厚生労働科学研究費補助金

食品の安心・安全確保推進研究事業

DNA 塩基配列変化を直接検出する遺伝毒性試験法の開発に関する研究

平成１８年度　総括研究報告書

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目　　次

Ⅰ．研究報告
DNA 塩基配列変化を直接検出する遺伝毒性試験法の開発に関する研究 ---- 1
増村健一

II．研究成果の刊行に関する一覧表 ------------ 5

III．研究成果の刊行物・別刷 ----------------- 7

平成18年度　厚生労働科学研究費補助金（食品の安心・安全確保推進研究事業）
研究報告書

研究課題名：DNA塩基配列変化を直接検出する遺伝毒性試験法の開発に関する研究

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研究要旨

内因性、外因性の遺伝毒性物質により誘発される突然変異の定量的解析は、ヒトの発がんリスク評価に重要である。突然変異の検出は標的遺伝子の表現型の変化に基づく方法が多いが、観察可能な表現型の変化をもたらす遺伝子の数は少なく、表現型に頼らず高感度かつ簡便に遺伝子突然変異を検出する手法の確立が望まれる。本研究では、制限酵素処理と1分子PCR法を組み合わせた直接検出法による新しい遺伝毒性試験法の開発をめざす。ヒト培養細胞株をENU処理したものと処理しないものに直接検出法を適用して得られる突然変異頻度を従来の方法の結果と比較することをめざした。充分な検出感度と効率的なアッセイ法が実現すれば、表現型に依存せずゲノムDNA中の任意の部位で直接突然変異を検出する次世代の遺伝毒性試験として応用できることが期待される。

A．研究目的

ヒトへの発がんリスクの評価を念頭に、内因性、外因性の遺伝毒性物質による突然変異の誘発を定量的に解析する場合、従来の方法は標的遺伝子の表現型の変化に基づく方法により突然変異を検出していた。この方法は、表現型の変化をもたらす遺伝子の数が少ないことから必ずしも目的とする突然変異が検出できるとは限らない点が問題である。また、表現型の変化によりバイアスがかかるなど検出される突然変異の正確さに疑問が残る場合もある。そのような理由から、表現型に頼らずに遺伝子突然変異を高感度かつ簡便に検出する手法の確立が望まれている。本研究では、制限酵素処理と1分子PCR法を組み合わせた直接検出法を用いて新しい遺伝毒性試験法を開発することを目的とする。原理を図1に示す。

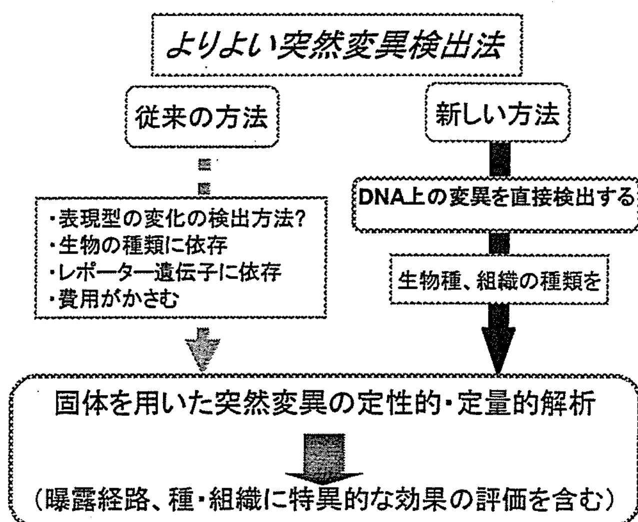

図1　突然変異の直接検出法の原理

B．研究方法

1）DNA の精製

ヒト培養細胞株 Nalm-6 の未処理細胞とエチルニトロソ尿素（ENU）で処理した細胞からフェノール/クロロホルム法を用いて、それぞれゲノム DNA（120 μg）を抽出した。

2）標的遺伝子の増幅

TP53 遺伝子第 6 イントロン内にある標的配列（制限酵素 *Taq*I の切断部位 5'-TCGA-3'）を含む 909 塩基対の DNA を、dUTP、dATP、dGTP、dCTP の存在下、片方の 5'末端をビオチン標識したプライマーのセット*を用いて上記精製ゲノム DNA（未処理）を鋳型に PCR 法により増幅した。PCR 産物はマイクロスピンカラム（S-400HR、Amersham Bioscience）で精製した。

*5'-biotin-CATCATACAGTCAGAGCCAACCTAGG-3'
5'-CTGTGGGTTGATTCCACACC-3'

3）プローブ DNA の調製

2）で増幅した dU を含む DNA 断片とストレプトアビジンが結合した磁気ビーズ（Dynabeads Streptavidin, Dynal Biotech）を混合して室温で 3 時間撹拌させた。この操作で DNA 断片のビオチンと磁気ビーズ上のアビジンが結合し、磁気ビーズの表面にプローブ DNA が吸着したものができる。

4）制限酵素処理

1）で抽出したゲノム DNA（未処理と ENU 処理）を 5 種類の制限酵素（*Pvu*II, *Rsa*I, *Eco*RI, *Eco*RV, *Bam*HI）で 37℃16 時間処理した後、エタノール沈殿により DNA 断片を精製した。（図 2）

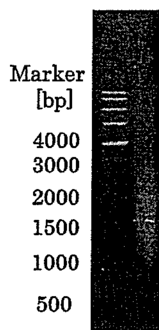

図 2　制限酵素処理

5）標的 DNA の回収

4）で切断した DNA 断片を 3）のプローブ DNA と 60℃で 16 時間ハイブリダイズさせ、形成された二本鎖 DNA を磁石により沈降させて、標的 DNA を選択的に回収した

6）ハイブリダイゼーションおよび突然変異を持つ断片の濃縮

標的を含む DNA とプローブ DNA がアニールしたと考えられる二本鎖 DNA を制限酵素 *Taq*I で処理し（65℃、1 時間）、95℃1 分で変性させて 50℃3 分で再アニールさせる操作を 5 回繰り返した。このとき 1 回ごとに *Taq*I を追加した。*Taq*I の標的配列に変異が入っていると *Taq*I による切断を免れるため、この操作で変異が入っていない DNA 断片が除かれてゆく。

7）プローブ DNA の除去

Uracil DNA glycosylase で 37℃2 時間処理することによりプローブ DNA（dU を含んでいる）を分解した。

8）定量的 PCR 法

回収された標的 DNA の数と、*Taq*I で分解されなかった標的 DNA の数を、それぞれ定量的 PCR 法により求めた。

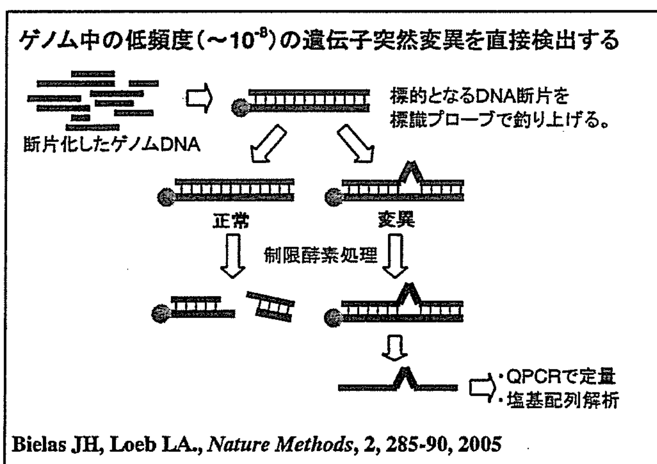

図 3　実験の流れ

C．研究結果

文献的にはゲノム DNA（120 μg）から 10^7～10^8 の標的 DNA が回収可能とされているが（表 1）、現在のところ回収効率は 10^4～10^5 であり、ENU 処理した細胞を用いても変異体を検出するに至っていない。

