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Demonstration of *ras* and *p53* Gene Mutations in Carcinomas in the Forestomach and Intestine and Soft Tissue Sarcomas Induced by *N*-Methyl-*N*-nitrosourea in the Rat

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The presence of *ras* family and *p53* gene mutations in rat forestomach, intestine and liver tumors and soft tissue sarcomas induced by *N*-methyl-*N*-nitrosourea (MNU) was examined using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) followed by direct sequencing analysis. In the forestomach squamous cell carcinomas (SCC), Ha-*ras* and *p53* mutations were detected in 2 (40%) and 4 (80%) of 5 cases, respectively. The figures for Ki-*ras* and *p53* gene mutations in adenocarcinomas of the large and small intestines were 3 (18.8%) and 5 (31.3%) of 16 cases. Soft tissue sarcomas in different sites were found to have mutations of Ki-*ras* in 7 (23.3%) and of *p53* in 9 (30%) of 30 cases. One forestomach SCC and 2 soft tissue sarcomas had double *p53* mutations in different exons. Single cases of forestomach SCC and intestinal adenocarcinoma had mutations in both Ki-*ras* and *p53* genes. No mutations were found in counterpart benign tumors or hepatocellular adenomas. The *p53* mutation spectrum revealed preferential clustering within exon 8 for the forestomach SCCs, and exons 5 and 8 for the intestinal adenocarcinomas, whereas the distribution was evenly spread through exons 5 to 8 in soft tissue sarcomas. All the detected *ras* or *p53* mutations were G:C to A:T transitions. These results indicate firstly that specific Ki-*ras*, Ha-*ras* and *p53* gene mutations in MNU-induced lesions are related to particular alkylation sites (G:C to A:T transitions) and secondly, although not essential, Ki-*ras*, Ha-*ras* or *p53* gene mutations may be involved in the progression stage of forestomach, intestine and soft tissue neoplasms induced by MNU.

Key words: *p53* — *ras* — Tumors — MNU — Rat

Carcinogenesis is believed to be a multi-step process, involving a progressive accumulation of genetic alterations.[1)] In human colon carcinogenesis, it is presumed that alteration of several genes, such as Ki-*ras* and *p53* genes, is required for development of malignancy. Thus, a high incidence of Ki-*ras* gene mutations has been detected in human colorectal carcinomas.[1–3)] In rats, colon tumors induced by 1,2-dimethylhydrazine or its metabolite azoxymethane are also characterized by frequent Ki-*ras* mutations, even at early stages.[4–7)] This is of importance, given their morphological similarities to human cases. Recent studies on *ras* point mutations in chemically induced rat tumors have also indicated tumors induced by the broad-spectrum, direct-acting carcinogen *N*-methyl-*N*-nitrosourea (MNU) to be often positive.[4, 5)]

Although *p53* point mutations or short deletions are detected frequently in human colon tumors,[1, 8, 9)] they are generally absent in rat colon models.[10)] Furthermore, while *p53* gene abnormalities have been found at an incidence of 42/127 (33.1%) in human soft tissue and bone sarcomas, exhibiting a wide mutational spectrum throughout codons 46–316,[11)] no information is available regarding such changes in soft tissue tumors in the rat. Although some renal mesenchymal tumors exhibit mutations, the reported incidences have varied greatly.[9, 12, 13)] *p53* gene knockout mice are particularly prone to development of non-epithelial tumors such as lymphomas, and soft tissue and neurogenic sarcomas rather than epithelial lesions.[14–16)] Thus, the possible involvement of *ras* and *p53* gene alterations and their induction in other models is of interest. The present study of MNU-induced tumors was conducted to cast light on this question.

MATERIALS AND METHODS

Treatment and tumors used Eighty-five, 5-week-old male Fischer F344 rats (Charles River Japan Inc., Atsugi) were intraperitoneally injected with MNU (Sigma, St. Louis, MO) at a dose of 20 mg/kg (dissolved in a citrate buffer adjusted to pH 6.0 at a concentration of 4 mg/ml) once a week for 4 weeks. The rats were killed when moribund due to tumor development after week 52 and

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all the survivors were killed at week 60 after the first MNU injection. Resected tumors were cut into 3 mm slices with a razor blade, immediately fixed in ice-cold acetone, embedded in paraffin, sectioned and mounted on slide glasses. For histological diagnosis, sections were stained with hematoxylin and eosin, and additionally, in the sarcoma cases, with polyclonal antibody to S-100 protein (Nichirei Co., Ltd., Tokyo) to allow differentiation of neurogenic tumors. A total of 67 benign and malignant tumors from 60 rats was used. The tumors included 11 forestomach tumors (4 papillomas and 5 squamous cell carcinomases (SCCs)), 20 intestinal tumors (2 adenomas and 7 adenocarcinomas from the small intestine and 2 adenomas and 9 adenocarcinomas from the large intestine), 30 soft tissue sarcomas (prostate, peri-prostate, retroperitoneal, peritoneal cavity and subcutaneous regions) and 8 hepatocellular adenomas.

