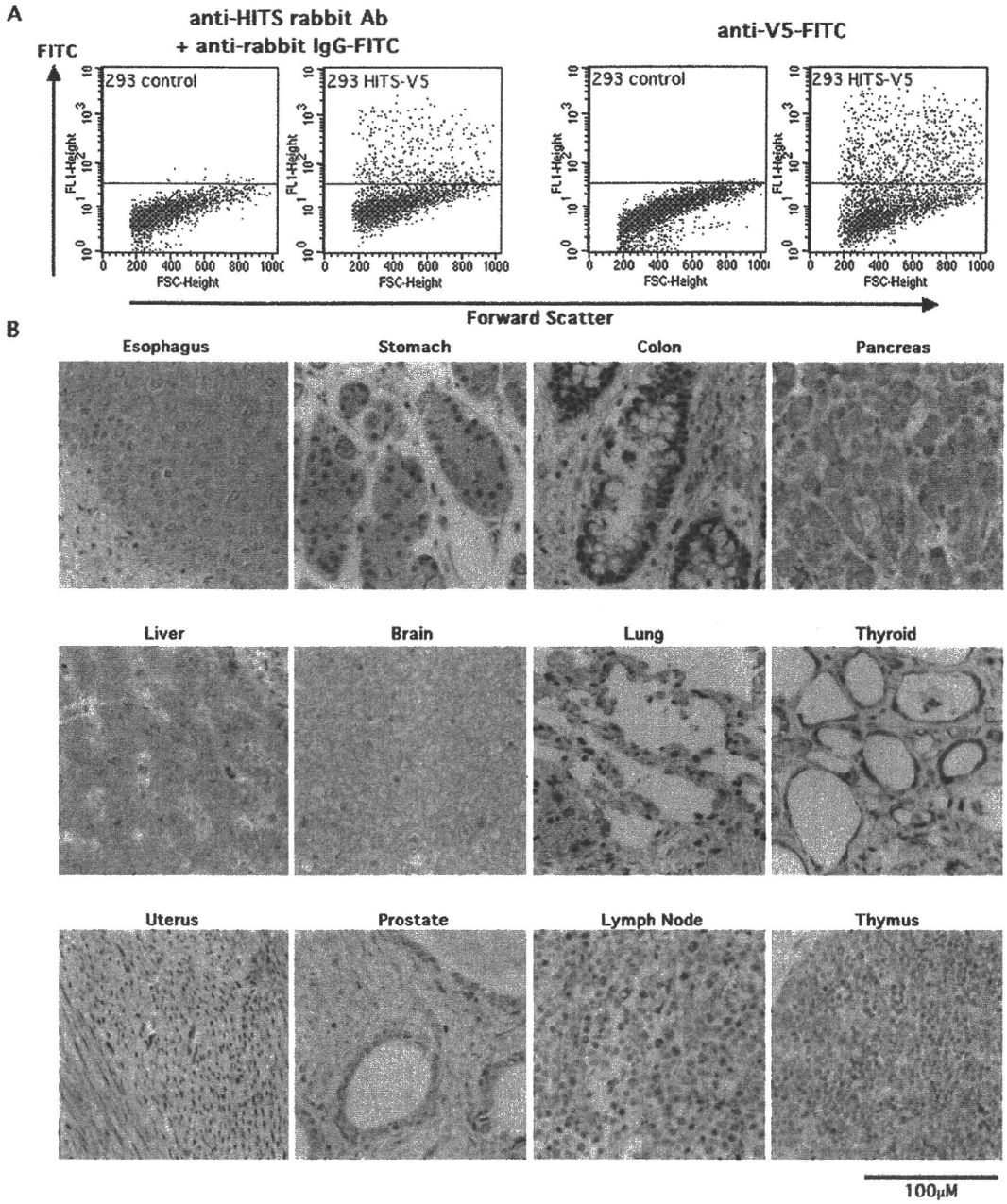

Figure 2. Characterization of the polyclonal antibody to HITS protein and immunohistochemical examination of expression and localization of HITS protein in normal human tissues. (A) To test specificity of the antibody against human HITS, 293 cells transiently transfected with pcDNA3.1 HITS-V5-His (293 HITS-V5) or control pcDNA3.1 V5-His (293 control) were immunostained with the purified anti-HITS antibody or anti-V5 monoclonal antibody. The stained cells were analyzed by FACS. X-axis, forward scatter; Y-axis, FITC (anti-rabbit IgG-FITC or anti-V5-FITC). (B) Immunohistochemical analysis of HITS expression. Paraffin sections of various human normal tissues were immunostained with anti-HITS antibody.

(UniProtKB/Swiss Prot: Q9H098) has a 131 aa sequence length which consists of an N-terminal (aa 1-66) conserved domain DUF1151 and a C-terminal (aa 61-112) variable coiled-coil region. Both FAM107A (TU3A) and FAM107B (HITS) have a nuclear localization signal (NLS) in the center of the protein sequences (Fig. 1A). Intracellular localization of HITS was determined by immunostaining of HITS-V5 fusion protein expressed in 293 cells with FITC-labeled anti-V5 Ab. As well as TU3A, which has been reported to be a nuclear protein, HITS was localized in the nuclear portion since its fluorescence signals largely coincided with DAPI signals (Fig. 1B).