	文献値	本研究
DNA の由来	Neonatal human dermal fibroblasts (FBS base medium)	Nalm-6 (ENU +/-)
精製したゲノム DNA	120 μg (3.4 x 10^7 target copies)	114.5 μg (3.3 x 10^7 target copies)
制限酵素処理後の精製方法	Microcon YM-50 (カラム)	エタノール沈殿
プローブ DNA を結合するダイナビーズ (M-280) の量	5 μg	50 μg
ハイブリダイゼーション、TaqI 処理、UDG 処理後のコピー数	少なくとも、2.1 x 10^6 copies in 200 μl	10^4 copies in 200 μl
定量 PCR 法	ウェル辺り 25,000 コピー (全部で 84 ウェル)	ターゲットの量が不足

表1　文献値との比較

D. 考　察

次年度は、遺伝毒性物質に曝露した *gpt* delta トランスジェニックマウスの DNA を用いて、従来のレポーター遺伝子を用いた変異検出法と、制限酵素処理と１分子 PCR による直接検出法との結果を比較する。

現在、(1)プローブ DNA と標的 DNA のハイブリダイゼーションの条件検討(2)細胞あたりのコピー数が約 100 であるミトコンドリア DNA 中の標的配列を使い、変異体を検出する工夫を進めている。

また本法を開発した米国ワシントン州立大学 Bielas 博士と電子メール等で連絡をとり手法の改善に努めている。

E. 結　論

無処理細胞の突然変異頻度は塩基当たり 10^{-8} と予想され、ENU 処理を行っても 10^{-6} 以下と予想される。改良点としては、変異体を検出するために回収効率を上げることが必要であることがわかった。

F. 健康危機情報

特になし。

G. 研究発表

本法を用いた突然変異検出に関する研究は現在進行中であり、該当する発表論文はないが、*gpt* delta トランスジェニックマウスを用いた主任研究者の 2006 年における論文は以下のとおりである。

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H. 知的所有権の取得状況

１．　特許取得　無し
２．　実用新案登録　無し
３．　その他　無し

研究成果の刊行に関する一覧表

雑誌

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Genes and Environment, Vol. 28, No. 3 pp. 84–91 (2006)

Regular article

Chemopreventive Effects of Nobiletin against Genotoxicity Induced by 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in the Lung of *gpt* delta Transgenic Mice

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Nobiletin, a major component of citrus polymethoxyflavones, possesses anticancer, antiviral and anti-inflammatory activities. To evaluate the chemopreventive potential against lung cancer induced by cigarette smoke, we examined suppressive effects of nobiletin against genotoxicity induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the most carcinogenic tobacco-specific nitrosamine, in the lung of *gpt* delta transgenic mice. Male and female *gpt* delta mice were fed nobiletin at a dose of 100 or 500 ppm in diet for seven days and treated with NNK at a dose of 2 mg/mouse/day, i.p. for four consecutive days. Dietary administration of nobiletin continued at the doses during the NNK treatments and in the following period before sacrifice at day 38. NNK treatments enhanced the *gpt* mutant frequency (MF) in the lung 19- and 9-fold, respectively, over the values of untreated female and male mice. Interestingly, nobiletin reduced the NNK-induced MFs by 25–45% in both sexes and the reduction at a dose of 100 ppm in females and 500 ppm in males was statistically significant ($P<0.05$). To further characterize the suppressive effects, we conducted bacterial mutation assay with *Salmonella typhimurium* YG7108 to examine whether nobiletin inhibits S9-mediated genotoxicity of NNK. Nobiletin as well as 8-methoxypsoralen, an inhibitor of CYP2A, reduced the genotoxicity of NNK by more than 50%. These results suggest that nobiletin may be chemopreventive against NNK-induced lung cancer and also that the chemopreventive efficacy may be due to inhibition of certain CYP enzymes involved in the metabolic activation of NNK.

Key words: nobiletin, NNK, chemoprevention, cigarette smoking, *gpt* delta transgenic mice

Introduction

Humans are exposed to a variety of exogenous and endogenous genotoxic agents. Of various hazardous environmental factors, cigarette smoke may be the most causative factor associated with the incidence of human cancer (1). Although cigarette smoke contains more than 4,000 compounds including 40 known human carcinogens, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (nicotine-derived nitrosamino ketone, NNK) is the most carcinogenic tobacco-specific nitrosamine (2,3). NNK is estimated to be present at levels of 17–430 and 390–1,440 ng, respectively, per cigarette in mainstream and sidestream of cigarette smoke (3). NNK induces lung tumors in rats, mice and hamsters and is classified into Class 2B carcinogen (possibly carcinogenic to humans) by International Agency for Research on Cancer (4). NNK is metabolically activated by CYP (P-450) enzymes, and the metabolites generate methylated and pyridyloxobutylated DNA, which can induce G:C-to-A:T and G:C-to-T:A mutations, respectively. O^6-Methylguanine in the lung may be a causative lesion of NNK leading to activation of Ki-*ras* proto-oncogene, an initiation of tumor development (5,6).

With smoking the major etiological factor for lung cancer, a number of naturally occurring and synthetic chemicals have been proposed as candidates of chemopreventive agents to protect smokers who are unwilling or unable to quit smoking. Examples of the candidates include inhibitors of metabolic activation of NNK, e.g., phenethyl isothiocyanate and curcumins (7–10), enhancers of detoxication enzymes, e.g., prodrugs of L-selenocystein (11), antioxidants, e.g., vitamine E and carotenoids (12,13) and inhibitors of signal transduction downstream from the activated oncogenes, e.g., perillyl alcohol and deguelin (14,15).

Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) is a

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polymethoxyflavone found in *Citrus depressa* Rutaceae, a popular citrus fruit in Okinawa, Japan (16). Interestingly, nobiletin seems to possess anticancer activities by inhibiting critical steps of carcinogenesis, i.e., initiation (13,17), promotion (18,19) and metastasis (16,20,21). In addition, nobiletin inhibits the P-glycoprotein drug efflux transporter, suggesting the ability to reverse multi-drug resistance of tumor cells (22).

To evaluate the chemopreventive efficacy against lung cancer induced by cigarette smoke, we examined suppressive effects of dietary administration of nobiletin in the lung of *gpt* delta mice treated with NNK. In this mouse model, base substitutions such as G:C-to-A:T or G:C-to-T:A can be detected by *gpt* selection. In fact, Miyazaki *et al.* (23) have employed the mice to demonstrate the chemopreventive effects of 8-methoxypsoralen against NNK-induced mouse lung adenoma. Besides *in vivo* genotoxicity assays, we conducted a bacterial mutation assay with *Salmonella typhimurium* YG7108 to examine whether nobiletin inhibits the genotoxicity of NNK in the presence of S9 metabolic activation system. The bacterial strain lacks O^6-methylguanine methyltransferase activity, so that it is highly sensitive to base substitution mutations by NNK and other alkylating agents (24,25). The results suggest that nobiletin clearly suppresses the genotoxicity of NNK *in vivo* and *in vitro*. We discuss the mechanisms underlying the suppressive effects and the possible usage of nobiletin as a chemopreventive agent against lung cancer induced by cigarette smoke.

Material and Methods

Materials: Nobiletin (>99.9% purity) was chemically synthesized according to the method described by Tsukayama *et al.* (26) with slight modifications. Sources of other chemicals used in this study are as follows: NNK, Toronto Research Chemicals (Toronto, Canada); benzo[a]pyrene (BP), Wako Pure Chemicals (Osaka, Japan); 8-methoxypsoralen and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), Sigma-Aldrich Japan K. K. (Tokyo, Japan). S9 prepared from male Sprague-Dawley rats pretreated with phenobarbital and 5,6-benzoflavone was purchased from Kikkoman Cooperation, Chiba, Japan.

