

N-RAS Mutations in Radiation-Induced Murine Leukemic Cells

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ABSTRACT: *N-ras* mutations were examined in DNA samples extracted from the spleen of CBA/Ca mice that developed myeloid leukemia (ML) following exposure to radiations of different qualities. A total of 17 ML cases, i.e. 5 cases of neutron-induced and 12 cases of photon- (3 γ -ray and 9 x-ray) induced ML were included in the study along with 12 DNA samples from the bone marrow cells of control mice. Polymerase chain reaction-single strand conformational polymorphisms (PCR-SSCP) and the direct sequencing of PCR products were used to analyze three regions of the *N-ras* gene: (i) a 128 base-pair (bp) long portion of exon I (codons 2-37); (ii) a 103 bp long portion of exon II (codons 48-82); and (iii) a 107 bp long portion of exon III (codons 118-150). PCR-SSCP mobility shifts indicated mutations within only exon II of the *N-ras* gene. Such mutations were more prevalent in samples from mice exposed to fast neutrons. The exact type and location of these mutations were then determined by direct DNA sequencing. Silent point mutations, i.e. base transitions at the third base of codons 57 (GAC→GAT), 62 (CAA→CAC), or 70 (CAG→CAA) were present only in mice that developed ML after exposure to fast neutrons. A base transversion at the third base of codon 61 (CAA→CAC) was also observed in some ML cases. DNA sequencing demonstrated that ML samples contained normal as well as mutated DNA sequences. The higher frequency of *N-ras* mutations in neutron-induced ML suggested that fast neutrons are more effective in inducing genomic instability at the *N-ras* region of the genome. More importantly, *N-ras* mutations are not the initiating event in radiation leukemogenesis. This conclusion was supported by the finding that *N-ras* mutations were detected only in mice with an overt leukemic phenotype but not in mice with minimal tissue infiltration of leukemic cells, suggesting that the disease may be present prior to the presence of *N-ras* mutations. Alternatively, *N-ras* may be present in these mice but a large number of normal spleen cells in these mice interferes with the detection of mutation in a small population of leukemic cells.

Keywords: *N-ras*, mutation, radiation, leukemogenesis, murine

INTRODUCTION

The ras proto-oncogenes (i.e. *H-ras*, *K-ras*, and *N-ras*) encode 21 kDa GTP-binding proteins. These are similar to the G-proteins which play a central role in signal transduction that controls proliferation and differentiation of many cell types (1). Activation of oncogenes in the ras family can occur through point mutations at codons 12 and 13 of exon I, 59 and 61 of exon II, or 146 of exon III, resulting in different amino acids. Oncogenic activation of the ras genes has been found to be one of the most frequent genetic alterations in

human and animal cancers (1). *N-ras* mutations are the most commonly detected alterations in spontaneous human and induced canine acute ML (1-4), but less common in chronic ML, regardless of carcinogen. In lymphomas, however, *N-ras* mutations are more predominant in those induced by chemical carcinogens and *K-ras* mutations are more likely to be found in those induced by radiation (3). Furthermore, a difference in the mutational spectra of *K-ras* has been reported between neutron- (at codon 146) and γ -ray-induced (at codon 12) thymic lymphomas in RF/J mice (5).

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N-ras mutations have been found in cells at different stages of human leukemia. The detection of *ras* mutations in myelodysplastic syndromes (MDS) (6,7), a “preleukemic” stage, suggests a role for *ras* mutations at an early stage of the leukemogenic process. An analogous line of evidence has been observed in human colorectal cancer and in animal mammary neoplasia (8). In some MDS cases, *N-ras* mutations were detected in both myeloid and lymphoid cells, indicating that such lesions originated from a pluripotent stem cell (9). There is, however, evidence showing that most *N-ras* mutations appear during the progression of MDS to ML (10-14). Such findings suggest that *N-ras* mutations are late events in the development of ML. In some leukemic patients, mutated *N-ras* genes were found in addition to the specific cytogenetic markers such as monosomy chromosome 7 or t(1;9)(q23;p13) (13-14). These observations suggest that *N-ras* mutations are necessary, but may not, by themselves, be sufficient for neoplastic transformation.