To investigate the expression and localization of HITS in normal human tissues, antibody against HITS was generated and verified its specificity by FACS (Fig. 2A) and Western blot analysis (Fig. 5A). Immunohistochemical examinations revealed that HITS was expressed in a broad range of normal tissues. In particular, intense immunoreactivity of antibody was observed in nuclei of colon, stomach and lung epithelial cells (Fig. 2B).

Expression of HITS in gastrointestinal tumors. As TU3A expression is reported to be downregulated in several human cancer types (1,2), we investigated the expression of HITS

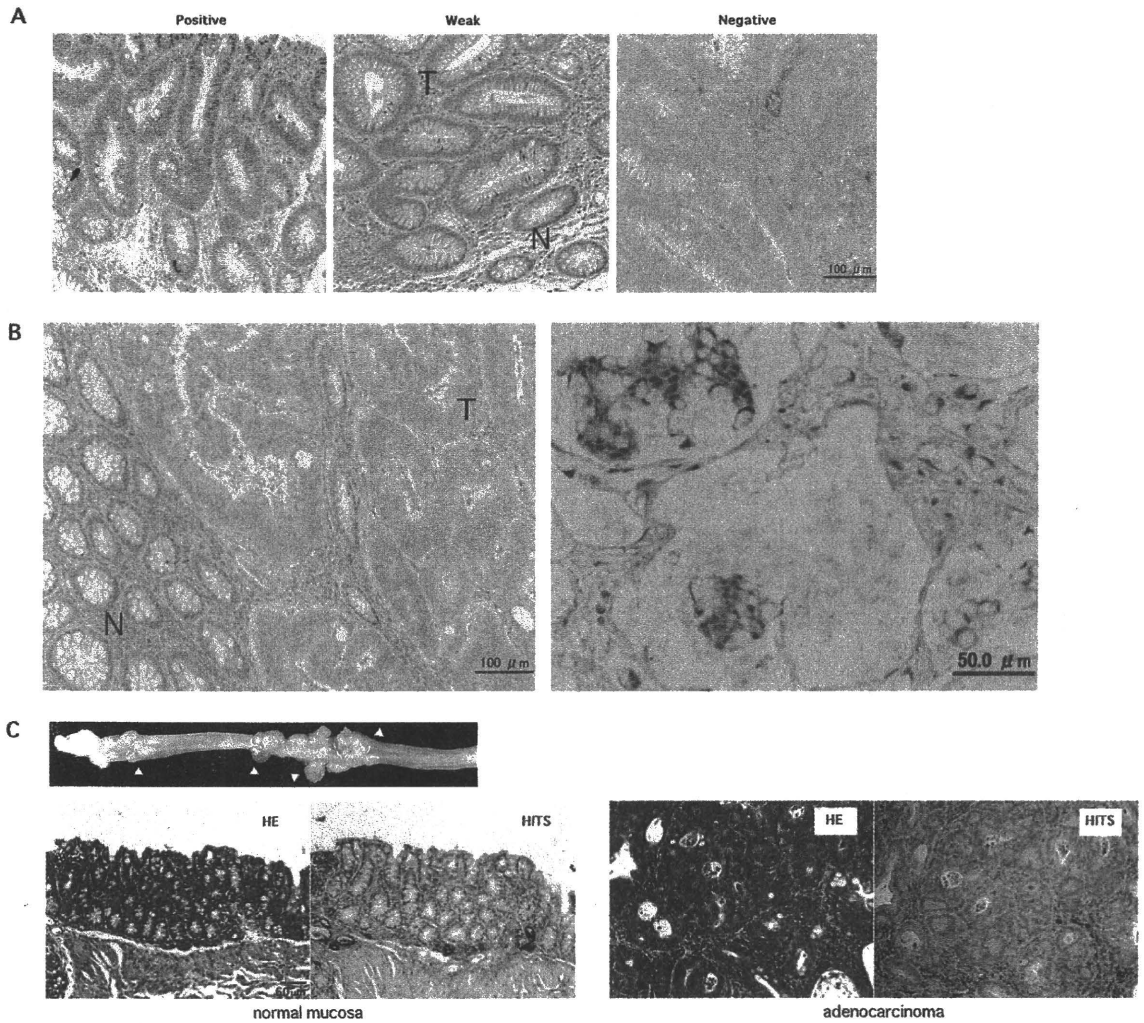

Figure 3. Expression of HITS in human colorectal tumors and colon cancer in a mouse model. (A) Expression of HITS in tubular adenoma of the human colon. Levels of HITS expression were classified into 3 categories, positive, weak (T, adenoma; N, normal mucosa) and negative, as defined in the text. (B) Expression of HITS was negative in a well differentiated adenocarcinoma (T) and positive in adjacent normal mucosa (N) (left) and in a mucinous adenocarcinoma (right) of human colon. The results are summarized in Table I. (C) Gross finding of a large bowel removed from an AOM-induced colon carcinogenesis model mouse (upper panel) showed multiple protruding tumors (arrow heads). Serial sections of non-neoplastic (normal) mucosa and adenocarcinoma were stained with HE and immunostained with anti-HITS antibody, respectively (lower panels). Note that no expression of HITS was found in adenocarcinoma cells.