Preparation of DNA Tumor tissue was carefully scraped with a small razor blade from 4 or 5 sections cut at 10 μm thickness and mounted on slide glasses, with reference to serially sectioned hematoxylin and eosin-stained preparations for orientation. DNA was then extracted using a Sepagene Kit (Sanko Junyaku Co., Ltd., Tokyo).

PCR-SSCP analysis of *ras* and *p53* genes Oligonucleotide primers for exons 1 and 2 of the Ki- and Ha- and N-*ras* oncogenes and exons 5 to 8 of the *p53* tumor suppressor gene were synthesized with a DNA synthesizer (Model 391, Applied Biosystems, Foster City, CA) and purified as previously described.[17,18] The sequences of the oligonucleotide primers for PCR-SSCP and direct sequencing analysis are shown in Table I. Most included intron nucleotides upstream or downstream of the primer to avoid amplification of *p53* pseudogenes.[19] PCR-SSCP analysis was performed with 1 μg of DNA, 0.7 units of Taq polymerase (Takara Biochemicals Co., Ltd., Kyoto) and 15 pmol of end-labeled primer pairs. Thirty cycles at 94°C, 55°C and 72°C for 30 s, 30 s and 1 min, respectively, were carried out using a Perkinson-Elmer Cetus DNA Thermal Cycler. Five microliter aliquots of PCR reaction mixtures were diluted with 45 μl of 95% formamide/20 m*M* EDTA/0.05% bromophenol blue/0.05% xylene cyanol, then heated at 80°C for 2 min, and 2 μl aliquots were applied to 6% polyacrylamide gels containing 90 m*M* Tris-borate buffer (pH 8.3), 4 m*M* EDTA and 5% glycerol. Electrophoresis was performed at 40 watts for 2–2.5 h followed by drying of the gel on filter paper and autoradiography.

Direct DNA sequencing DNA from abnormally shifted alleles on SSCP gels was carefully cut out and dissolved in 50 μl of distilled water for 30 min at 80°C, then 15 μl aliquots were amplified by asymmetric PCR with 2.5 units of Taq DNA polymerase (Takara Biochemicals Co., Ltd.) using 50 and 5 pmol of paired primers. Fifty cycles of 94°C, 55°C, and 72°C for 0.5, 1, and 2 min, respectively, were performed. After phenol/chloroform extraction and ethanol precipitation, unincorporated nucleotides and primers were removed from the amplified DNAs by filtration through a polysulfone filter with a

Table I. Sequences of Oligonucleotide Primers Used for the PCR-SSCP and DNA Sequencing Analyses of the *ras* and *p53* Genes in the Rat

ras		*p53*	
Ki-*ras*			
Exon 1		Exon 5	
K12A	5′-GGCCTGCTGAAAATGACTGA-3′	P5-1	5′-GATTCTTTCTCCTCTCCTAC-3′
K12C	5′-CGTAGGATCATATTCATCCA-3′	P5-2	5′-ACAGGCAGTGCCAGTGCTCA-3′
Exon 2		Exon 6	
Kr611	5′-GACTCCTACAGGAAACAAGT-3′	P6-U	5′-CCTCTGACTTATTCTTGCTC-3′
K61Br	5′-CACAAAGAAAGCCCTCCCCA-3′	P6-2	5′-CTCAGGTGGCTCATACGGTA-3′
Ha-*ras*		Exon 7	
Exon 1		P7-1	5′-GGCTCCGACTATACCACTAT-3′
H12Ar	5′-AAGCGATGACAGAATACAAG-3′	P7-D	5′-AACCTGGCACACAGCTTCCT-3′
H12Br	5′-CTCACCTCTATAGTGGGATC-3′	Exon 8	
Exon 2		P8-U	5′-CTTGTGCTGTGCCTCCTCTT-3′
Hr612	5′-TTTGCAGGACTCCTACCGGA-3′	P8-5	5′-CCAATAATAACCTTGGTACC-3′
H61Br	5′-GGTCACCTGTACTGATGGAT-3′		
N-*ras*			
Exon 1			
Nr121	5′-TCGTAATTGCTGCTTTCC-3′		
N12B	5′-GGGCCTCACCTCTATGGTG-3′		
Exon 2			
Nr610	5′-CCAGGATTCTTACCGAAA-3′		
Nr613	5′-GATGGCAAACACACAGAGGA-3′		

