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***Rb* and *p53* Gene Deletions in Lung Adenocarcinomas
from Irradiated and Control Mice¹**

Yueru Zhang and Gayle E. Woloschak²

Center for Mechanistic Biology and Biotechnology
Argonne National Laboratory
9700 South Cass Avenue
Argonne, IL 60439-4833

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²To whom correspondence should be addressed, at Center for Mechanistic Biology and Biotechnology, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, Illinois 60439-4833; Telephone: 708-252-3312; Fax: 708-252-3387

³The abbreviations used are dCTP, deoxycytidine triphosphate; LET, linear energy transfer; *mRb*, mouse retinoblastoma gene; β_2 -M, β_2 -microglobulin; *Rb*, retinoblastoma gene.

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ABSTRACT

This study was conducted on mouse lung adenocarcinoma tissues that were formalin-treated and paraffin-embedded 25 years ago to investigate the large gene deletions of *mRb* and *p53* in B6CF₁ male mice. A total of 80 lung tissue samples from irradiated mice and 40 lung samples from nonirradiated controls were randomly selected and examined in the *mRb* portion of this study. The results showed a significantly ($P < 0.05$) higher percentage of *mRb* deletions in lung adenocarcinomas from mice exposed to 60 once-weekly γ -ray doses than those from mice receiving 24 once-weekly γ -ray doses at low doses and low dose rates; however, the percentage was not significantly different ($P > 0.05$) from that for spontaneous lung adenocarcinomas or lung adenocarcinomas from mice exposed to single-dose γ irradiation at a similar total dose. *mRb* fragments 3 (71%) and 5 (67%), the parts of the gene that encoded the pocket binding region of Rb protein to adenovirus E1A and SV40 T-antigen, were the most frequently deleted fragments. *p53* gene deletion analysis was carried out on normal lungs and lung adenocarcinomas that were initially found to bear *mRb* deletions. Exons 1, 4, 5, 6, and 9 were chosen to be analyzed. The data showed that 30 (97%) of 31 normal lungs and lung adenocarcinomas had *p53* deletions. Exons 4 (83%) and 5 (90%) were the most frequently deleted among tested exons. Mice exposed to neutrons 60 times on a once-weekly schedule had a higher percentage of complete *p53* deletions (5/8; 63%) than those exposed to γ -rays 60 times on a once-weekly schedule (2/8; 25%). We conclude that *p53* deletions may be one of the major mutational events in the tumorigenesis of lung adenocarcinomas in the irradiated B6CF₁ mice.

INTRODUCTION

Many tumor suppressor gene products are known to play critical roles in the control of normal cell growth and the maintenance of genomic stability (1). The *p53* and retinoblastoma (*Rb*³) genes are the tumor suppressor genes that have been studied most extensively. Gene alterations, such as deletion of the entire gene or missense point mutations, are known to result in uncontrolled cell proliferation, which contributes to malignancy (2,3). Alterations in *p53* are the most common (about 50%) molecular abnormalities described to date in human neoplasias (4-6).

Retinoblastoma is a form of human childhood retinal cancer that occurs with a frequency of 1 in 20,000 newborns (7,8). There are several "Rb-gene-related" tumors, including retinoblastoma (9), osteosarcoma (10), sarcomas (11), small-cell lung carcinoma (12), non-small-cell lung cancer (13), carcinoma of the breast (14), ovarian carcinoma (15), and leukemia (16).

Wild-type *p53* is an unstable protein with a very short cellular half-life (15-30 min) so its level in normal cells is very low (11,12). In responding to the DNA damage-inducing agents such as radiation, cells accumulate *p53* protein. Elevated levels of *p53* can cause either rapid induction of G1 arrest (allowing cells to undergo repair) or apoptosis (1). Under conditions that threaten the integrity of the genome, *p53* accumulates and induces expression of a *WAF1/CIP1* gene to produce protein p21 (13-15); p21 can bind to cyclin/*cdk* and inhibit its phosphorylation function. Because phosphorylation of Rb protein is cyclin/*cdk*-dependent, increased accumulation

of p53 protein can indirectly block the Rb protein phosphorylation process by stimulating the expression of the inhibitor p21.

The association between exposures to relatively high doses (about 1 Gy) of low linear energy transfer (LET) radiation, such as γ rays, and the incidence of many solid tumors (of the lung, stomach and female breast) has been shown clearly (16). For high-LET radiation, the carcinogenic effects of radiation dose protraction are uncertain and vary by tumor type (17), which indicates an organ-specific carcinogenesis process. Ullrich (18) observed a reduced carcinogenic effect for ovarian tumors in mice exposed to fission neutrons at a low dose rate relative to high dose rates with total doses up to 40 cGy. Significant enhancement of mammary tumorigenic effects of neutrons was observed after low-dose-rate or low-dose (2.5–20 cGy) exposures. An incidence of lung tumors four times greater was evident for protracted $^{239}\text{PuO}_2$ exposure, compared with a single inhalation at similar total cumulative doses in mice (19).