in stomach and colorectal tumors (Figs. 3 and 4). Levels of HITS protein expression in tumor tissues were classified into 3 categories, positive (comparable to normal epithelium), weak (expressed less than normal counterpart) and negative (no expression) (Fig. 3A). Human colorectal adenomas (22 cases) and adenocarcinomas (52 cases) were immunohistochemically analyzed, and the results are summarized in Table I. While more than half of adenomas were positive, only 6% of adenocarcinomas were positive for HITS expression. Two of three HITS-positive carcinomas were histologically mucinous adenocarcinomas (Fig. 3B right and Table I). In AOM induced colon carcinogenesis mouse model, HITS expression was markedly decreased in carcinoma cells (Fig. 3C). Thus, HITS expression was downregulated in association with the progression of colon carcinogenesis.

HITS showed a distinct expression pattern in gastric carcinomas that were histologically classified into two major groups (15). In most of intestinal type gastric carcinomas, expression of HITS was negative or weak (Fig. 4A and Table I) as defined above, while its expression in >80% of diffuse type carcinomas was comparable to the foveolar ducts in the matched normal mucosa (Fig. 4B and Table I). Staining in mixed type of intestinal and diffuse carcinoma clearly represents this specificity of HITS expression pattern (Fig. 4C). Since the classification of HITS expression level in mixed type is difficult to judge, it is excluded from the sum in Table I. Mucinous adenocarcinoma was positive for expression of HITS, similar to colon mucinous adenocarcinoma (Fig. 3B right and 4D). Consistent with immunohistochemical observation, semi-quantitative RT-PCR analysis showed that HITS mRNA expression was negative or weak in intestinal type and positive in diffuse type adenocarcinomas (Fig. 4E).

Functions and biological properties of HITS in cancer cells. To clarify the functional properties of HITS protein, we employed an approach to tetracycline inducible expression system (Tet-ON). Human cervical cancer cell line, HeLa, was transfected with tetracycline-inducible expression

