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19年度　総括研究報告書

主任研究者　増村　健一

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目　　次

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主任研究者：増村健一　国立医薬品食品衛生研究所　主任研究官

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。直接検出法によってヒトおよびマウスのゲノムDNA中から突然変異を検出することを目指した。また、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。検出感度の向上と効率的なアッセイ法が実現すれば、表現型に依存せず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

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。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。

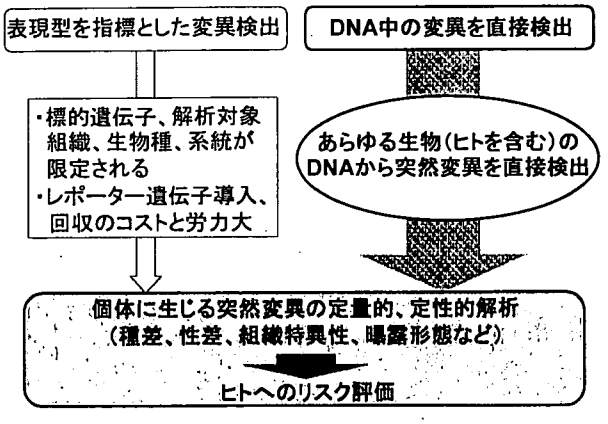

図1　突然変異の直接検出法の意義

B．研究方法

1）DNA の精製と断片化

ゲノム DNA は、ヒト培養細胞株 Nalm-6 およびマウス肝臓組織よりフェノール/クロロホルム法を用いてを抽出した。同様にヒト大腸がん由来のゲノム 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。DNA 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。

2）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 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設定した。

3）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等によって精製した。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 時間撹拌し、DNA 断片と磁気ビーズを結合させたものを磁気ビーズ標識プローブ DNA として使用した。

4）標的 DNA の回収

1）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。

5）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。

6）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。

7）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。

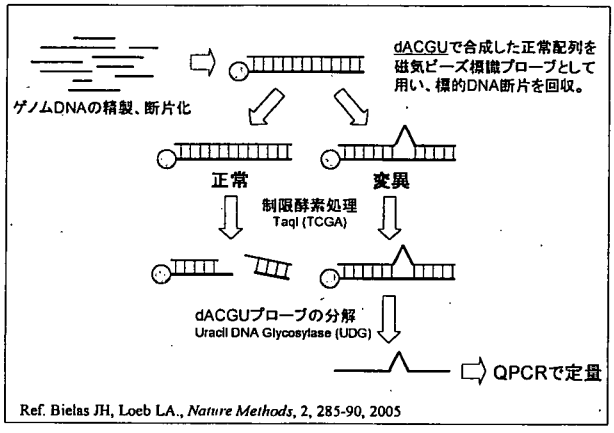

図 2　直接検出法の原理と実験の概要

（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

昨年度はヒト培養細胞（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、標的 DNA を回収する際の収率が 0.1%以下と低く、変異 DNA は

検出されなかった。今年度はヒトゲノム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（定量的PCRにおけるΔCt＝3～6）が得られた。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、ヒト大腸がんのゲノム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、マウス肝臓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結果、ミトコンドリアDNAを標的とした際の検出感度は約3 x 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検出を妨げ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り、標的DNA断片と磁気ビーズ標識プローブとのハイブリダイゼーション条件の検討が必要である。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。

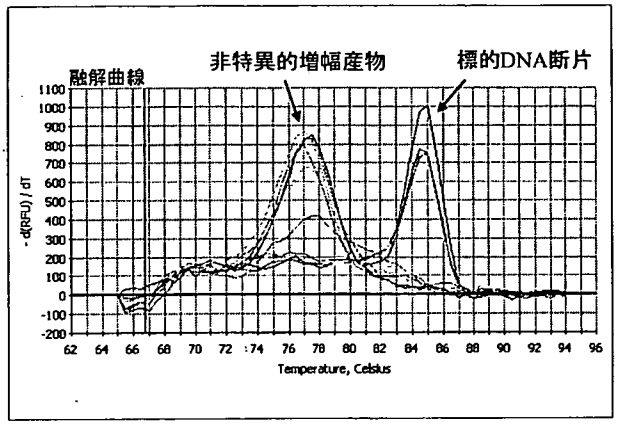

図３　融解曲線による増幅産物の判定

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 x 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（$10^{-5\sim-6}$/遺伝子）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

塩基当たり 10^{-8} と予想されるゲノムDNA中の突線変異を直接検出するためには、(１)標的DNA断片の回収効率の向上、(２)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（３）制限酵素処理段階で生じる擬陽性の克服が重要である。

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発表論文はないが、主任研究者の2007年における論文は以下のとおりである。

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2．学会発表

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Mariko Onishi, Masako Omori, Min Wei, Ken-ichi Masumura, Takehiko Nohmi, Hideki Wanibuchi, Shoji Fukushima, Existence of thresholds for carcinogenicity and in vivo mutagenicity of 1,4-dioxane in liver of rats, 66th Annual Meeting of the Japanese Cancer Association (2007.10)

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

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分担研究報告書

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
（至適DNA配列の検索および制限酵素処理の条件検討）

分担研究者：ピーター・グルーズ　国立医薬品食品衛生研究所　主任研究官

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。本研究では、表現型の変化を突然変異の指標とせず、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。低頻度の突然変異を検索するために、ゲノムDNA中よりもコピー数が多く自然突然変異頻度が高いミトコンドリアDNAを用いて変異DNAの検出を試みた。現状では不十分な制限酵素処理に起因すると思われる擬陽性のバックグラウンドが高いため、変異DNAを検出するには制限酵素処理の特異性と検出感度の向上が必要である。

A．研究目的

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。しかし、表現型の変化をもたらす遺伝子の数が少なく、表現型を検出可能な対照臓器も限定されるという問題がある。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。さらに、ゲノムDNAよりコピー数の多いミトコンドリアDNAを用いて検討を行った。

B．研究方法

1）DNAの精製と断片化

ゲノムDNAはマウス肝臓組織よりフェノール/クロロホルム法を用いてを抽出した。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。DNA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。

2）標的配列の設定

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においては呼吸鎖電子伝達系に関わる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

ーセットを設定した。

3）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）、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 時間撹拌し、DNA 断片と磁気ビーズを結合させたものを磁気ビーズ標識プローブ DNA として使用した。