The results presented here are derived from a study of 40,000 B6CF₁ hybrid mice (C57BL/6 female \times BALB/c male) conducted at Argonne National Laboratory from 1971 to 1986 on the effects of acute and chronic radiation injury (20). The experiments tested many different aspects of radiation effects, including single doses, protracted doses with daily or weekly whole-body exposure, and both ^{60}Co γ -ray and ^{235}U fission-spectrum neutron irradiation. One of the conclusions of this study was that a higher incidence of lung adenocarcinoma was found in the B6CF₁ male mice exposed to protracted neutron irradiation than in those receiving the same dose in a single exposure (20–22). Previous data from our laboratory for mice that were exposed to

single-dose-irradiation demonstrated that the 5' region of the *mRb* gene was deleted in 1 (17%) of 6 lung adenocarcinomas from mice exposed to γ -ray irradiation in 6 (33%) of 18 spontaneous lung tumors, and in none (0%) of 6 lung adenocarcinomas from neutron-irradiated mice (23). The purpose of this study was to detect large gene deletions of tumor suppressor genes (*mRb* and *p53* genes) in mice bearing lung adenocarcinomas following protracted doses of radiation. *p53* gene mutations can be both early and late events in tumorigenesis. Such mutations have been considered to be an early event in lung cancer (24).

MATERIALS AND METHODS

Paraffin-Embedded Tissues. Paraffin blocks containing normal lungs and lung adenocarcinomas from B6CF₁ hybrid mice (C57BL/6 \times BALB/c) that had been exposed to ⁶⁰Co γ -ray radiation or ²³⁵U fission-spectrum neutrons (0.85-MeV mean energy and 2.5% γ -ray contamination) were obtained. Sham-irradiated normal lung tissues and spontaneous lung adenocarcinomas were used as controls in this study. Ten lung samples were randomly selected from each category (Table 1). We gratefully thank B. J. Wright for her expert help on the sample selections.

The lung adenocarcinomas from B6CF₁ mice bearing *mRb* deletions and some normal controls were chosen for the *p53* experiments. Some of the lungs had no confirmed *mRb* deletions when the experiment was repeated; those tissues, however, were also included in the experiment.

Chemical Reagents and Equipment. The polymerase chain reaction (PCR) was performed in a DNA thermal cycler Perkin-Elmer Cetus 480 for *mRb* gene fragments and Perkin-Elmer Cetus 9600 for *p53* gene; (Norwalk, CT). All PCR reagents were purchased from Perkin-Elmer Cetus (Branchburg, NJ). Proteinase K was obtained from Sigma Chemical Co. (St. Louis, MO). Speed-hybridization solution (Bios; New Haven, CT) was used for prehybridization and hybridization reactions. *p53* primers were designed by our laboratory (T. Paunesku *et al.*, Argonne National Laboratory, unpublished information). To avoid amplification of the *p53* pseudogene in mice, half of the sequence from each primer contained intronic sequences to ensure that all of the PCR reactions were actually amplifying the functional *p53* gene, rather than the pseudogene.

Plasmids. We thank Dr. T. P. Dryja (Harvard University, Boston, MA), who provided us with *mRb* cDNA plasmid probe (25); and Dr. Jane R. Parnes (Stanford University Medical Center) for providing the β_2 -microglobulin cDNA plasmid (pBRcB-3) (26). Mouse *p53* plasmid was generously provided by Dr. A. J. Levine (Princeton University, Princeton, NJ) (27).

***mRb* cDNA Plasmid Probe Labeling.** The Nick Translation System Kit (NEK-004); DuPont, Boston, MA, was used to label the mouse *Rb* cDNA plasmid probe with [α - 32 P] deoxycytidine triphosphate (dCTP)(1 mCi/100 μ l). The probe was counted by Probe Count (Oncor, Inc., Gaithersburg, MD), and approximately $50\text{--}100 \times 10^6$ dpm was used per 500-cm² blot.

p53 cDNA probe was obtained by PCR amplification (with *p53* primer sense 1 and antisense 9) by using mouse *p53* cDNA plasmid as the DNA template, which generated a large PCR fragment (1,097 bp). Initial denaturation was at 95 °C for 5 min, followed by 30 cycles of PCR reaction: 30-min denaturation at 95 °C, 30-min annealing at 55 °C, and 1-min extension at 72 °C; the last cycle was performed at 72 °C for 7 min. This probe was labeled by using the NEN Random Primer Extension Labeling System (NEP-103L; Dupont, Boston, MA); about 50×10^6 dpm was used per 500-cm² blot.

