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# Radon, tobacco-specific nitrosamine and mutagenesis in mammalian cells

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**Abstract**

The mutagenicity of 4-methylnitrosamine-1-3-pyridyl-1-butanone (NNK), either alone or in combination with low dose alpha particle irradiation, was examined using the human–hamster hybrid ($A_L$) cell assay. NNK induced a dose-dependent toxicity in $A_L$ cells. In combination with a 25 cGy dose of alpha particles, the induced survival fraction fell within the statistical range of the calculated values assuming an additive interaction of the two agents. In addition, NNK is mutagenic in $A_L$ cells at the *CD59* locus. Furthermore, a low dose of NNK, when combined with radon alpha particles, resulted in a combined mutagenic effect in $A_L$ cells that was consistent with an additive model but less than additive at higher NNK concentrations. The majority of NNK induced *CD59*$^-$ mutants (77.6%) lost at least one additional marker in addition to the *CD59* which encodes the cell surface antigen. When combined with alpha particles, the proportion of mutants with additional marker loss increased with increasing dose of NNK. Our study further confirms that NNK is mutagenic in mammalian cells, induces mostly deletions, and provides an in vitro assessment of the combined genotoxic effects of NNK and alpha particles at low environmentally relevant doses. This finding should be helpful in understanding the molecular mechanism of the mutagenic process as a result of multi-agent interaction. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* NNK; Alpha particle; $A_L$ cell; *CD59*; PCR

## 1. Introduction

It has been recognized for more than four decades that tobacco smoking is causally associated with several types of human cancer such as lung, oral cavity, and esophageal cancer. Cigarette smoke is a mixture of about 3800 chemical substances containing at least 40 known human carcinogens [1]. Studies have indicated that 4-methylnitrosamine-1-3-pyridyl-1-butanone (NNK) is the most carcinogenic among tobacco-specific nitrosamines, and there is approximately 80–770 ng NNK per cigarette, depending on the type of tobacco [2]. Although previous studies have shown that NNK is carcinogenic in mice, rats, and hamsters [3], little information is available re-

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garding the clastogenic effects of tobacco-specific nitrosamines in mammalian cell cultures.

Radon, a secondary decay product of uranium-238, is a colorless, odorless gas that decays with a half-life of 3.82 days into a series of solid, short-lived radionucleosides, including polonium-218 and polonium-214 that emit alpha particles during decay. Radon is ubiquitous in indoor environments, including homes and schools and, in general, at concentrations hundreds of fold lower than in underground mines. Residential exposure to radioactive radon and its decay products has been estimated to account for 10%–12% of all lung cancer deaths in the United States [4]. Epidemiological studies have shown that uranium miners exposed to high levels of radon progeny have the largest incidence of radiation-induced lung cancers of any exposed population [5].

Assessment of the carcinogenic and mutagenic effects of two or more environmental agents in combination is an important health issue, as the risk from joint exposure may be substantially higher than predicted from the sum of the individual agents. While tobacco smoke remains the single most important compounding factor in lung cancer incidence among uranium miners, analysis of the current epidemiological data cannot formulate a definitive interaction model between smoking and radon exposure.

Mutation may play a causal role in cancer induction either by activating silent oncogene(s) or by eliminating the activity of tumor suppressor gene(s). While the first process can be mediated by point mutations, the latter can also be brought about by multilocus deletions. In order to understand the underlying mechanisms of mutagenesis, it is necessary to analyze the molecular pattern of mutations. In this paper, we report our findings on mutagenesis in human–hamster hybrid ($A_L$) cells treated with graded doses of NNK either alone or in combination with a single 25 cGy dose of alpha particles. We further examine the molecular pattern using multiplex PCR. Our data indicate that NNK induces mostly deletion mutations at moderately high doses in mammalian cells. In combination with a 25 cGy dose of alpha particles, NNK at low non-cytotoxic doses induces a mutagenic yield that is consistent with an additive interaction. However, at a higher dose of NNK, the combined mutagenic yield is less than additive.

## 2. Materials and methods

### 2.1. Cell culture

The $A_L$ hybrid cells that contain a standard set of Chinese hamster ovary-K1 chromosomes and a single copy of human chromosome-11 were used. Chromosome-11 encodes cells surface markers that render $A_L$ cells sensitive to killing by a special monoclonal antibody in the presence of complement. Rabbit serum complement was from HPR (Denver, PA). Antibody specific to the *CD59* (*S1*) antigen was produced from hybridoma culture as described [6,7]. Cells were maintained in Ham's F-12 medium supplemented with 8% heat-inactivated fetal bovine serum, 25 μg/ml gentamycin, and 2× normal glycine ($2 \times 10^{-4}$ M) at 37°C, in a humidified 5% $CO_2$ incubator, and were passaged as described [8–10].