Treatment of *gpt* delta mice: Male and female *gpt* delta C57BL/6J transgenic mice, obtained from Japan SLC, Inc. (Shizuoka, Japan), were maintained in Animal Facility of Kanazawa Medical University, according to the institutional animal care guidelines. The animals were housed in plastic cages with free access to tap water and powdered basal diet CRF-1 (Oriental Yeast, Tokyo, Japan) under controlled conditions of temperature at 23±2°C, humidity of 10% and lighting (12 h light-dark cycle). Twenty female and 25 male *gpt* delta mice were each divided into four experimental and one control groups (Fig. 1). When the mice were 8 weeks of age, they were fed diet supplemented with nobiletin at a concentration of 100 ppm (Group 2) or 500 ppm (Groups 3 and 4) for 38 days. Groups 1 through 3 were treated with a single i.p. injection of NNK dissolved in saline at a dose of 2 mg/mouse/day for four consecutive days from day 7 through day 10. Groups 4 and 5 were treated with saline as vehicle. Mice were sacrificed under ether anesthesia at day 38. The lung was removed, placed immediately in liquid nitrogen, and stored at −80°C until analysis.

DNA Isolation, *in vitro* packaging and *gpt* mutation assay: High-molecular-weight genomic DNA was extracted from the lung using the RecoverEase DNA Isolation Kit (Stratagene, La Jolla, CA). λEG10 phages were rescued using Transpack Packaging Extract (Stratagene, La Jolla, CA). The *gpt* mutation assay was performed according to previously described methods (27,28). *gpt* MFs were calculated by dividing the number of colonies growing on agar plates containing chloramphenicol and 6-thioguanine by the product of the number of colonies growing on plates containing chloramphenicol and the dilution factor.

Bacterial mutation assay: The mutagenicity assay was carried out with a pre-incubation method with modifications (29). Nobiletin or 8-methoxypsoralen was dissolved in DMSO and the solution (50 μL) was mixed with S9 mix (0.5 mL). They were kept on ice for 5 min and mixed with the solution (50 μL) of chemicals, i.e., NNK, BP or MNNG, dissolved in DMSO. Then, they were mixed with overnight culture (0.1 mL) and incubated for 20 min at 37°C. When the mutagenicity of MNNG was assayed, 1/15M phosphate buffer pH7.4 (0.5 mL) was added instead of S9 mix. The reaction mixture containing bacteria, nobiletin (or 8-methoxypsoralen) and the chemical with or without S9 mix was poured onto agar plates with soft agar and incubated for two days at 37°C. Each chemical was assayed with 6–8 doses on triplicate or duplicate plates. Tester strains for the mutation assays were *S. typhimurium* YG7108 for NNK and MNNG, and *S. typhimurium* YG5161 (30) for BP. Relevant genotypes of the strains are as follows: YG7108 (24,25) as *S. typhimurium* TA1535 but is $\Delta ada_{ST}\,\Delta ogt_{ST}$; YG5161 (30) as *S. typhimurium* TA1538 harboring plasmid pYG768 carrying the *dinB* gene of *Escherichia coli*.

Statistical analysis: All data are expressed as mean ± standard deviations. Differences between groups were tested for statistical significance using a Student's *t*-test. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

Results

Dietary administration of nobiletin suppresses mutations induced by NNK in the lung of *gpt* delta mice: To examine the suppressive effects of nobiletin against genotoxicity induced by NNK, female and male *gpt* delta mice were fed nobiletin in diet at a dose of 100 or 500 ppm for a week and treated with NNK (Fig. 1). Dietary administration of nobiletin continued during the NNK treatments and in the following period before sacrifice at day 38. NNK treatments enhanced *gpt* MF in the lung 19 times in females and 9 times in males over the control levels (Tables 1 and 2). Since the MFs ($\times 10^{-6}$) of untreated controls were similar between females and males (3.0 ± 1.3 versus 3.1 ± 2.0), NNK-induced MF was higher in females (58.1 ± 16.7) than in males (26.5 ± 11.8). Nobiletin itself was non-genotoxic (Group 4). Nobiletin appeared to reduce the MFs in both sexes. In females, the dietary administration of nobiletin at 100 and 500 ppm (Groups 2 and 3) reduced the NNK-induced MF by 34 and 32%, respectively, and the reduction at 100 ppm was statistically significant ($P<0.04$). In males, nobiletin at 100 and 500 ppm reduced the MF by 25 and 45%, respectively, and the reduction at 500 ppm was statistically significant ($P<0.04$). These results indicate that nobiletin suppresses NNK-induced genotoxicity in the lung of *gpt* delta mice.

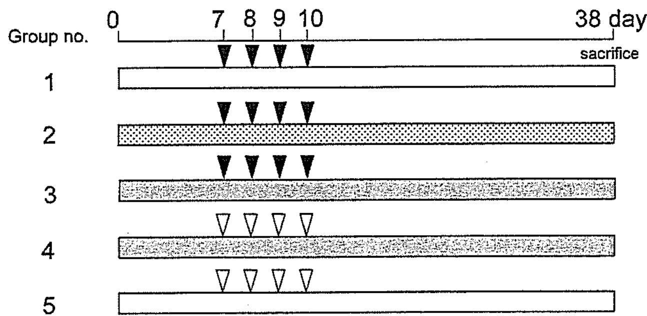

Fig. 1. An experimental design to examine chemopreventive effects of nobiletin against genotoxicity of NNK in the lung of *gpt* delta mice. Twenty female and 25 male eight-week-old *gpt* delta mice were each divided into five groups. Groups 1 through 3 were treated with a single i.p. injection of NNK at a dose of 2 mg/mouse/day for four consecutive days from day 7 through day 10. Groups 2 and 3 were fed diet supplemented with nobiletin at doses of 100 ppm and 500 ppm, respectively, for 38 days. Groups 4 and 5 were treated with saline as vehicle, and Group 4 was fed diet with nobiletin at a dose of 500 ppm for 38 days. Mice were sacrificed at day 38, and the *gpt* MF in the lung were determined. □, basal diet; ▒, nobiletin in diet at a dose of 100 ppm; ▩, nobiletin in diet at a dose of 500 ppm; ▼, NNK (2 mg/mouse/day, i.p.); ▽, saline.

Nobiletin inhibits genotoxicity of NNK in the presence of S9 activation in *S. typhimurium* YG7108: To further characterize the suppressive effects of nobiletin against genotoxicity of NNK, we conducted bacterial mutation assays to examine whether nobiletin inhibits genotoxicity of NNK in the presence of S9 activation enzymes (Fig. 2A). NNK at a dose of 500 μg/plate induced mutations in *S. typhimurium* YG7108 and produced about 900 His^+ revertants/plate, which was 40–50 times higher than the value of spontaneous mutations. Nobiletin itself was non-genotoxic either with or without S9 activation (Fig. 2A, C and D).

