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p53 Gene Mutation and Loss of Heterozygosity of Chromosome 11 in Methylcholanthrene-induced Mouse Sarcomas

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Mutations of the *p53* tumor suppressor gene are the most prevalent genetic alteration observed in a wide variety of human cancers. In this study we examined 63 methylcholanthrene (MCA)-induced sarcomas from C57BL/6N×C3H/HeN F_1 (BCF_1) or C3H/HeN×C57BL/6N F_1 (CBF_1) mice for *p53* gene mutations and loss of heterozygosity (LOH) of chromosome 11. Mutation analysis was done on exons 5 to 8 of the *p53* gene by polymerase chain reaction-single strand conformation polymorphism analysis. This identified 53 potential mutations in 45 sarcomas. Mutations were further confirmed by direct sequencing of the region. Forty-nine of the 53 cases (94%) were missense mutations, while the rest included two nonsense mutations, one silent mutation and one insertional mutation. Spectra of base substitutions were: 25 cases (47%) of G:C→T:A transversion, 13 cases (25%) of G:C→A:T transition (CpG site 15%), 13 cases (24%) of G:C→C:G transversion, a case (2%) of A:T→T:A transversion and a case (2%) of insertion. In addition, analysis of 5 polymorphic markers of mouse chromosome 11 revealed LOH in ten cases (22%) among those carrying *p53* mutations. In nine of these 10 cases, the loss involved all 5 markers. In addition, the loss was biased toward the C57BL allele (9 cases). The present study establishes the pattern of mutation of the *p53* gene in MCA-induced mouse sarcomas.

Key words: *p53* — LOH — MCA — Mouse sarcoma

The *p53* tumor suppressor gene has been implicated in the pathogenesis of a wide variety of human cancers.[1, 2] Functions of p53 protein include induction of G1 arrest of the cell cycle and apoptosis after DNA damage.[3, 4] Mutations of the *p53* gene reported thus far seem to cluster predominantly around five highly conserved amino acid domains which are coded for by exons 5 to 8.[5, 6] Numerous studies have been conducted on mutation analysis of the *p53* gene in rodents with chemically induced tumors.[7–18] These analyses revealed that the frequency of *p53* alterations in rodent experimental tumors varied greatly depending on the chemical agents and tumor types.

Analysis of *p53* mutations in experimental tumors may offer interesting and important information to elucidate the mechanism of mutagenesis and the biological significance of the mutation in carcinogenesis. We established a series of methylcholanthrene (MCA)-induced sarcomas in C57BL/6N×C3H/HeN F_1 (BCF_1) mice.[19] MCA is a well-defined carcinogen that binds to the Ah receptor and activates cytochrome P450.[20, 21]

In this study, we analyzed the spectrum of *p53* gene mutations in MCA-induced mouse sarcomas. In contrast to the results of earlier studies, the present analysis revealed that *p53* gene mutation is quite common in MCA-induced mouse sarcomas.

MATERIALS AND METHODS

Sarcoma induction MCA-induced sarcomas analyzed in this study were described previously.[19] Briefly, BCF_1 or C3H/HeN×C57BL/6N (CBF_1) mice were injected subcutaneously at several regions on the back with 0.5–1 mg of MCA dissolved in olive oil. When tumors had grown to 1 cm in diameter, they were excised and examined histologically. Sixty-three independent tumors were obtained, of which five were from CBF_1 mice (tumors numbered CB), and 26 from BCF_1 mice (tumors numbered BC). A portion of each tumor was minced with scissors and transferred to a 3-cm culture dish. The samples were grown for 10 days in order to minimize contamination with stromal cells. All of the tumors of the present study were transplantable to syngeneic mice.