cut-off M.W. of 10,000 (Ultrafree C3GC, Millipore Japan Ltd., Tokyo). Primers for sequencing were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and the sequencing reaction was performed with Sequenase Version 2.0 (United States Biochemical Corp., Cleveland, OH) according to the manufacturer's instructions.

RESULTS

Tumor incidence and histology Sixty (70.6%) out of the total of 85 rats had at least one tumor. These included 4 papillomas (4.7%) and 5 SCCs (5.9%) in the forestomach, 2 adenomas (2.4%) and 7 adenocarcinomas (8.2%) in the small intestine, and 2 adenomas (2.4%) and 11 adenocarcinomas (12.9%) in the large intestine. Two cases had adenocarcinomas in both intestines. There were 30 soft tissue sarcomas (35.3%) including 12 periprostate (14.1%), 7 retroperitoneal (8.2%), 5 peritoneal (5.9%) and 5 (5.9%) subcutaneous tissues. Seven rats (8.2%) had 2 histologically different tumors, mostly intestinal adenocarcinomas with sarcomas. Several sarcomas resembled peripheral nerve sheath tumors, but none of them showed positive staining for S-100 protein, in contrast to clear positivity for peripheral nerve tissue involved in sarcomas. Therefore they were diagnosed as pleomorphic sarcomas, which may include peripheral nerve tumors of low differentiation. Eight cases (9.4%) of liver adenoma were also induced.

***Ras* gene mutations** SSCP and direct sequencing analysis revealed the presence of Ki-*ras*, Ha-*ras* or *p53* gene mutations at relatively low frequencies in tumors induced by MNU. None of the tumors exhibited N-*ras* mutation. Two out of 5 (40%) forestomach SCCs showed mutations of Ha-*ras* codon 12 (GGA to GAA) (Table II),

Table II. The *ras* and *p53* Gene Mutation Spectra in Forestomach Squamous Cell Carcinomas

Case	*ras*		*p53*		
	Codon	Nucleotide change	Exon	Codon	Nucleotide change
1	Ha-*ras* 12	GGA→GAA		—	
2	Ha-*ras* 12	GGA→GAA	8	277	GGG→GAG
3		—	8	277	GGG→GAG
4		—	8	275	TGT→TAT
5		—	8	275	TGT→TAT

—, no mutation.

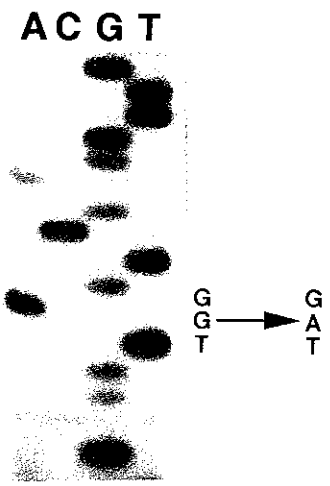

Fig. 1. DNA sequencing autoradiograph of codons 12 and 13 of the Ki-*ras* gene in large intestine. Normal large intestinal mucosa shows the wild-type sequence, while the adenocarcinoma demonstrates a transition mutation (GGT to GAT) in codon 12.

Table III. The *ras* and *p53* Gene Mutation Spectra in Intestinal Adenocarcinomas

Case	Site	*ras*		*p53*		
		Codon	Nucleotide change	Exon	Codon	Nucleotide change
1	S	Ki-*ras* 12	GGT→GAT[a)]	8	275	TGT→TAT[a)]
2	L	Ki-*ras* 12	GGT→GAT		—	
3	L	Ki-*ras* 12	GGT→GAT		—	
4	S		—	5	179	CGT→TGT
5	L		—	5	174	TGC→TAC[b)]
				8	277	GGG→GAG
6	L		—	5	179	CGT→TGT
7	L		—	8	277	GGG→GAG

S, small intestine; L, large intestine; —, no mutation.
a) Mutations in both *ras* and *p53* genes.
b) Twin-site mutations within the *p53* gene.