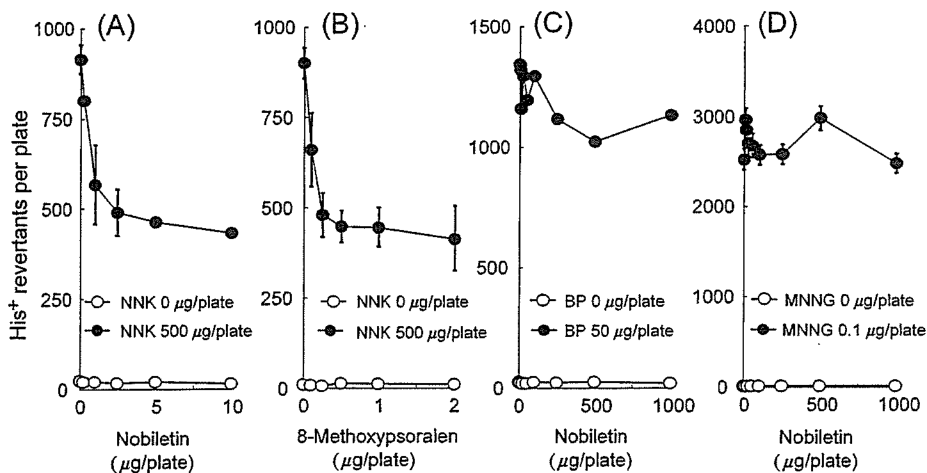

Fig. 2. Suppressive effects of nobiletin against genotoxicity of NNK in the presence of S9 mix in *S. typhimurium* YG7108. Closed circles represent the numbers of His^+ revertants/plate induced by the following compounds: NNK (500 μg/plate) in the presence of S9 mix along with the increasing doses of nobiletin (A), NNK (500 μg/plate) in the presence of S9 mix along with the increasing doses of 8-methoxypsoralen (B); BP (50 μg/plate) in the presence of S9 mix along with the increasing doses of nobiletin (C); MNNG (0.1 μg/plate) in the absence of S9 mix along with the increasing doses of nobiletin. Open circles represent the numbers of His^+ revertants/plate when the non-genotoxicity of nobiletin (A, C and D) and 8-methoxypsoralen (B) were confirmed. Strains used are *S. typhimurium* YG7108 (A, B and D) and *S. typhimurium* YG5161 (C). Averages and standard deviations are presented in A, B and D where three plates were used for the assays. Averages are presented in C where two plates were used for the assay.

Table 1. Suppressive effects of nobiletin against genotoxicity of NNK in the lung of female *gpt* delta mice

Group number*	Animal I.D.	Total colonies	No. of mutants	*gpt* MF ($\times 10^{-6}$)	Average ±S.D.†	*P*-value‡
1	F001	898,500	68	75.7		
NNK alone	F002	1,017,000	57	56.1		
	F003	1,464,000	53	36.2		
	F004	1,054,500	68	64.5		
		4,434,000	246	55.5	58.1±16.7	
2	F005	1,134,000	36	31.8		
NNK	F006	1,353,000	48	35.5		
+Nobiletin	F007	1,152,000	54	46.9		
(100 ppm)	F008	916,500	37	40.4		
		4,555,500	175	38.4	38.6±6.6	0.036§
3	F009	1,369,500	33	24.1		
NNK	F010	798,000	36	45.1		
+Nobiletin	F011	1,606,500	66	41.1		
(500 ppm)	F012	1,027,500	48	46.7		
		4,801,500	183	38.1	39.3±10.4	0.052
4	F013	1,059,000	3	2.8		
Nobiletin	F014	1,377,000	4	2.9		
(500 ppm)	F015	1,092,000	6	5.5		
alone	F016	900,000	6	6.7		
		4,428,000	19	4.3	4.5±1.9	<0.001
5	F018	2,856,000	6	2.1		
No treatments	F019	1,560,000	4	2.6		
	F020	1,809,000	9	5.0		
	F021	2,013,000	5	2.5		
		8,238,000	24	2.9	3.0±1.3	<0.001

*Group 1, mice treated with NNK (2 mg/mouse/day × 4 days) alone; Group 2, mice treated with NNK plus nobiletin at a dose of 100 ppm in diet; Group 3, mice treated with NNK plus nobiletin at a dose of 500 ppm in diet; Group 4, mice fed nobiletin at a dose of 500 ppm in diet without NNK treatments; Group 5, mice without treatments with NNK or nobiletin. The Group No. corresponds with group No. in Fig. 1.

†Average ± standard deviation of *gpt* MF of four mice.

‡Differences between *gpt* MF of each group and that of Group 1 were tested for statistical significance using a Student's *t*-test.

§Statistically significant ($P<0.05$) against Group 1. The values in Groups 4 and 5 are also statistically significant. But the mice in Groups 4 and 5 are not treated with NNK so that the values are not marked with §.

An addition of nobiletin in the reaction mixture containing NNK and S9 mix reduced the genotoxicity of NNK in a dose-dependent manner, and the number of His^+ revertants/plate decreased by more than 50% at the highest dose of nobiletin, i.e., 10 μg/plate. There was no obvious reduction of background lawn of bacteria at any dose of nobiletin, suggesting that nobiletin was not very much toxic under the experimental conditions. Similar dose-dependent reduction of the genotoxicity of NNK was observed with 8-methoxypsoralen (Fig. 2B). An addition of 8-methoxypsoralen into the reaction mixture containing NNK and S9 mix reduced the number of His^+ revertants/plate by more than 50%. Despite the similar inhibitory effects, the dose necessary to reduce the genotoxicity of NNK by 50% was 5- to 10-fold higher with nobiletin than with 8-methoxypsoralen (2.5 μg/plate for nobiletin versus 0.25–0.5 μg/plate for 8-methoxypsoralen). In contrast, nobiletin exhibited weak or virtually no inhibitory effects on the genotoxicity of BP or MNNG, respectively (Fig. 2C and D). An addition of nobiletin reduced the genotoxicity of BP in the presence of S9 activation by 20%, while it did not modulate the genotoxicity of MNNG in the absence of S9 enzymes.

Discussion

Lung cancer continues to be the leading cause of cancer death in developed countries. Dietary compounds with potential to inhibit lung cancer may be a promising and practical approach for reducing the risk of lung cancer caused by smoking. In this study, we examined the chemopreventive efficacy of nobiletin

Table 2. Suppressive effects of nobiletin against genotoxicity of NNK in the lung of male *gpt* delta mice

Group number*	Animal I.D.	Total colonies	No. of mutants	*gpt* MF ($\times 10^{-6}$)	Average ±S.D.†	*P*-value‡
1	M001	960,000	21	21.9		
NNK alone	M002	987,000	32	32.4		
	M003	1,320,000	57	43.2		
	M004	876,000	20	22.8		
	M005	1,892,000	23	12.2		
		6,035,000	153	25.4	26.5±11.8	
2	M007	1,156,000	16	13.8		
NNK	M008	991,000	19	19.2		
+Nobiletin	M009	828,000	20	24.2		
(100 ppm)	M010	828,000	23	27.8		
	M011	840,000	12	14.3		
		4,643,000	90	19.4	19.9±6.1	0.147
3	M013	700,000	16	22.9		
NNK	M014	1,404,000	11	7.8		
+Nobiletin	M015	1,052,000	14	13.3		
(500 ppm)	M016	760,000	10	13.2		
	M017	1,000,000	15	15.0		
		4,916,000	66	13.4	14.4±5.4	0.035‖
4	M019	1,028,000	4	3.9		
Nobiletin	M020§	388,000	4	10.3		
(500 ppm)	M021	1,640,000	6	3.7		
alone	M022	708,000	3	4.2		
	M023	972,000	2	2.1		
		4,348,000	15	3.5	3.5±1.0	0.003
5	M024§	705,000	14	19.9		
No treatments	M025	1,410,000	8	5.7		
	M026	1,410,000	5	3.6		
	M027	1,928,000	3	1.6		
	M028	2,032,000	3	1.5		
		6,780,000	19	2.8	3.1±2.0	0.003