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis DNA was isolated according to the procedure described previously.[19, 22] Oligonucleotide primers for exons 5 to 8 of the *p53* tumor suppressor gene were synthesized.[23] The sequences are shown in Table I. All primers included a portion of intron

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Table I. Oligonucleotide Primers Used for PCR-SSCP and Direct Sequencing of *p53* Exons 5–8

Exon	Primer sequence (sense/antisense)	Product size (bp)
5	5′-TCT CTT CCA GTA CTC TCC TC-3′ 5′-GAG GGC TTA CCA TCA CCT TC-3′	204
6	5′-TTG CTC TTA GGC CTG GCT C-3′ 5′-AAT TAC AGA CCT CGG GTG GC-3′	133
7	5′-TCT TCC CCA GGC CGG CTC TG-3′ 5′-GCC TTC CTA CCT GGA GTC TT-3′	130
8	5′-TCC CGG ATA GTG GGA ACC TT-3′ 5′-GCC TGC GTA CCT CTC TTT GC-3′	155

Table II. PCR Primers for Polymorphic Markers on Chromosome 11[a)]

Locus	cM[b)]	Primer sequence[c)] (sense/antisense)	Allele size[d)]
D11Mit229	10.9	5′-TGT TTG CTT GGT TTT GTT TTG-3′ 5′-ATC CCG GTT TTA CAT CAG ACA-3′	C>B
D11Mit349	27.3	5′-AGT ATC AGA TCC AGT TGG AGG-3′ 5′-GTA GAA AAA GAT ACC CAG TGT CAG C-3′	B>C
D11Mit320	39.3	5′-CCC ATA TAG TGA AGC AAG AAA CG-3′ 5′-TTA TAG TGT ATG CAT CCA GGT GTG-3′	C>B
D11Mit41	48.1	5′-CTG CTA AAG TGG GGT TAA ATG C-3′ 5′-CGA CTG AGC AAG TTG TAT TTC TG-3′	C>B
D11Mit258	65.6	5′-AAA CAG AGA TAA ACC ACG GGG-3′ 5′-TGT GGA ACT AAC TCT CAG AAG GC-3′	C>B

a) *p53* locus: 38.4 centiMorgans (cM) from centromere of chromosome 11, Integrated MIT SSLP and Copeland/Jenkins RFLP Genetic Maps.
b) Distance from centromere of chromosome 11 in centiMorgans (cM).
c) DNA sequence: Whitehead Institute, MIT Center for Genome Research, Genetic and Physical Maps of the Mouse Genome.
d) Abbreviations: B, C57BL/6N; C, C3H/HeN.

in order to avoid amplification of the *p53* pseudogene. PCR-SSCP analysis was done according to the standard procedure.[24)] Briefly, primers were end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. Genomic DNA was amplified for 30 cycles in 10 μl of a reaction mixture containing 100 ng of template DNA, 4 μM end-labeled primers, 200 μM each of dNTPs (dATP, dCTP, dGTP, dTTP) and 0.05 units of Taq DNA polymerase. Each cycle consisted of 94°C for 1 min, 52°C for 1 min and 72°C for 30 s. Reaction mixtures were treated with 10 μl of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol, then denatured at 90°C for 2 min. An aliquot (1 μl/lane) was applied to a 6% non-denaturing polyacrylamide gel containing 5% glycerol, and electrophoresed at 30–40 Watts for 2–3 h at room temperature with fan cooling. The gel was dried and exposed to X-ray film.

Direct sequencing of *p53* mutations Putative mutant bands of the *p53* gene detected by PCR-SSCP analysis were eluted from the dried gel and used for re-amplifica-

tion. PCR-amplified products were cycle-sequenced by the dye terminator method (373 DNA Sequencer, Applied Biosystems; Dye Terminator Cycle Sequence FS Reaction Kit, Perkin-Elmer Cetus, Norwalk, CT). Primers for DNA sequencing were the same as those for PCR-SSCP.

