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Rare Occurrence of *ras* and *p53* Gene Mutations in Mouse Stomach Tumors Induced by *N*-Methyl-*N*-nitrosourea

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The incidence of point mutations of H-, K- and N-*ras* and *p53* oncogenes in male BALB/c mouse stomach tumors induced with *N*-methyl-*N*-nitrosourea (MNU) was examined by direct sequencing and PCR single-strand conformation polymorphism (PCR-SSCP). A mutation of GGT to AGT at K-*ras* codon 12 was found by SSCP in one adenocarcinoma from a total of 19 specimens including 5 adenocarcinomas, 9 adenomatous hyperplastic regions, 1 squamous cell carcinoma and 4 normal-like stomach regions from 4 mice. No mutations were detected by direct sequencing of H-, K- and N-*ras* oncogenes at exons 1 (codons 12 and 13) and 2 (codon 61) in a total of 26 specimens comprising 10 adenocarcinomas, 10 adenomatous hyperplastic regions, 2 squamous cell carcinomas and 4 normal-like stomach regions from 6 mice. No mutations were detected by direct sequencing of *p53* oncogene at exons 5, 6, 7 and 8 in a total of 30 specimens including 13 adenocarcinomas, 8 adenomatous hyperplastic regions, 2 squamous cell carcinomas, 1 papilloma and 6 normal-like stomach regions from 7 mice. These results suggest that *ras* and *p53* oncogenes do not play a role in mouse stomach carcinogenesis induced by MNU.

Key words: *ras* — *p53* — *N*-Methyl-*N*-nitrosourea — Mouse — Stomach tumor

Point mutations in oncogenes have been associated with carcinogenesis in various organs. The DNA alkylating agent *N*-methyl-*N*-nitrosourea (MNU) has been shown to induce *ras* and *p53* gene mutations in a diverse range of species and organs. A high frequency of point mutation was reported in H-*ras* in rat mammary carcinogenesis[1] and in *ras* in murine thymic lymphomas,[2] while *ras* mutations were rare in neoplastic mouse endometrial lesions.[3] MNU-induced *p53* mutations are relatively rare.[4,5] Point mutations in oncogenes induced by MNU in mouse or rat stomach have not been reported before.

We have studied various changes during experimental stomach carcinogenesis.[6–10] In this present study we examined point mutations in *ras* and *p53* oncogenes in mouse MNU-induced stomach carcinogenesis, which has been developed as an animal model in our laboratory.[11,12] Point mutations in oncogenes in mouse and rat stomach carcinogenesis have not been reported before. We are interested in mutations that occur in the early stage of gastric carcinogenesis and which may trigger carcinogenesis. We consider that a cell harboring mutations may expand clonally to form growing tumors; such mutation could be analyzed by direct sequencing. As the mouse stomach tumors were small, we applied multiple polymerase chain reaction (PCR) amplification and direct sequencing[13] to obtain multiple data from one DNA specimen. Since no mutations were thus found, we then looked for minor changes by PCR single-strand conformation polymorphism (PCR-SSCP) analysis. PCR-SSCP analysis of DNA from 19 specimens revealed one K-*ras* codon 12 mutation. These results suggest that *ras* and *p53* oncogenes do not play a role in mouse stomach carcinogenesis induced by MNU.

MATERIALS AND METHODS

Animals Male BALB/c mice (Charles River Japan, Inc., Atsugi), 6 weeks old, were housed in an air-conditioned animal room at 23±2°C and 50% humidity with food and water available *ad libitum*. The animals were given MNU (Sigma Chemical Co., St. Louis, MO) by gastric intubation at a dose of 0.5 mg/mouse once a week for 10 weeks.[11] Mice were killed after 36 weeks and stomachs were removed, fixed in ice-cold acetone, embedded in paraffin and sectioned (10 μm). Tissue of the tumor area from 10 serial slides was dissected with a syringe needle under microscopy and placed in 50 μl of non-ionic detergent solution for DNA extraction. Normal-like areas were identified from hematoxylin and eosin-stained sections.