*Group 1, mice treated with NNK (2 mg/mouse/day × 4 days) alone; Group 2, mice treated with NNK plus nobiletin at a dose of 100 ppm in diet; Group 3, mice treated with NNK plus nobiletin at a dose of 500 ppm in diet; Group 4, mice fed nobiletin at a dose of 500 ppm in diet without NNK treatments; Group 5, mice without treatments with NNK or nobiletin. The Group No. corresponds to Group No. in Fig. 1.
†Average ± standard deviation of *gpt* MF of four or five mice.
‡Differences between *gpt* MF of each group and that of Group 1 were tested for statistical significance using a Student's *t*-test.
§Two unusually high *gpt* MF of M020 and M024 were excluded for the calculation of average by the Smirnov-Grubb's outlier test.
‖Statistically significant ($P < 0.05$) against Group 1. The values in Groups 4 and 5 are also statistically significant. But the mice in Groups 4 and 5 are not treated with NNK so that the values are not marked with ‖.

against genotoxicity of NNK in the lung of *gpt* delta mice. NNK exposure significantly enhanced the *gpt* MFs in the lung of mice (Tables 1, 2). There was a marked sex difference in the genotoxicity of NNK where females exhibited about twice higher sensitivity than males. This may be due to gender-related differences in the metabolic activation enzymes for NNK (31). The high sensitivity in female than in male mice may be relevant in humans because women are more sensitive to the genotoxic effects of NNK than men (32). Interestingly, dietary administration of nobiletin substantially reduced the *gpt* MFs in both sexes, and the reduction at a dose of 100 ppm in females and 500 ppm in males was statistically significant ($P < 0.05$). Administration of nobiletin at 500 ppm also reduced the genotoxicity in females at a similar extent to that observed with nobiletin at 100 ppm. Ikeda *et al.* reported that NNK induces G:C-to-A:T, G:C-to-T:A, A:T-to-T:A, A:T-to-G:C in the lung of *gpt* delta mice (unpublished obervations). Since G:C-to-A:T can activate Ki-*ras* oncogene, the reduction of *gpt* MF may correlate with the reduction of lung tumors (5). Thus, we suggest that nobiletin may be a

chemopreventive agent against NNK-induced lung tumorigenesis in mice. Nobiletin inhibits metastasis (20,21) and suppresses inflammation and promotion (18,33–36). Hence, it may prevent events that occur in multi-step of lung carcinogenesis, i.e., initiation, promotion and progression/metastasis, induced by cigarette smoke. However, certain compounds that can reduce NNK-induced tumors do not necessarily reduce lung tumors in smoke-exposed animals (37). Thus, further examination is needed to evaluate the chemopreventive efficacy of nobiletin against lung tumors induced by cigarette smoke.

In addition to *in vivo* results, we observed reduction of NNK-induced mutations by nobiletin in the present of S9 activation enzymes *in vitro*. Interestingly, nobiletin exhibited a specificity inhibiting the genotoxicity of chemicals in *S. typhimurium*. Although nobiletin inhibited the genotoxicity of NNK, it inhibited the genotoxicity of BP with S9 activation only slightly and did not inhibit the genotoxicity of MNNG without S9 activation. Since MNNG induces O^6-methylguanine leading to G:C-to-A:T mutations (38), we suggest that nobiletin may not enhance the repair activity against O^6-methylguanine or promote error-free translesion bypass across the lesion. Instead, we suggest that nobiletin may suppress the genotoxicity of NNK by inhibiting the activity of CYP (P-450) enzymes involved in the metabolic activation of NNK (39–41). In fact, 8-methoxypsoralen, a specific-inhibitor of CYP2A, similarly suppressed the genotoxicity of NNK in the presence of S9 enzymes (23). The inhibitory effect of nobiletin may be specific to certain CYP enzymes including CYP2A because the genotoxicity of BP, which is activated *via* CYP1A1 (42), was weakly inhibited by nobiletin. However, since both nobiletin and 8-methoxypsoralen inhibited the genotoxicity of NNK only by 50%, we suggest that other CYP enzymes may be responsible for the remaining genotoxicity of NNK in the S9 enzymes. Although nobiletin did not effectively affect the genotoxicity of BP in the present study, Conney *et al.* (43) observed that nobiletin stimulates human liver microsomes and activates both the hydroxylation of BP and the metabolism of aflatoxin B_1 to mutagens. Nobiletin also stimulates oxidative metabolism of zoxazolamine by rat liver microsomes (44) and acetaminophen by human liver microsome (45). These reports suggest that nobiletin has a potential to modulate CYP enzyme activities.

In summary, we examined the chemopreventive efficacy of nobiletin against the genotoxicity of NNK in the lung of female and male *gpt* delta mice. Dietary administration of nobiletin significantly reduced the genotoxicity of NNK in both sexes. In addition, the chemical was able to reduce NNK-induced genotoxicity in *S. typhimurium* YG7108 in the presence of S9 activating enzymes. Our findings suggest that nobiletin could inhibit the activities of certain CYP enzymes involved in the metabolic activation of NNK, thereby suppressing the genotoxicity in the lung of mice.

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IL-10 deficiency leads to somatic mutations in a model of IBD

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Individuals with inflammatory bowel disease (IBD) are at increased risk of developing gastrointestinal cancer. Here, we have tested the possibility that chronic inflammation could trigger mutations. For this, we have used IL-10-deficient (*IL-10*$^{-/-}$) mice, which spontaneously develop intestinal inflammation, in combination with a transgenic gpt gene and *red/gam* gene (*gpt*$^{+}$*IL-10*$^{-/-}$), which is a well-characterized mutation reporter locus. The total mutation frequency in the colon of *gpt*$^{+}$*IL-10*$^{-/-}$ mice was about five times higher than that in normal *gpt*$^{+}$*IL-10*$^{+/+}$ mice. In the particular case of G:C to A:T transitions, the frequency of mutations in *gpt*$^{+}$*IL-10*$^{-/-}$ mice was 4.1 times higher than that in control mice. Interestingly, the frequency of small deletions and insertions was also strikingly increased (~10 times). The majority of the deletion or insertion mutations were observed in the monotonous base runs or adjacent repeats of short tandem sequences. In contrast, the frequency of large deletions, detected by loss of the Spi marker present in the *red/gam* transgene, was similar among the mouse strains. Finally, as a control, the mutation frequency in non-inflamed tissues, such as the liver, were similar between *gpt*$^{+}$*IL-10*$^{-/-}$ mice and *gpt*$^{+}$*IL-10*$^{+/+}$ mice. Our data demonstrate that the chronic inflammatory environment in the colon promotes the generation of mutations.

Abbreviations: FAP, familial adenomatous polyposis; Spi, sensitive to P2 interference; 6-TG, 6-thioguanine; TGF-β1, transforming growth factor-β1; UC, ulcerative colitis

Introduction

Ulcerative colitis (UC) and Crohn's disease are chronic inflammatory bowel diseases (IBD) associated with a high risk of gastrointestinal cancer. This risk begins to increase ~10 years after the onset of the disease and increases with the extent and duration of the inflammatory process (1). Gastrointestinal cancer in individuals with IBD appears to develop through a multistep process involving genomic instability and the progressive accumulation of genomic alterations (2–4). However, it has not been fully elucidated what kinds of genomic mutations are critical for tumorigenesis.

It has been reported that interleukin-10 knockout (*IL-10*$^{-/-}$) mice spontaneously develop intestinal inflammation characterized by discontinuous transmural lesions affecting the small and large intestine and by the dysregulated production of proinflammatory cytokines (5). Inflammatory changes first appear in the cecum and ascending and transverse colon of such 3-weeks-old mice, and thereafter spread to the remainder of the colon and rectum (5). Prolonged disease with transmural lesions and a high incidence of colorectal adenocarcinomas are also observed. However, in germ-free conditions, *IL-10*$^{-/-}$ mice never develop inflammation nor adenocarcinomas (5).