Loss of heterozygosity (LOH) analysis of chromosome 11 Allelic losses of chromosome 11 in tumor DNAs were analyzed using the five polymorphic markers along the chromosome. The primers for PCR and the size difference of the two alleles are shown in Table II.[25)]

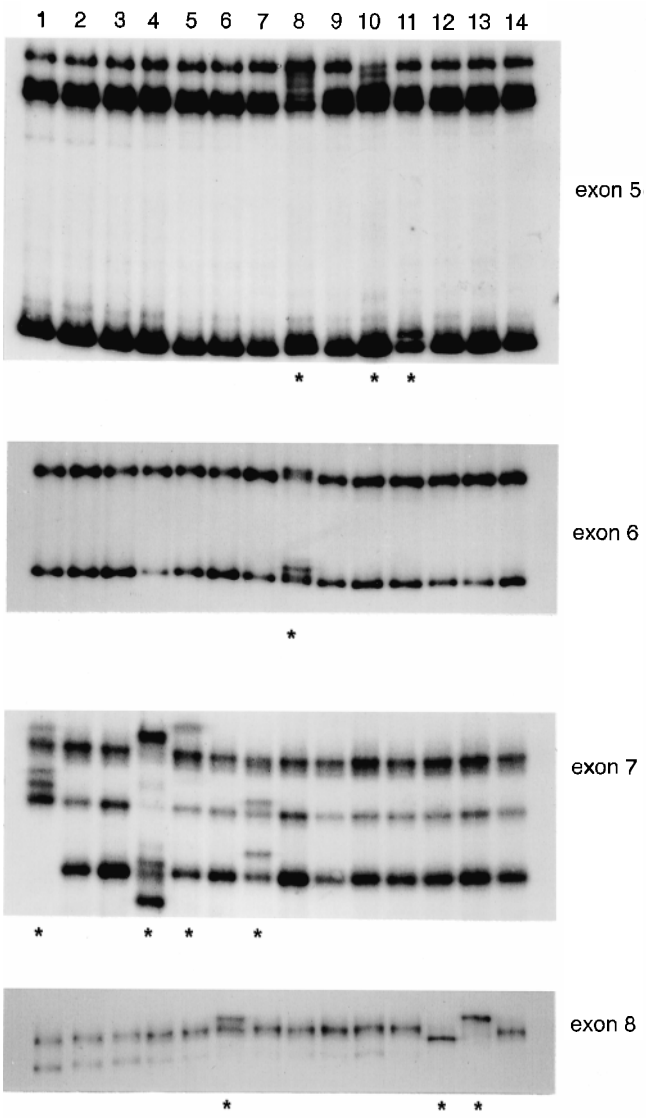

Fig. 1. PCR-SSCP analysis of *p53* gene mutations in MCA-induced mouse sarcomas. Lanes 1 to 14 are cases 8, 40, 29, 19, 20, 36, 17, 53, 50, 48, 62, 57, 49 and 59. Exons examined are 5 (top), 6 (second), 7 (third) and 8 (bottom). A closed star (★) indicates samples with mobility shifts.

RESULTS

Spectrum of *p53* mutations Genomic DNAs from 63 MCA-induced mouse sarcomas were examined by PCR-SSCP analysis of exons 5 to 8 of the *p53* gene (Fig. 1). PCR products showing mobility shifts on SSCP gel were eluted and subjected to direct sequencing (Fig. 2). The results are summarized in Table III. In total, 45 out of 63 sarcomas carried mutation of the *p53* gene. Among these, 53 mutations were identified. Missense mutations were the most prevalent and 49 of the 53 were of this type. The rest included two cases of nonsense mutations, one silent mutation and one insertion. Mutations were found frequently at codons 172, 242, 245, 246 and 270, and these codons correspond to the hot spots of mutation in the human *p53* gene.

Table III summarizes the results of *p53* mutations and LOH in the MCA-induced sarcomas. Double mutations in a single tumor were found in 6 cases (case 1, 12, 20, 28, 41 and 43) and triple mutations in one case (case 53).

