

Evidence for an Uncommon Microsatellite Instability on Mouse Chromosomes 2 and 4 and its Possible Role in Radiation Leukemogenesis

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ABSTRACT: Although microsatellite instability (MSI), usually detected by DNA length polymorphisms, has been implicated in the induction of solid tumors in both humans and animals, its role in leukemogenesis is unclear. The goal of this study was to investigate whether there is an association between MSI and radiation leukemogenesis in CBA/Ca mice. Microsatellite lengths at 55 loci, mapped to eight different mouse chromosomes, were examined in two groups of DNA samples: 1) 10 normal DNA samples collected from the bone marrow cells of control male CBA/Ca mice, and 2) 17 DNA samples isolated from the spleens of mice that developed myeloid leukemia (ML) after exposure to neutrons, or X rays, or γ rays. Microsatellite markers were amplified using the non-radioisotopic multiplex-touchdown PCR protocols developed in our laboratory, and the sizes of amplicons were examined on 6% non-denaturing polyacrylamide gels. Although no correlation between microsatellite length polymorphisms and radiation leukemogenesis was observed at the 55 CBA/Ca mouse loci tested in this study, an uncommon MSI, manifested as the absence of DNA bands after PCR amplification at 2 loci (D2MIT140 and D4MIT104), was observed in both control and ML samples. However, the frequency of ML samples showing this type of MSI is statistically significant ($p < 0.05$). Although there is no direct evidence that this type of MSI predisposes mice to the development of leukemia, the results suggests that genes flanking the D2MIT140 and D4MIT104 are susceptible to spontaneous mutation and perhaps to damage caused by ionizing radiation.

Keywords: microsatellites, instability, leukemia, radiation, PCR

INTRODUCTION

Microsatellites are composed of tandemly repeated sequences of di-, tri-, or tetranucleotides which are interspersed throughout the genome of eukaryotes (1-3). The entire length of the repeated sequences ranges can be a few hundred bases in length. The most common repeated sequence is the (CA)_n dinucleotide. Due to their abundance in the genome and polymorphic nature, microsatellites have been used as genetic

markers in genetic mapping and linkage analysis for several years (4,5). In humans, microsatellite instability (MSI), determined by length polymorphisms and frequently referred to as the mutator phenotype, has been implicated in the development of diverse diseases such as cancer, neurodegenerative disorder, and myotonic dystrophy (6,7).

An intense interest in microsatellites has emerged following the finding that the length of microsatellite CA repeats at specific loci in

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cancer cells from patients with hereditary nonpolyposis colorectal (HNPCC) was different from that in normal tissues from the same individuals (8). It was shown later that a defect in DNA mismatch repair mechanisms, resulting in replication errors, is responsible for length polymorphisms of microsatellites in these cancer cells (9). Although MSI has been found in a variety of solid tumors, the role of this phenomenon is less clear in hematopoietic neoplasms (10-15). In this regard, a more comprehensive database, with more microsatellite markers covering wider regions of the human genome, is essential before conclusions can be made.

It has been observed that rodent cells are approximately 10^4 - 10^6 fold more sensitive to neoplastic transformation than human cells, both *in vivo* and *in vitro* because of an intrinsic high genome instability (16). Alterations in microsatellite length may contribute to this phenomenon. Recently, microsatellite length polymorphisms have been observed in chemically induced mouse sarcoma (17) and in rat colon and esophageal tumors (18,19), but not in corresponding normal tissues. These findings, together with those from human studies, prompted us to initiate a study to determine whether there is an association between murine MSI and leukemogenesis.

In this study the lengths of 55 microsatellite markers that map to eight different mouse chromosomes were examined in radiation-induced ML samples, obtained from the spleen of CBA/Ca mice that had developed ML following exposure to different types of ionizing radiation (i.e., neutrons, X rays, or γ rays) and in cells isolated from the bone marrow of normal mice at different ages. Due to the compelling evidence showing the involvement of mouse chromosome 2 in murine radiation leukemogenesis (20-26), the emphasis of this initial study was on microsatellite markers localized on chromosome 2 (chromosome 2) extending from regions 2A3 to 2H, i.e., 8 to 85 centimorgan (cM) (27). Additional, microsatellite markers flanking

several known oncogenes (e.g., *Mos*, *Lyn*, *Bcl* and *Myc*), the *p53* tumor suppressor genes, and genes related to growth factors (e.g., *Il-2*, *IL-4*), which map to other chromosomes (Table 1), were also included in the study. Among the 55 microsatellite markers examined, no length polymorphisms were found in leukemic cells. However, our results indicate the presence of an uncommon MSI at 2 of 55 microsatellite markers in some radiation-induced ML samples and in some control mice.