### 2.2. Toxicity studies with NNK and alpha particles

A stock solution of NNK (Midwest Research Institute, Kansas City, MO) at 100 mg/ml was prepared in dimethyl sulfoxide (DMSO). Working concentrations were prepared by diluting the stock with complete F-12 medium. Exponentially growing cultures of $A_L$ cells were treated with NNK in the presence of S9 (ICN Biomedicals, Lisle, IL) for either 1 day or 7 days followed by irradiation with a 25 cGy dose of $^4$He ions (150 keV/μm). These high energy particles have a LET value comparable to the alpha particles emitted by radon progenies. The $^4$He ions were accelerated using a 4 MeV van de Graff Accelerator at the Radiological Research Accelerator Facility as described previously [11,12]. A single 25 cGy dose of $^4$He ions was chosen for the study since it corresponded to an average of one particle traversal per nucleus based on microdosimetric analysis [13]. The dose of S9 used in this study (184 μg/ml) was non-lethal, non-mutagenic, and capable of metabolically activating benzo(*a*)pyrene in $A_L$ cells based on our preliminary experiments (data not shown). After treatment, cultures were washed twice with balanced salt solution, trypsinized to remove them from the culture flasks or mylar dishes, and replated into 100-mm diameter petri dishes for colony formation. Cultures were incubated for 7–8 days, at

which time they were fixed with formaldehyde and stained with Giemsa. The number of colonies was counted to determine the surviving fraction as described [8,10].

### 2.3. Quantification of mutations at the CD59 (S1) locus

After treatment, cultures were replated into T75 flasks and cultured for 7 days. This expression period was needed to permit surviving cells to recover from the temporary growth lag caused by NNK with or without alpha particles and to multiply sufficiently so that the progeny of the mutated cells were no longer expressing lethal amounts of the *CD59* surfaced antigen. To determine mutant fractions, aliquots containing $5 \times 10^4$ cells per dish were plated into six 60-mm dishes in a total of 2 ml of growth medium as described [8–10]. The cultures were incubated for 2 h to allow for cell attachment, after which 0.3% *CD59* antiserum and 1.5% (vol/vol) freshly thawed complement were added to each dish. After overnight incubation, this medium was removed, and the cultures were further incubated in standard growth medium for 7–8 days. At this time, the cells were fixed and stained, and the number of *CD59*$^-$ mutant colonies was scored. Controls included identical sets of dishes containing antiserum alone, complement alone, or neither agent. The cultures derived from each treatment dose were tested for mutant yield for two consecutive weeks to ensure full expression of the mutations. The mutant fraction at each dose (Mf) was calculated as the number of surviving colonies divided by the total number of cells plated after correction for any non-specific killing due to complement alone. The mutant yield (My) is the slope of the dose–response curve and is independent of the background mutant level.

### 2.4. Analysis of mutant spectrum by multiplex PCR

Cloning of *CD59*$^-$ mutants and PCR analysis were performed as described previously [9,10]. Briefly, independently derived colonies from each treatment groups including controls from each experiment were isolated by cloning and expanded in cultures, and DNA was extracted using a salt-out method described by Miller et al. [14]. To ensure their clonal origin, either a single colony or, at times, two well-separated colonies per culture dish were isolated.

For *CD59*$^-$ mutant analysis, five DNA marker genes on chromosome-11 (Wilms' tumor, parathyroid hormone, catalase, *RAS*, and apolipoprotein A-1) were chosen for multiplex PCR analysis because of their mapping positions relative to the *CD59* gene, which encodes the *CD59* antigen (Refs. [6,7,15], Fig. 1), and the availability of PCR primers for the coding regions of these genes [16–18]. PCR amplifications were performed for 30 cycles using a DNA thermal cycler model 480 (Perkin-Elmer/Cetus) in