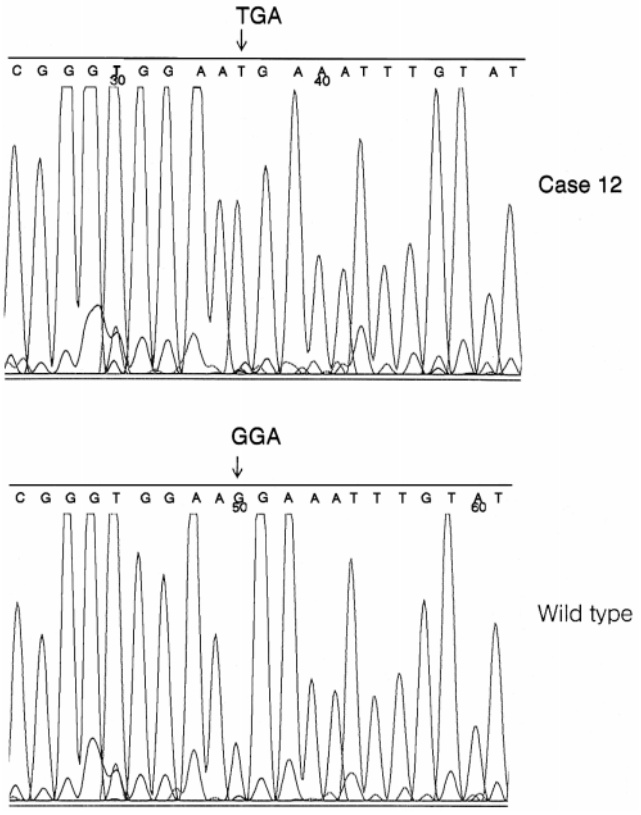

Fig. 2. Histogram of DNA sequencing of *p53* gene. The region around codon 196 (exon 6) is shown for sarcoma case 12, which carried a GGA→TGA transversion.