Table IV. The *ras* and *p53* Gene Mutation Spectra in Soft Part Sarcomas

Case	Histology	*ras*		*p53*		
		Codon	Nucleotide change	Exon	Codon	Nucleotide change
Prostate and peri-prostate region						
1	pleomorphic sarcoma	Ki-*ras* 12	GGT→GAT		—	
2	fibrosarcoma	Ki-*ras* 12	GGT→GAT		—	
3	fibrosarcoma		—	5	179	CGT→TGT
4	fibrosarcoma		—	8	264	GGA→AGA
5	fibrosarcoma		—	8	283	GAG→AAG
6	pleomorphic sarcoma		—	5	175	CCC→TCC[a]
				7	242	GGG→GAG
Retroperitoneal region						
7	fibrosarcoma	Ki-*ras* 12	GGT→GAT		—	
8	fibrosarcoma	Ki-*ras* 12	GGT→GAT		—	
9	fibrosarcoma	Ki-*ras* 12	GGT→GAT		—	
Peritoneal cavity and mesenterium						
10	pleomorphic sarcoma	Ki-*ras* 63	GAG→AAG			
11	fibrosarcoma	Ki-*ras* 58	ACA→ATA			
12	fibrosarcoma		—	8	277	GGG→GAG
Subcutaneous region						
13	pleomorphic sarcoma		—	6	195	GAA→AAA
14	fibrosarcoma		—	6	209	GTG→ATG
15	fibrosarcoma		—	8	275	ACT→ATT
16	fibrosarcoma		—	6	209	TGT→TAT[a]
				7	256	ACT→ATT

—, no mutation.
a) Twin-site mutations within the *p53* gene.

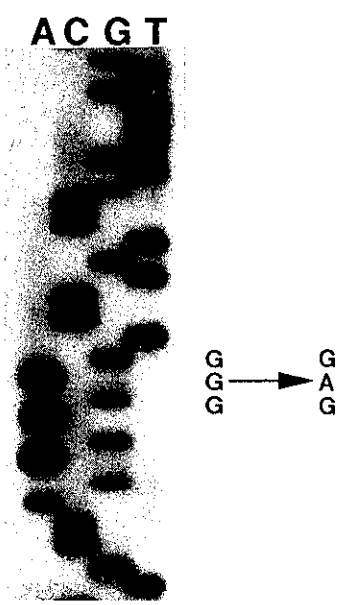

Fig. 2. DNA sequencing autoradiograph of the *p53* gene around codon 277 (exon 8) in fibrosarcoma of the peritoneal cavity, showing a transition mutation (GGG to GAG).

while 3 out of 16 (18.8%) intestinal adenocarcinoma cases, 1 small and 2 large intestine, were found to have common Ki-*ras* codon 12 (GGT to GAT) mutations (Fig. 1, Table III). Similarly, 5 out of 30 (23.3%) sarcoma cases in the prostate/peri-prostate and retroperitoneal regions had Ki-*ras* codon 12 (GGT to GAT) mutations, whereas intraperitoneal cavity sarcomas exhibited unusual mutations in Ki-*ras*; one in codon 63 (GAG to AAG) and another in codon 58 (ACA to ATA) (Table IV). Therefore, the total number of *ras*-mutated soft tissue sarcomas was 7 out of 30 cases (23.3%). All of the Ha- and Ki-*ras* mutations were G:C to A:T transitions. One SCC and one intestinal adenocarcinoma had concurrent mutations in both *ras* and *p53* genes. No mutations were detected in benign lesions such as forestomach papillomas, intestinal adenomas and hepatocellular adenomas.

***p53* gene mutations** In the forestomach SCCs, 4 (80%) out of 5 exhibited mutations, all being GGG to GAG transition within exon 8 (Table II). In the intestinal adenocarcinoma cases, 5 out of 16 (31.3%) had mutations in exons 5 or 8. One small intestine case had one mutation in each of exons 5 and 8. Tumor sites and types of mutation are presented in Table III. Out of a total of 30 sarcoma cases, 9 (30%) exhibited mutations. Al-

though exon 8 was most frequently involved, no hot spot was apparent (Table IV). Two cases had two mutations, one pleomorphic sarcoma in codons 175 (CCC to TCC) and 242 (GGG to GAG), and one fibrosarcoma in codons 209 (ACT to ATT) and 256 (GAA to AAA). A fibrosarcoma exhibiting mutation in codon 242 (GGG to GAG) is illustrated in Fig. 2 (case 12 in Table IV). As with the ras mutations, all of the detected alterations were G:C to A:T transitions. As with *ras*, no mutation was observed in the benign tumors.