MATERIALS AND METHODS

DNA Samples

High molecular weight DNA was isolated, using a standard phenol/chloroform protocol (28), from 2 groups of samples:

1. Radiation-induced ML samples. A total of 17 radiation-induced ML cases were included in this study. These ML cases were collected in the Medical department during our studies on radiation leukemogenesis in CBA/Ca mice. Details of each ML case, including the radiation used are presented in Table 2. The criteria for diagnosis and the cytogenetic findings have previously been published (23). Whole spleens of mice with ML following exposure to different types of ionizing radiation were aseptically removed, washed twice with McCoy's 5A medium (Gibco, Gaithersburg, MD). Single-cell suspensions of the dissected spleen were prepared in McCoy's 5A medium and cryopreserved until used.

2. Control samples. A total of 10 male CBA/Ca mice (12-20 weeks old) obtained from the BNL breeding colony were used as a control group. The bone marrow cells collected from femurs and tibiae were used for DNA isolation. Our methods for animal care and collection of bone marrow cells have been presented elsewhere (29).

Microsatellite Markers and PCR Analysis

A total of 55 microsatellite markers mapping to eight different mouse chromosomes (Table 1) were included in this study [ref. 30-32 and the mouse MapPairs database, Research Genetics, Inc., Huntsville, AL (April, 1995 release)]. The size in basepairs (bp) of the amplification product (amplicon) for each microsatellite marker is based on that of the closely related C3H mouse (33). They ranged in size from 78 to 329 bp (Table 1). The *Fisher's exact test* was used to determine whether there was a statistically significant difference in the frequency of animals with MSI at each locus between control and leukemic mice. The criterion for statistical significance was a P value of < 0.05 .

The combination of **multiplex** (34) and **touchdown** (35) PCR strategy developed in our laboratory, termed **MT-PCR** (36), with the "hot-start" (37) format (96°C for 3 min) was used throughout the study. The majority of MT-PCR contained 3 different pairs of primers with comparable touchdown annealing temperatures (Table 1). PCR reactions were carried out in 20 μ L of solution containing 1X PCR buffer (Perkin-Elmer, Norwalk, CT), 200 μ M of each dNTP, 0.5 μ M of each primer, 0.5 units AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT), 2 mM MgCl₂, 20 ng of mouse genomic DNA template. A PCR without DNA template was used as a negative control in all MT-PCR experiments. The initial annealing temperature was 65°C which was subsequently decreased at the rate of 1°C for every cycle of the amplification reaction until the targeted (TOUCHDOWN) annealing temperature was reached (either 60°C or 55°C), depending on the A+T/G+C ratio of the primers. Denaturation and extension conditions for each "TOUCHDOWN" cycle were 96°C for 30 s and 72°C for 30 s, respectively. After five to ten "TOUCHDOWN" cycles, 20 PCR cycles were performed under the following conditions: denaturation at 96°C for 30 s, annealing at 55°C (or 60°C) for 30 s,

extension at 72°C for 30 s. After the final extension, the samples were incubated at 72°C for an additional 7 min. Amplified microsatellite markers were size separated in a 6% non-denaturing polyacrylamide (30:0.8 acrylamide:bisacrylamide, Integrated Separation System, Natick, MA) slab gel using 1X TBE buffer. Electrophoresis was performed at 85 V (constant) for 1 hr. *Hae*III-digested pBR322 DNA fragments were used as the size standards. Subsequently, DNA bands were visualized by staining the gels with 0.5 μ g/mL ethidium bromide for 15 min, followed by a brief rinse in deionized water, and observing fluorescence with long wavelength UV light. Each DNA sample was tested at least twice. If results showed some deviation in size or intensity of the amplified DNA bands, two more sets of the amplification reactions for those markers were carried out independently to confirm results.