In order to define cellular and molecular mechanisms leading to leukemogenic transformation, a series of studies has been conducted in our laboratory using CBA/Ca mice. This mouse has been an excellent experimental animal for leukemogenesis studies (15-16) because of the: (a) low spontaneous incidence of ML (1%), (b) responsiveness to radiation, (c) lack of a viral component of the disease, and (d) histopathological characteristics that are similar to those seen in human leukemia. In this study, we used the polymerase chain reaction-single strand conformational polymorphisms (PCR-SSCP) technique as a rapid method to screen for the *N-ras* mutations in leukemic cells from mice that developed ML following exposure to ionizing radiation with different qualities, i.e. “monoenergetic” fast neutrons (0.22-14.7 MeV) or photons (X rays or γ rays). The detection of mutations using the PCR-SSCP technique depends on the fact that induced base alterations often lead to changes in the three-dimensional conformation of single-stranded DNA segments causing shifts in mobility during electrophoresis through non-denaturing polyacrylamide gels. Basically, the SSCP technique allows

the mutated form of the gene to be distinguished against a background containing the wild-type homologue. Direct sequencing of PCR products was used to determine the nature and the position of mutations that lead to changes in mobility. In this study, we focused our analyses on: i) a 128 base-pair (bp), portion of exon I (codons 2-37); ii) a 103 bp, portion of exon II (codons 48-82), and a 107 bp, portion of exon III (codons 118-150).

MATERIALS AND METHODS

DNA Samples

Using a standard phenol/chloroform protocol (17), high molecular weight DNAs were isolated from: 1) bone marrow cells of 12 control CBA/Ca male mice (70-140 days old) collected by flushing the femurs and tibiae with 1X Dulbecco phosphate-buffered saline (pH 7.1) (Gibco, Gaithersburg, MD), and 2) spleens of 17 mice with radiation-induced ML. The age of each ML case at diagnosis and the descriptions of radiations that induced each ML case are shown in Table 1. The criteria for diagnosis of ML have been presented elsewhere (18).

Polymerase Chain Reactions (PCR)

Oligonucleotide primers were synthesized on the Milligen/Biosearch 8750 DNA synthesizer. Nucleotide sequences of the primers for each exon of the mouse *N-ras* gene are given below:

Exon (Codon)	Length (bp)	Primer Sequence
I (2-37)	128	+ :5'-GACTGAGTACAACTGGTGG-3'
		- :5'-GGGCCTCACCTCTATGGTG-3'
II (48-82)	103	+ :5'-GGTGAAACCTGTTTGTGGA-3'
		- :5'-ATACACAGAGGAAGCCTTCG-3'
III (117-150)	107	+ :5'-GTGTGACTTGCCAACAAGG-3'
		- :5'-AGCATACTGTCGGGTCTT-3'

Table 1. DNA Sequences at Codons 57, 61, 62 and 70 of the Mouse N-ras Gene in DNA Isolated from Radiation-Induced Myeloid Leukemic Cells

Case #	Age (days)	Radiation	CODON			
			57 GAC asp	61 CAA gln C CA A his	62 GAG glu A GA G	70 CAG gln A CA G
1	693	Neutron (0.5 Gy, 0.44 MeV)	GAT	C CA A his	A GA G	CAA
2	423	Neutron (0.1 Gy, 1.5 MeV)	GAT	C CA A his	A GA G	CAA
3	625	Neutron (0.4 Gy, 0.22 MeV)	GAC		A GA G	A CA G
4	464	Neutron (0.4 Gy, 0.22 MeV)	GAC		A CA G	A CA G
5	743	Neutron (0.05 Gy, 0.22 MeV)	GAC	C CA A his	A GA G	CAG
6	735	γ rays (2.0 Gy, ¹³⁷ Cs)	GAC		GAG	CAG
7	711	γ rays (1.5 Gy, ¹³⁷ Cs)	GAC		GAG	CAG
8	787	γ rays (1.5 Gy, ¹³⁷ Cs)	GAC		GAG	CAG
9	472	X rays (2.0 Gy, 250 kVp)	GAC		GAG	GAG
10	448	X rays (2.0 Gy, 250 kVp)	GAC		GAG	CAG
11	347	X rays (2.0 Gy, 250 kVp)	GAC		GAG	CAG
12	514	X rays (2.0 Gy, 250 kVp)	GAC	C CA A his	GAG	CAG
13	556	X rays (2.0 Gy, 250 kVp)	GAC	C CA A his	GAG	CAG
14	533	X rays (2.0 Gy, 250 kVp)	GAC		GAG	CAG
15	644	X rays (2.0 Gy, 250 kVp)	GAC		GAG	CAG
16	743	X rays (2.0 Gy, 250 kVp)	GAC		GAG	CAG
17*		X rays (2.0 Gy, 250 kVp)	GAC		GAG	CAG