4）標的 DNA の回収

1）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。

5）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。

6）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。

7）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。

（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。

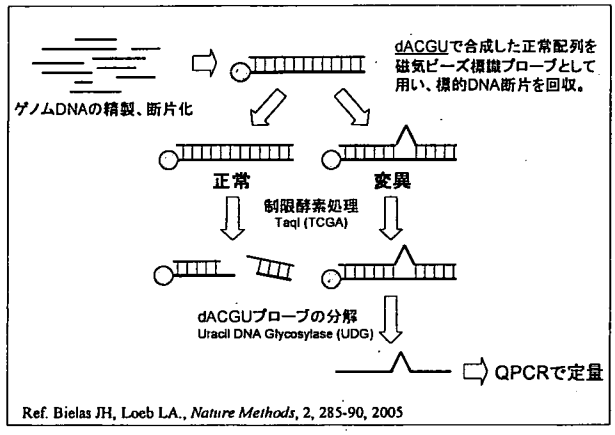

図 1　直接検出法の実験概要

C．研究結果

制限酵素処理と 1 分子 PCR 法を組み合わせて、ヒト細胞集団にランダムに生じる遺伝子突然変異を直接検出する方法（Nature Methods, 2, 285-290, 2005）を応用して遺伝毒性検出法として用いることが可能か検討を行った。マウスゲノム DNA を用いて標的 DNA 断片の回収を行ったところ、磁気ビーズ標識プローブを用いた回収過程での効率が低く、変異 DNA の検出には充分でないと考えられた。そこで変異 DNA を検出するためにコピー数が多く自然突然変異頻度が高いミトコンドリア DNA を用いて検討を行った。マウスミトコンドリア DNA の CYTB 遺伝子内にある制限酵素 *Taq*I の認識配列（5'-TCGA-3'）を標的としてプライマーセットの設定を行った（図 1）。

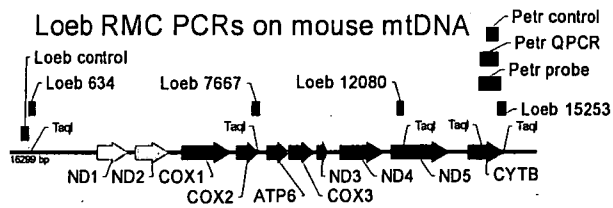

図 1　マウス mtDNA 標的配列

マウス肝臓から抽出したミトコンドリア DNA 2 x 10^8 コピーを用いて直接検出法の検討を行った。磁気ビーズ標識プローブに

よって 5 x 10^5 コピーの標的 DNA 断片が回収された。定量的 PCR によって 2.4 x 10^4 塩基対分の検索を行った結果、7つの変異 DNA 候補が得られたが、*Taq*I 処理で確認したところいずれも切断されたことから、擬陽性反応と考えられた（図2）。ミトコンドリア DNA を標的とした際の検出感度を計算すると約 3 x 10^{-5}/塩基となり、従来のレポーター遺伝子を用いた変異検出法よりも低かった。*Taq*I 処理の際の不十分な切断に起因する擬陽性が検出の際にバックグラウンドとなり、変異 DNA 検出を妨げていると考えられた。

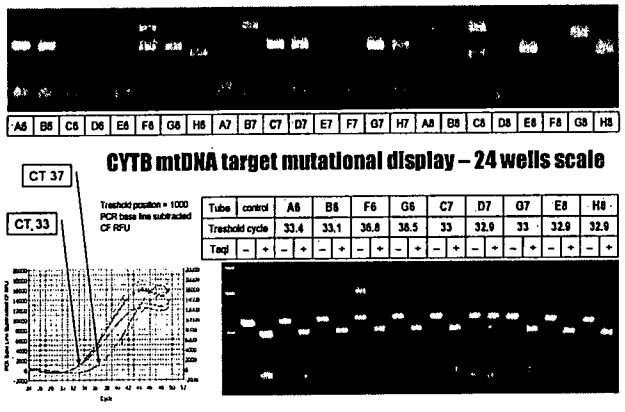

図2　*Taq*I 処理による擬陽性の判定

D．考　察

ミトコンドリア DNA の変異は発がんや老化に関与していることが知られている。ミトコンドリア DNA はヒストン等の保護がないため活性酸素に曝露されやすく、ミトコンドリア呼吸鎖による活性酸素種の発生は個体の寿命にも影響していると考えられる。加えて、ゲノムと異なり細胞あたりおよそ数千コピーのミトコンドリアが存在し、非分裂細胞においてもミトコンドリアの分裂および DNA 複製は行われている。従って変異頻度もゲノム DNA と比べて顕著に高い。これらのことから、ミトコンドリア DNA は遺伝毒性の指標として適当であると考えられる。直接検出法によって低頻度の変異 DNA を検出するためには、標的 DNA の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が改善される可能性がある。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 x 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（$10^{-5\sim-6}$/遺伝子）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である。

E．結　論

DNA 中の低頻度の突線変異を直接検出するためには、標的 DNA 断片の回収効率を高めるとともに、不十分な制限酵素処理に由来する擬陽性 PCR 産物を著しく低減させることが重要である。

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発表論文はないが、分担研究者の 2007 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

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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別紙４

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Combined genotoxic effects of radiation and a tobacco-specific nitrosamine in the lung of *gpt* delta transgenic mice

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Available online 7 September 2006

Abstract

It is important to evaluate the health effects of low-dose-rate or low-dose radiation in combination with chemicals as humans are exposed to a variety of chemical agents. Here, we examined combined genotoxic effects of low-dose-rate radiation and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the most carcinogenic tobacco-specific nitrosamine, in the lung of *gpt* delta transgenic mice. In this mouse model, base substitutions and deletions can be separately analyzed by *gpt* and Spi$^-$ selections, respectively. Female *gpt* delta mice were either treated with γ-irradiation alone at a dose rate of 0.5, 1.0 or 1.5 mGy/h for 22 h/day for 31 days or combined with NNK treatments at a dose of 2 mg/mouse/day, i.p. for four consecutive days in the middle course of irradiation. In the *gpt* selection, the NNK treatments enhanced the mutation frequencies (MFs) significantly, but no obvious combined effects of γ-irradiation were observable at any given radiation dose. In contrast, NNK treatments appeared to suppress the Spi$^-$ large deletions. In the Spi$^-$ selection, the MFs of deletions more than 1 kb in size increased in a dose-dependent manner. When NNK treatments were combined, the dose–response curve became bell-shaped where the MF at the highest radiation dose decreased substantially. These results suggest that NNK treatments may elicit an adaptive response that eliminates cells bearing radiation-induced double-strand breaks in DNA. Possible mechanisms underlying the combined genotoxicity of radiation and NNK are discussed, and the importance of evaluation of combined genotoxicity of more than one agent is emphasized.