RESULTS

The MT-PCR protocol developed in our laboratory was designed to allow simultaneous amplification of several microsatellite markers in the same reaction by choosing primer pairs with compatible touchdown annealing temperatures. Our current set of primers (listed in Table 1) includes 55 different markers which in every case generates a single major amplified microsatellite band in a non-denaturing polyacrylamide gel. Figure 1 shows the typical efficacy of the MT-PCR protocol with three pairs of primers present during amplification to amplify simultaneously three separate microsatellites. In nearly every case the amplicons produced by each of the 55 primer pairs from 10 control and 17 ML DNA samples were of the expected size relative to published data as determined by their rate of migration alongside molecular size markers, with two exceptions. These were D2MIT37, which produced a band migrating at 123 bp instead of 141 bp, and D2MIT126 whose amplicon product was calculated to be 160 bp instead of the expected 112 bp. No other deviations in band

size or ratio of products were observed.

While these results demonstrated that there was no obvious change in the size of amplicons obtained from control or ML samples among the 55 loci selected for the study, a rare MSI, i.e., an absence of amplicons after PCR amplification, was observed in several DNA samples at D2MIT140 (107 bp) and D4MIT104 (241 bp). Figure 2 presents an example of an electrophoresis pattern showing this phenomenon at D2MIT140. As summarized in Table 1, the D2MIT140 amplicon was not produced in 1 of

10 normal control DNA samples (collected from a 12 weeks old mouse), and in 6 of 17 leukemic DNA samples. Likewise, amplicons for D4MIT104 were not detected in 2 of 10 control DNA samples (collected from a 12 and 20 weeks old mice) and in 11 of 17 ML samples. At each locus, the frequency of ML samples that failed to give the amplified DNA products was statistically different from those in controls, *p* values of 0.024 and 0.028 for D2MIT140 and D4MIT104, respectively.

Table 1. Microsatellite Markers Used in This Study

Chromosome (cM)	Locus	Amplicon Size (bp)	Touchdown Annealing Temperature (°C)	Number of Animals with Microsatellite Changes	
				Control (N=10)	Myeloid Leukemia (N=17)
1 (41)	MMBC12	132	60	0	0
2 (8)	D2MIT316	121	60	0	0
2 (18)	D2MIT83	114	60	0	0
2 (18)	D2MIT151	134	55	0	0
2 (18)	D2MIT180	147	55	0	0
2 (20)	D2MIT6	178	55	0	0
2 (28)	D2NdS1	192	55	0	0
2 (28)	D2MIT85	116	60	0	0
2 (32)	D2MIT8	188	55	0	0
2 (34)	D2MIT9	190	55	0	0
2 (34)	D2MIT34	138	60	0	0
2 (34)	D2MIT61	142	60	0	0
2 (37)	D2MIT90	100	60	0	0
2 (37)	D2MIT91	174	60	0	0
2 (37)	D2MIT182	151	60	0	0
2 (39)	D2MIT56	114	60	0	0
2 (44)	D2MIT37	123	55	0	0
2 (44)	D2MIT271	148	55	0	0
2 (45)	D2MIT35	140	55	0	0
2 (45)	D2MIT93	142	60	0	0