* ML cells from the second transplantation into syngeneic mice.

Genomic DNA (25 ng) was amplified in 25 μ L of the standard reaction mixture suggested by Perkin-Elmer Cetus for 30 cycles at 96, 52, and 72°C for 1, 1, and 2 min, respectively. After 30 cycles of amplification, the PCR mixtures were incubated at 72°C for an additional 7 min. The amplified DNAs were electrophoresed on a 5% non-denaturing polyacrylamide (30:8 acrylamide: BisA, Integrated Separation System, Natick, MA) slab gel using 1XTBE. DNA bands were visualized by soaking the gels in 0.5 μ g/mL ethidium bromide and observing fluorescence with long wavelength UV light. Thereafter, DNAs were isolated from crushed gel slices by overnight incubation at 37°C in 10 volumes of the elution buffer containing 20 mM Tris.Cl pH 8.0, 10 mM EDTA, 50 mM NaCl, 0.1% SDS. Gel fragments were removed by centrifugation and the DNA recovered by precipitation with 0.1 vol of 3M sodium acetate (pH 5.2) and 2 volumes of absolute ethyl alcohol.

Single Strand Conformational Polymorphism (SSCP) Analysis

The method developed by Orita et al (19) was followed with slight modifications. Briefly, electrophoretically purified PCR products (approx. 20 ng) were labeled by incubation with γ -³²P ATP (150 mCi/mL, NEN, Dupont, Wilmington, DE) and polynucleotide kinase (Promega, Madison, WI). The DNA samples were diluted 100-fold with formamide-dye solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), heated for 2 min at 80°C, and electrophoresed on an 8% non-denaturing polyacrylamide gel (19:1 acrylamide:BisA, Amresco, Solon, OH) without urea or glycerol. Electrophoresis was performed at 400 volts for 3 hrs at room temperature to separate the individual strands of the DNAs. The gel was dried onto filter paper using a BioRAD gel drier and then placed on x-ray film for 48-72 hr at -80°C with Quanta 3 (Dupont) intensifying screens.

Direct DNA Sequencing

The portion of exon II of the *N-ras* gene (codons 48-82) was analyzed by direct DNA sequencing using amplified DNA samples isolated from the 17 cases of leukemias obtained from irradiated mice as well as from 12 control animals. The sequencing primers were the same as those for PCR. After annealing to heat denatured purified PCR products, the annealed primer was extended in the presence of [α -³⁵S]dATP using the T7 sequenase chain-termination DNA sequencing kit (Amersham, Arlington Heights, IL). The reactions were incubated for 5 min at 37°C. In some case the *Escherichia coli* single-stranded binding protein was included in the sequencing reactions (1.5 μ g/mL) to increase the read-length of the sequencing ladder (20, 21). Reactions were stopped by addition of formamide dye mix to the samples and incubation on ice for 5 min. Thereafter, the samples were heated to 80°C for 15 min prior to electrophoresis on a standard 8% polyacrylamide gel (19:1 acrylamide:Bis, Amresco, Solon OH) containing 8 M urea. Sequencing gels were electrophoresed at 30 W with constant voltage and current for 3 hr. The gels were dried for 3 hr and exposed to x-ray film for 72-96 hr at room temperature.