Recently, a new transgenic mouse line, *gpt* delta (*gpt*$^{+}$), was established to facilitate the detection and analysis of mutations *in vivo* (6). The striking feature of *gpt*$^{+}$ mice is their ability to reveal deletions and point mutations. About 80 copies of lambda EG10 shuttle vector DNA carrying the *red/gam* gene of lambda phage and the *gpt* gene of *Escherichia coli* are integrated in chromosome 17. Relatively large deletions in the *red/gam* gene are individually identified by sensitive to P2 interference (Spi)$^{-}$ selection, and base substitutions or small frameshifts in the *gpt* gene are individually identified by 6-thioguanine (6-TG) selection, respectively (6,7).

IL-10$^{-/-}$ mice and *gpt*$^{+}$ mice are of C57BL/6J background, although the vendors of these mice were each different. Therefore, the recombinant mice, *gpt*$^{+}$*IL-10*$^{-/-}$, are much like *IL-10*$^{-/-}$ mice or *gpt*$^{+}$ mice. In this paper, to elucidate the role of inflammation on the accumulation of mutations in colonic DNA, we analyzed *gpt*$^{+}$*IL-10*$^{-/-}$ and *gpt*$^{+}$*IL-10*$^{+/+}$ mice by 6-TG selection, Spi^{-} selection and direct sequencing method. Then we compared the patterns and frequencies of mutations in colonic DNA.

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Materials and methods

Mice

The experimental protocol was approved by the committee of animal research of the Tohoku University School of Medicine, Sendai, Japan. *IL-10*$^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME) and *gpt*$^{+}$ mice were obtained from SLC (Hamamatsu, Japan). To investigate the role of inflammation in the mutagenicity, the recombinant mice, *gpt*$^{+}$*IL-10*$^{-/-}$, were established by crossing *gpt*$^{+}$ with *IL-10*$^{-/-}$ mice. Mice were housed in plastic cages in an environmentally controlled room (24°C, 12 h/12 h light/dark cycle). Chow (Nippon Nosan, Yokohama, Japan) and tap water were given *ad libitum* during the experiment. At 15 weeks or 40 weeks of age, eight *gpt*$^{+}$*IL-10*$^{-/-}$ mice and eight *gpt*$^{+}$*IL-10*$^{+/+}$ mice (four 15-weeks mice and four 40-weeks mice, for each type, all siblings) were weighed and killed by cervical dislocation. The colon was removed and divided into proximal and distal portions.

DNA isolation and in vitro *packaging*

Genomic DNA was extracted from the colon using RecoverEase™ DNA Isolation Kit (Stratagene, La Jolla, CA) according to the manufacture's recommendations. Lambda EG10 phages were rescued from genomic DNA by the *in vitro* packaging method using Transpack® Packaging Extract (Stratagene) following the instructions.

Mutation assay and sequencing analysis

The 6-TG selection was carried out as described previously (6). DNA sequencing of the *gpt* gene was performed with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The PCR primers of the *gpt* gene were primer-1 (5′-TACCACTTTATCCCGCGTCAGG-3′) and primer-2 (5′-ACAGGGTTTCGCTCAGGTTTGC-3′) (6).

The sequencing primers were primer-A (5′-GAGGCAGTGCGTAAAAAGAC-3′) and primer-B (5′-CTATTGTAACCCGCCTGAAG-3′).

The Spi^{-} selection was performed as described previously (7). Phage lysates of the recovered Spi^{-} mutants were used as templates for PCR analysis. The PCR primers were primer-001 (5′-CTCTCCTTTGATGCGAATGCCAGC-3′), primer-002 (5′-GGAGTAATTATGCGGAACAGAATCATGCCAGC-3′), primer-005 (5′-CGTGGTCTGAGTGTGTTACAGAGG-3′), primer-006 (5′-GTTATGCGTTGTTCCATACAACCTCC-3′) and primer-012 (5′-CGGTCGAGGGACCTAATAACTTCG-3′). The appropriate primers for DNA sequencing were selected on the basis of the results of the aforementioned PCR analysis (7).

Statistical analysis

Data were expressed as mean ± standard error (SE). Differences between two groups were tested for statistical significance using Student's *t*-test. A *P*-value < 0.05 denoted the presence of a statistically significant difference.

Results

At 15 weeks of age, the average weight of the *gpt*$^{+}$*IL-10*$^{-/-}$ mice was 23.7 ± 3.1 g, and that of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice was 28.1 ± 2.1 g ($P < 0.05$). Two of the four *gpt*$^{+}$*IL-10*$^{-/-}$ mice and none of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice showed bloody stools or prolapse of the anus. At 40 weeks of age, the average weight of the *gpt*$^{+}$*IL-10*$^{-/-}$ mice was 26.2 ± 4.7 g, and that of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice was 30.6 ± 3.0 g ($P < 0.05$). One of the four *gpt*$^{+}$*IL-10*$^{-/-}$ mice and none of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice showed bloody stools or prolapse of the anus. The excised colons from the *gpt*$^{+}$*IL-10*$^{-/-}$ mice were slightly thick and edematous compared with those of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice. As reported previously (5), the *gpt*$^{+}$*IL-10*$^{-/-}$ mice developed inflammation in SPF conditions.

The 6-TG mutant frequency in the total colon of the *gpt*$^{+}$*IL-10*$^{-/-}$ mice was 13.4×10^{-6}, which was about five times higher than that of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice (2.8×10^{-6}) (Figure 1). In both the proximal and distal colon of the *gpt*$^{+}$*IL-10*$^{-/-}$ mice, the 6-TG mutant frequencies were significantly higher than those of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice (11.8×10^{-6} versus 3.3×10^{-6}, $P = 0.004$, 15.0×10^{-6} versus 2.3×10^{-6}, $P = 0.01$, respectively).

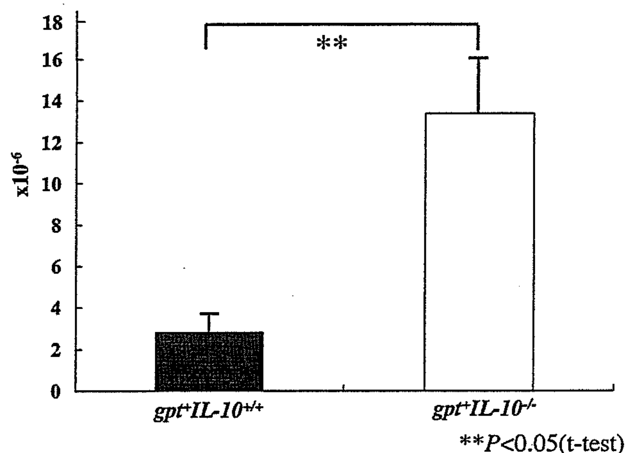

Fig. 1. Mutation frequency of 6-TG selection in the total colon of *gpt*$^{+}$*IL-10*$^{-/-}$ mice (filled square) and *gpt*$^{+}$*IL-10*$^{+/+}$ mice (unfilled square). The mutation frequencies of 6-TG selection in the total colon of *gpt*$^{+}$*IL-10*$^{-/-}$ mice were significantly higher than those in the total colon of *gpt*$^{+}$*IL-10*$^{+/+}$ mice. $P < 0.05$, statistically significant difference versus *gpt*$^{+}$*IL-10*$^{+/+}$. Bars represent mean values and SE.