PCR Procedures. Detection of *mRb* gene deletions was carried out by PCR, followed by Southern blot analyses. The method was adapted from previous experiments from the laboratory (23), with fragments representing different exons (1–6) of the *mRb* gene. *p53* deletions were also detected by PCR and Southern blots. The initial denaturation was done at 95 °C for 5 min, followed by 40 cycles of PCR: 20-min denaturation at 94 °C, 20-min annealing at 42 °C, and 40-min extension 65 °C; the last cycle was at 65 °C for 7 min. For all experiments, for a sample to be considered as having a deleted fragment, these repetitions had to be negative by Southern blot and by comparison to control.

β_2 -Microglobulin (β_2 -M) was used as an internal control. It is a small polypeptide (99 amino acids; 11,800 molecular weight) that is encoded by a single gene per haploid genome in the mouse. The primers were designed according to the published sequence of Parnes and Seidman (26):

Sense: 5'-GCTCACACTGAATTCACCCC-3'

Anti: 5'-CCCAGTAGACGGTCTTGGGC-3'

The use of these primer pairs generates a 91-bp fragment, which can be hybridized with [α - 32 P]dCTP-labeled β_2 -M cDNA clone pBRcB-3 probe (labeled by nick translation). The PCR amplification of the β_2 -M gene fragment was carried out simultaneously with *mRb* fragments or *p53* exons under the same PCR conditions. All samples failing to amplify β_2 -M were deleted from the study because the DNA likely was not sufficiently intact for PCR analyses.

Statistical Analyses. The statistical analyses for the data were done by one-tailed "Fisher's exact test" (2×2 contingency tables; SigmaStat, version 2.0). The critical value (α) was 0.05 (5% significance level).

RESULTS

mRb. Usually, the amount of PCR product amplified from the DNA of paraffin-embedded samples was not sufficient to be seen directly under UV light with ethidium bromide staining of the agarose gel. For this reason, Southern blots were chosen to detect the presence or absence of *mRb* bands. Figure 1 is a typical Southern blot for detection of *mRb* deletions. All of the bands are the PCR products from normal lungs of mice exposed to 60 once-weekly γ irradiations. Deletions were scored by monitoring the intensity of each band. Those bands with an intensity of 50% or less relative to other bands from the same tumor sample were considered deleted. Figure 1 shows a complete *mRb* deletion in sample S0473; partial *mRb* deletions in sample S0897, with fragments 2-6 being deleted, and in sample S1840, with fragments 3, 5, and 6 being deleted; and no deletion in sample S0214. The internal control gene,

β_2 -microglobulin (β_2 -M) gene, the β_2 -M gene, was amplified and detected by a similar procedure (Figure 2).

Table 2 summarizes the *mRb* deletion data from each tested category. A significantly higher percentage of *mRb* deletion was found in lung adenocarcinomas from mice exposed to 60 once-weekly γ irradiations (4/10; 40%), compared with those of mice exposed to 24 once-weekly γ irradiations (0%) ($P = 0.043 < \alpha = 0.05$; Fisher's exact test). The results with 60 once-weekly γ irradiations were not significantly different in the *mRb* deletion percentage from that in spontaneous lung adenocarcinomas (5/20; 25%; $P = 0.431 > \alpha = 0.05$). Notably, both total dose and dose rate from 60 once-weekly γ -ray exposures (600 cGy; 0.5 cGy/min) were higher than those from 24 once-weekly γ -ray exposures (417 cGy; 0.386 cGy/min) (Table 1). This could be a factor causing a higher percentage of *mRb* deletions in the lungs of mice exposed to the 60 once-weekly γ -ray protocol. The percentage of *mRb* deletion in lung adenocarcinomas from mice exposed to once-weekly doses of neutron irradiation (3/10; 30%) was not significantly different compared with those of mice exposed to single-dose (0/6; 0%) ($P = 0.250$) or 24 once-weekly (0/10; 0%) ($P = 0.105$) neutron irradiations or compared with spontaneous lung adenocarcinomas (5/20; 25%) ($P = 1.000$).

For the irradiated normal lungs, no significantly different dose-rate effects were observed with regard to *mRb* deletion in normal lungs from mice exposed to protracted doses of radiation compared with single-dose irradiation with neutrons or γ rays. The percentages of *mRb* deletions in normal lungs from mice exposed to 60 once-weekly neutron irradiations (3/10; 30%) were not

significantly different from the normal lungs of mice exposed to single-dose neutron irradiation (0/6, 0%; $P = 0.250$). The same results were also found in normal lungs from mice exposed to 60 once-weekly γ irradiation (3/10; 30%) when comparing the percentages of *mRb* deletion in normal lungs from mice exposed to single-dose γ irradiation (0%) ($P = 0.250$) or 24 once-weekly γ irradiations (0%) ($P = 0.105$).

Surprisingly, no *mRb* deletions were observed in the lung adenocarcinomas of mice receiving 24 once-weekly dose of γ rays or neutrons. The fact that these mice did not show at least the same *mRb* deletion frequency as spontaneous lung adenocarcinomas suggests that the tumorigenesis of lung adenocarcinomas from irradiated mice may involve a different mechanism.