Statistical Analysis

For each type of radiation, i.e. densely (neutrons) and sparsely (photons), ML cases were divided into two groups on the basis of the presence or absence of mutations in *N-ras* exon II. A difference in the incidence of particular *N-ras* mutations was compared by Fisher's exact test. The criterion for statistical significance used here was a P value of ≤ 0.05 .

RESULTS

SSCP Analysis

SSCP analysis did not detect mobility shifts in exon I or exon III of the *N-ras* gene in the 17

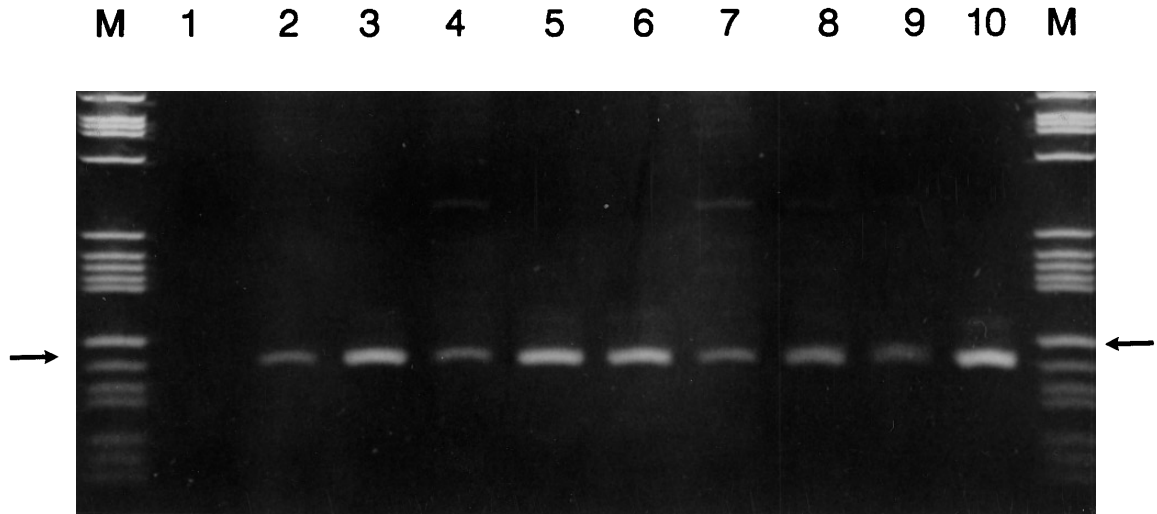


Figure 1a. Specific PCR amplification of Exon II of the mouse *N-ras* gene. Lanes marked M contain Hae III-digested pBR 322 DNA as molecular length markers. The arrows indicate the position of the 103 bp long PCR band from Exon II. Lane 1 represents control PCR reaction (no DNA template), lanes 2-3 represent PCR products of DNA isolated from x-ray-induced ML. Lanes 4-5 represent PCR products of DNA isolated from γ -ray-induced ML. Lanes 6-8 represent PCR products of DNA isolated from neutron-induced ML. Lanes 9-10 represent PCR products of DNA isolated from control mice.