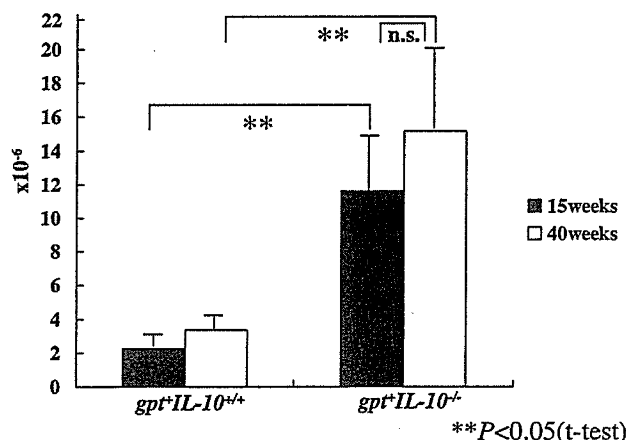

Fig. 2. Mutation frequency of 6-TG selection in the total colon of *gpt*$^{+}$*IL-10*$^{-/-}$ mice and *gpt*$^{+}$*IL-10*$^{+/+}$ mice (filled square, 15 weeks; unfilled square, 40 weeks). The mutation frequencies of 6-TG selection in the total colon of *gpt*$^{+}$*IL-10*$^{-/-}$ mice were significantly higher than those in the total colon of *gpt*$^{+}$*IL-10*$^{+/+}$ mice, at 15 weeks or 40 weeks of age. The mutation frequencies of 6-TG selection in the total colon of 40 weeks *gpt*$^{+}$*IL-10*$^{-/-}$ mice were not significantly higher than those in the total colon of 15 weeks *gpt*$^{+}$*IL-10*$^{-/-}$ mice. $P < 0.05$, statistically significant difference versus *gpt*$^{+}$*IL-10*$^{+/+}$. Bars represent mean values and SE.

At 15 weeks of age, the 6-TG mutant frequency of the total colon in the *gpt*$^{+}$*IL-10*$^{-/-}$ mice was 11.6×10^{-6}, which was about five times higher than that of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice (2.3×10^{-6}) (Figure 2). In the sequencing analysis of the *gpt*$^{+}$*IL-10*$^{-/-}$ mice, 51.5% of the mutants were single base substitutions (G:C to A:T transition, 14.7%; A:T to G:C transition, 1.5%; G:C to T:A transversion, 7.4%; G:C to C:G transversion, 14.7%; A:T to T:A transversion, 8.8%; A:T to C:G transversion, 4.4%), 35.3% were 1 bp deletions and 13.2% were 1–3 bp insertions. In contrast, 91.6% of the mutants in the *gpt*$^{+}$*IL-10*$^{+/+}$ mice were single base substitutions (G:C to A:T transition, 33.3%; A:T to G:C transition, 16.7%; G:C to T:A transversion, 33.3%; G:C to C:G transversion, 8.3%), 8.3%

Table I. Distribution of the different kinds of mutations in the colon (15 weeks, 6-TG selection)

	$gpt^{+}IL\text{-}10^{-/-}$			$gpt^{+}IL\text{-}10^{+/+}$		
	Number	Percentage (%)	Mutation frequencies ($\times 10^{-6}$)	Number	Percentage (%)	Mutation frequencies ($\times 10^{-6}$)
Transition	11	16.2	1.88	6	49.7	1.12
G:C to A:T	(10)	(14.7)	(1.71)	(4)	(33.0)	(0.75)
A:T to G:C	(1)	(1.5)	(0.17)	(2)	(16.7)	(0.38)
Transversion	24	35.3	4.1	5	41.3	0.94
G:C to T:A	(5)	(7.4)	(0.86)	(4)	(33.0)	(0.75)
G:C to C:G	(10)	(14.7)	(1.71)	(1)	(8.3)	(0.19)
A:T to T:A	(6)	(8.8)	(1.02)	(0)	(0)	(0)
A:T to C:G	(3)	(4.4)	(0.51)	(0)	(0)	(0)
Deletion	24	35.3	4.1	1	8.3	0.19
1 bp	(24)	(35.3)	(4.1)	(1)	(8.3)	(0.19)
>2 bp	(0)	(0)	(0)	(0)	(0)	(0)
Insertion	9	13.2	1.53	0	0	0
Total	68	100	11.61	12	100	2.25

Table II. List of deletions or insertions in the colon (15 weeks, 6-TG selection)

$gpt^{+}IL\text{-}10^{-/-}$			$gpt^{+}IL\text{-}10^{+/+}$		
Position	Sequence change	Number	Position	Sequence change	Number
Deletion					
283~	GGT→GG	1	315~	AAAA→AAA	1
309	-T	1			
315~	AAAA→AAA	1			
325~	CTTT→TTT	1			
332~	CCA →CC	1			
342~	AAAA→AAA	1			
348~	GG→G	3			
395~	ATA→AA	1			
414~	GTGGG→GGGG	1			
416~	GGG→GG	1			
419~	ATA→AA	1			
423~	GGG→GG	1			
431	-T	1			
438~	CCCGCC→CCCCC	4			
444~	AA→A	1			
450~	GGT→GG	4			
Insertion					
256~	GTT→GTTGTT	1			
305~	AAA→AAAA	1			
325~	TTT→TTTT	1			
380~	TT→TTT	1	Not detected		
407~	AA→AAA	2			
416~	GGGA→GGGAA	2			

The position numbers indicate the locations where mutations were found. The numbering starts from the first nucleotide of the *gpt* gene.

were 1 bp deletions and none were insertions or complex mutants (Table I). The frequency of transition mutations in the colitis mice was 1.7 times higher than that of the control mice, the transversion was 4.4 times higher and the 1 bp deletion was 21.6 times higher (Table I). Furthermore, 13.2% of the mutants in the colitis mice were insertions, in marked contrast with the result that insertions were not observed in the control mice. In the $gpt^{+}IL\text{-}10^{-/-}$ mice, 93.8% of the 1 bp deletions and insertions occurred in the monotonous base runs or adjacent repeats of short tandem sequences (Table II). In both the proximal and distal colon of the $gpt^{+}IL\text{-}10^{-/-}$ mice, the 6-TG mutant frequencies were higher than those of the $gpt^{+}IL\text{-}10^{+/+}$ mice (11.1×10^{-6} versus 2.8×10^{-6}, 12.1×10^{-6} versus 1.7×10^{-6}, respectively), but the differences did not reach significance.

At 40 weeks of age, the 6-TG mutant frequency of the total colon in the $gpt^{+}IL\text{-}10^{-/-}$ mice was 15.2×10^{-6}, which was about five times higher than that of the $gpt^{+}IL\text{-}10^{+/+}$ mice (3.3×10^{-6}) (Figure 2). In the sequencing analysis of the $gpt^{+}IL\text{-}10^{-/-}$ mice, 84.1% of the mutants were single base substitutions (G:C to A:T transition, 46%; A:T to G:C transition, 11.1%; G:C to T:A transversion, 19%; G:C to C:G transversion, 1.6%; A:T to T:A transversion, 3.2%; A:T to C:G transversion, 3.2%), 9.5% were 1–3 bp deletions and 6.4% were 1–2 bp insertions. In contrast, 93.4% of the mutants in the $gpt^{+}IL\text{-}10^{+/+}$ mice were single base substitutions (G:C to A:T transition, 52.2%; A:T to G:C transition, 2.2%; G:C to T:A transversion, 32.6%; G:C to C:G transversion, 6.5%), 4.3% were 1 bp deletions and 2.2% were 2 bp insertions (Table III). The frequency of transition mutations in the colitis mice was 1.8 times higher than that of the control mice; the transversions were 1.3 times higher. In the transitions, the frequency of G:C to A:T in the $gpt^{+}IL\text{-}10^{-/-}$ mice was 4.1 times higher than that of the control mice, and 4.1 times higher than that of the 15-weeks $gpt^{+}IL\text{-}10^{-/-}$ mice. Furthermore, the small deletions of the $gpt^{+}IL\text{-}10^{-/-}$ mice were 10.3 times higher (Table III), and the small insertions were 13.4 times higher than those of the control mice. In the $gpt^{+}IL\text{-}10^{-/-}$ mice, 90% of the deletions and insertions occurred in the monotonous base runs or adjacent repeats of short tandem sequences (Table IV). In both the proximal and distal colon of the $gpt^{+}IL\text{-}10^{-/-}$ mice, the 6-TG mutant frequencies were higher than those of the $gpt^{+}IL\text{-}10^{+/+}$ mice (12.4×10^{-6} versus 3.9×10^{-6}, 17.9×10^{-6} versus 2.8×10^{-6}, respectively), but the differences did not reach significance.