Table 3 shows the incidence of the deleted *mRb* fragments from each sample. The data demonstrate that γ irradiation tends to cause a wider range of *mRb* deletions (3/7 [43%] with complete *mRb* deletion) than neutron irradiation (1/8 [13%] with complete *mRb* deletion). Although the *mRb* deletion pattern looks random throughout the gene, the deletions occurred most frequently in *mRb* fragments 3 (71%) and 5 (67%) and least frequently in *mRb* fragment 6 (38%).

p53. Experiments were designed to examine large *p53* gene deletions in irradiated normal lungs and lung adenocarcinomas from B6CF₁ mice that were initially found to have deleted *mRb* fragments. Because later repeat experiments did not confirm some of the initial *mRb* deletions observed, those samples with no *mRb* deletions were also analyzed for *p53* deletions.

Meanwhile, four normal lungs from sham-irradiated controls were randomly chosen for the *p53* deletion study (Table 2).

An autoradiograph of a Southern blot to detect *p53* deletions is shown in Figure 3. The method used for scoring deletions was the same as that for the *mRb* gene. For example, S2123, a sample of normal lung from a mouse exposed to 60 once-weekly neutron irradiations had *p53* deletions in exon 4 (204 bp), exon 5 (133 bp), and exon 6 (116 bp) and no deletions in exon 1 (103 bp) or exon 9 (120 bp). Sample S2363 from a spontaneous lung tumor had *p53* gene deletions in exons 4 and 5 and no deletions in exons 1, 6, or 9. To ensure that all of the exons were capable of being amplified with the *p53* primer pairs in this PCR reaction, B6CF₁ mouse DNA purified from normal fresh kidney tissue was simultaneously amplified. To control for the integrity of the DNA from each paraffin embedded sample, β_2 -M fragments (91 bp) were also amplified from all samples.

Deletion data on exons 1, 4, 5, 6, and 9 of the *p53* gene (generated as in Figure 3) from all tested samples were collected and listed in Table 3. First, *p53* deletions were detected in 30 (97%) of 31 tested normal lungs and lung adenocarcinomas. Only one (sample S1840, normal lung tissue exposed to 60 once-weekly γ -ray doses) did not show any deletion in the tested exons. Secondly, 10 (32%) of 31 had complete deletions of all exons tested; 7 (70%) of the 10 were from mice that had been irradiated by 60 once-weekly γ -ray doses (2/8; 25%) or by 60 once-weekly neutron irradiation (5/8; 63%). For the remaining three lungs that had complete *p53* deletions, one (sample S0835) was from a sham-irradiated normal lung, and two (samples C3495 and S0762) were from spontaneous lung adenocarcinomas. Third, the most frequently deleted

exons were exon 4 (83%) and exon 5 (90%). Exon 9 (43%) was the least likely to be deleted among all exons studied. Finally, all samples that had no *mRb* deletion showed partial or complete *p53* gene deletions. Only sample S1840, the normal lung of a mouse receiving 60 once-weekly γ -ray doses had *mRb* deletion (fragments 3, 5, and 6) with the *p53* gene intact.

DISCUSSION

Dose Rate Effects: Single Dose vs. Protracted Doses. The original pathologic data from protracted high-LET neutron irradiation demonstrated an "inverse dose-rate effect" for the incidence of lung adenocarcinoma in B6CF₁ male mice (22). This means that the number of days of life lost per centigray of dose was greater in animals exposed to protracted doses of neutrons than in those receiving the same total dose in a single exposure. In fact, many reports about this phenomenon are found in the literature (28–30).

A significant difference in the deletion percentage of the *mRb* gene was found for lung adenocarcinomas from mice receiving 60 once-weekly γ -ray doses (40%) when compared to those receiving 24 once-weekly γ -ray doses (0%; $P < 0.05$); however, the percentage in those receiving 60 once-weekly γ irradiation was not significantly different from that of mice that had received single-dose γ -ray exposure (17%), nor from that in spontaneous lung adenocarcinomas (25%; $P > 0.05$) (Table 2). This significant difference could be attributed to the higher total dose and dose rate (600 cGy; 0.5 cGy/min) in the 60 once-weekly γ -ray doses than the total dose and dose rate (417 cGy; 0.368 cGy/min) in the 24 once-weekly γ -ray exposures. According to Grahn *et*

al. (22), the incidence of spontaneous lung adenocarcinomas in B6CF₁ male mice was very high (first occurrence around 200 days of age, and a frequency of 53% in 800-day-old control males), but the prevalence of lung adenocarcinoma was even greater in irradiated mice. Thus, some of the *mRb* gene deletions may be the result of spontaneous alterations.