Keywords: Combined genotoxic effects; Radiation; NNK; Lung cancer; *gpt* delta mice; Deletion

1. Introduction

Environmental factors play important roles in the etiology of human cancer [1]. Of various environmental hazardous compounds, cigarette smoke is the most causative factor associated with the increase in cancer risk in humans. Tobacco smoking plays a major role in the etiology of lung, oral cavity and esophageal cancers, and a variety of chronic degenerative diseases [2]. Although cigarette smoke is a mixture of about 4000 chemicals including more than 60 known human carcinogens, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (nicotine-derived nitrosamino ketone, NNK) is the most carcinogenic tobacco-specific nitrosamine [3,4]. NNK induces lung tumors in mice,

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rats and hamsters, and International Agency for Research on Cancer has concluded that exposure to NNK and NNN (*N'*-nitrosonornicotine) is carcinogenic to humans [5]. NNK is metabolically activated by CYP (P-450) enzymes in the lung and generates O^6-methylguanine in DNA, which leads to G:C to A:T mutations, and the subsequent activation of Ki-*ras* proto-oncogene, an initiation of tumor development [6,7].

Radiation, on the other hand, is one of the most causative physical factors that induce human cancer. Radiation induces double-strand breaks (DSBs) in DNA, which lead to chromosome aberrations and cell deaths, and generates a variety of oxidative DNA damage [8]. Because of the genotoxicity, radiation at high doses clearly induces various tumors in humans [9]. Even at low doses, residential exposure to radioactive radon and its decay products may account for about 10% of all lung cancer deaths in the United States and about 20% of the lung cancer cases in Sweden [10,11].

Since humans are exposed to a variety of chemical and physical agents that may induce cancer, these factors may interact with each other and the action of one agent may be influenced by exposure to another agent [12]. The risk from combined exposure to more than one agent may be substantially higher or lower than predicted from the sum of the individual agents. In fact, low-dose radiation can induce an adaptive response, causing rodent or human cells to become resistant to genotoxic damage by subsequent higher doses of radiation [13]. Pre-exposure to alkylating agents at low doses induces another adaptive response that provides mechanisms by which the exposed bacterial cells can tolerate the higher challenging doses of genotoxic agents [14]. In addition, mitomycin C, bleomycin, hydrogen peroxide, metals and quercetin may also induce an adaptive response [15].

To explore the mechanisms underlying the interactive effects of chemical and physical agents on carcinogenesis, we examined the combined genotoxic effects of NNK and γ-irradiation in the lung of *gpt* delta transgenic mice [16]. In this mouse model, point mutations and deletions are separately analyzable by *gpt* and Spi^- selections, respectively [17]. Point mutations such as base substitutions are induced by a number of chemical carcinogens including NNK [18]. Spi^- selection detects deletions in size between 1 bp and 10 kb [19]. Deletions in size more than 1 kb, which we call large deletions in this study, are efficiently induced by γ-ray, X-ray and carbon-ion irradiation [20], and are thought to be generated by non-homologous end joining (NHEJ) of DSBs in DNA [21].

We report here that low-dose-rate γ-irradiation enhanced the mutation frequencies (MFs) of the large deletions in the lung of *gpt* delta mice in a dose-dependent manner. When combined with NNK treatments, however, the MF at the highest radiation dose, i.e., 1.02 Gy, was reduced by more than 50%, suggesting that NNK treatments may induce an adaptive response against radiation-induced deletion mutations. We discuss possible mechanisms of the adaptive response and emphasize the importance of the risk assessment of combined genotoxic effects of radiation and chemicals in vivo.

2. Materials and methods

2.1. Treatment of mice

gpt delta C57BL/6J transgenic mice were maintained in the conventional animal facility of National Institute of Radiological Sciences, Chiba, Japan, according to the institutional animal care guidelines. They were housed in autoclaved aluminum cages with sterile wood chips for bedding and given free access to standard laboratory chow (MB-1, Funabashi Farm Co., Japan) and acidified water under controlled lighting (12 h light/dark cycle). Seven-week-old female *gpt* delta mice were divided to eight groups each consisting of six mice. Three groups were γ-irradiated at a dose rate of 0.5, 1.0 or 1.5 mGy/h for 22 h/day for 2 weeks (Fig. 1). After the irradiation, three groups of mice were treated with a single i.p. injection of NNK (Toronto Research Chemicals, Toronto, Canada) dissolved in saline at a daily dose of 2 mg/mouse for four consecutive days. The irradiation continued during the 4-day treatments, and the mice were kept in the cage for another 2 weeks with irradiation. Three control groups were γ-irradiated as described but received saline instead of NNK. The whole irradiation period was 31 days, and the total estimated doses were 0.34, 0.68 and 1.02 Gy, respectively. Another control group of mice was treated with NNK as described but without γ-irradiation. The third control was kept in the cage for 31 days without γ-irradiation or NNK treatments. The source of radiation was ^{137}Cs, and the dose rate was estimated by a fluorescent glass dosimeter. The non-irradiated control groups were placed behind a concrete wall of 1 m thickness. The mice were sacrificed by cervical vertebral dislocation. The liver and lung were removed, placed immediately in liquid nitrogen, and stored at −80 °C until analysis.

2.2. DNA isolation and in vitro packaging of DNA

High-molecular-weight genomic DNA was extracted from the lung and the liver using the RecoverEase DNA Isolation Kit (Stratagene, La Jolla, CA). Lambda EG10 phages were rescued using Transpack Packaging Extract (Stratagene, La Jolla, CA).

