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～19年度　総合研究報告書

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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検出法は標的遺伝子の表現型の変化に基づく方法が多いが、測定可能な表現型の変化をもたらす遺伝子の数は限られており、対象組織も限定されている。発がん等の要因となる遺伝子突然変異の定量的解析はヒトのリスク評価において重要であり、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が望まれる。本研究では、DNA 中の突然変異を直接検出する方法の開発を目的とした。ヒトおよびマウスのゲノム DNA 中から制限酵素処理と定量的 PCR 法を組み合わせた直接検出法によって変異 DNA 分子を検出することを試みた。さらに、コピー数の多いミトコンドリア DNA への適用についても検討を行った。現状では標的 DNA の回収効率と検出感度が充分でないことが課題である。効率的なアッセイ法が実現すれば、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。

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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、従来の方法では標的遺伝子の表現型の変化に基づいて突然変異を検出している。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。加えて、個体を用いる場合は表現型を検出可能な対象臓器が限定されるという問題もある。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

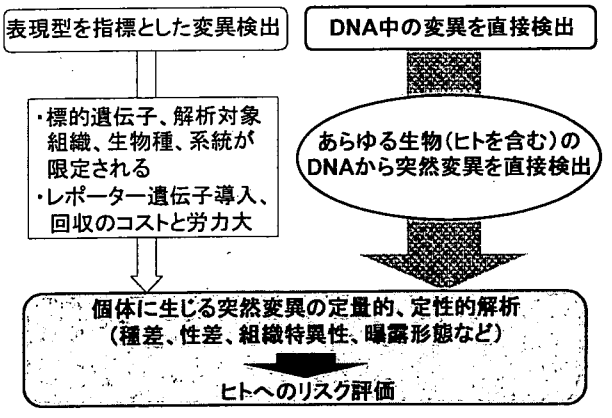

図１　突然変異の直接検出法の意義

確立が望まれている。本研究では、制限酵素処理と定量的PCR法を組み合わせてDNA中の突然変異を直接検出する方法の開発を目的とした。

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を抽出した。また、マウス肝臓凍結組織よりフェノール/クロロホルム法を用いてゲノムDNAを抽出した。同様にヒト大腸がん組織由来のゲノムDNAを用いた。ミトコンドリアDNAは、マウス肝臓組織よりmtDNAエクストラクターCT Kit (Wako)を用いて抽出した。

2）DNAの断片化

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、エタノール沈殿またはMicrocon YM-50によりDNA断片を濃縮・精製した。（図2）。

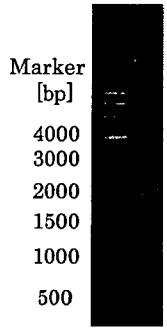

図2　DNAの断片化

3）標的配列の設定

ヒトゲノムDNAにおいてはP53遺伝子第6イントロン内、マウスゲノムDNAにおいてはP53遺伝子第1イントロン内、マウスミトコンドリアDNAにおいてはCYTB遺伝子内にある制限酵素 *Taq*I の認識配列（5'-TCGA-3'）を標的とした。標的配列を含む約1 kbの領域に、標的配列を回収するためのprobe、回収されたDNA断片を定量するconrtol、制限酵素処理後に変異DNAを検出するtargetの3種を増幅するPCRプライマーセットをそれぞれ設定した。

4）プローブDNAの調製

標的配列を含む約1 kbのDNA(probe)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セットを用いて、精製ゲノムDNA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）、アガロースゲル電気泳動後のバンド切り出し、QIAquick PCR purification kit (QIAGEN)等を用いて精製した。精製後のビオチン標識産物をストレプトアビジン結合磁気ビーズ（Dynabeads Streptavidin, Dynal Biotech）と混合して室温で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。これを磁気ビーズ標識プローブDNAとして使用した。

5）標的DNAの回収

2）で切断したDNA断片を4）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）制限酵素処理

回収された標的DNA＋プローブDNAの二本鎖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た。この操作を1回ごとに *Taq*I を追加しつつ 5回繰り返し、*Taq*I の認識配列に変異を持たない断片を分解した。*Taq*I の認識配列に変異が入っていると切断を免れるため、処理後には変異DNA断片のみが残ることになる。

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(control)と、*Taq*I で分解されなかった標的DNAの数(target)を、定量的PCR法により測定した。

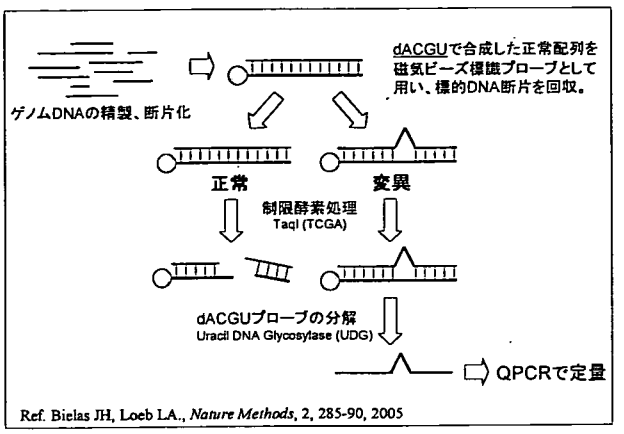

図３　直接検出法の概要

（倫理面の配慮）

本研究では培養細胞および精製DNAを使用するため問題ない。

C．研究結果

１８年度は、ヒトゲノムDNAにおける標的配列をP53遺伝子のイントロン６内に設定し、ヒト培養細胞（ENU処理および無処理細胞）を用いて変異DNAの検出を試みたが、解析した10^4～10^5コピー中に変異DNAは検出されなかった。ENU処理した細胞を用いても突然変異を検出するに至らなかった。磁気ビーズ標識プローブDNAを用いて標的DNAを回収する際の収率が0.1%以下と低かったために低頻度の突然変異を検出できなかったと考えられた。１９年度は、ヒトゲノムDNAについてプローブデザイン変更とハイブリダイゼーションの条件検討を行った。これらによって数%～10%の回収率が得られ、実用レベルに近づいた。