Table III. Summary of *p53* Mutations in MCA-induced Mouse Sarcomas

Case	Sarcoma	Exon	Codon	Base change	Amino acid change	Base change pattern	LOH	Lost allele
1	CB6296	7	241	GGG→AGG	Gly→Arg	G:C→A:T	–	
		8	277	AGA→AGT	Arg→Ser	A:T→T:A		
2	CB6328	7	241	GGG→TGG	Gly→Trp	G:C→T:A	–	
3	CB6329	7	245	CGC→CCC	Arg→Pro	G:C→C:G	–	
4	CB6330	5	155	CGC→CTC	Arg→Leu	G:C→T:A	–	
5	BC7199-1	5	172	CGC→CAC	Arg→His	G:C→A:T	–	
6	BC7199-3	5	172	CGC→CAC	Arg→His	G:C→A:T	–	
7	BC7200-1	8	276	GGG→GTG	Gly→Val	G:C→T:A	–	
8	BC7210-1	7	241	GGG→TGG	Gly→Trp	G:C→T:A	+	C57BL
12	BC7212-3	6	196	GGA→TGA	Gly→Stop	G:C→T:A	–	
		8	264	CGG→TGG	Arg→Trp	G:C→A:T		
13	BC7213-1	7	246	CGA→CTA	Arg→Leu	G:C→T:A	–	
14	BC7213-2	7	241	GGG→GTG	Gly→Val	G:C→T:A	–	
16	BC7214-3	5	170	GTG→TTG	Val→Leu	G:C→T:A	–	
17	BC7274-1	7	246	CGA→CTA	Arg→Leu	G:C→T:A	–	
18	BC7352-1	5	172	CGC→CAC	Arg→His	G:C→A:T	–	
19	BC7353-1	7	241	GGG→GTG	Gly→Val	G:C→T:A	–	
20	BC7353-3	7	246	CGA→CCA	Arg→Pro	G:C→C:G	–	
		8	300	AGC→AAC	Ser→Asn	G:C→A:T		
21	BC7354-1	8	276	GGG→TTGG	Gly→Stop		+	C57BL
24	BC7354-4	7	239	TGC→TTC	Cys→Phe	G:C→T:A	–	
25	BC7371-1	8	279	CGC→CCC	Arg→Pro	G:C→C:G	–	
26	BC7371-2	8	270	CGT→CCT	Arg→Pro	G:C→C:G	–	
28	BC7373-1	5	130	CTA→TTA	Leu→Leu	G:C→A:T	–	
		6	212	AGC→ACC	Ser→Thr	G:C→C:G		
29	BC7412-1	6	210	CGC→CTC	Arg→Leu	G:C→T:A	–	
34	BC7415-2	8	264	CGG→CCG	Arg→Pro	G:C→C:G	+	C57BL
36	BC7421-2	8	270	CGT→CTT	Arg→Leu	G:C→T:A	–	
38	BC7422-2	6	212	AGC→CTC	Ser→Leu	G:C→T:A	+	C57BL
39	BC7422-4	5	172	CGC→CAC	Arg→His	G:C→A:T	+	C3H
40	BC7423-5	8	263	GGA→GTA	Gly→Val	G:C→T:A	–	
41	BC7424-5	5	152	AGC→AGG	Ser→Arg	G:C→C:G	+	C57BL
		5	153	CGT→CTT	Arg→Leu	G:C→T:A	–	
42	BC7425-1	7	242	GGC→TGC	Gly→Cys	G:C→T:A	–	
43	BC7425-5	5	172	CGC→CAC	Arg→His	G:C→A:T	–	
		7	241	GGG→TGG	Gly→Trp	G:C→T:A	–	
44	BC7426-2	5	172	CGC→CAC	Arg→His	G:C→A:T	+	C57BL
46	CB6334	7	241	GGG→GTG	Gly→Val	G:C→T:A	–	
47	BC7200-2	7	245	CGC→CAC	Arg→His	G:C→A:T	–	
48	BC7214-2	5	170	GTC→ATC	Val→Ile	G:C→A:T	+	C57BL
49	BC7273	8	270	CGT→CAT	Arg→His	G:C→A:T	+	C57BL
51	BC7353-2	7	242	GGC→TGC	Gly→Trp	G:C→T:A	–	
52	BC7412-3	6	196	GGA→TGA	Gly→Stop	G:C→T:A	–	
53	BC7413-1	5	156	GCC→CCC	Ala→Pro	G:C→C:G	–	
		6	212	AGC→ACC	Ser→Thr	G:C→C:G		
		6	213	GTG→TTG	Val→Leu	G:C→T:A		
54	BC7413-2	8	270	CGT→CCT	Arg→Pro	G:C→C:G	–	
55	BC7413-4	8	264	CGG→CCG	Arg→Pro	G:C→C:G	–	
57	BC7415-4	8	278	GAC→TAC	Asp→Tyr	G:C→T:A	–	
58	BC7419-4	8	278	GAC→TAC	Asp→Tyr	G:C→T:A	–	
60	BC7421-4	5	155	CGC→CTC	Arg→Leu	G:C→T:A	–	
62	BC7423-3	5	155	CGC→CCC	Arg→Pro	G:C→C:G	–	
63	BC7424-4	5	155	CGC→CCC	Arg→Pro	G:C→C:G	+	C57BL

Table IV. Spectrum of *p53* Mutations in MCA-induced Mouse Sarcomas

Exon	G:C→A:T		G:C→T:A	G:C→C:G	A:T→G:C	A:T→T:A	A:T→C:G	Ins.	Total
	non-CpG	CpG site							
5	2	6	4	4	0	0	0	0	16
6	0	0	5	2	0	0	0	0	7
7	1	1	11	2	0	0	0	0	15
8	1	2	5	5	0	1	0	1	15
Total	4	9	25	13	0	1	0	1	53
(%)	(8)	(17)	(47)	(24)	(0)	(2)	(0)	(2)	(100)