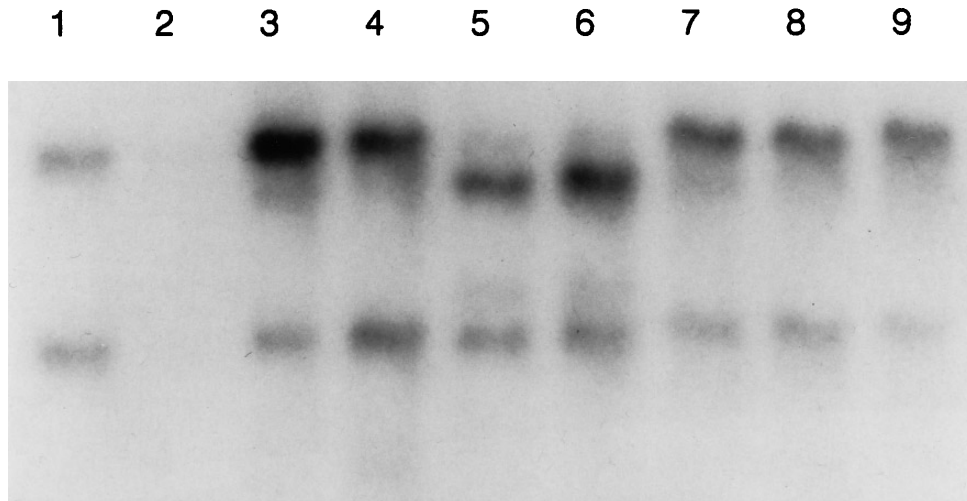


Figure 1b. Detection of point mutations in Exon II of mouse *N-ras* by PCR-SSCP analysis. Gel-purified products were γ^{32} -P-labeled, electrophoresed on 8% polyacrylamide gel (19:1 acrylamide:BisA) without urea and without glycerol in cool air stream. Lane 1 is the PCR product of control mouse DNA cells, lane 2 is the internal control lane (no PCR product), lanes 3-7 are PCR products of neutron-induced ML cells, and lanes 8-9 are PCR products of x-ray-induced ML cells.

leukemic DNA samples. In contrast, mobility shifts of exon II DNA bands covering codons 48-82 were observed in all 5 cases of neutron induced ML and 2 of 12 cases of photon-induced ML. These results suggest the involvement of mutations in exon II, but not in exon I or exon III of the *N-ras* gene in radiation leukemogenesis. No evidence for deletion or insertion of bases in these regions of the mouse genome was observed, i.e. in every sample we analyzed (normal controls and ML), the amplified exon I, II and III bands were the expected sizes (22). Fig. 1a shows the relative migration of the 103 bp portion of exon II DNA band that was then subjected to SSCP analysis (Fig. 1b). As shown in Fig. 1b, the PCR product from control mouse DNA gave rise to two well separated single-strand species. In marked contrast, three or four separated bands were seen in the seven ML cases. The differences in the SSCP band patterns from ML samples indicate that the base alterations were most likely heterogenous. In addition, some bands within a pattern were more predominant than others, presumably because they were derived from a majority sequence in the amplified DNA.

Direct Sequencing of PCR Products

Direct sequencing of all 12 control DNA samples demonstrated that no mutations were present in the 103 bp portion of *N-ras* exon II spanning codons 48-82. Point mutations (base transitions at the 3rd base in codons 57, or 62, and/or 70) were detected in the 5 cases of neutron-induced ML, however, all are silent mutations which are either clearly heterozygous (Fig. 2b, in which a wild-type sequence was simultaneously observed) or essentially homozygous (Fig. 2c). These silent mutations were not observed in γ - or x-ray-induced ML. In addition to silent mutations, a missense point mutation (an A→C base transversion at the 3rd base in codon 61 [CAA-Gln] was observed in DNA from 3 of 5 neutron-induced

ML cases [Table 2, cases 1, 2, and 5] and in 2 of 12 x-ray-induced ML [Table 2, cases 12 and 13]). This mutation encodes histidine instead of glutamine. DNA samples with this mutation were heterogenous because the wild-type CAA triplet was also observed in the sequencing ladder (Table 2). Fisher's exact test indicated that the frequency of neutron-induced ML having *N-ras* mutation at codon 61 was statistically significant, as compared to that in ML induced by photons ($P \leq 0.05$).