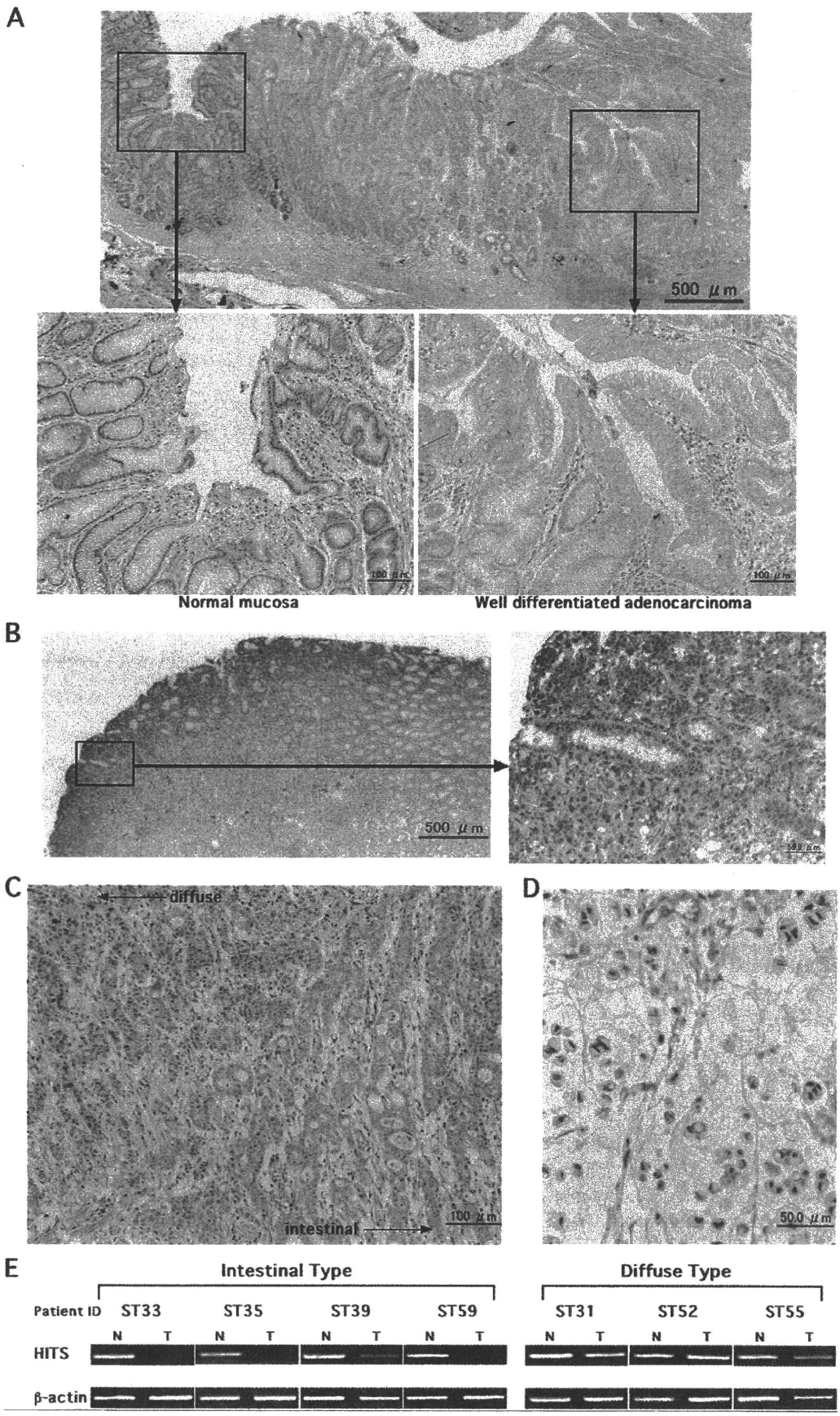

Figure 4. Expression of HITS protein and mRNA in human gastric cancer. Immunohistochemical expression of HITS protein was examined in intestinal type (A), diffuse type (B), mixed type of both intestinal and diffuse (C), and mucinous (D) adenocarcinomas. Higher magnification images of the squared regions are shown in (A) and (B). The results are summarized in Table I (mixed type is excluded from the sum). (E) Semi-quantitative RT-PCR analysis of HITS mRNA expression in the matched pairs of normal (N) and tumor (T) tissues obtained from the patients with intestinal type and those with diffuse type gastric adenocarcinomas, respectively. Amount of cDNA in each sample was monitored by ß-actin mRNA expression.

Table I. Expression of HITS protein in human stomach and colorectal tumors.

Organ	Tumor type	Histological types[a]	No. of cases	HITS expression in tumor		
				Positive (%)	Weak (%)	Negative (%)
Colon and rectum	Tubular adenoma		22	13 (59)	8 (36)	1 (5)
	Adenocarcinoma	W/D	14	0 (0)	7 (50)	7 (50)
		M/D	33	1 (3)	13 (39)	19 (58)
		Muc	4	2 (50)	2 (50)	0 (0)
		U/D	1	0 (0)	0 (0)	1 (100)
Stomach	Adenocarcinoma	Intestinal	22	1 (3)	9 (41)	12 (55)
		Diffuse	12	10 (83)	2 (17)	0 (0)
		Muc	2	2 (100)	0 (0)	0 (0)

[a]Histological types according to WHO Classification (10,11). M/D, moderately differentiated; Muc, mucinous; U/D, undifferentiated; W/D, well differentiated.