On the contrary, the normal lungs of mice receiving protracted doses of neutrons did not have statistically significant ($P > 0.05$) *mRb* deletion frequencies (20% in 24 once-weekly neutron irradiation; 30% in 60 once-weekly neutron irradiation), compared with those of mice exposed to single-dose neutron irradiation (0%) (Table 2). The experiments were designed so that normal lungs of mice receiving 24 once-weekly neutron doses were compared with those of mice receiving single-dose neutron exposure at the same total dose (60 cGy), so the differences in *mRb* deletion frequency may be attributed to dose protraction. Notably, normal lungs of mice receiving 60 once-weekly neutron doses had a lower total dose (40 cGy) than the 24 once-weekly neutron or the single-dose neutron exposures but have shown the highest *mRb* deletion frequencies (30%) among these three groups.

The histopathologic data (Table 3) also revealed the possible involvement of lymphoma in the lungs. For example, sample S0835, a normal lung from a sham-irradiated control, indicated a frequency (1/10; 10%) of *mRb* deletion that could be caused by the presence of undetected lymphoma in the lung. Similarly, no *mRb* deletions were observed in the normal lungs from mice exposed to a single-dose γ irradiation or to 24 once-weekly irradiation, while 30% of the normal lungs of mice receiving 60 once-weekly γ irradiation showed *mRb* deletions.

However, the percentage in mice receiving 60 once-weekly γ irradiation was not significantly different compared with normal lungs exposed to single-dose γ irradiation or 24 once-weekly γ irradiation.

Radiation Quality: γ -ray vs. Neutron Radiation. The data from Table 3 demonstrate a difference in the *mRb* deletion spectrum in mice receiving different qualities of radiation. Comparisons of the percentages of complete deletions of *mRb* genes in normal lungs and lung adenocarcinomas from mice receiving protracted γ rays (3/7; 43%) with those receiving protracted neutrons (1/8; 13%) have shown that protracted γ -ray doses tend to produce a higher percentage of *mRb* deletions than those of protracted neutrons. This fact is surprising because high-LET neutron radiation is commonly reported to induce more large gene deletions than low-LET γ rays (31). Estimates of DNA damage by different qualities of radiation have revealed that a single γ -ray track produced an average of one single-strand break (ssb), but high-LET α particles produced 200 ssb. γ -Rays produced 0.04 double-strand breaks (dsb), and an α particle produced 35 dsb (a neutron should be between these two values) (32). High-LET radiation can deposit very large numbers of ionizations within a short stretches of DNA, inducing qualitatively different types of damage than those commonly found from low-LET radiation (33). The damage caused by high-LET radiation is often very great and much more difficult to repair than that from low-LET radiation (31,34); however, there are exceptions for a few genes, such as *hprt* gene mutations in hamster cells, in which no differences in the high- vs. low-LET mutation spectrum were observed (35,36).

Unlike previous data on lung adenocarcinomas following single-dose irradiations (23) that showed that all *mRb* deletions were at the 5' end of the gene (fragments 1-3), our data demonstrate deletions throughout the whole gene, with the highest deletion frequencies in fragments 3 (71%) and 5 (67%) and the lowest in fragment 6 (38%). According to Kaelin *et al.* (37), the pocket binding region of the *Rb* gene with adenovirus E1A and SV40 T-antigen was at amino acids (a.a.) sequences 379-928 and 379-792, respectively. Based on *mRb* gene sequencing data (25), PCR fragment 3 (a.a. 469-494), fragment 4 (a.a. 635-689), and fragment 5 (a.a. 795-831) were included in the *Rb* protein pocket binding region (38). Their deletion would ultimately influence the *mRb* protein pocket binding function. The function of the amino acid region covered by *mRb* fragments 1 and 2 is currently unknown; fragment 6, which has the lowest deletion frequency, is in the 3' nontranslated region.

***Rb* Deletion in Mouse Lung Tumorigenesis.** What role does *mRb* deletion play during the process of lung tumorigenesis in irradiated B6CF₁ mice? By comparing the *mRb* deletion frequency in spontaneous lung adenocarcinomas (25%) to the frequencies in lung adenocarcinomas of mice receiving 60 once-weekly neutron (30%) or 60 once-weekly γ irradiation (40%), our data suggest that radiation caused no significant increase of *mRb* deletions in the lung adenocarcinomas of B6CF₁ male mice. This finding agrees with a transgenic study done by Jacks *et al.* (39), which found that heterozygous (*Rb*^{+/-}) mice were not predisposed to large number of tumors; only a limited tumor incidence (such as pituitary tumor) was observed in the mice that lost heterozygosity.

On the other hand, both heterozygotes and homozygotes for the null allele of the *p53* gene had a very high rate of tumor incidence (40). Several recent studies have shown the cooperative relationship between the *Rb* gene and the *p53* gene in tumorigenesis (41–43). Harvey *et al.* (41) demonstrated that mice deficient in both *Rb* and *p53* showed accelerated tumorigenesis, especially in endocrine tumor types, compared with mice deficient only in *Rb* or *p53*. Hiyama *et al.* (44) showed that 16 (27%) out of 60 primary lung tumors had telomere length alterations, and 10 (63%) of 16 had allelic loss of both the *p53* and *Rb* genes. Therefore, the increased incidence of lung adenocarcinoma incidence in irradiated B6CF₁ male mice may be due to other mechanisms, such as *p53* alteration or some oncogene activation. *mRb* gene deletion may play a role by combining with *p53* alterations to accelerate lung tumorigenesis.