2.3. gpt mutation assay

The *gpt* mutagenesis assay was performed according to previously described methods [17]. Briefly, *Escherichia coli*

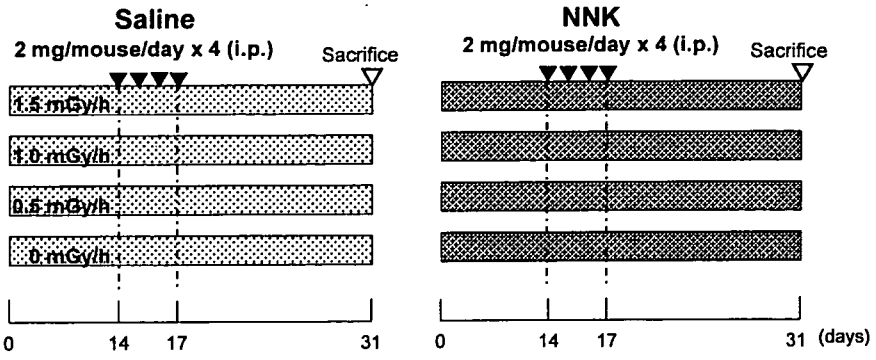


Fig. 1. An experimental design to examine the combined genotoxicity of γ-irradiation and NNK treatments in the lung of mice. Female 7-week-old *gpt* delta mice were divided into eight groups each composed of six mice. Three groups of mice were irradiated at a dose rate of 0.5, 1.0 or 1.5 mGy/h for 22 h/day for 14 days and treated with NNK at a daily dose of 2 mg/mouse for four consecutive days. The irradiation continued during the NNK treatments and the following 14 days before sacrifice. The total radiation doses were 0.34, 0.68 and 1.02 Gy, respectively. Control three groups of mice were γ-irradiated but without NNK treatments. Another control group was treated with NNK but without γ-irradiation. The third control was kept in the cage for 31 days without γ-irradiation or NNK treatments. Transgene λEG10 DNA was rescued from the lung of mice, and the base substitutions and deletions were analyzed by *gpt* and Spi$^-$ selection, respectively.

YG6020 expressing Cre recombinase was infected with the rescued phage. The bacteria were then spread onto M9 salts plates containing chloramphenicol (Cm) and 6-thioguanien (6-TG), and incubated for 72 h at 37 °C for selection for the colonies harboring a plasmid carrying the Cm acetyltransferase (*cat*) gene and a mutated *gpt* gene. The 6-TG-resistant colonies were streaked again onto the same selection plates for confirmation of the resistant phenotype. All the confirmed *gpt* mutants recovered from the lung were sequenced and the identical mutations from the same mouse counted one mutant. The *gpt* MFs in the lung were calculated by dividing the number of the *gpt* mutants after sequencing by the number of rescued plasmids, which was estimated from the number of colonies on plates containing Cm but without 6-TG. Since no *gpt* mutants recovered from the liver were sequenced, the MFs in the liver were calculated by dividing the number of confirmed 6-TG-resistant colonies by the number of rescued plasmids.

2.4. PCR and DNA sequencing analysis of 6-TG-resistant mutants

A 739 bp DNA fragment containing the *gpt* gene was amplified by polymerase chain reaction (PCR) using primers 1 and 2 [17]. The reaction mixture contained 5 pmol of each primer and 200 mM of each dNTP. PCR amplification was carried out using Ex Taq DNA polymerase (Takara Bio, Shiga, Japan) and performed with a Model PTC-200 Thermal Cycler (MJ Research, Waltham, MA). PCR products were analyzed by agarose gel electrophoresis to determine the amount of the products. DNA sequencing of the *gpt* gene was performed with BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using sequencing primer gptA2 (5′-TCTCGCGCAACCTATTTTCCC-3′). The sequencing reaction products were analyzed on an Applied Biosystems model 310 genetic analyzer (Applied Biosystems, Foster City, CA).

2.5. Spi$^-$ mutation assay

The Spi$^-$ assay was performed as described previously [17]. The lysates of Spi$^-$ mutants were obtained by infection of *E. coli* LE392 with the recovered Spi$^-$ mutants. The lysates were used as templates for PCR analysis to determine the deleted regions. Sequence changes in the *gam* and *redAB* genes, and the outside of the *gam/redAB* genes were identified by DNA sequencing analysis [22]. The appropriate primers for DNA sequencing were selected based on the results of PCR analysis. The entire sequence of λEG10 is available at http://dgm2alpha.nhis.go.jp.

2.6. Statistical analysis

All data are expressed as mean ± standard deviations of the MFs of six mice for lung and those of four mice for liver. 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.

3. Result

3.1. gpt MFs in the lung of NNK-treated and γ-irradiated gpt delta mice

We measured the *gpt* MFs in the lung of *gpt* delta mice untreated or treated with NNK in the absence or the presence of γ-irradiation (Fig. 2). NNK treatments significantly enhanced the MFs over the control groups. The mean MFs ($\times 10^{-6}$) of NNK-treated versus saline-treated groups were 14.3 ± 6.9 versus 4.2 ± 4.0, 20.7 ± 5.1 versus 4.7 ± 3.0, 15.2 ± 7.3 versus 2.0 ± 2.1 and 17.2 ± 7.9 versus 2.7 ± 1.4 at the dose rates of 0, 0.5, 1.0 and 1.5 mGy/h, respectively. The γ-irradiation

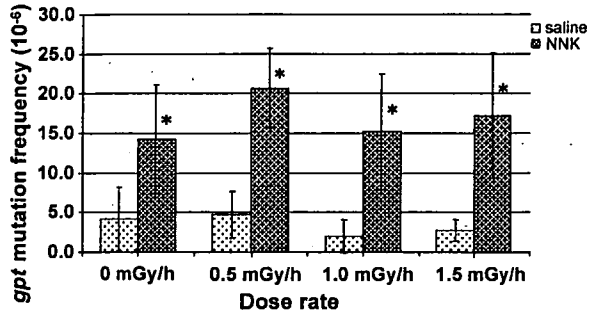


Fig. 2. *gpt* MFs in the lung of mice untreated or treated with NNK in the absence or the presence of γ-irradiation. An asterisk (*) denotes $p < 0.05$ ($n = 6$) in a Student's *t*-test of MF of NNK-treated vs. the corresponding untreated mice. Vertical bars show the standard deviations with mice as the unit of comparison.

alone, i.e., the saline-treated group, did not enhance the *gpt* MF under the conditions. Hence, the increases in MFs are due to NNK treatments. Although the individual MFs slightly varied, there was no significant difference among the four MFs of the NNK-treated groups. Thus, we suggested that the irradiation did not modify the genotoxicity of NNK in the lung of mice.