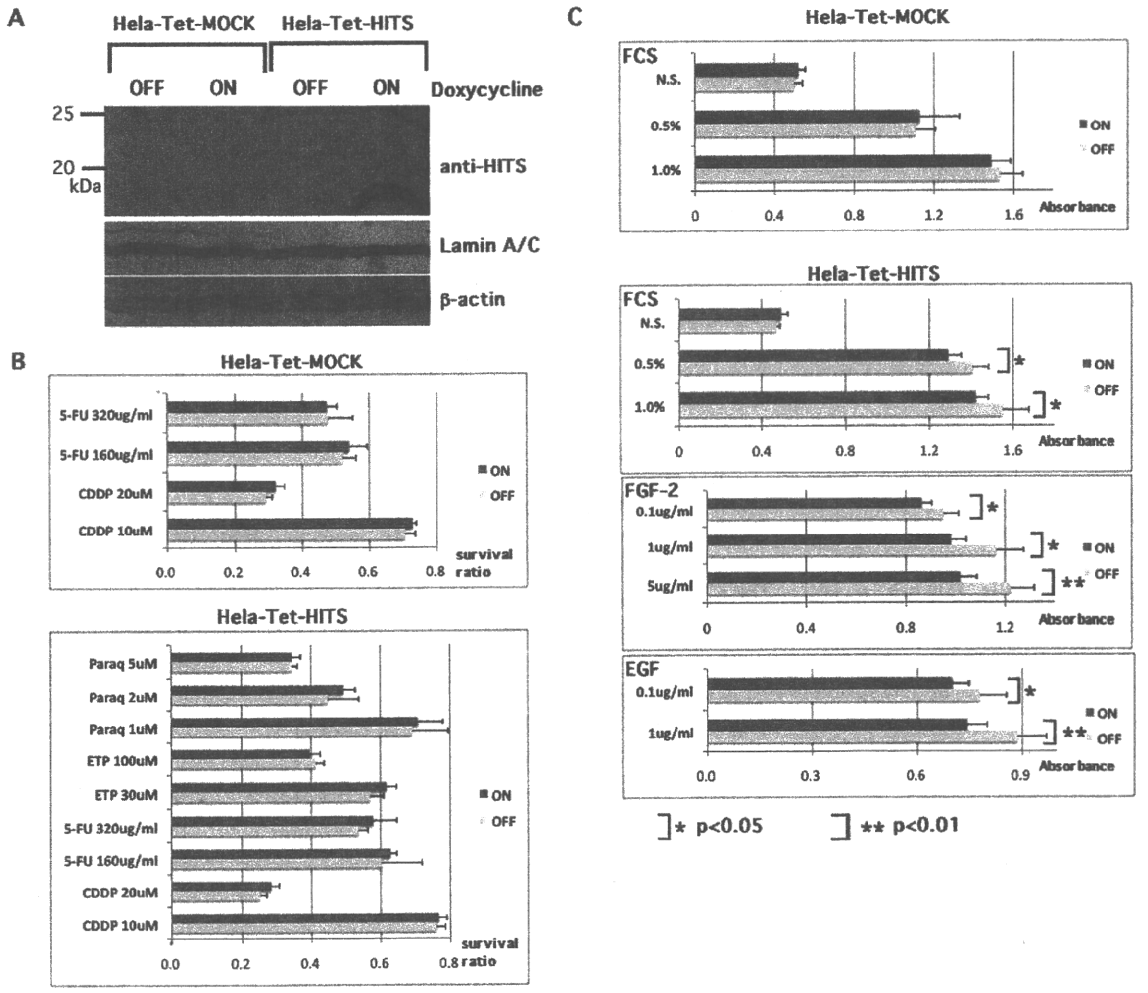

Figure 5. Generation of HeLa cells with tetracycline (Tet)-inducible expression of HITS and the effects of the inducible expression of HITS on tumor cells sensitivities to the drugs and their responses to growth factors. (A) Protein samples prepared from HeLa-Tet-HITS and HeLa-Tet-MOCK cells treated with (ON) or without (OFF) 1 μg/ml doxycycline were examined for expression of HITS, Lamin A/C (nuclear protein control) and ß-actin by Western blot analysis. (B) Effects of HITS on HeLa cell sensitivity to the drugs. HeLa-Tet-MOCK and -HITS cells with (ON) or without (OFF) doxycycline were treated with paraquat, etoposide, 5-FU or CDDP at indicated concentrations. After treatment for 2 days, relative cell survival was calculated by the ratio against untreated cells in MTT assay. (C) Effects of HITS on HeLa cells responses to growth factors. HeLa-Tet-MOCK and -HITS cells with (ON) or without (OFF) doxycycline were maintained under serum depletion for 2 days, and then cultured with FCS (0.5, 1.0%), human recombinant FGF-2 (0.1-5 μg/ml), EGF (0.1-1 μg/ml) and no growth factor (NS). Cell proliferation after 2 days of treatment was measured by MTT assay. In (B) and (C), values are mean ± standard deviations in octuplicates.