DNA extraction The tissue was treated in non-ionic detergent solution (10 m*M* Tris·HCl, pH 8.4, 2.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.5% Nonidet P-40, 0.5% Tween

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Table I. Primers Used for PCR Amplification of the Mouse *ras* Genes

Region amplified	Primer name (codons)	Primer sequence
H-*ras* codon 12–13	H12S (2–10)	5′-ACAGAATACAAGCTTGTGGTGGTGGGC
(108 bp)	H12AS (37–30)	5′-CTCTATAGTGGGATCATACTCGTC
H-*ras* codon 61	H61S (38–45)	5′-GACTCCTACCGGAAACAGGTAGTC
(138 bp)	H61AS (83–76)	5′-GGCAAATACACAGAGGAAGCCCTC
K-*ras* codon 12–13	K12S (4–11)	5′-TATAAACTTGTGGTGGTTGGAGCT
(102 bp)	K12AS (37–30)	5′-CTCTATCGTAGGGTCGTACTCATC
K-*ras* codon 61	K61S (38–45)	5′-GACTCCTACAGGAAACAAGTAGTA
(141 bp)	K61AS (84–77)	5′-TATGGCAAATACACAAAGAAAGCC
N-*ras* codon 12–13	N12S (4–11)	5′-TACAAACTGGTGGTGGTTGGAGCA
(99 bp)	N12AS (36–29)	5′-TATGGTGGGATCATATTCATCCAC
N-*ras* codon 61	N61S (38–45)	5′-GATTCTTACCGAAAGCAAGTGGTG
(144 bp)	N61AS (85–78)	5′-ATTGATGGCAAATACACAGAGGAA

The expected length of each amplification product is given in parentheses below the "Region amplified." Under "Primer name," primers corresponding to *ras* sequences on the sense strand are labeled "S," whereas primers corresponding to *ras* sequences on the antisense strand are labeled "AS." The *ras* codon numbers represented in each primer are given in parentheses. H12AS, H61S, H61AS, K12AS, K61S, K61AS, N12AS, N61S and N61AS were described by Manam and Nichols.[13]

Table II. Primers Used for PCR Amplification of the Mouse *p53* Genes

Region amplified	Primer name	Primer sequence
p53 exon 5	*p53* intron 4S	5′-ACACCTGATCGTTACTCGGCTTGTC
(184 bp)	*p53* intron 5AS	5′-ATAAGTCAGAAGCCGGGAGATGGG
p53 exon 6	*p53* intron 5S	5′-CCTCAACACCGCCTGTGGGGTTAG
(255 bp)	*p53* intron 6AS	5′-GAAAGTCAACATCAGTCTAGGCTG
p53 exon 7	*p53* intron 6S	5′-CATTCCCGGCTGCTGCAGGTCACC
(252 bp)	*p53* intron 7AS	5′-TCGTGGAACAGAAACAGGCAGAAG
p53 exon 8	*p53* intron 7S	5′-TTTACACACAGTCAGGATGGGGCC
(269 bp)	*p53* intron 8AS	5′-AAGAGGTGACTTTGGGGTGAAGCTC

The expected length of each amplification product is given in parentheses below the "Region amplified." Exons 5, 6, 7 and 8 contain, respectively, 111 bp, 113 bp, 110 bp and 137 bp, as well as upstream and downstream introns. Under "Primer name," primers corresponding to *p53* sequences on the sense strand are labeled "S," whereas primers corresponding to the *p53* sequences on the antisense strand are labeled "AS."

20, proteinase K 0.4 mg/ml) at 55°C for 3 h with gentle shaking, heated at 95°C for 10 min and centrifuged at 15,000 rpm for 10 min.[14] The supernatant contained the DNA for analysis.