Chromosome (cM)	Locus	Amplicon Size (bp)	Touchdown Annealing Temperature (°C)	Number of Animals with Microsatellite Changes	
				Control (N=10)	Myeloid Leukemia (N=17)
2 (47)	MMILIB	257	55	0	0
2 (49)	D2MIT126	160	60	0	0
2 (53)	D2MIT13	180	60	0	0
2 (53)	D2MIT41	118	55	0	0
2 (53)	D2MIT42	142	55	0	0
2 (53)	D2MIT43	242	60	0	0
2 (57)	D2MIT129	110	55	0	0
2 (57)	D2MIT131	150	55	0	0
2 (66)	D2MIT133	203	55	0	0
2 (66)	D2MIT104	148	60	0	0
2 (66)	D2MIT395	122	55	0	0
2 (70)	D2MIT105	108	55	0	0
2 (75)	D2MIT106	154	55	0	0
2 (76)	D2MIT135	240	55	0	0
2 (76)	D2MIT192	146	55	0	0
2 (77)	D2MIT46	118	55	0	0
2 (83)	D2MIT261	113	55	0	0
2 (84)	D2MIT26	195	55	0	0
2 (85)	D2MIT140	107	55	<u>1</u>	<u>6</u>
3 (32)	IL2	232	55	0	0
4 (29)	D4NDS3	132	55	0	0
4 (45)	D4NDS1	100	55	0	0
4 (53)	D4NDS2	95	55	0	0
4 (30)	D4MIT17	144	55	0	0
4 (3)	D4MIT104	241	60	<u>2</u>	<u>11</u>
4 (4)	D4MIT317	92	55	0	0
4 (39)	D4MIT15	329	60	0	0
4 (10)	D4MIT261	123	60	0	0
4 (2)	D4MIT316	112	60	0	0

Chromosome (cM)	Locus	Amplicon Size (bp)	Touchdown Annealing Temperature (°C)	Number of Animals with Microsatellite Changes	
				Control (N=10)	Myeloid Leukemia (N=17)
5 (11)	MMIL6A	78	55	0	0
7 (74)	MMINT2	161	55	0	0
11 (59)	D11MIT54(MMHGX23R)	138	55	0	0
11 (9)	D11MIT107(MMP53IN4)	142	55	0	0
11 (25)	D11MIT111(MMIL4G12)	32	60	0	0
15 (18)	MMYCE12	107	55	0	0

Table 2. Microsatellite Changes and *N-ras* Mutations in Radiation-Induced ML Cells

Case	Radiation	Age at Diagnosis (Days)	D2MIT140	D4MIT104	N-ras
1	Neutron (0.5 Gy, 0.44 MeV)	693	-	+	+
2	Neutron (0.1 Gy, 1.5 MeV)	423	-	-	+
3	Neutron(0.4 Gy, 0.22 MeV)	625	-	+	+
4	Neutron (0.4 Gy, 0.22 MeV)	464	-	+	+
5	Neutron (0.05 Gy, 0.22 MeV)	743	-	+	+
6	γ rays (2.0 Gy, ¹³⁷ Cs)	735	-	-	-
7	γ rays (1.5 Gy, ¹³⁷ Cs)	711	-	+	-
8	γ rays (1.5 Gy, ¹³⁷ Cs)	787	+	+	-
9	X rays (2.0 Gy, 250 kVp)	472	+	+	-
10	X rays (2.0 Gy, 250 kVp)	448	+	-	-
11	X rays (2.0 Gy, 250 kVp)	347	-	-	-
12	X rays (2.0 Gy, 250 kVp)	514	-	+	+
13	X rays (2.0 Gy, 250 kVp)	556	+	-	+
14	X rays (2.0 Gy, 250 kVp)	533	-	-	-
15	X rays (2.0 Gy, 250 kVp)	644	+	+	-
16	X rays (2.0 Gy, 250 kVp)	743	-	+	-
*17	X rays (2.0 Gy, 250 kVp)		+	+	-

+ = The presence of microsatellite changes or *N-ras* mutations.

- = The absence of microsatellite changes or *N-ras* mutations.

* = ML cells from the second transplantation into syngeneic mice.

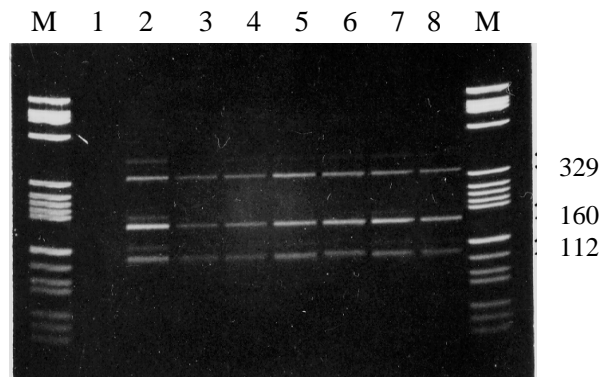


Figure 1. "Multiplex Touchdown-PCR" products of D4MIT316 (112 bp), D2MIT126 (160 bp), and D4MIT15 (329 bp) of leukemic DNA samples (lanes 2-5) and control DNA samples (lanes 6-8). Molecular marker (HaeIII digested pBR332 DNA) are shown in lanes marked M. Lane 1 is a control "MT-PCR" without mouse template DNA. The touchdown annealing of this MT-PCR is 60°C. The sequence of primers, as suggested by Research Genetics, Inc., Huntsville, AL (April, 1995 release) were:

D4MIT316:

Forward: 5'-GAATGAATGACTGTCCATCTTATCC-3'

Reverse: 5'-AGCATGTTGAAAGTTTGTGTTCC-3'

D2MIT126:

Forward: 5'-GCTGAACTGAGCAAATTCCTGG-3'

Reverse: 5'-TGACAATGGGAAGTTATGTGTATG-3'

D4MIT15:

Forward: 5'-AGGAATACTGAATGTGGACTTTGC-3'

Reverse: 5'-TCCCTTGATTAAACAGAAGACCTG-3'

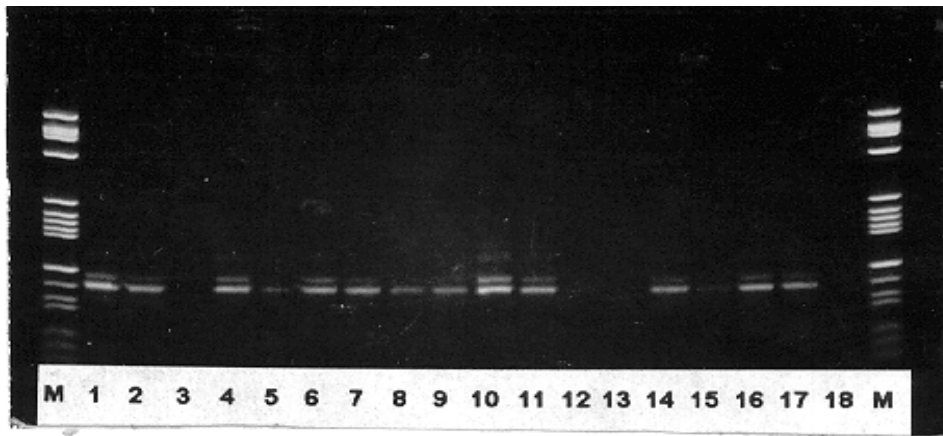


Figure 2. Representative of amplicons for the D2MIT140 marker (107 bp). Among control DNA samples (lanes 1, 2, 3, 16, and 17), no amplified DNA band is detected in the DNA sample isolated from a 10 weeks old mouse (lane 3). Similarly, among the leukemic DNA samples (lanes 4 to 15), no amplified DNA bands are shown in lanes 12,13, and 15). Lane 18 represents a control PCR containing no mouse DNA template. Molecular markers (HaeIII digested pBR332 DNA) are shown in lanes marked M. The touchdown annealing temperature of this PCR is 55°C. The primers of this marker (Research Genetics, Inc., Huntville, AL, release April, 1995) were:

Forward: 5'-CTGCCTCCTGTTTTGAAAGC-3'

Reverse: 5'-GACATGTATACACGTGTGCGC-3'

DISCUSSION

Results from this study indicate no direct association between microsatellite length polymorphisms and radiation leukemogenesis at 55 selected loci within the mouse genome. However, an uncommon MSI, manifested as the total lack of amplicon product was detected at two loci; one on chromosome 2 and one on chromosome 4. It seems very unlikely that technical problems would have contributed to this phenomenon as consistent results were obtained in four separate experiments. The lack of PCR amplified microsatellites indicates the absence of complimentary DNA to one or both primers in both alleles of the DNA template. Although the frequency of ML samples without the amplified DNA bands was statistically different from that in control DNA samples, the precise mechanism leading to the failure to amplify these regions is unknown.