また、がん組織では通常組織と比較して高頻度でランダムなDNA変異が誘発される、いわゆるmutator phenotypeを示すことが報告されている(Bielas et al., 2006)ことから、変異DNAを効率的に検出するためにヒトがん由来のDNAを用いて検討を行った。ヒト大腸がん由来のゲノムDNAから標的DNA断片を回収して制限酵素*Taq*I処理による選択を行った結果、変異DNAを検出することができた。

さらに、突然変異検出用*gpt* deltaトランスジェニックマウスの肝臓組織から抽出したゲノムDNAおよびミトコンドリアDNAを標的として直接検出法の検討を行った。標的配列をマウスのゲノムDNA（P53遺伝子のイントロン１内）およびミトコンドリアDNA(CYTB遺伝子内)に設定し、変異DNAの検出を試みた。ミトコンドリアDNAを標的とした際の検出感度は約3×10^{-5}/塩基であった。いずれの場合においても*Taq*I処理の際の不十分な切断に起因する擬陽性が変異DNA検出の妨げになっていると考えられた。

D．考　察

低頻度の変異DNAを検出するためには回収効率を上げることが重要である。コピー数の多いミトコンドリアDNAを標的とする際は、磁気ビーズを使用しない方法を用いることで回収率の一層の改善が期待される。また、変異DNAを定量的PCR法で検出する際は、変異DNAを非特異的増幅産物と区別するために、PCRの増幅曲線と融解曲線とを合わせて判別することが必要である。そのため、より特異性が高いPCR酵素の使用が望まれる。

現時点では、ミトコンドリアDNAを標的とした際の検出感度は約3×10^{-5}/塩基であり、従来のレポーター遺伝子を用いた変異検出法における自然突然変異頻度に及ばない。検出感度を改善するためには、不完全な*Taq*I処理に起因する擬陽性反応の克服が重要な課題と考える。

E．結　論

ゲノムDNA中の低頻度の突線変異(塩基当たり10^{-8}）を検出するためには、標的DNA断片の回収効率の向上、定量的PCRの検出効率に影響を与える非特異的増幅産

物の低減、および制限酵素処理段階で生じる擬陽性の克服が重要である。今後は検出感度と特異性の改善を目指し、直接検出法の有効性ならびに汎用性を検討する。

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論文はないが、2006-2007 年における研究発表は以下のとおりである。

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ﾒﾗｰｾﾞによる酸化的損傷ヌクレオチドの取り込み　第30回日本分子生物学会年会・第80回日本生化学会大会　合同大会
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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

別紙４

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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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* protooncogene, 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.

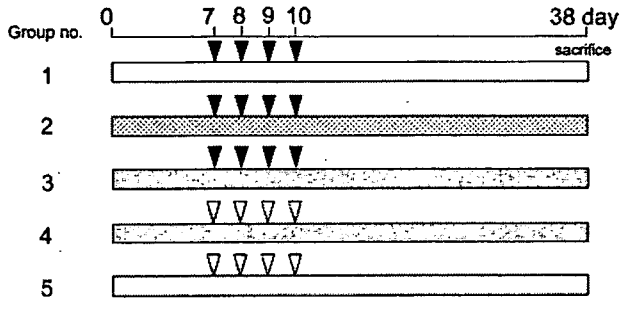

Fig. 1. An experimental design to examine chemopreventive effects of nobiletin against genotoxicity of NNK in the lung of *gpt* delta mice. Twenty female and 25 male eight-week-old *gpt* delta mice were each divided into five groups. Groups 1 through 3 were treated with a single i.p. injection of NNK 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).

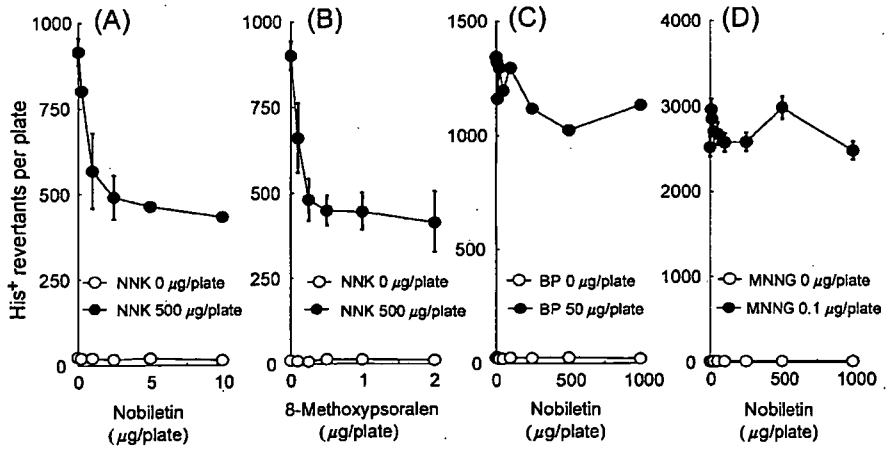

Fig. 2. Suppressive effects of nobiletin against genotoxicity of NNK in the presence of S9 mix in *S. typhimurium* YG7108. Closed circles represent the numbers of His^+ revertants/plate induced by the following compounds: NNK (500 μg/plate) in the presence of S9 mix along with the increasing doses of 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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