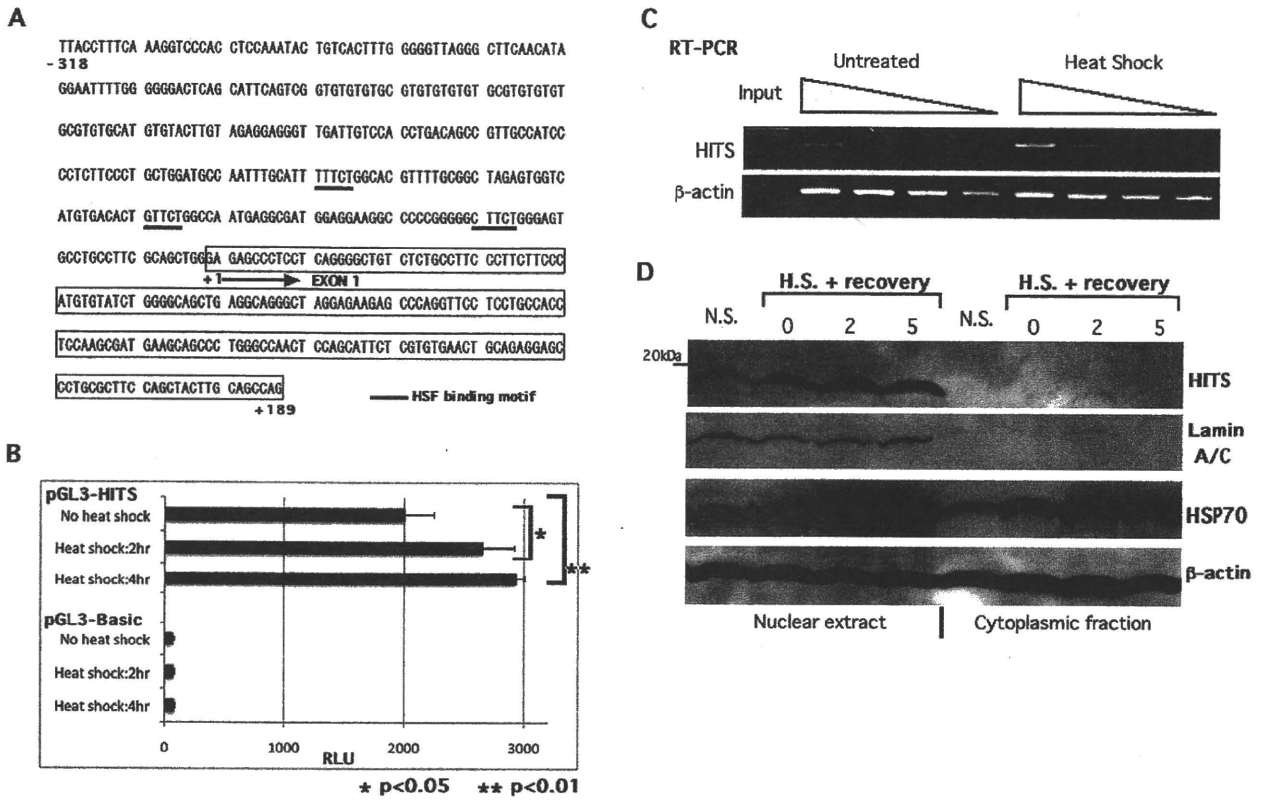

Figure 6. Sequences of the promoter region of HITS gene and heat shock-induced expression of HITS in cultured cells. (A) Genomic sequence of the proximal promoter of HITS -318 from transcription initiation site to +189 in exon 1 is shown. HSF binding sites were underlined. (B) To examine the effects of heat shock treatment on HITS transcription, 293 cells transfected with pGL3-HITS (-316 to +189) and pGL3-Basic, respectively were incubated at 42°C for 2 h and recovered at 37°C for indicated periods, and processed for luciferase assay. The relative light units (RLU) were calculated after normalization against Renilla luciferase activities of the pRL-TK internal control vector. Values are mean ± standard deviations in quadruplicates. (C) Effects of heat shock treatment on mRNA expression of HITS in cultured cells. Jurkat cells were incubated at 42°C for 2 h and recovered at 37°C for 4 h (heat shock) or sham treated (untreated), and subjected to RT-PCR analysis. For RT-PCR analysis, cDNA was amplified using specific primer pairs of HITS and ß-actin. Amounts of input (3-fold serial dilution of cDNA) are shown above the panels. DNA microarray analysis of these samples is shown in Table II. (D) Effects of heat shock treatment on expression of HITS protein in cultured cells. THP-1 cells were treated with heat shock (HS) at 42°C for 2 h followed by recovery at 37°C for indicated hours and with no heat shock stimulation (NS). Cellular extracts were separated into nuclear and cytoplasmic fractions, and examined by Western blotting for expression of HITS, Lamin A/C (nuclear protein control), HSP70 (heat shock marker) and ß-actin.

vector with full length HITS or without an insert (MOCK). In Western blot analysis, HITS was detected as an ~18-kDa protein that was strongly induced by doxycycline treatment on HeLa-Tet-HITS cells, but not on HeLa-Tet-MOCK cells (Fig. 5A). Since TU3A has been reported to inhibit cell growth and induce apoptosis (2,6), we tested the effects of HITS on drug resistances and responses to growth factors using this inducible expression system. At first, HeLa-Tet-MOCK and -HITS cells were treated with various cytotoxic agents such as paraquat (oxidative stress), etoposide (inhibitor of the topoisomerase II), fluorouracil (5-FU: anti-metabolite) and cisplatin (CDDP: cross-linking of DNA). Drug resistance was determined by relative numbers of survival cells after 48-h exposure to the respective agents. The results showed no significant differences in cell survival between the doxycycline-induced (ON) and non-induced (OFF) cells in both HeLa-Tet-MOCK and -HITS cell lines (Fig. 5B). Next, we compared the cellular response to growth factors (FCS, FGF-2 and EGF) employing the same system. The results showed that proliferative responses to all growth factors tested were attenuated by the inducible expression of HITS, although the inhibitory effects by HITS were not very potent but statistically significant (Fig. 5C).

Table II. Heat shock-induced changes in expression of FAM107 family genes and HSPs determined by DNA microarray analysis.

Gene	Untreated signal	Heat-shock signal	Fold change
DKFZp434P116 (HITS)	652	2098	3.22
FAM107A (TU3A)	35	53	1.65
Heat shock 27 kDa protein 1	813	4194	5.16
Heat shock 40 kDa protein 1	3231	9417	2.91
Heat shock 70 kDa protein 1B	1228	5964	4.86
Heat shock 105 kDa B	4259	12099	2.84

Fold increases of signal intensity of the FAM107 family genes and HSPs after heat-shock treatment are shown.