***Ras* and/or *p53* gene mutations in different types of tumors induced in the same rats** Two out of 7 rats with

Table V. *ras* and/or *p53* Gene Mutations in Different Tumors Induced in the Same Rats

Reference	Tumor A		Tumor B	
	Site and histology	Mutation	Site and histology	Mutation
Case 3 in Table II	Forestomach SCC	*p53* (8, 277, GGG→GAG)	Retroperitoneal pleo. sarcoma[a]	—[b]
Case 3 in Table III	Small intestine adenocarcinoma	Ki-*ras* (12, GGT→GAT)[c] *p53* (8, 275, TGT→TAT)	Subcutaneous pleo. sarcoma	—
Case 4 in Table III	Large intestine adenocarcinoma	*p53* (5, 174, TGC→TAC)[c] *p53* (8, 277, GGG→GAG)	Subcutaneous fibrosarcoma	—
Case 5 in Table III	Small intestine adenocarcinoma	*p53* (5, 179, GGT→TGT)[d]	Prostate region pleo. sarcoma	Ki-*ras* (12, GTT→GAT)[e]
Case 6 in Table III	Large intestine adenocarcinoma	*p53* (5, 179, GGT→TGT)	Forestomach SCC	*p53* (8, 277, GGG→GAG)
Case 7 in Table IV	Small intestine adenocarcinoma	—	Retroperitoneal fibrosarcoma	Ki-*ras* (12, GGT→GAT)
Not listed	Small intestine adenocarcinoma	—	Prostatic region fibrosarcoma	—

a) pleo., pleomorphic.
b) — indicates no mutation.
c) Two mutations within the same tumor.
d) (Exon number, codon number, nucleotide).
e) (Codon number, nucleotide).

Table VI. Summary of Incidence of the *ras* and *p53* Gene Mutations

Tumor	No. of tumors examined	No. of cases with mutation (%)	
		ras	*p53*
Forestomach			
papilloma	4	0	0
SCC[a]	5	2 (40%)	4 (80%)[b]
Intestines			
adenoma	4	0	0
small intestine	2	0	0
large intestine	2	0	0
adenocarcinoma	16[c]	3 (18.8%)	5 (31.3%)[b, d]
small intestine	7	1	2
large intestine	9	2	3
Soft tissue			
sarcoma	30	7 (23.3%)	9 (30%)[e]
Liver			
adenoma	8	0	0

a) Squamous cell carcinomas.
b) One case had mutations in both *ras* and *p53* genes.
c) Two cases had tumors in both small and large intestines.
d) One case had twin-site mutations within the *p53* gene.
e) Two cases had twin-site mutations within the *p53* gene.

2 histologically different tumors had mutations of either *ras* or *p53* in each of the tumors. They are a small intestine adenocarcinoma with peri-prostate region pleomorphic sarcoma, and a large intestine adenocarcinoma with forestomach SCC. One case lacked any mutation of either gene in the two tumors (Table V).

Duplicated *ras* and *p53* mutations within the same tumor

One case each of forestomach SCC and small intestine adenocarcinoma had mutations in both *ras* and *p53* genes (case 2 in Table II and case 1 in Table III). Summarized data for all mutations are shown in Table VI.

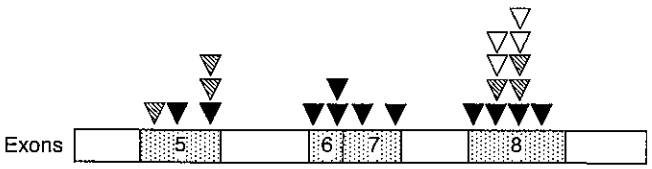

Fig. 3. The distributions of *p53* gene point mutations in tumors induced in this study. A cluster region in exon 8 was found for forestomach squamous cell carcinoma cases, while mutations were found in exons 5 and 8 for intestinal carcinomas, and throughout all exons for soft tissue sarcomas. ▽, forestomach squamous cell carcinomas; ▼, intestinal adenocarcinomas; ▼, soft tissue sarcomas.