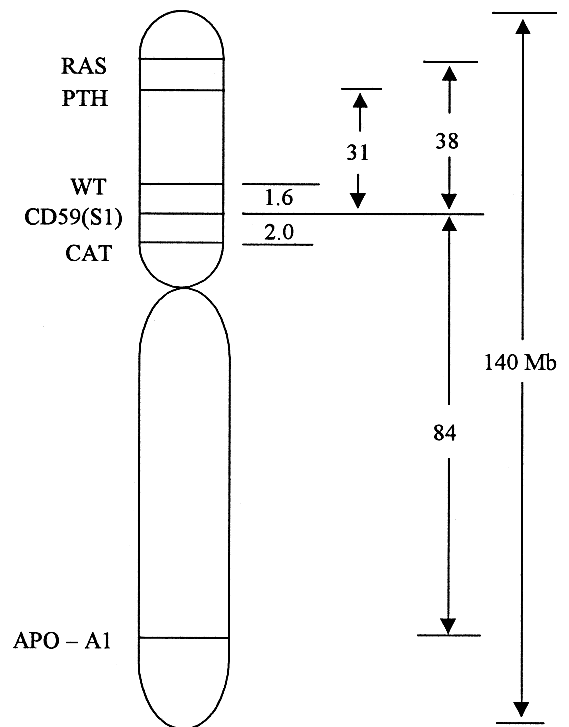


Fig. 1. Diagram of human chromosome-11 showing the *CD59* gene used in defining the *CD59*$^-$ phenotype and the relative positions of other markers used in the multiplex PCR analysis to determine the extent of the *CD59* mutations. The *CD59* gene maps to 11p13.5. The two nearest markers flanking *CD59*, *CAT* and *WT* are separated by approximately 3.6 megabase pairs (Mbp) so that the *CD59*$^-$ mutants that retained these neighboring markers could result from a base change to deletions as large as 3.6 Mbp.

20 μl reaction mixtures containing 0.2 μg of the *Eco*RI-digested DNA sample in 1 × Stoffel fragment buffer, all four dNTPs (each at 0.2 mM), 3 mM $MgCl_2$, 0.2 mM each primer, and 2 units of Stoffel fragment enzyme [8,12]. Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. After the last cycle, the samples were incubated at 72°C for an additional 20 min, electrophoresed on 3% agarose gels, and stained with ethidium bromide.

### 2.5. Statistical analysis

The survival and mutagenic data for either alpha particles or NNK alone were compared with those obtained using the two carcinogens concomitantly. The mode of interaction, either additive, super-additive, or antagonistic, was assessed as a function of effect level at a confidence level of ±1 SD as described previously [13,19].

All numerical data were calculated as mean and SD, comparisons of survival fractions and induced mutation frequencies between treated groups and controls were made by Student's *t*-test. A *p*-value of 0.05 or less between groups was considered to be significance of the differences.

## 3. Results

### 3.1. Toxicity of NNK and alpha particles in $A_L$ cells

NNK induced a dose-dependent toxicity in $A_L$ cells, as shown in Fig. 2, where the survival fractions after either a 1- or 7-day continuous exposure in the presence of microsomal S9 fraction are plotted against drug concentration, the survival data fit well to a log–linear curve. NNK treatment of $A_L$ cells for a 24-h period was largely non-toxic as shown in Fig. 2. In cultures treated with NNK continuously for 7 days and in which fresh S9 was added every other day, the toxicity was significantly increased with a mean lethal dose ($D_0$) of about 500 μg/ml. After a single dose of 25 cGy alpha particle irradiation, the surviving fraction was about 0.70. When cells were pretreated with NNK for 7 days followed by exposure to a 25 cGy dose of alpha particles, the resultant survival fraction fell within the statistical range of the calculated values assuming an additive interaction of the two agents (Fig. 3).

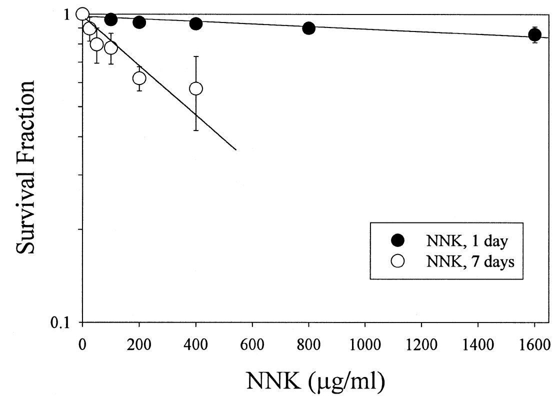


Fig. 2. Survival response of $A_L$ cells treated with graded doses of NNK plus S9 (184 μg/ml) exposed for either 1 or 7 days. Data are pooled from four to six experiments. Error bars show means ± S.E.M.

### 3.2. Mutagenicity of NNK and alpha particles

Since the majority of the chromosome-11 is not required for survival of $A_L$ cells, the entire gene except for a required small segment near the *RAS* gene (11p15.5) can serve as a target for mutagens. As such, the $A_L$ cell assay is highly sensitive to agents that induce predominately multilocus deletions, as demonstrated previously [10,12]. In addition,, the *CD59* surface antigen is an effective genetic marker since its presence or absence can readily measured in a complement-mediated cytotoxicity assay.