While there is no clear evidence that the loss of microsatellites at these specific loci predisposes mice to leukemogenesis, it is reasonable to hypothesize that these regions of the genome may be mutable (fragile) sites contributing to susceptibility for spontaneous mutation or to radiation-induced damage. This concept is supported by the previous finding that D2MIT140 and D4MIT104 microsatellite markers map to regions of the mouse genome that are frequently involved in chromosomal rearrangements during the early stages of ML induction in mice exposed to ionizing radiation (24). Multiple fragile sites, containing telomere-like repeated sequences on mouse chromosome 2, have previously been implicated in radiation leukemogenesis (25). The D2MIT140 and D4MIT104 markers have been mapped to mouse chromosome 2H and chromosome 4A, respectively. Translocations between mouse chromosome 2 and chromosome 4, particularly 4A such as t(2;4)(H:A) and t(2;4)(B:A), were detected in the bone marrow cells of mice examined at different times following radiation exposure, i.e., 3, 12, and 18 months (24).

The mouse 2H is syntenic to human 20q11-13 (27). Deletions at 20q11-13 have been frequently detected in patients with myeloproliferative disorders and myelodysplastic syndromes (38,39). Moreover, several candidate tumor suppressor genes associated with growth regulation and apoptosis have been mapped to human 20q: a hematopoietic cell kinase gene, a Bcl2-related gene, a topoisomerase I gene, and a CD40 gene (40-42). The mouse 2H may also harbor tumor suppressor gene(s). The synteny of the mouse 4A is to the proximal end of human 8q, approximately 8q11-13 (43,44). Although the direct involvement of human 8q11-13 or mouse 4A in carcinogenesis has not been demonstrated (45), several prominent oncogenes with associated kinase activity have been mapped to these regions, including *Mos* and *Lyn* (46-48). *Mos* (Moloney murine sarcoma virus) is an upstream activator of mitogen-activated protein kinase (MAPK), a protein with serine/threonine kinase activity. Activation of the *Mos* oncogene induces fibrosarcoma in the mouse (ref. 49 for a review). *Lyn* is a *scr*-related oncogene encoding a tyrosine kinase that often is found to be associated with hematopoietic cell surface receptors (50) and with regulation of neutrophils (51). Interestingly, our previous cytogenetic data demonstrated that irradiated mice with translocations involving 4A had high neutrophil counts (24). The synergistic effects of *Lyn*-tyrosine kinase activity and *IL6* or *GM-CSF* in promoting cell proliferation have also been demonstrated in a human leukemic (AML-193) cell line (52). Thus, rapidly growing information on *Mos* and *Lyn* in combination with the close association of protein kinase with growth and differentiation provide plausible reasons to suggest that alterations in *Mos* and *Lyn* may be contributing factors for the malignant transformation of hematopoietic cells by radiation exposure. However, other molecular mechanisms associated with radiation leukemogenesis cannot be ruled out because all the radiation-induced ML cases did not acquire these abnormalities. Nevertheless, the 2H and

the 4A regions of the mouse genome may be radiation sensitive sites. The type of MSI detected in this study is not associated with *N-ras* mutations found in some of the ML cases reported recently (53, Table 2). This finding indicates that mechanisms leading to *N-ras* mutations and MSI are two separate genetic events. Similar results were observed in dimethylbenz[a]anthracene-induced mouse skin tumors (54). In contrast, association between MSI mutations of some oncogenes such as *c-myc* and *K-ras* has been found in mouse sarcoma induced by methylcholanthrene (17). These inconsistencies may partly be due to tissue and tumor specificity of MSI in the mouse.

Results from our studies and those obtained from human leukemia studies demonstrated that MSI, determined by length polymorphisms, is not a common genetic lesion in hematopoietic neoplasms. These results suggest that other types of genetic instabilities may be more critical for neoplastic transformation of exposed hematopoietic stem cells. Cytogenetic evaluation of radiation-induced murine ML cells strongly supports the concept that gene loss on one copy of chromosome 2 is directly involved in radiation leukemogenesis (20-26). In principle, microsatellite markers can be used to detect gene loss, manifested as loss of heterozygosity (LOH), providing that ML cells are obtained from heterozygous hybrid mice (55). In our study, however, the detection of LOH is not feasible because the CBA/Ca mouse is a highly inbred strain and identification of hemizygous gene loss in homozygous individuals is very difficult, if not impossible.

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