To confirm the results in the lung, we analyzed the *gpt* MFs in the liver of the NNK-treated and saline-treated groups. The mean MFs ($\times 10^{-6}$) of NNK-treated versus saline-treated groups were 134 ± 48 versus 8.1 ± 3.8, 105 ± 31 versus 8.7 ± 3.5, 101 ± 18 versus 8.0 ± 4.2 and 128 ± 76 versus 6.8 ± 0.6 at the dose rates of 0, 0.5, 1.0 and 1.5 mGy/h, respectively. Although NNK treatments induced mutations much more strongly in the liver than in the lung, there were no significant modulating effects of radiation on the NNK-induced mutations in the liver.

The irradiation might modulate specific types of mutations without affecting the total *gpt* MFs. To examine the possibility, we determined the mutation spectra of the *gpt* gene in the lung and examined whether the radiation affected specific types of mutations (Table 1). NNK treatments induced G:C to A:T, G:C to T:A, A:T to T:A and A:T to C:G mutations. In particular, A:T to T:A mutations were induced more than 20-fold by NNK treatments. We observed, however, no remarkable variations of mutation spectra associated with the dose rates of combined radiation. Thus, we concluded that the irradiation did not enhance or suppress the base substitutions induced by NNK in the lung of *gpt* delta mice significantly.

3.2. Spi⁻ MFs in the lung of NNK-treated and γ-irradiated gpt delta mice

Next, we measured the Spi⁻ MFs in the lung of *gpt* delta mice untreated or treated with NNK in the absence or the presence of γ-irradiation. The mean Spi⁻ MFs ($\times 10^{-6}$) of NNK-treated versus saline-treated groups were 5.15 ± 2.34 versus 4.11 ± 0.98, 5.47 ± 1.98 versus 5.06 ± 3.50, 5.36 ± 1.56 versus 4.09 ± 0.80 and 5.39 ± 2.56 versus 4.65 ± 1.78 at the dose rates of 0, 0.5, 1.0 and 1.5 mGy/h, respectively. These results suggest that neither NNK treatments nor the irradiation enhanced the Spi⁻ MFs in the lung significantly.

To investigate the combined effects of NNK and γ-irradiation on specific types of deletion mutations, we identified all the Spi⁻ mutations by DNA sequencing analysis (Table 2). Of various classes of deletions observed, only the MFs of large deletions in the size of more 1 kb increased in a dose-dependent manner in the saline-treated group. To examine the dose–response in more detail, we determined the MFs of the large deletions

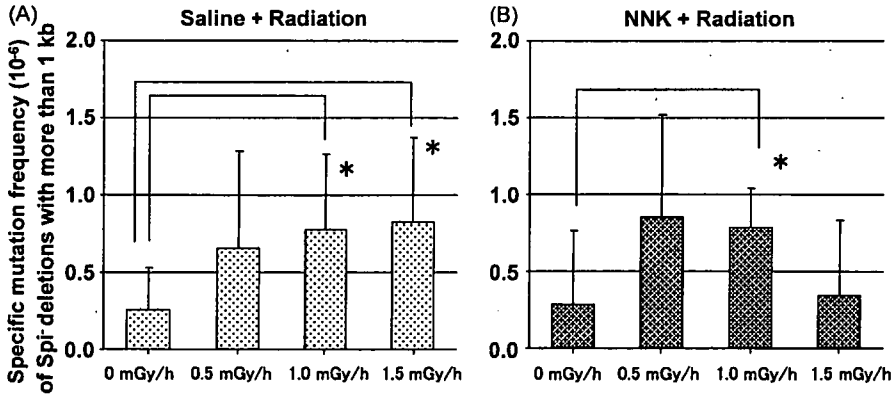


Fig. 3. Specific MF of large deletions with the size of more than 1 kb in the lung of unirradiated or γ-irradiated mice. The mice were not treated (A) or treated with NNK (B). An asterisk (*) denotes $p < 0.05$ ($n = 5$) in a Student's *t*-test of MF of γ-irradiated vs. the corresponding unirradiated mice. Vertical bars show the standard deviations with mice as the unit of comparison.

Table 1
gpt mutation spectra in the lung of NNK-treated and γ-irradiated *gpt* delta mice

Treatment: saline	0 mGy/h			0.5 mGy/h			1.0 mGy/h			1.5 mGy/h		
	No.	MF ($\times 10^{-6}$)	%	No.	MF ($\times 10^{-6}$)	%	No.	MF ($\times 10^{-6}$)	%	No.	MF ($\times 10^{-6}$)	%
Base substitution												
Transition												
G:C → A:T	15(6)	1.81	43	12(6)	1.76	38	5(2)	0.61	29	8(4)	0.81	30
A:T → G:C	2	0.24	6	4	0.59	13	1	0.12	6	2	0.20	7
Transversion												
G:C → T:A	1	0.12	3	5(2)	0.73	16	1	0.12	6	6(1)	0.61	22
G:C → C:G	1	0.12	3	0	0.00	0	0	0.00	0	2(2)	0.20	7
A:T → T:A	1	0.12	3	1	0.15	3	1	0.12	6	1	0.10	4
A:T → C:G	3	0.36	9	1	0.15	3	1	0.12	6	1	0.10	4
Deletion	8	0.97	23	6	0.88	19	7	0.85	41	6	0.61	22
−1 bp	3			2			5			3		
>2 bp	5			4			2			3		
Insertion	3	0.36	9	3	0.44	9	1	0.12	6	1	0.10	4
Others	1	0.12	3	0	0.00	0	0	0.00	0	0	0.00	0
	35	4.23	100	32	4.69	100	17	2.06	100	27	2.73	100