***p53* Deletion.** Our data have demonstrated a high frequency of *p53* gene deletions in irradiated lungs and lung adenocarcinomas of B6CF₁ male mice (Table 2). Among tested samples, 10 (32%) of 31 had complete gene deletions. Exons 4 (83%) and 5 (90%) were the most frequently deleted among the tested exons. Many studies have shown the mutation type of *p53* in tumors is predominantly a missense mutation (80%) (G:C to T:A transversions in human lung tumor) at the "hot spots" of the *p53* central core region (45), which is encoded by exons 5–8, and is responsible for sequence-specific DNA binding. Any missense mutation or deletion in this region can cause either a change of protein structure or the loss of the entire gene product, which results in the complete loss of tumor suppression function of the *p53* gene (45). A report has been published about strain-specific nucleotide polymorphism in codon 234 (in exon 6) of the *p53* protein as Ile in the C57BL/6 mouse strain and Met in the BALB/c mouse strain (46).

More recent work from our laboratory has demonstrated that the reported Ile on codon 234 in the C57BL/6 mouse is actually an oncogenic change associated with the transformed SVT2 cell line (70). Thus, the core domain of p53 was evolutionarily conserved among the species.

While a point mutation was the predominant type of *p53* alteration, our data demonstrate that the major large gene deletion in *p53* occurs within its central core domain. In fact, many investigators have reported *p53* deletions in both human and mouse tumors, such as urothelial tumors, bladder cancer, hepatocellular carcinomas, and mouse skin and bone tumors (47-49). Human lung tumors had a high frequency of *p53* mutations in small-cell lung cancer (70% and, non small-cell lung cancer (47%, including 33% in adenocarcinomas) (29,50).

Differences in radiation quality were also observed from the *p53* deletion data (Table 3). All of the complete deletions of the *p53* gene occurred in the normal lungs and lung adenocarcinomas from mice exposed to 60 once-weekly γ or 60 once-weekly neutron irradiations. Among these mice, the ones exposed to 60 once-weekly neutron irradiations (5/8; 63%) had a higher percentage of complete deletions than did mice exposed to 60 once-weekly γ irradiations (2/8; 25%) or spontaneous lung adenocarcinomas (2/6; 33%). Similarly, spontaneous lung adenocarcinomas had both complete (2/6; 33%) and partial (4/6; 67%) *p53* deletion. Among these mice, exon 4 (4/6; 67%), exon 5 (6/6; 100%), and exon 6 (4/6; 67%) were the most frequently deleted exons.

Many studies in the literature show *p53* deletions following radiation exposure. For example, Boice *et al.* (51) observed an increased incidence of breast cancer following diagnostic or therapeutic radiation. In another study using fractionated γ -ray irradiation at a clinically used dose (total dose of 30 Gy), the normal human mammary epithelial cell was transformed, and those transformed cells showed a complete lack of *p53* protein, deletion of one allele of the *p53* gene, and a 26-bp deletion in the third intron of the other allele (52). Ootsuyama *et al.* (53) reported that 20 (31%) out of 65 tumors had *p53* mutations; 11 (55%) of the 20 were minute deletions in the cDNA of *p53* from repeated ^{90}Sr - ^{90}Y β -irradiated mouse skin and bone tumors. In contrast to our findings, Thraves *et al.* (54) showed that cellular *p53* (exons 4–9) and *ras* gene mutations were not involved in the transformation of immortalized human epithelial cells to a malignant phenotype by single-dose fission-neutron irradiation. Because our study used tissues from mice exposed to protracted doses, the *p53* deletion frequency could be expected to be different when compared with that of lungs from mice exposed to single-dose irradiation.

Limitations of the Assays. The reason for using Southern blot analyses in these experiments was the difficulty of obtaining an adequate amount of PCR products from the paraffin blocks for direct gel analyses. Combining PCR with Southern blotting made it possible to observe rather easily the limited amount of *mRb* gene PCR product through autoradiography.

Nevertheless, some limitations in the *mRb* deletion detection assay, are unavoidable as indicated by Churchill *et al.* (23). In particular, this assay only detected part of the whole *mRb*

gene; the information about introns and the regions between those fragments were not included in this study.

Our *p53* assay also has certain limitations: (1) The studies of large *p53* deletions were performed only on lungs and lung adenocarcinomas that were initially found to be bearing *mRb* deletions. We did not carry out a study of *p53* deletion independent of *mRb* deletions. (2) Only a few of the sham-irradiated normal lungs were included in this test; all were found to have *p53* deletions (Table 2). (3) Other exons of the *p53* gene (exons 2, 3, 7, 8, 10, and 11) have not been included in our experiments. (4) The introns of *p53* gene have not been tested.