Induction of *CD59*$^-$ mutants by NNK either alone or in combination with alpha particles is shown in Fig. 4. The induced mutant frequency for cells treated with S9 alone for 7 days was similar to the control. The average background mutant frequency in these experiments was approximately 50 per $10^5$ survivors. NNK induced a dose-dependent increase in mutant yield over the range of doses examined. The single 25 cGy dose of alpha particles induced a net mutant fraction (total mutant yield minus background) averaging 74.5 ± 5.8 per $10^5$ survivors. In cultures pretreated with either a 25 or 100 μg/ml

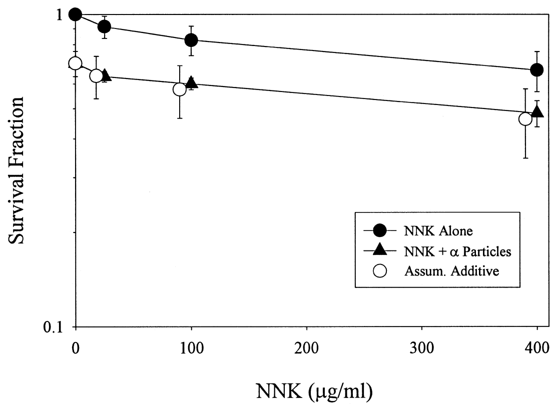


Fig. 3. Survival fraction of $A_L$ cells treated with graded doses of NNK plus S9 (184 μg/ml) for 7 days followed by a 25cGy dose of alpha particle irradiation. Survival fraction of a single 25cGy dose was 0.70. Data are pooled from three to four experiments. Error bars show means ± S.E.M.

dose of NNK, concurrent treatment with alpha particles induced a combined mutant frequency that was consistent with an additive effect, i.e., the combined treatment resulted in a mutant yield which fell within the statistical range assuming an additive interaction between the two mutagens. However, with a 400 μg/ml dose of NNK, the combined mutant yield in $A_L$ cells exposed to concurrent alpha radiation was significantly less than an additive interaction ($p < 0.01$). One plausible explanation is that the treatment induced mostly multilocus deletions that are incompatible with cell survival. In other words, it is possible that many types of mutations induced by 400 μg/ml NNK combined with alpha particles were poorly recovered in these assays because they were lethal. To assess this possibility, we examined the spectrum of mutants induced by the various treatments.

### 3.3. Analysis of mutant spectra

The *CD59* surface antigenic marker is encoded by the *CD59* gene mapped to chromosome 11p13. A total of 192 mutants, including 35 spontaneous ones, were analyzed. Fig. 5 shows the cumulative deletion maps of these $CD59^-$ mutants. Previous studies have shown that a small segment of the human chromosome-11 near the *RAS* gene is required for survival of the $CD59^-$ mutant [20]. The obligate presence of this region identified here by the *RAS* probe in all the mutants provides a convenient internal PCR control. Consistent with previous studies, the majority of spontaneous $CD59^-$ mutants showed no detectable changes in any of the marker genes examined; 69% of these spontaneous $CD59^-$ mutants had retained all of the markers analyzed. In contrast, only 35% of mutants from alpha particle irradiation retained all of the marker genes examined, i.e., 65% of them lost at least one additional marker gene. The proportion of mutants suffering loss of additional chromosomal markers increased with increasing concentration of NNK. Eighty-eight percent (23/26) of the mutants induced by a 400 μg/ml NNK lost at least one additional marker and of which 19% (5/26) lost all four markers examined which spanned both the short and long arms of the human chromosome-11. In combination with alpha particle irradiation, the proportion of NNK-induced mutants suffering loss of additional markers increased such that for mutants induced by a 400 μg/ml dose of NNK in combination with alpha particles, 97% (33/34) of the mutants lost at least one additional marker as compared with 88% (23/26) with NNK alone. Furthermore, 24% (8/34) of these mutants induced by the combined treatments

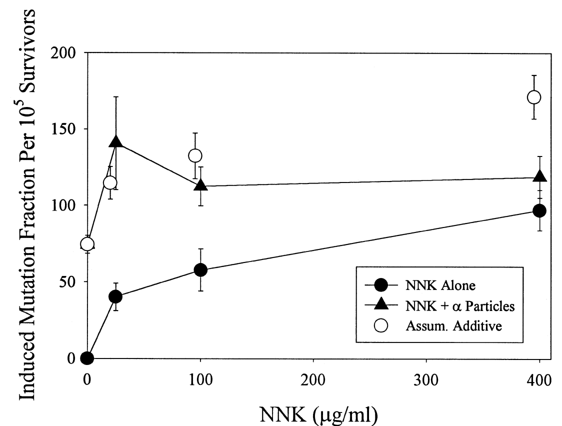


Fig. 4. Induction of $CD59^-$ mutants in $A_L$ cells treated with graded dose of NNK plus S9 (184 μg/ml) for 7 days followed by a single 25cGy dose of alpha particle irradiation. Induced mutation frequency = total mutant yield minus background. Average spontaneous mutation frequencies from these three to five experiments averaged 50 per $10^5$ survivors. Error bars show means ± S.E.M.