Induction of HITS expression by heat shock stimulation. Downregulation of HITS expression in cancer cells seems controlled in transcriptional level because the results from

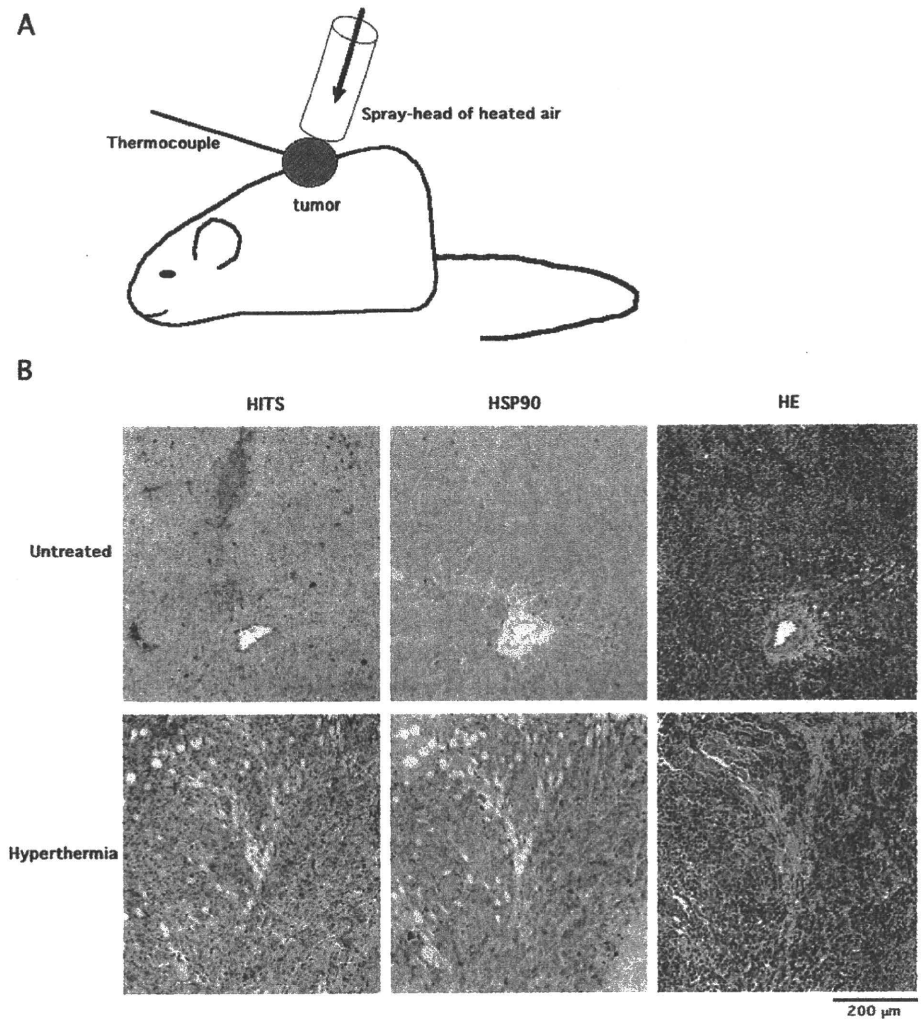

Figure 7. Effects of hyperthermia on expression of HITS and HSP90 in Rat Walker 256 sarcoma cells transplanted in the rat. (A) Schematic representation of hyperthermia using an air heating machine. Rats were placed on the platform of the machine (AOYUE 615) under anesthesia, and the tumor area was covered by the spray-head where heated air was sprayed out. The temperature of the tumor center was monitored by the thermocouple and adjusted to the desired temperature. (B) Rat Walker 256 sarcoma graft of ~2 cm in diameter was sham treated (upper) or treated (lower) with hyperthermia at 43°C for 2 h. Six hours after treatment, the tumors were removed. Serial paraffin sections of the respective tumors were stained with HE and immunostained for HITS and HSP90, respectively.

semi-quantitative RT-PCR analysis showed decreased amount of HITS mRNA in intestinal type gastric adenocarcinomas (Fig. 4E). Then we analyzed the promoter region of HITS gene to identify factors that regulate the transcription of the gene. By database search through http://www.cbrc.jp/research/db/TFSEARCHJ.html and http://tfbind.ims.u-tokyo.ac.jp, we found three heat shock elements with an NGAAN motif that recruits heat shock transcription factor (HSF) in the proximal region of HITS gene promoter (Fig. 6A).

To evaluate the effect of heat shock on HITS transcription, first we performed luciferase assays with pGL3 construct with 5'-flanking region (-316 - +189) of HITS gene containing three HSF binding sites. Basal transcriptional activity of pGL3-HITS is ~30-fold higher than control pGL3-Basic without an insert (Fig. 6B). In comparison with untreated cells, luciferase activity of pGL3-HITS was increased up to ~1.5-fold by heat shock treatment followed by 4-h recovery at 37°C (Fig. 6B). Enhancement of HITS mRNA expression by heat shock treatment was also observed by semi-quantitative RT-PCR and DNA microarray analysis, estimating ~3-fold increase in mRNA level that is comparable to the effects of other HSPs (Fig. 6C and Table II). Heat shock-induced HITS expression in protein level was detected by Western blot analysis, exhibiting induction of ~18-kDa proteins probed with the anti-HITS Ab in nuclear fractions (Fig. 6D).

Upon these heat shock responses, we further investigated the induction of HITS expression *in vivo* by immunohistochemical analysis of rat tumor model treated with hyperthermia. Thermotherapy kills or weakens tumor cells, with little effects on healthy cells (16). After treatment at 43°C for 2 h followed by 6-h recovery, HITS as well as HSP90 was markedly induced in the tumor cells compared to those in untreated controls (Fig. 7).