Despite the limitations in our assays of large gene deletions, our data demonstrated that (1) the *p53* gene was frequently deleted in this group of mice; (2) protracted doses of neutrons or γ rays have not demonstrated statistically significant differences in *mRb* deletion compared with single-dose exposures; (3) complete *p53* deletions were found in highly protracted doses in 60 once-weekly treatments, especially from neutrons. To further investigate the effects of dose, dose rate, and radiation quality on *p53* deletion in B6CF₁ mice, the remaining lung tissues need to be tested.

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Table 1 *Radiation methods*^a

Radiation	Dosage regimen	Total dose (cGy)	Dose rate (cGy/min)	Weekly dose (cGy/wk)	Text symbol
Neutrons	Single dose	60	3.000	3.000	1×1,n
	24 once-weekly	60	0.056	2.500	24×1,n
	60 once-weekly	40	0.033	0.667	60×1,n
γ rays	Single dose	569	28.45	28.45	1×1,γ
	24 once-weekly	417	0.386	17.375	24×1,γ
	60 once-weekly	600	0.500	10.000	60×1,γ

^aData from Grahn *et al.* (20).

Table 2 Incidence of partial or total mRb deletion in mouse lung adenocarcinoma

Dosage regimen	Normal lung (sham-irradiated)	Spontaneous lung tumor	Normal lung (n)	Lung tumor (n)	Normal lung (γ)	Lung tumor (γ)
single dose ^a	0/6 (0%)	6/18 (33%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	1/6 (17%)
24x1	0/10 (0%)	5/20 (25%)	2/10 (20%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
60x1	1/10 (10%)	—	3/10 ^b (30%)	3/10 ^c (30%)	3/10 ^d (30%)	4/10 ^e (40%)

^a Data from Churchill *et al.* (23).

^b Not significantly different from normal lungs receiving 60x1 sham-irradiation ($P = 0.582 > \alpha = 0.05$; Fisher's exact test) or 1x1, n irradiation ($P = 0.250$) or 24x1, n irradiation ($P = 1.000$).

^c Not significantly different from lung adenocarcinomas of mice exposed to 1x1, n irradiation ($P = 0.250$), 24x1, n irradiation ($P = 0.105$), or spontaneous lung adenocarcinomas ($P = 1.000$).

^d Not significantly different from normal lungs of mice exposed to 1x1, γ ($P = 0.250$) OR 24x1, γ irradiation ($P = 0.105$).

^e Significantly different from lung adenocarcinomas of mice exposed to 24x1, γ irradiation ($P = 0.043 < \alpha = 0.05$) but not significantly different from spontaneous lung adenocarcinomas ($P = 0.431$).

Table 3 *p53* and *mRb* tumor suppressor gene deletions in B6CF₁ mouse lung adenocarcinoma^a

Paraffin blocks	Cause of Death ^b	Radiation/ treatment	Exons of <i>mRb</i> deleted	Exons of <i>p53</i> deleted
BCF1 (+ control)			None	None
S0835 (N)	Lymphoma	0	1,2,3,4,6	1,4,5,6,9
C2462 (N)	Angiosarcoma	0	None	1,4,6
S0644 (N)	Lymphoma	0	None	4,5
S2379 (N)	Acute pancreatitis	0	None	1,4,5
C1343 (T)	Lung adenocarcinoma	24×1,γ	None	1,4,5,9
S0214 (N)	Lymphoma	60×1,γ	None	4,5
S0473 (N)	Fibrosarcoma	60×1,γ	1,2,3,4,5,6	1,5
S0897 (N)	Lymphocytic- lymphoblastic lymphoma	60×1,γ	2,3,4,5,6	4,5,6
S1840 (N)	Lymphocytic- lymphoblastic lymphoma	60×1,γ	3,5,6	None
S1134 (T)	Lung adenocarcinoma	60×1,γ	2,3	1,5,6
S1406 (T)	Lung adenocarcinoma	60×1,γ	1,2,3,4,5,6	1,4,5,6,9
S1458 (T)	Lung adenocarcinoma	60×1,γ	1,2,3,4,5,6	1,4,5,6,9
S2109 (T)	Lung adenocarcinoma	60×1,γ	3	1,4,5,9
C3576 (T)	Lung adenocarcinoma	Spontaneous	3	5
C4492 (T)	Lung adenocarcinoma	Spontaneous	5	1,4,5,6
C3495 (T)	Lung adenocarcinoma	Spontaneous	3,4,5	1,4,5,6,9
S0762 (T)	Lung adenocarcinoma	Spontaneous	1,2,3,4,5,6	1,4,5,6,9
S1591 (T)	Lung adenocarcinoma	Spontaneous	None	5