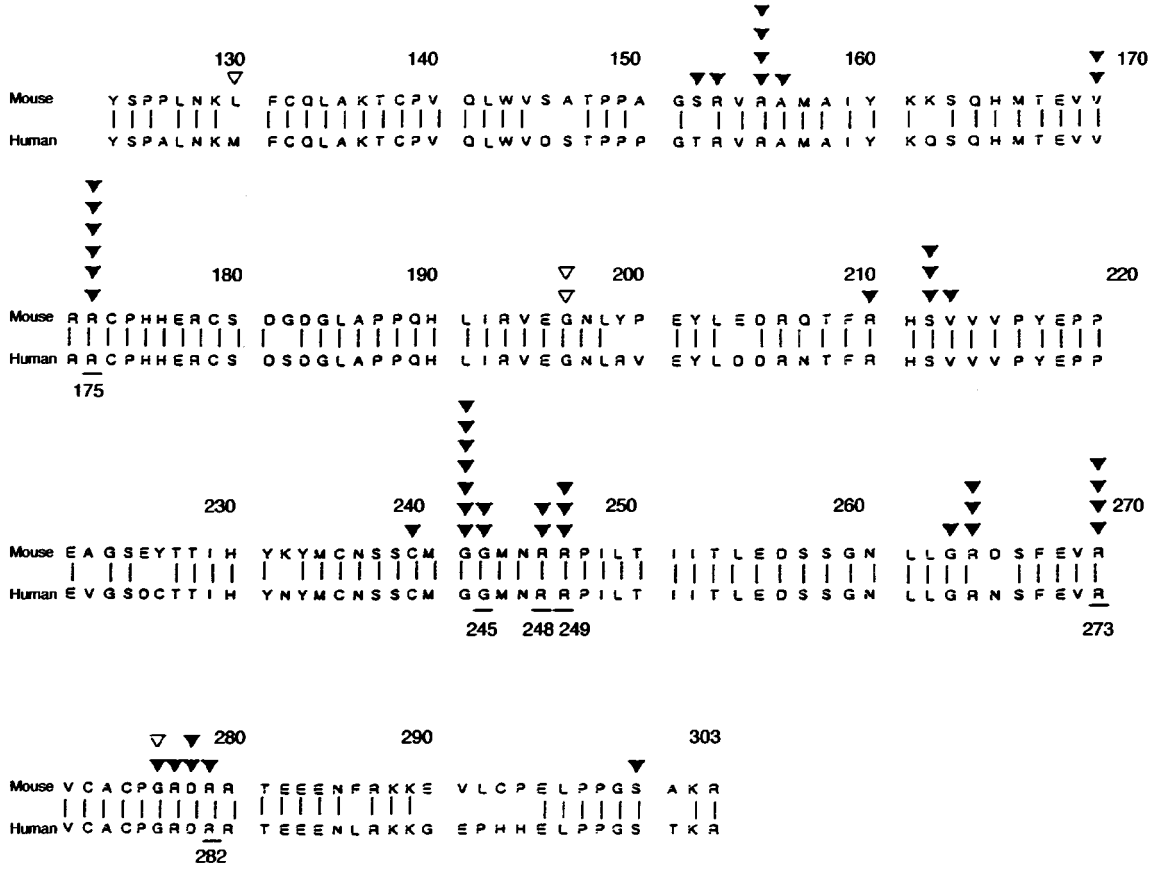

Fig. 3. Location of mutations in the amino acid sequence of mouse p53 protein. Open reverse triangles (▽) and closed reverse triangles (▼) indicate nonsense and missense mutations, respectively. The amino acid sequence of human p53 protein is shown under the corresponding mouse sequence. Underlines indicate mutational hot spots and their codon numbers in human cancers.

Five of these 7 cases involved different exons. It is likely that these mutations occurred on different alleles of the gene. In two cases (case 41 and 53), double mutations were located in consecutive codons of the same exon, which suggests that these mutations were on the same allele. The spectrum of *p53* mutations is summarized in Table IV. The most prevalent type was G:C→T:A transversion (25 cases, 47%).