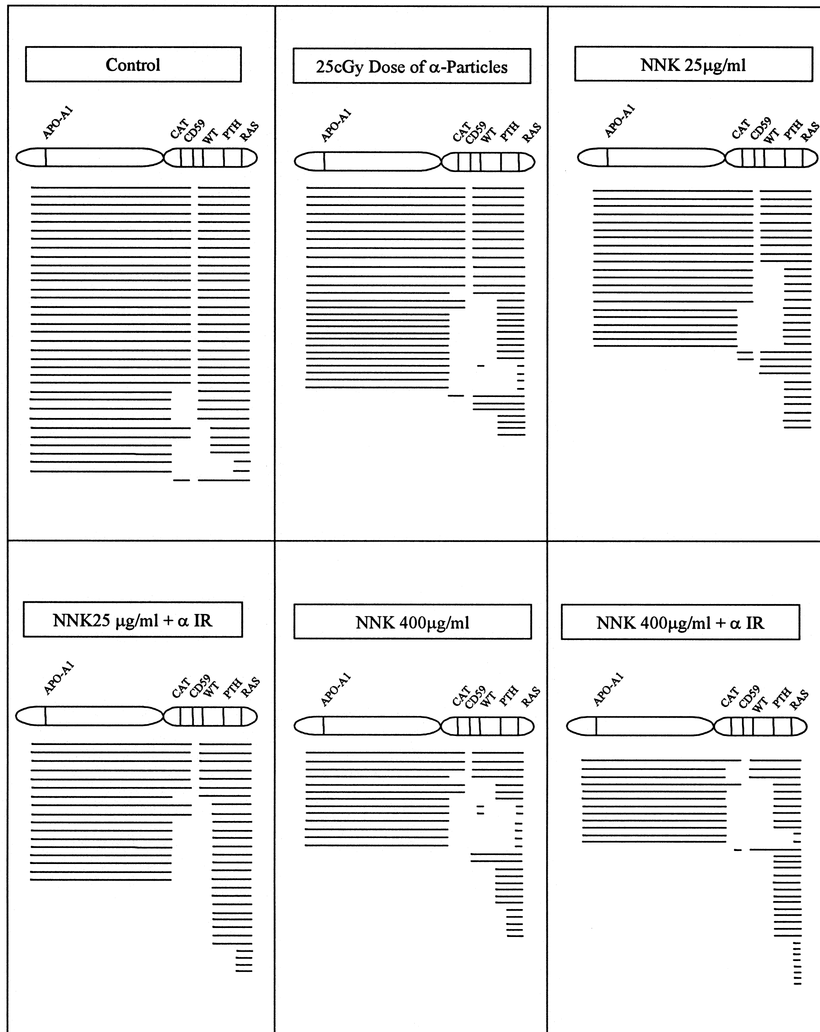


Fig. 5. Cumulative deletion spectra of *CD59*$^{-}$ mutants either of spontaneous origin or from cells treated with graded dose of NNK plus S9 for 7 days alone or followed by a single 25cGy dose of alpha particle irradiation.

lost all four markers compared with 19% (5/26) induced by NNK alone.

## 4. Discussion

Tobacco products are responsible for a significant proportion of human cancers. A recent estimate indicates that cigarette smoking causes approximately 80%–90% of lung cancers, 60%–90% of oral cancers, 70%–80% of esophageal cancers, 80%–90% of larynx cancers, 30% of pancreatic cancers, 40%–50% of bladder cancers, 10%–50% of kidney cancers, and 30% of cervical cancers in the United States [21]. As one of the strongest carcinogens found in tobacco smoking, NNK has been shown to induce tumors in mice, rats, and hamsters. However, the carcinogenic and mutagenic mechanism(s) of NNK are still unclear. It has been shown that the amount of NNK in tobacco smoke are high enough that the total estimated doses to smokers and long term snuff-dippers

are similar in magnitude to the total doses required to produce cancer in laboratory animals. These exposures thus represent a realistic risk to cigarette smokers and non-smokers exposed for years to environmental tobacco smoke. Recent studies have shown that the proportion of smokers affected in the U.S. population is highest among African Americans (35%) and Hispanics (20%), and they also have the highest mortality rate for smoking-related cancers [22]. The fact that cigarette smoke can interact with other environmental pollutant such as asbestos in a synergistic fashion in cancer induction highlight the complexity in risk assessment and emphasizes the urgent need for basic research on the fundamental mechanisms involved.