S2123 (T)	Lung adenocarcinoma	Spontaneous	None	4,5,6
S1229 (T)	Lung adenocarcinoma	Spontaneous	5	N/A
C0591 (N)	Lymphocytic-lymphoblastic lymphoma	24×1,n	None	1,4,5,9
C3176 (N)	Vascular tumors	24×1,n	1,3	1,4,5,6
C4260 (N)	Lymphoblastic-lymphoma	24×1,n	1	4,6
C1515 (T)	Lung adenocarcinoma	24×1,n	None	4,6
S0239 (N)	Angiosarcoma	60×1,n	2,4,5	4,5
S0584 (N)	Lymphocytic-lymphoblastic lymphoma	60×1,n	1,2,3,4,5,6	1,4,5,6,9
S2051 (N)	Lymphocytic-lymphoblastic lymphoma	60×1,n	None	5
S1166 (N)	Lymphocytic-lymphoblastic lymphoma	60×1,n	3,4,5	1,4,5,6,9
S0550 (T)	Lung adenocarcinoma	60×1,n	2,5	1,4,5,6,9
S0263 (T)	Lung adenocarcinoma	60×1,n	1,2,3,4,5	1,4,5,6,9
S0945 (T)	Lung adenocarcinoma	60×1,n	1,2	1,4,5,6,9
S2363 (N)	Lymphocytic-lymphoblastic lymphoma	60×1,n	None	4,5
Total number (deleted)			21	30
Deletion frequency (%)			F1: 10/21 (48%) F2: 12/21 (57%) F3: 15/21 (71%) F4: 11/21 (52%) F5: 14/21 (67%) F6: 8/21 (38%)	Exon 1: 19/30 (63%) Exon 4: 25/30 (83%) Exon 5: 27/30 (90%) Exon 6: 18/30 (60%) Exon 9: 13/30 (43%)

^aT, mouse lung adenocarcinoma; N, normal mouse lung; None, no detectable deletion; N/A, not available.

^bData from Grahn *et al.* (20).

FIGURE LEGENDS

Fig. 1. Typical Southern blot for *mRb* deletion detection from 25-year-old paraffin-embedded mouse lung tissues. All of the bands were the PCR products that which hybridized with [α - 32 P]dCTP p53 plasmid probe. Samples in this picture are normal lung tissues exposed to 60 once-weekly 60 Co γ -ray doses. F1–F6 indicate the six different *mRb* gene fragments from PCR amplifications. F4 (St.) is the standard fragment 4 with a known amount of DNA. S0214 shows a typical pattern of six *mRb* gene fragments; sample S0473 shows a complete *mRb* gene deletion; and the *mRb* gene in samples S0897 (fragments 2–6), and S1840 (fragments 3, 5, and 6) is partially deleted.

Fig. 2. Southern blot showing PCR products of β_2 -M fragment (91 bp). [α - 32 P]dCTP radiolabeled β_2 -M probe was hybridized to the fragment of β_2 -M gene from each tested mouse lung and lung adenocarcinoma. Lane 1: β_2 -M fragment from normal kidney of B6CF₁ mouse; lane 2: β_2 -M fragment amplified from β_2 -M plasmid; lanes 3–10: β_2 -M fragments amplified from lung adenocarcinomas from mice exposed to 24 once-weekly 60 Co γ -ray doses (i.e., C2309, C2025, C1788, C1570, C1343, C1314, C1021, and C0310, respectively).

Fig. 3. Southern blot showing *p53* gene deletions in *mRb*-deleted lung from B6CF₁ mouse adenocarcinoma. Random radiolabeled *p53* probe was hybridized to *p53* exons 1, 4, 5, 6, and 9, which were amplified from each mouse lung and lung adenocarcinoma. B6CF₁ is control of

p53 primers. Sample S0644 (exons 4 and 5 deleted) is normal lung with sham-irradiation. Sample S1591 (exon 5 deleted) and S2123 (exons 4, 5, and 6 deleted) were spontaneous lung adenocarcinomas. Samples S2051 (exon 5 deleted) and S2363 (exons 4 and 5 deleted) were normal lungs exposed to 60 once-weekly neutron irradiations.



F₁ F₂ F₃ F₄ F₅ F₆ F₁ F₂ F₃ F₄ F₅ F₆ F₁ F₂ F₃ F₄ F₅ F₆ F₁ F₂ F₃ F₄ F₅ F₆ F₄ St.

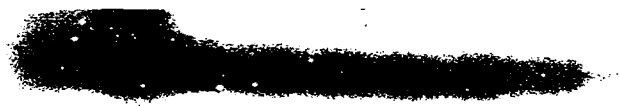
S0214 S0328 S0473 S0897



F₁ F₂ F₃ F₄ F₅ F₆ F₁ F₂ F₃ F₄ F₅ F₆ F₁ F₂ F₃ F₄ F₅ F₆ F₁ F₂ F₃ F₄ F₅ F₆ F₆

S1198 S1294 S1560 S1840 C1343

Figure 1. Zhang and Woloschak



1 2 3 4 5 6 7 8 9 10

Figure 2. Zhang and Woloschak



Figure 3. Zhang and Woloschak