Treatment: NNK	0 mGy/h			0.5 mGy/h			1.0 mGy/h			1.5 mGy/h		
	No.	MF ($\times 10^{-6}$)	%	No.	MF ($\times 10^{-6}$)	%	No.	MF ($\times 10^{-6}$)	%	No.	MF ($\times 10^{-6}$)	%
Base substitution												
Transition												
G:C → A:T	24(2)	5.11	36	45(8)	8.02	39	32(5)	5.85	39	54(6)	8.51	50
A:T → G:C	0	0.00	0	7	1.25	6	6	1.10	7	2	0.32	2
Transversion												
G:C → T:A	9(2)	1.92	13	10(1)	1.78	9	7(1)	1.28	8	7(1)	1.10	6
G:C → C:G	0	0.00	0	2	0.36	2	0	0.00	0	3	0.47	3
A:T → T:A	13	2.77	19	26	4.64	22	17	3.11	21	17(1)	2.68	16
A:T → C:G	15	3.19	22	12	2.14	10	8	1.46	10	12(1)	1.89	11
Deletion	5	1.06	8	12	2.14	10	9	1.65	11	12	1.89	11
−1 bp	5			6			4			5		
>2 bp	0			6			5			7		
Insertion	1	0.21	2	0	0.00	0	4	0.73	5	1	0.16	1
Others	0	0.00	0	2	0.36	2	0	0.00	0	1	0.16	1
	67	14.26	100	116	20.68	100	83	15.18	100	109	17.18	100

No. stands for the number of mutations.

of each mouse and calculated the mean MF and standard derivations. The mean MFs ($\times 10^{-6}$) and standard derivations were 0.25 ± 0.28, 0.66 ± 0.63, 0.77 ± 0.49 and 0.82 ± 0.55 at the dose rates of 0, 0.5, 1.0 and 1.5 mGy/h, respectively (Fig. 3A). The values at 1.0 and 1.5 mGy/h were about three-fold higher than the value at 0 mGy/h, and the differences were statistically significant ($p = 0.04$). In contrast, the dose–response curve of large deletions in NNK-treated group was a bell shaped (Fig. 3B). The mean MFs ($\times 10^{-6}$) and standard derivations of large deletions in the NNK-treated group were 0.29 ± 0.47, 0.85 ± 0.66, 0.78 ± 0.26 and 0.35 ± 0.48 at the dose rates of 0, 0.5, 1.0 and 1.5 mGy/h, respectively. It should be noted that the

Table 2
Spi⁻ mutation spectra in the lung of NNK-treated and γ- irradiated *gpt* delta mice

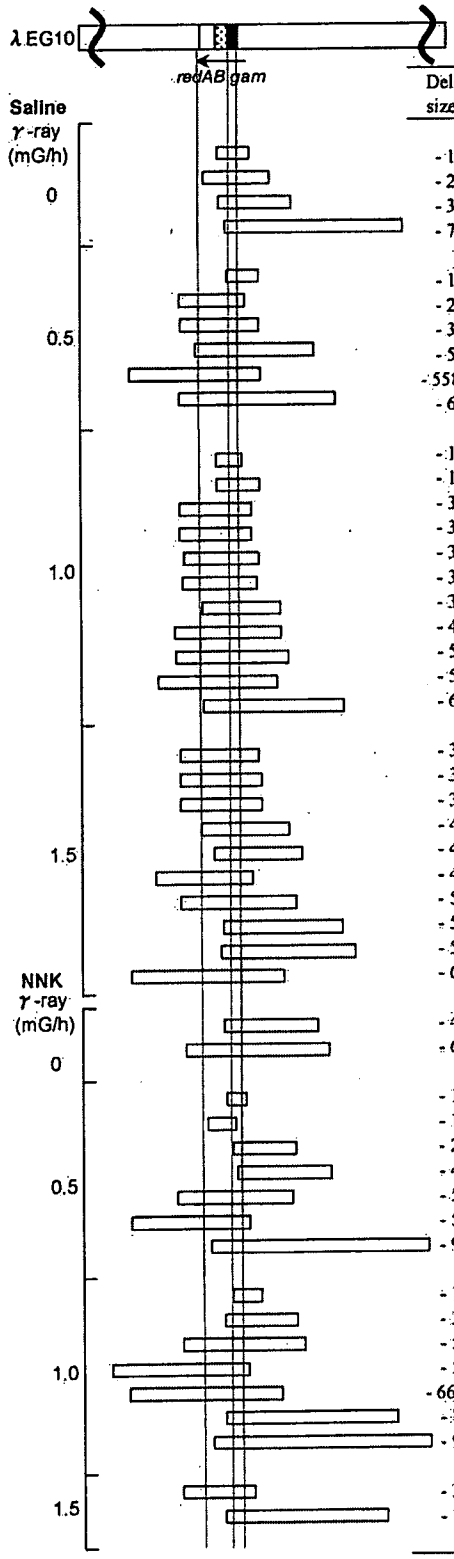
Treatment: saline	0 mGy/h			0.5 mGy/h			1.0 mGy/h			1.5 mGy/h		
	No.	MF ($\times 10^{-6}$)	%	No.	MF ($\times 10^{-6}$)	%	No.	MF ($\times 10^{-6}$)	%	No.	MF ($\times 10^{-6}$)	%
1 bp deletion												
Simple												
Guanine	9	0.49	12	7	0.59	12	5	0.34	8	4	0.30	7
Adenine	4	0.22	5	0	0.00	0	2	0.14	3	1	0.08	2
In run												
Guanine	13	0.71	17	15	1.27	25	12	0.82	20	13	0.99	21
Adenine	31	1.70	41	19	1.60	32	22	1.50	37	25	1.91	41
With b.s.	0	0.00	0	0	0.00	0	0	0.00	0	0	0.00	0
>2 bp deletion	15	0.82	20	17	1.43	28	17	1.16	28	13	0.99	21
2 bp ~ 1 kb	2	0.11	3	7	0.59	12	3	0.20	5	1	0.08	2
>1 kb	5	*0.27*	7	7	*0.59*	12	11	*0.75*	18	10	*0.76*	16
Complex	8	0.44	11	3	0.25	5	3	0.20	5	2	0.15	3
Insertion	3	0.16	4	2	0.17	3	2	0.14	2	5	0.38	8
	75	4.11	100	60	5.06	100	60	4.09	100	61	4.65	100