The Spi^{-} mutant frequency of the total colon in the $gpt^{+}IL10^{-/-}$ mice was not significantly different from the $gpt^{+}IL10^{+/+}$ mice (15 and 40 weeks; 1.5×10^{-6} versus 1.4×10^{-6}, $P = 0.9$, 15 weeks; 1.1×10^{-6} versus 0.8×10^{-6}, $P = 0.4$, 40 weeks; 1.8×10^{-6} versus 2.0×10^{-6}, $P = 0.8$) (Figures 3 and 4). In sequencing analysis, the pattern of the mutations was identical in both types of mice (Table V, VI).

Discussion

Assaying mutations using transgenic mice is a powerful tool for obtaining information about the pattern and frequency of

Table III. Distribution of the different kinds of mutations in the colon (40 weeks, 6-TG selection)

	$gpt^{+}IL\text{-}10^{-/-}$			$gpt^{+}IL\text{-}10^{+/+}$		
	Number	Percentage (%)	Mutation frequencies ($\times 10^{-6}$)	Number	Percentage (%)	Mutation frequencies ($\times 10^{-6}$)
Transition	36	57.1	8.66	25	54.3	1.81
G:C to A:T	(29)	(46)	(6.98)	(24)	(52.2)	(1.74)
A:T to G:C	(7)	(11.1)	(1.68)	(1)	(2.2)	(0.07)
Transversion	17	27	4.1	18	39.1	1.31
G:C to T:A	(12)	(19)	(2.88)	(15)	(32.6)	(1.09)
G:C to C:G	(1)	(1.6)	(0.24)	(3)	(6.5)	(0.22)
A:T to T:A	(2)	(3.2)	(0.49)	(0)	(0)	(0)
A:T to C:G	(2)	(3.2)	(0.49)	(0)	(0)	(0)
Deletion	6	9.5	1.44	2	4.3	0.14
1 bp	(5)	(7.9)	(1.2)	(2)	(4.3)	(0.14)
>2 bp	(1)	(1.6)	(0.24)	(0)	(0)	(0)
Insertion	4	6.4	0.97	1	2.2	0.07
Total	63	100	15.17	46	100	3.34

Table IV. List of deletions or insertions in the colon (40 weeks, 6-TG selection)

gpt $+IL\text{-}10^{-/-}$			gpt $+IL\text{-}10$ $+/+$		
Position	Sequence change	Number	Position	Sequence change	Number
Deletion					
223~	AAA→AA	1	83~	AA→A	2
250~	TTCATCGTT→ TTCGTT	1			
308~	TGT→TT	1			
315~	AAAA→AAA	1			
423~	GGG→GG	1			
451~	GG→G	1			
Insertion					
3~	G→GG	1	264~	GA→GAGA	1
8~	AAAAA→AAAAAA	1			
58~	GC→GCGC	1			
387~	CCC→CCCC	1			

The position numbers indicate the locations where mutations were found. The numbering starts from the first nucleotide of the *gpt* gene.

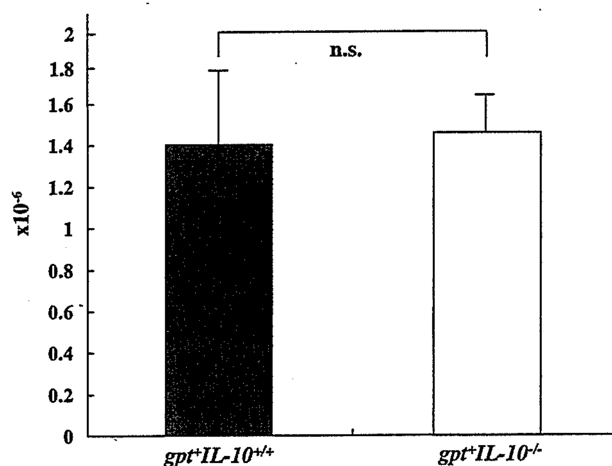

Fig. 3. Mutation frequency of Spi⁻ selection in the colon of $gpt^{+}IL\text{-}10^{-/-}$ mice (filled square) and $gpt^{+}IL\text{-}10^{+/+}$ mice (unfilled square). The mutation frequencies of Spi⁻ selection in the total colon of $gpt^{+}IL\text{-}10^{-/-}$ mice were not significantly higher than those in the total colon of $gpt^{+}IL\text{-}10^{+/+}$ mice. $P < 0.05$, statistically significant difference versus $gpt^{+}IL\text{-}10^{+/+}$. Bars represent mean values and SE.

inflammation-induced mutations. A feature of the assay using gpt^{+} mice is the incorporation of two distinct selections for detecting different types of mutations: Spi⁻ selection for relatively large deletions and 6-TG selection for base substitutions and small frameshifts (6,7). In this study, we examined the *in vivo* mutation spectrum induced by chronic inflammation by comparing $gpt^{+}IL\text{-}10^{-/-}$ mice with $gpt^{+}IL\text{-}10^{+/+}$ mice.

The *APC* gene is a tumor suppressor gene, and carcinomas from familial adenomatous polyposis (FAP) patients or non-FAP patients exhibit a high frequency of mutations in the *APC* gene. In FAP tumors or sporadic tumors, small deletions and insertions of the *APC* gene are most frequent (8). In the present study, small deletions and insertions strikingly increased in the $gpt^{+}IL\text{-}10^{-/-}$ mice. Regarding the point mutations of the *APC* gene in FAP tumors or sporadic tumors, G:C to A:T transitions were most prevalent (8), which was similar to the $gpt^{+}IL\text{-}10^{-/-}$ mice. The frequency of G:C to A:T transitions in the 40-weeks $gpt^{+}IL\text{-}10^{-/-}$ mice was 4.1 times higher than that of the 40-weeks control mice, and 4.1 times higher than that of the 15-weeks $gpt^{+}IL\text{-}10^{-/-}$ mice. It is suggested that G:C to A:T transitions in the inflamed colon accumulate with time. Furthermore, point mutations and allelic loss of the *APC* gene have been reported in UC-related dysplasia and cancer, although there is a controversy about the frequencies (9). In that report, five of the seven *APC* mutations were frameshifts and two were point mutations. Of the five frameshifts, four were deletions, and three of these occurred within homocopolymer tracts and one was a 4 bp direct repeat (AAGA). On this point, the mutation spectrum of our result was similar to that of *APC* mutations.

The *p53* gene is a member of a family of tumor suppressor genes, and inactivation of this protein plays a crucial role in the emergence and further progression of a multitude of human malignancies, including carcinoma of the colon and rectum. It was reported that the *p53* mutation can be detected in early colitic cancer and dysplasia of UC patients' colon, in contrast to sporadic colon cancer (10). Previous studies demonstrated that over 50% of UC samples had increased frequency of G:C to A:T transition mutations of the *p53* gene (10–14). In our study, it is suggested that G:C to A:T transitions in the inflamed