Distribution of the 53 mutations in MCA-induced mouse sarcomas is shown in Fig. 3, where the amino acid sequence of the mouse protein is aligned with that of human p53 protein. Some of the hot spots of *p53* gene

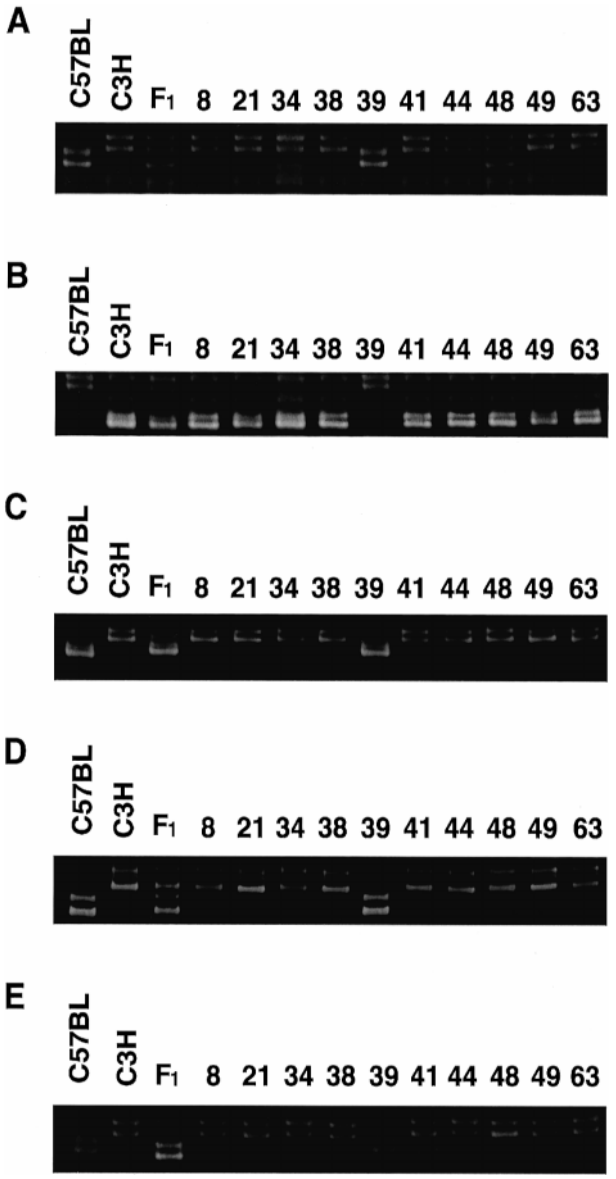

Fig. 4. LOH analysis of mouse chromosome 11. Lanes indicate analysis of control DNA from C57BL/6N, C3H/HeN and F_1 mice together with that from sarcomas; cases 8, 21, 34, 38, 39, 41, 44, 48, 49 and 63. Panels A, B, C, D and E show the analyses of D11Mit229, D11Mit349, D11Mit320, D11Mit41 and D11Mit258, respectively. Among these, D11Mit320 is the closest marker to the *p53* gene. The loss of the C57BL/6N allele was evident in cases 8, 21, 34, 38, 41, 44, 48, 49 and 63, while case 39 showed loss of the C3H/HeN allele.

mutation in the present study matched those of human tumors. However, some hot spots, such as mouse codons 155, 212 and 264, did not have human counterparts.

LOH analysis of chromosome 11 Forty-five of MCA-induced mouse sarcomas carrying *p53* mutations were examined for LOH of chromosome 11, on which the gene is located.[25] Sarcomas were examined for LOH using polymorphic markers of chromosome 11 (Table II). Fig. 4 illustrates representative cases of such analysis with D11Mit320, which is located near the *p53* gene (within 1 cM). Only 10 among 45 sarcomas (22%) showed LOH at this locus. Except for one case (case 48), all other markers examined were also lost, which suggests the involvement of a large region. Nine of 10 sarcomas with LOH were accompanied with single *p53* mutation, which indicated mutation of one allele and loss of the other allele. Interestingly, nine of the 10 losses involved the C57BL allele in BC sarcomas.