DISCUSSION

The combination of PCR and SSCP techniques allowed us to screen rapidly for potential mutations in the *N-ras* gene in radiation-induced ML cells. During SSCP analysis DNA strand separation during electrophoresis on non-denaturing gels is dependent on the actual DNA sequence (19). Single base changes often result in detectable differences in the electrophoretic mobilities of both strands of a given allele. In our hands, PCR-SSCP of exons I and III did not reveal the presence of any mutations, however, we were able to detect bands with altered electrophoretic mobilities from exon II which were then confirmed by direct sequencing of the amplified DNA samples. The data indicated that *N-ras* mutations are not the initiating event in radiation leukemogenesis because these mutations were detected only in mice with a full-blown leukemic phenotype and not in those with minimal tissue infiltration of leukemic cells (Table 2, cases 16 and 17). The finding that most *N-ras* mutations were detected along with a wild-type background supports this hypothesis. On the other hand, it is possible that a high ratio of normal spleen to leukemic cells in mice with minimal tissue infiltration hindered the detection of *N-ras* mutations. Nevertheless, these data suggest that, even though *N-ras* mutations, may not be sufficient to elicit the malignant phenotype, they may provide an additional proliferative

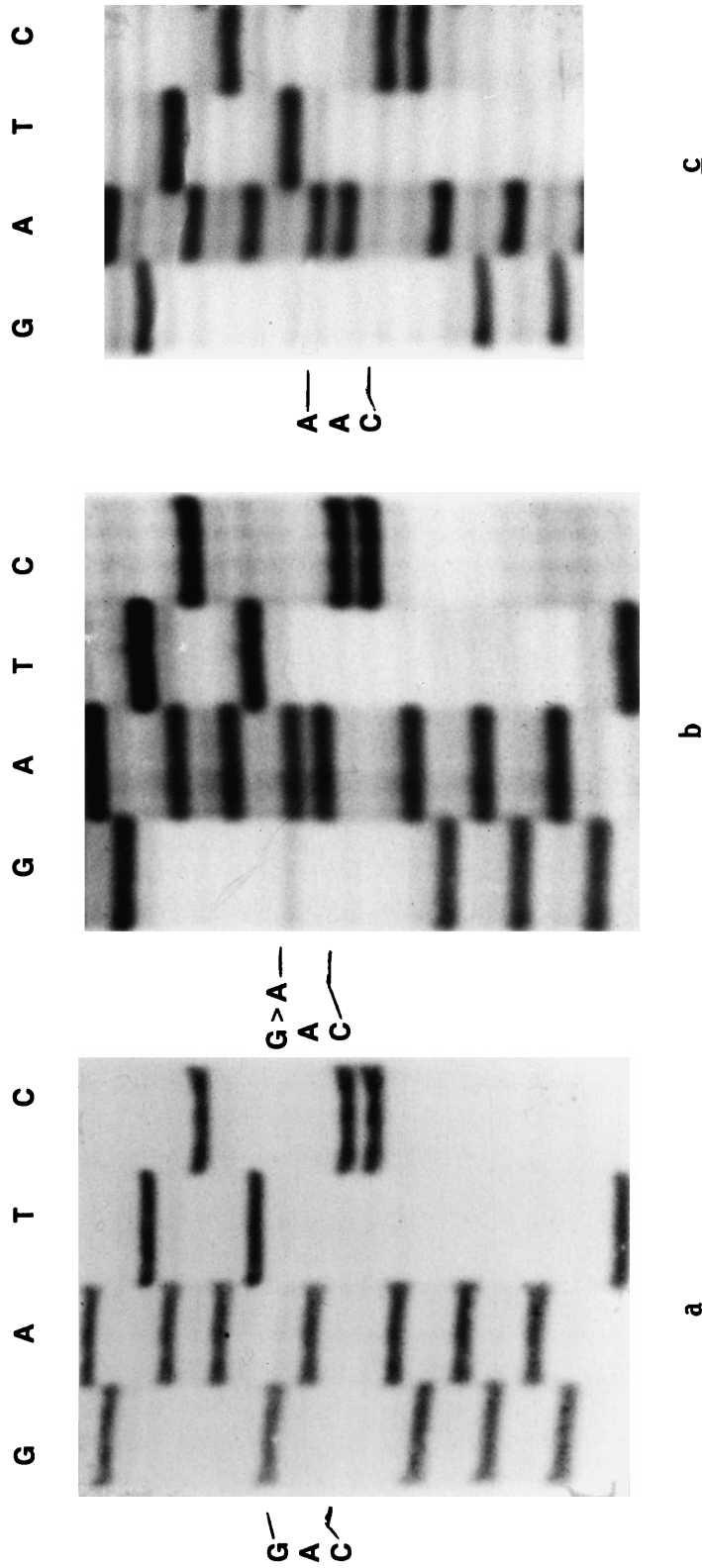


Figure 2. The normal sequence of codon 70 of the mouse *N-ras* gene (a) as detected by direct sequencing of PCR products. An appearance of a base transition at the third base of codon 70 of the mouse *N-ras* gene with (b) and without (c) wild type sequence is shown.