Tobacco smoke has been shown to be a compounding factor in the induction of lung cancers among underground miners exposed to high doses of radon alpha particles. Radon is ubiquitous in indoor environments. It is estimated that residential exposure to radon and its progenies may account for 10%–12% of all lung cancer deaths in the U.S. [4]. Epidemiological studies based on cohorts of underground miners have been performed in an attempt to determine the mode of interaction between smoking and radon exposure. However, the variance of the interaction estimate was an order of magnitude greater than the statistical variance of the individual agents under a no-interaction model [23,24]. Therefore, it was difficult to formulate a definitive interaction model between smoking and radon exposure. Animal studies have also shown that exposure to high levels of cigarette smoke decreased the risk of radon induced lung cancer in dogs [25], but a synergistic effect of smoking and radon was found in rats [26]. In contrast to occupational exposure where the majority of the target lung epithelial cells were likely to be traversed by multiple alpha particles [27], the overwhelming majority of lung cells will never be exposed to more than a single particle over a lifetime in domestic exposure [28].

The single 25 cGy dose of an average of $^4$He ions chosen in the present study corresponds to a single alpha traversal per nucleus based on the measured cross-section area of 108 $\mu m^2$ for the $A_L$ hybrid cells [9]. Our current finding of an additive interaction in mutagenic yield in $A_L$ cells exposed to a low dose of NNK and alpha particles is consistent with our previous oncogenic transformation studies in 10T1/2 cells between alpha particles and cigarette smoke condensate [13].

Individual difference in susceptibility to cancer causing agents is one of the most important determining factors in human risk estimation related to environmental carcinogenesis. There is evidence based on both rodent and human studies that NNK can be activated into a DNA reactive metabolite by α-hydroxylation of its methylene and methyl group to yield various DNA methylating and pyridyloxobuylating species [29]. Studies with NNK induced lung tumors among A/J mice indicated that the methylating moieties induce primarily G–A transition whereas pyridylozobuylating agents induce mainly G–T transvertion in codon 12 of the K-*ras* oncogene. However, report on the genotoxity of NNK in mammalian cells is rather limited. NNK has been shown to be largely non-toxic, non-mutagenic at the *hprt* locus in human lymphoblastoid cells [30] and in splenic lymphocytes from rats exposed in vivo to NNK [31]. The negative findings have been largely attributed to the inability of the lymphoid tissue to metabolize NNK since cells transfected with human cytochrome *P*450 cDNA demonstrated high *hprt* mutant yields [30].

In the present study, we treated $A_L$ cells with graded doses of NNK in the presence of S9 rat liver microsomal fraction. In our preliminary studies, we had shown that S9 at the dose used was non-toxic, non-mutagenic, and effective in metabolizing the polycyclic aromatic hydrocarbon, benzo(*a*)pyrene in $A_L$ cultures. We show here that NNK is mutagenic in $A_L$ cells at the *CD59* locus. Furthermore, a low dose of NNK, when combined with radon alpha particles results in a combined effect in $A_L$ cells that is consistent with an additive model, but a less than additive response was observed at a higher NNK concentration. The majority of NNK induced $CD59^-$ mutants (77.6%) lost at least one additional marker examined. When combined with alpha particles, the proportion of mutants with additional marker loss increased with increasing dose of NNK. Our study further confirms that NNK is mutagenic in mammalian cells and induces mostly deletions. The data are comparable to the *hprt* mutant spectra induced by equivalent doses of NNK (data not shown). The mutant spectra for *CD59* mutants induced by the

single 25 cGy dose of alpha particles are consistent with previous reports that high linear energy transfer radiation induces predominately multilocus deletions in mammalian cells at the various genetic loci examined [9,12,32]. While mutations at codon 249 of exon 7 in the *p53* gene had previously been suggested to be a hotspot mutation in lung cancers among Colorado uranium miners [33], there is recent evidence that such point mutations may not be common among other miner cohorts studied [34,35]. Consequently, if loss of the *p53* tumor suppressor function is a target for radon alpha particles, it is likely to occur by chromosomal loss via intrachromosomal deletions [35,36].