DISCUSSION

MNU is well known to be a broad-spectrum carcinogen inducing tumors in many organs in rodents. The mammary carcinomas induced by MNU have a high incidence of Ha-*ras* gene point mutation.[20,21] In the present study, only forestomach SCC showed Ha-*ras* gene mutations, those in other tumors exclusively involving the Ki-*ras* form. Our results are generally in agreement with the earlier finding of a lack of Ha-*ras* gene mutations in MNU-induced liver and urinary bladder tumors.[18,22,23] The indicated tissue specificity and frequent presence of Ha-*ras* gene mutations in esophageal lesions induced by *N*-nitrosomethylbenzylamine suggest a link with tumors of squamous cell origin.[22] However, since this is the first report to show Ha-*ras* gene point mutation in forestomach SCC induced by MNU, further studies are required for precise characterization.

The appreciable incidences of Ki-*ras* mutations found for intestinal adenocarcinomas and soft tissue sarcomas in the present study, as well as the types of nucleotide changes, are in line with those reported earlier for intestinal tumors induced by MNU, 1,2-dimethylhydrazine and azoxymethane.[4–7] Although the role of Ki-*ras* gene mutations in chemically induced tumors in the rat has yet to be fully elucidated and the incidence is very variable,[6,18,24,25] our results provide further support for some involvement in intestinal carcinogenesis.[5]

Although various numbers of *p53* mutations have been reported in tumors induced by other carcinogens,[17,26–28] to our knowledge, this is the first report to show appreciably high incidences of *p53* mutation in nitrosamine-induced forestomach and soft tissue tumors in the rat.[10,13,29–31] It is well known that there are mutational hot spots of the *p53* gene in human tumors.[9,32] In the rat, the data have generally been insufficient for evaluation primarily because of low incidences, but our present results clearly indicate that the mutation spectrum is dependent on the tumor type. Thus, the forestomach SCCs exhibited mutations only in exon 8, and intestinal adenocarcinomas preferentially in exons 5 and 8, while the soft tissue sarcomas exhibited a rather wider distribution. However, even in the latter case, exon 8 was the site in 4 out of 11, and the overall data indicate clustering of *p53* mutations in MNU-induced tumors in this exon, the most frequent site being codon 277 (6 cases) and then 275 (4 cases) (Fig. 3). Our results are not in line with the mutation sites reported by Ohgaki *et al.*, possibly due to the differences of applied PCR primers, in which intron nucleotides were not included.[12]

It should be noted that all the observed Ha-*ras*, Ki-*ras* *ras* and *p53* point mutations were G:C to A:T transitions. It has been suggested that the persistence of O^6-alkylguanines, through fixation by cellular replication or misrepair, may result in gene mutation events. In particular, the presence of O^6-methylguanine is considered to cause mispairing with deoxythymidine during DNA replication, leading to base substitutions from G to A. Therefore, DNA containing O^6-methyldeoxyguanine would be expected to cause G:C to A:T transitions during its replication. Abundant alkyl-DNA adducts have been detected in many tissues of rats treated with MNU, and observed mutations are directly in line with the known O^6-methylguanosine formation by MNU.[33–35] Our results on the frequency of *p53* mutations involving G:C to A:T transitions are similar to those observed for human soft tissue sarcomas, in which G:C to A:T transition was more frequent than G:C to T:A transversion.[11] This may suggest that development of soft tissue sarcoma is related to exposure to environmental alkylating agents.

Simultaneous occurrence of *p53* and *ras* mutations has been observed in many human colorectal cancers.[1,36] However, in this study, only two tumors, a forestomach SCC and an intestinal adenocarcinoma, had mutations in both *ras* and *p53* genes. It is well accepted that neoplastic development is a multistage process,[37,38] and it is unlikely that a single gene alteration is sufficient to induce malignant tumors. Results with benign tumors indicating lack of both *ras* and *p53* mutations also support multistep progression of carcinogenesis. Furthermore, 7 rats had tumors in different sites, mostly soft tissue sarcomas and intestinal carcinomas (6 out of 7). Only two cases

showed *p53* and/or *ras* mutations in both lesions and one case did not demonstrate any mutation (Table VI). Accordingly, although the currently observed alterations may be important for tumor development, there must be some other pathway independent of Ki-*ras* or *p53* alterations.

In conclusion, the present results indicate that malignant tumors induced by MNU exhibited Ki-*ras*, Ha-*ras* or *p53* gene mutations, generally leading to G:C to A:T transitions with some organ and exon site specificity. Given the lack of such changes in benign tumors, they would appear to be progression-related. How this can be explained by the initial limited exposure to the carcinogen requires clarification.

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