DISCUSSION

Point mutations of the *p53* gene were frequent in MCA-induced mouse sarcomas, as previously reported by Halevy *et al*.[26] They identified seven *p53* mutations, of which four were G:C→T:A transversion. In this study, we examined mutation in the *p53* gene in 63 MCA-induced mouse sarcomas, and we detected 53 mutations in 45 out of 63 sarcomas. This frequency is one of the highest among chemically induced rodent tumors so far examined.[7–18] In addition, our study of MCA-induced sarcomas revealed some similarities in the distribution of *p53* gene mutation between human and mouse. Mutations were observed at mouse codons 172 (6 cases), 242 (2 cases), 245 (2 cases), 246 (3 cases), 270 (4 cases) and 279 (1 case), and these corresponded to human *p53* gene hot spots 175, 245, 248, 249, 273 and 282. In addition, mutations were frequently detected at codons 241 (7 cases), and 278 (2 cases). In the case of human cancers, about half of the G-to-A transitions were at the CpG site.[27] Eight of 13 cases of G:C→A:T transition in the present study were at the CpG site of codon 172 (6 cases), 245 (1 case), or 270 (1 case). All of these cases correspond to CpG site hot spots of human cancers.

MCA belongs to the family of polycyclic aromatic hydrocarbons and is a well-known carcinogen to rodents. Polycyclic aromatic hydrocarbons are metabolically activated and the metabolites bind to the 2-amino group of guanine in DNA to produce bulky carcinogen-DNA adducts which produce predominantly the G:C→T:A transversions.[28]

We previously examined K-*ras* mutations in MCA-induced mouse sarcomas (H. Watanabe *et al*., unpublished data). Interestingly, the spectrum of K-*ras* mutations was similar to that of *p53*, and G:C→T:A transversion predominated (50%). Thus, the spectrum of mutation by MCA seems to be similar for both the K-*ras* gene and the *p53* gene. A large variety of mutations has been identified in the *p53* gene in human cancers.[29] Different cancer

types exhibit different patterns of *p53* gene mutations.[27, 29] The high frequency of *p53* mutations in MCA-induced mouse sarcomas offers a unique opportunity to elucidate the role of *p53* gene mutation in the etiology of carcinogenesis. Analysis of human bone and soft tissue sarcomas identified 42 somatic alterations of the *p53* gene, of which 21 were point mutations.[30] The spectrum of these point mutations was different from that of our MCA-induced mouse sarcomas.

p53 gene knock-out mice develop sarcomas and lymphomas.[31] Analysis of mutations of the wild-type allele in sarcomas arising in *p53* heterozygous mice by Southern analysis revealed loss of the wild-type allele in the majority of cases.[32] Human colorectal cancers were shown to suffer frequent LOH of the *p53* gene,[33] while such LOH was rare in chemically induced mouse colon tumors.[13] Rodent tumor models have been examined thoroughly for LOH of chromosome 11.[15, 34–36] We have examined LOH on chromosome 11, where the *p53* tumor suppressor gene is located. We detected 10 cases of LOH (22%) of chromosome 11 among 45 sarcomas with *p53* mutations. Nine of these 10 cases were accompanied with single *p53* mutation. A case of double mutations occurred at consecutive codons, 152 and 153 (case 41), and this case also carried LOH of chromosome 11. Multiple mutations of one allele and LOH of another allele are consistent with the two-hit theory. However, cases with LOH and mutation, and cases with mutations on both alleles were rather infrequent, and the two-hit mechanism may not be applicable to the majority of cases. Therefore, most MCA-induced sarcomas may be due to the dominant negative *p53* gene mutation mechanism.[37]

Our present analysis revealed the preferential loss of regions of mouse chromosome 11 derived from C57BL/6N in MCA-induced sarcomas of BCF_1 mice. It is not known whether the preference has bias toward maternal origin, or whether there is a strain difference of the allele. Preferential LOH of the maternally derived alleles was reported in several human tumors for *WT1*, *IGF2* and *KIP2*.[38–40] Strain preference in LOH has been noted in some mouse tumors.[41, 42] Analysis of a larger number of CBF_1 tumors should clarify the mechanism of the allelic preference of LOH of chromosome 11 in MCA-induced mouse sarcomas.

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