Since the majority of the chromosome-11 is not required for the survival of $A_L$ cells, the entire gene except for a required small segment near the *RAS* gene (11p15.5) can serve as a target for mutagens [9,12,20]. As such, the $A_L$ cell assay is highly sensitive to agents that induce predominately multilocus deletions, as demonstrated previously [10,12]. In this regard, our finding is consistent with the recent report that NNK induced a significant increase in exon loss of the *hprt* gene among NNK induced mutants in a metabolically competent human lymphoblastoid MCL-5 cells [37]. Our in vitro finding provides the first report on the combined genotoxic effects of NNK and alpha particles and should be helpful in understanding the interactive mechanism of diverse environmental carcinogens at low doses.

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## References

[1] S.S. Hecht, S.G. Carmella, P.G. Foiles, S.E. Murphy, Biomarks for human uptake and metabolic activation of tobacco-specific nitrosamines, Cancer Res. 54 (1994) 1912s–1917s, (suppl.).

[2] R.R. Baker, Mechanisms of smoke formation and delivery, Recent Adv. Tob. Sci. 6 (1980) 184–224.

[3] D. Hoffmann, S.S. Hecht, Nicotine-derived *N*-nitrosamines and tobacco-related cancer: current status and future directions, Cancer Res. 45 (1985) 935–944.

[4] J.H. Lubin, J.D. Boice, Jr., C. Edling, R.W. Hornung, G. Howe, E. Kunz, R.A. Kusiak, H.I. Morrison, E.P. Radford, J.M. Samet, M. Tirmarche, A. Woodward, S.X. Yao, D.A. Pierce, Lung cancer and Radon: a joint analysis of 11 underground miners studies. No. 94-3644, U.S. National Institutes of Health, Bethesda, MD, 1994.

[5] A.S. Whittemore, A. McMillan, Lung cancer mortality among U.S. uranium miners: a reappraisal, J. Natl. Cancer Inst. 71 (1983) 489–499.

[6] C.A. Waldren, C. Jones, T.T. Puck, Measurement of mutagenesis in mammalian cells, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 1358–1362.

[7] C.A. Waldren, L. Correll, M.A. Sognier, T.T. Puck, Measurement of low levels of X-ray mutagenesis in relation to human disease, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 4839–4843.

[8] T.K. Hei, C.A. Waldren, E.J. Hall, Mutation induction and relative biological effectiveness of neutrons in mammalian cells, Radiat. Res. 115 (1988) 281–291.

[9] T.K. Hei, L.J. Wu, S.X. Liu, D. Vannais, C.A. Waldren, Mutagenic effects of a single and an exact number of alpha particles in mammalian cells, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 3765–3770.

[10] T.K. Hei, C.Q. Piao, Z.Y. He, D. Vannais, C.A. Waldren, Chrysotile fiber is a strong mutagen in mammalian cells, Cancer Res. 52 (1992) 6305–6309.

[11] T.K. Hei, C.Q. Piao, J.C. Willey, T. Sutter, E.J. Hall, Malignant transformation of human bronchial epithelial cells by radon-simulated alpha particles, Carcinogenesis 15 (1994) 431–437.

[12] L.X. Zhu, C.A. Waldren, D. Vannais, T.K. Hei, Cellular and molecular analysis of mutagenesis induced by charged particles of defined linear energy transfer, Radiat. Res. 145 (1996) 251–259.

[13] C.Q. Piao, T.K. Hei, The biological effectiveness of radon daughter alpha particles I. radon, cigarette smoke and oncogenic transformation, Carcinogenesis 14 (1993) 497–501.

[14] S.A. Miller, D.D. Dykes, H.F. Polesky, A simple salting out procedure for extracting DNA from human nucleated cells, Nucleic Acids Res. 16 (1988) 1215.

[15] T.T. Puck, P. Wuchier, C. Jones, F.T. Kao, Genetics of somatic mammalian cells: lethal antigens as genetic markers for study of human linkage groups, Proc. Natl. Acad. Sci. U.S.A. 68 (1971) 3102–3106.

[16] J. Pelletier, W. Bruening, C.E. Kashtan, S.M. Mauer, J.C. Manivel, J.E. Striegel, D.C. Houghton, C. Junien, R. Habib, L. Fouser, R.N. Fine, B.L. Silverman, D.A. Haber, D. Housman, Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys–Drash syndrome, Cell 67 (1991) 437–447.

[17] T.J. Vasicek, B.E. McDevitt, M.W. Freeman, B.J. Fennick, O.N. Hendy, J.T. Potts Jr., A. Rich, H.M. Kronenburg, Nucleotide sequence of the human parathyroid hormone gene, Proc. Natl. Acad. Sci. U.S.A. 80 (1983) 2127–2131.