erative advantage to leukemic cells. More than one type of mutation was observed in the same animal among the neutron-induced ML (Table 1). This finding, in combination with our previous histological, hematological, and cytogenetic data on these ML cases (18), demonstrated that phenotypically and cytogenetically similar leukemic cells can have a heterogeneous genotype. A similar phenomenon has also been found in human leukemia (10). The authors suggested that each type of *N-ras* mutation belongs to a different clone and that *N-ras* mutations were not an initiating event in leukemogenesis. Rather, they contributed to the outgrowth of more malignant subclones.

N-ras sequence changes in exon II were observed in 7 cases of radiation-induced ML, however only 5 of these had a mutation in codon 61. As mentioned earlier, codon 61 of the *N-ras* gene is one of the hotspots for oncogenic activation in many types of human and animal cancers and a mutation in this codon would cause a change of the *N-ras* protein sequence and protein function (1). The other two cases of radiation-induced ML with base changes acquired only silent mutations. These results demonstrate that, although PCR-SSCP analysis is a simple and sensitive method for the detection of nucleotide sequence changes, a band shift may be associated with mutations affecting coding potential as well as silent polymorphisms (23). Thus, the exact type of mutations needs to be determined in samples exhibiting shifted bands in the SSCP gels as described using sequencing techniques.

Although age-matched control DNA samples are not available in this study, it is unlikely that aging might have contributed to *N-ras* mutations in ML samples. This argument is supported by the fact that *N-ras* mutations were not detected in other ML cases within a similar age-group or older (Table 1). Interestingly, 5 of 5 neutron-induced ML cases acquired *N-ras* mutations. In contrast, only 2 of 16 photon-induced ML cases presented

with this type of lesion. These data are consistent with the concept that densely ionizing radiation is more effective, per unit of energy deposited, than sparsely ionizing radiation in inducing genomic instability manifested as gene mutation (24). As mentioned earlier, results from our study, together with those from other groups (10-14), suggest that *N-ras* mutations detected in ML cells are not the primary genetic lesions caused by a direct hit of ionizing radiation at the time of exposure (70-84 days old mice). Neutrons cause a wider spectrum of initial genetic damage (instability) which in turn escalates the occurrence of new mutations, including those in *N-ras*. The mouse *N-ras* gene has been previously mapped to chromosome 3 (25). Thus, *N-ras* mutations observed in ML cases included in this study do not correlate with the cytogenetic feature of these cells i.e. a deletion on one copy of mouse chromosome 2 (18, 26-28). It is, nevertheless, not surprising that these point mutations do not relate to chromosomal abnormalities detected in these cells because cytogenetic analysis can detect only genotypic changes that affect regions greater than 3,000 kilobases (29). However, it is possible that genomic instability resulting from chromosome 2 lesions may trigger the activation of others oncogenes and tumor suppressor as well.

In this study, it was, however, impossible to determine precisely the time at which these mutations occurred or to assess accurately the role of *N-ras* in the progression of leukemia because neither the information on the *N-ras* germline sequence for each mouse prior to irradiation nor the temporal pattern of *N-ras* mutations during leukemogenic transformation was available. However, results from this study reinforces the concept that *N-ras* mutations are not the initiating event in radiation leukemogenesis and that leukemia rises from the accumulation of genomic instability. Moreover, results from our study not only were comparable to those from human studies but also demonstrated the usefulness of the CBA/Ca mouse model for molecular studies of leukemogenesis.

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