Discussion

TU3A has been proven to be commonly downregulated or deleted in many types of cancer, such as non-small cell lung, renal cell and prostate cancers and astrocytoma (1,2,4-6). Accordingly TU3A is characterized as a candidate of tumor

suppressor gene (TSG) because of its typical expression pattern as a TSG and the fact that introduction of TU3A suppresses tumor cell proliferation and induces apoptosis (2,6,17,18). Similar to TU3A, as a member of FAM107, HITS was shown to be decreased or absent in gastric and colorectal cancers and to inhibit tumor cell proliferative responses to growth factors in the present study.

Immunohistochemical examination of HITS in stomach and colorectal tumors exhibited distinct characteristics consistent with a hypothesis that HITS is a TSG. First, expression of HITS decreased in tumors during the process of colorectal adenoma-to-carcinoma sequence (Fig. 3 and Table I). As alterations in the oncogenes and tumor suppressor genes such as adenomatous polyposis coli (APC), K-ras and p53 were frequently involved in a multistep genetic model of colorectal tumorigenesis (19-23), loss of the HITS expression may be involved in the development of colorectal cancers. Second, clear difference in HITS expression was observed between intestinal and diffuse types of gastric adenocarcinomas (Fig. 4 and Table I). Intestinal type cancer is postulated to develop through a sequential pathway from normal mucosa, chronic atrophic gastritis, intestinal metaplasia and then to dysplasia (24-26). On the contrary, diffuse type cancer does not typically arise from recognizable precancerous lesions, and has a higher association with familial occurrence, suggesting a genetic susceptibility (27-29). According to our results that HITS expression was decreased in intestinal type but not in diffuse type gastric adenocarcinomas, HITS may be inactivated or deleted in the development of intestinal type carcinoma from precancerous lesions such as gastric glandular atrophy and intestinal metaplasia. Third, HITS expression was positive in both stomach and colon mucinous adenocarcinomas (Figs. 3B right and 4D), which have been reported to show distinct biological behavior and worse clinical outcome compared to intestinal type adenocaricnomas (30-32).

The most interesting feature concerning HITS expression in tumors was its transcriptional induction by heat shock stimulation. A series of studies has shown that high temperatures can damage cancer cells, usually with minimal injury to normal cells (16). Hyperthermia therefore renders cancer cells to undergo apoptosis in direct response to heat stimulation, while normal cells acclimate to it and maintain their physiological status. Even if the cancer cells do not die immediately, they may become more susceptible to ionizing radiation or to certain chemotherapies. Therefore, hyperthermia is always used with other modalities of cancer therapy, such as radiation and chemotherapy (33). Contrary to clinical benefits of hyperthermia treatment of cancer, induction of HSPs is an undesired consequence in cancer cells (7,8).

HSPs and their relatives are molecular chaperons that facilitate folding of normal proteins and protect them from misfolding and aggregation (34). Their increased expression in tissues that are exposed to various proteotoxic stressors is an adaptive response to sustain cell survival. Therefore, the increased expression of chaperon proteins in many tumor types represents the ability of malignant cells to hold their neoplastic nature in a hostile tumor microenvironment (35,36). In breast cancer, overexpression of HSP70 and HSP90 correlates with poor prognosis (37,38). Overexpression of HSPs also contributes to drug resistance and a poor response to combination chemotherapy (39-42). HSF1 is the master regulator of the heat shock response in eukaryotes, a highly conserved protective mechanism against heat stimulation (43). It functions to facilitate cell survival under different pathophysiological conditions. HSF1 is a powerful multifaceted modifier of cellular malignant transformation by orchestrating a biological network of core cellular functions including proliferation, survival, protein synthesis and glucose metabolism (44-46). Contrary to these oncogenic activities reported in several HSPs and HSF1, HITS seems to function as a tumor suppressor. This is indicated by our observations that forced expression of HITS inhibited the proliferative responses to growth factors, and did not give rise to resistance to various stresses by cytotoxic agents including anti-cancer drugs (Fig. 5).

In conclusion, the present study suggests that HITS, as a member of FAM107, is a potential TSG, with a unique feature of its transcriptional induction by heat shock stimulation. HITS would be useful for tumor diagnosis and for monitoring therapeutic effects of hyperthermia. The molecular mechanism underlying the biological functions of FAM107 is yet to be investigated. C-terminal variable regions of FAM107 carry a coiled-coil domain that has been identified in many nuclear proteins including transcription factors, suggesting a role of FAM107 in regulating gene transcription. An N-terminal conserved domain DUF1151 may play a role in interacting with other proteins to transduce cellular signals. Investigating molecular interactions between FAM107 family proteins and other proteins as well as generating genetic mutant animals are necessary to clarify molecular and physiological functions of FAM107. We are presently investigating fruit flies with genetic alterations of CG9328, Drosophila homolog of FAM107 (Fig. 1A), and have observed putative genetic interaction between CG9328 and Ras-MAPK mediated signaling cascade. Such investigation will lead to the elucidation of the physiological function and the putative mechanism of tumor suppressor effects of FAM107 family of proteins.

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