Treatment: NNK	0 mGy/h			0.5 mGy/h			1.0 mGy/h			1.5 mGy/h		
	No.	MF ($\times 10^{-6}$)	%	No.	MF ($\times 10^{-6}$)	%	No.	MF ($\times 10^{-6}$)	%	No.	MF ($\times 10^{-6}$)	%
1 bp deletion												
Simple												
Guanine	5	0.61	12	4	0.46	8	4	0.50	9	4	0.48	9
Adenine	3	0.37	7	0	0.00	0	4	0.50	9	1	0.12	2
In run												
Guanine	9	1.10	21	19	2.17	40	9	1.12	21	14	1.68	31
Adenine	12	1.47	29	10	1.14	21	9	1.12	21	15	1.80	33
With b.s.	0	0.00	0	0	0.00	0	2	0.25	5	0	0.00	0
>2 bp deletion	12	1.47	29	10	1.14	21	11	1.37	26	11	1.32	24
2 bp ~ 1 kb	6	0.74	14	2	0.23	4	3	0.37	7	7	0.84	16
>1 kb	2	*0.25*	5	7	*0.80*	15	7	*0.87*	16	2	*0.24*	4
Complex	4	0.49	10	1	0.11	2	1	0.12	2	2	0.24	4
Insertion	1	0.12	2	5	0.57	10	4	0.50	9	0	0.00	0
	42	5.15	100	48	5.47	100	43	5.36	100	45	5.39	100

No. stands for the number of mutations. Specific MFs of large deletions more than 1 kb in size are italicised.

MF at 1.0 mGy/h (0.78×10^{-6}) was about three-fold higher than that of 0 mGy/h ($p = 0.04$) but the MF at 1.5 mGy/h (0.35×10^{-6}) was very similar to that of 0 mGy/h (0.29×10^{-6}). The p values of the differences of MFs between saline-treated and NNK-treated groups at dose rates of 0, 0.5, 1.0 and 1.5 mGy/h were 0.44, 0.32, 0.48 and 0.09, respectively. From the results, we suggested that NNK treatments suppressed the induc-

Fig. 4. Molecular nature of large deletions recovered from the lung of *gpt* delta mice untreated or treated with NNK in the absence or the presence of γ-irradiation. Horizontal bars represent the deleted regions of mutants. Most of the mutants lack the entire *gam* gene and part of the *redAB* genes, but some lack the *gam* gene and the upstream region. The *gam* and *redAB* genes make an operon and the transcription starts from the upstream of the *gam* gene. Short homologous sequences in the junctions of the mutants are underlined. Underlined sequences, i.e., T or CTTA, in the middle of two sequences are inserted sequences in the junctions.



Delesion size (bp)	Sequence of the junction of deletion		No.of mutant	Mouse I.D.
-1242	TGAATGGTTCGCGGCGGC	GTGTTTGTGCATCCATCT	2	2,34
-2912	AGAGCAGGCGGTACGCAT	TAATCCTTAACTTTGCCC	1	34
-3393	GGTAAATCCGGCATGTAC	AGCCAGGGGATCTCTCCC	1	34
-7626	CAGCCAGCGTTGCGAGTG	CGGTTGCTTTATGCATGG	1	3
-1608	GCTATCACGTTGTGAACT	TCGGATTTAGTGCGCTTT	2	9,11
-2912	AGAGCAGGCGGTACGCAT	TAATCCTTAACTTTGCCC	1	9
-3650	ACTGTTGGTTAATACGCT	AAAGCTCCAAGCTCAACA	1	41
-5339	GATGCGGCTAACGTATGA	TGCCTTTGCGGCTTGTAA	1	11
-5582 + 1	CGCCTGTCTCTGCCTAAT T	ATACTCTCTAATCTTGGC	1	10
-6789	CATCCTGAACCCATTGAC	ACCACAGCGTGATGGGCT	1	10
-1164	ATGGTTCGCGGCGGCATT	TTGCGCTTACCCCAACCA	1	50
-1922	CACTGAATCATGGCTTTA	AGAAGCAATAACCCGCAG	1	50
-3219	TTCTATTTCTGATGCTGA	GTATCACACACCCCAAAG	1	19
-3326	TGATGTATTGCTGGTTTC	CTGGTTTTGCGCTTACCC	1	19
-3360	GATTTCAGAATTAGCCTG	GGGTATCATGTAGCCGCT	1	49
-3473	GCGGCGTTTGATGTATTG	TGTGGCAGTTGTAGTCCT	1	19
-3966	TAACTTCCGGAGCCACAC	GAAGACGCGATCTCGCTC	1	17
-4716	TCCTTTGATGCGAATGCC	GCATAATTACTCCTGATA	1	18
-5053	AATTCATGGAAAAGGTCT	GCGTAAACTCCGCTGTCA	1	50
-5244	GTATCAGCAATGTTTATG	GTAGCTTGGCTTCTACCT	1	17
-6140	ATCGGGGATTCAGTAACA	CGCCTTCTGTCACCACCA	1	10
-3645	GGTCTGCGTCAAATCCCC	GGTTATCTGTATGTTTTT	1	27
-3833	AATCGATGGTGTTACCAA	AACCCGGCGCTGAGGCCG	1	57
-3879	TTGCTGGTTTCTTTCCCG	CTGAGGCCGGGTTATTCT	1	27
-4014	TTTTCGATGAACTCCGGC	CTTACCGGGTTATCCCCA	1	26
-4035	CTTCCCAGCCAGCGTTGC	CCTGCACCAGCGTGGTAA	1	27
-4416	TAAAATAAATCCCCGCGA	TTGGCACATTGGCAGCTA	1	27
-5194	ATAACTCTGTTGAATGGC	ATATGATGACAATGTCGC	1	58
-5366	TAACGCAGATCGGATGAT	CCGCCAGTGGCGCACTAT	1	58
-5905	GGAACAGAGCGGCAATAA	CTGCCTGATTAGCGGCGA	1	25
-6629	CCGCAATTTCTTTTGTGG	CCTTCAACAAACAAAAAA	1	26
-4283	TGATGATGCGGGACCAGC	GACCACGCCTTTGGGGCG	1	38
-6283	TTGCCAGGCTTAAATGAG	ATCTAAGGCCGTCACCTG	1	5
-1035	ATCACCTTTAAATGCCGT	AACCCCCCGCGATTGGCA	1	15
-1315	CAGTGCTTTAGCGTTAAC	ATAGCTGTGAAAATATCG	1	45
-2724	TGCTGGCCCCGTGGCGTT	CTCCAGCCCCTATCCCCC	1	45
-4318	CCTTTTTCCATGTCGTCT	CAGCGGGTACGGTTGGCG	1	13
-5146	CGAGAAGATGTTGAGCAA	TCTGCCAGTTCTGCCTCT	1	15
-5273	GCTGACGCCGCCTTGCCC	CCCGCGATTGGCACATTG	1	14
-9265	TGTGCCACGCATCATCCC	ATGGATGCAGCCTCCAGA	1	15
-1373	GTGAGCAATGGTTTCAAC	TGGCGATAGTGGGTATCA	1	23
-3466	AGGATTCATTGTCCTGCT	GTTCGCCACCCAACCCGC	1	53
-5445	GTTTAACATGCCGATTGC	AGCGCGCGTAATCCGGCG	1	54
-5974	ATCCGGATGAAGCCGCTT	GCAATGTATGTCGTTTCA	1	21
-6666 + 4	GCCGTAAGTCTTGATCTC CTTA	CCTCATCAGTGGCTCTAT	1	22
-7430	TTTGAATGGTTCGCGGCG	CGTAGCGGTGGAAGTGGC	1	21
-9265	TTGTGCCACGCATCATCC	ATGGATGCAGCCTCCAGA	1	21
-3219	GTAATAGCGATGCGTAAT	AGGAAAACAGGAAAGGGG	1	61
-7081	TCAGCCAGCTTCCCAGCC	GGTGGAAGTGGCGATGAA	1	30