[18] S.K. Karathanasis, V.I. Zannis, J.L. Breslow, Isolation and characterization of the human apolipoprotein A-1 gene, Proc. Natl. Acad. Sci. U.S.A. 80 (1983) 6147–6151.

[19] M. Zaider, Concepts for describing the interaction of two agents, Radiat. Res. 123 (1990) 257–262.

[20] S.M. McGuinness, S.M. Shibuya, A.M. Ueno, D. Vannais, C.A. Waldren, Mutant quantity and quality in mammalian cells ($A_L$) exposed to cesium-137 gamma radiation: effect of caffeine, Radiat. Res. 142 (1995) 247–255.

[21] D.R. Shopland, H.J. Eyre, T.F. Pechachek, Smoking-attributable cancer mortality in 1991: is lung cancer now leading cause of death among smokers in the United States?, J. Natl. Cancer Inst. 83 (1991) 1142–1148.

[22] C.E. Bartecchi, T.D. Mackenzie, R.W. Schrier, The human cost of tobacco use, N. Engl. J. Med. 330 (1994) 907–912.

[23] S. Greenland, Basic problems in interaction assessment, Environ. Health Perspect. 101 (1993) 59–66, (suppl.).

[24] L. Damber, L.G. Larsson, Combined effects of mining and smoking in the causation of lung carcinoma, Acta Radiol. Oncol. 21 (1982) 305–313.

[25] F.T. Cross, R.F. Palmer, R.E. Filipy, G.E. Dagle, B.O. Stuart, Carcinogenic effects of radon daughters, uranium ore dust and cigarette smoke in beagle dogs, Health Phys. 42 (1982) 33–52.

[26] F.T. Cross, Radioactivity in cigarette smoke issue, Health Phys. 46 (1984) 205–208.

[27] National Research Council Committee on Health Risks of Exposure to Radon (BEIR VI), Health Effects of Exposure to Radon: Time for Reassessment?, National Academy Press, Washington, DC, 1994.

[28] J.H. Lubin, Z. Liang, Z. Hrubec, G. Pershagen, J.B. Schoenberg, W.J. Blot, J.B. Klotz, Z.Y. Xu, J.D. Boice Jr., Radon exposure in residences and lung cancer among women: combined analysis of three studies, Cancer Causes Control 5 (1994) 114–128.

[29] D. Hoffman, B. Spiegelhalder, Tobacco specific nitrosamines, Crit. Rev. Toxicol. 21 (1991) 234–294.

[30] C.L. Crepsi, B.W. Penman, H.V. Gelboin, F.J. Gonzalez, A tobacco smoke-derived nitrosamine, 4-methylnitrosamine-1-3-pyridyl-1-butanone, is activated by multiple human cytochrome *P*450s including the polymorphic human cytochrome *P*4502D6, Carcinogenesis 12 (1991) 1197–1201.

[31] J.G. Jansen, A.J. de Groot, C.M. van Teijlingen, A.D. Tates, H. Vrieling, A.A. van Zeeland, Induction of *hprt* gene mutations in splenic T-lymphocytes from the rat exposed in vivo to DNA methylating agents is correlated with formation of $O^6$-methylguanine in bone marrow and not in the spleen, Carcinogenesis 17 (1996) 2183–2191.

[32] H.H. Evans, Failla memorial lecture. The prevalence of multilocus lesions in radiation-induced mutants, Radiat. Res. 137 (1994) 131–144.

[33] J.A. Taylor, M.A. Watson, T.R. Devereux, R.Y. Michels, G. Saccomanno, M. Anderson, *p53* mutation hotspot in radon-associated lung cancer, Lancet 343 (1994) 86–87.

[34] K.H. Vahakangas, J.M. Samet, R.A. Metcalf, J.A. Welsh, W.P. Bennett, D.P. Lane, C.C. Harris, Mutations of *p53* and *ras* genes in radon-associated lung cancer from uranium miners, Lancet 339 (1992) 576–580.

[35] M. Hollstein, H. Bartsch, H. Wesch, E.H. Kure, R. Mustonen, K. Muhlbauer, A. Spiethoff, K. Wegener, T. Wiethege, K. Muller, *p53* gene mutation analysis in tumors of patients exposed to α-particles, Carcinogenesis 18 (1997) 511–516.

[36] T.K. Hei, J. Bedford, C.A. Waldren, *p53* mutation hotspot in radon-associated lung cancer, Lancet 343 (1994) 1158.

[37] F. Garganta, G. Krause, G. Scherer, Rapid characterization of mutations in amplified human *hprt* cDNA by polyacrylaminde gel electrophoresis, Mutat. Res. 406 (1998) 33–43.