tion of large deletions at a dose rate of 1.5 mGy/h of γ-irradiation.

To further characterize the large deletions induced by the irradiation, we identified the size and junctions of all the 51 deletion mutants (Fig. 4). The size of deletions distributed from 1035 to 9265 bp. About half of the mutants had short homologous sequences up to 11 bp in the junctions while another half had no such short homologous sequences. Two mutants had 1 or 4 bp insertions in the junctions. There was no hot spot of the junctions so that only 2 out of 51 deletions were identified in two mice. There were no obvious differences between large deletions induced by radiation alone and those induced by radiation plus NNK treatments. These results suggest that radiation-induced DSBs in DNA caused large deletions either in the absence or the presence of NNK treatments.

4. Discussion

Humans are exposed to a variety of exogenous and endogenous genotoxic agents. Thus, biological effects of radiation at low doses or low-dose-rate should be evaluated in combination with chemical exposure [12]. In fact, survey of chromosome aberrations in habitats in high-background radiation area in China indicates that cigarette smoking has stronger effects on induction of chromosome aberrations than has the elevated level of natural radiation [23]. Epidemiological studies on underground mineworkers exposed to high levels of radon or plutonium suggest the complexity of interactions between radiation and cigarette smoke in induction of lung tumors [24,25]. Hence, it is important to understand the fundamental mechanisms underlying the interactive genotoxicity and carcinogenicity of cigarette smoking and radiation for the risk assessment on human health.

To elucidate the mechanisms involved, we examined the combined genotoxicity of low-dose-rate γ-irradiation and a tobacco-specific nitrosamine NNK in the lung of *gpt* delta mice. In this study, we focused on whether γ-irradiation would modulate NNK-induced base substitutions and whether NNK treatments would modulate radiation-induced deletions. The mice were irradiated at dose rates of 0.5, 1.0 and 1.5 mGy/h for 22 h for 2 weeks and treated with NNK, i.e., 2 mg/mouse/day for four consecutive days, with irradiation (Fig. 1). The mice were irradiated at the same dose rates for another 2 weeks before sacrifice. Base substitutions and deletions in the lung detected by *gpt* and Spi$^-$ selection, respectively, were analyzed at the molecular levels. We chose the dose rates, i.e., 0.5, 1.0 and 1.5 mGy/h of γ-ray, since Sakai et al. [26] report the suppression of carcinogenicity of 3-methylchoranthrene in ICR female mice by chronic low-dose-rate irradiation of γ-ray at 0.95 mGy/h. According to the report, there is an optimum dose rate of about 1 mGy/h to observe the suppressive effects, and the higher or lower dose rates fail to suppress the tumor induction.

In the present study, NNK treatments significantly enhanced the *gpt* MF (Fig. 2). We observed, however, no modulating effects, i.e., enhancement or suppression, of γ-irradiation at any given dose rate, on the NNK-induced mutations (Fig. 2). This conclusion holds true even when we analyzed the detailed mutation spectra (Table 1). NNK treatments induced similar pattern of base substitutions, i.e., G:C to A:T, G:C to T:A, A:T to T:A and A:T to C:G regardless of the dose rates of combined radiation. In contrast, we observed a suppressive effect of NNK treatments on the radiation-induced deletions. γ-Irradiation enhanced the MF of large deletions in the size of more than 1 kb in a dose-dependent manner (Fig. 3A and Table 2). When combined with NNK treatments, however, the dose–response curve became bell-shaped and the MF at the highest dose rate, i.e., 1.5 mGy/h, was reduced by more than 50% (Fig. 3B and Table 2). The total radiation dose at the highest dose rate was 1.02 Gy. The size of the large deletions was between about 1 and 9 kb, and about half of the large deletions had short homologous sequences in the junctions while other did not (Fig. 4). These features are similar to those of large deletions induced by high dose irradiation with heavy ion, X-ray and γ-ray [20]. Thus, we suggest that NNK induced an adaptive response that eliminated the cells bearing radiation-induced DSBs in DNA.

Previous studies show that low-dose radiation can induce an adaptive response, which causes cells to become resistant to damage by subsequent high doses of radiation [13,27]. Although the exact mechanisms of the adaptive response are not well understood, it is assumed that some proteins are induced by low-dose radiation and they recognize and remove the cells bearing DSB in DNA. Tucker et al. [28] report that the frequency of *Dlb-1* mutations in the small intestine in female F1 mice obtained by crossing SWR/J and C57BL/6 increases along with the total radiation doses of γ-ray, but it saturates and slightly decreases at high doses, i.e., 2–3 Gy (55 mGy/day × 42 or 63 days). Interestingly, our results also suggest that the MFs of the large deletions saturated slightly at the highest dose of 1.02 Gy (Fig. 3A). Thus the adaptive response might be induced slightly at the highest radiation dose even without NNK treatments. Nevertheless, concomitant NNK treatments much clearly suppressed the occurrence of large dele-