PCR primers PCR primers for 1st amplification and cycle sequencing (FITC-primers) were synthesized in a Model 392 DNA synthesizer (Perkin Elmer Applied Biosystems Division, Foster City, CA) and are listed in Tables I–IV.

Amplification and DNA sequencing H-, K- and N-*ras* exons 1 and 2 were amplified simultaneously, using a similar method to that previously described,[13] in 2 tubes containing 100 μl of a mixture of 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 0.6 μM of each 5′- and 3′-primers, 2 units of Taq DNA polymerase and 5 μl of DNA solution for 30 cycles (96°C 1 min, 56°C 1 min, and 74°C 1 min). PCR products were purified with QIAquick-spin (QUIAGEN Inc., Chatsworth, CA). Exons 5, 6, 7 and 8 of *p53* were amplified simultaneously by PCR using a similar method. Amplified DNA was sequenced using Vent (exo-) DNA polymerase (New England Biolabs, Inc., Beverly, MA) and FITC-primers with an ALF DNA sequencer (Pharmacia LKB Biotech. AB, Uppsala, Sweden).

Non-radioactive SSCP PCR-amplified DNA fragments were electrophoresed in non-denatured MDE gel (AT Biochem, Malvern, PA), using a similar method to that described by Ballhausen and Kraus[15] and by Hongyo *et al.*[16] DNA fragments were stained with SYBR Green I (Molecular Probes, Inc., Eugene, OR) and detected by a

Table III. FITC-Primers Used for DNA Sequencing of the Mouse *ras* Genes

Region sequenced	Primer name	Primer sequence
H-*ras* codon 12–13	H12AS (31–24)	5′-CTCGTCCACAAAATGGTTCTGGAT
H-*ras* codon 61	H61S (43–49)	5′-CAGGTGGTCATTGATGGGGAG
K-*ras* codon 12–13	K12AS (32–25)	5′-GTACTCATCCACAAAGTGATTCTG
K-*ras* codon 61	K61S (43–50)	5′-CAAGTAGTAATTGATGGAGAAACC
N-*ras* codon 12–13	N12AS (31–24)	5′-TTCATCCACAAAGTGGTTCTGGAT
N-*ras* codon 61	N61S (41–48)	5′-CGAAAGCAAGTGGTGATTGATGGT

Under "Primer name," primers corresponding to *ras* sequences on the sense strand are labeled "S," whereas primers corresponding to *ras* sequences on the antisense strand are labeled "AS." The *ras* codon numbers represented in each primer are given in parentheses. H12AS, H61S, N12AS and N61S were described by Manam and Nichols.[13]

Table IV. FITC-Primers Used for DNA Sequencing of the Mouse *p53* Genes

Region sequenced	Primer name	Primer sequence
p53 exon 5	*p53* exon 5AS	5′-AGATGGGAGGCTGCCAGTCCTA
p53 exon 6	*p53* exon 6S	5′-GTTAGGACTGGCAGCCTCCCAT
	p53 exon 6AS	5′-CTAGGCTGGAGTCAACTGTCTCT
p53 exon 7	*p53* exon 7S	5′-CACCTGTAGTGAGGTAGGGAGC
	p53 exon 7AS	5′-AGAAGCTGGGGAAGAAACAGGCT
p53 exon 8	*p53* exon 8S	5′-GGGCCCAGCTTTCTTACTGCCT
	p53 exon 8AS	5′-GCTCAACAGGCTCCTCCGCCT

Under "Primer name," primers corresponding to *p53* sequences on the sense strand are labeled "S," whereas primers corresponding to the *p53* sequences on the antisense strand are labeled "AS."

UV detector set at 250 nm. Mutant (FM128C-2) and normal (FM128C-1) bands were cut from the gel, reamplified, cycle-sequenced by using Vent (exo-) DNA polymerase and sequenced on an ALF DNA sequencer. The reamplified product was digested by BfaI (New England Biolabs, Inc.) and electrophoresed on 6% polyacrylamide gel electrophoresis.

Histology Histological procedures and analysis were conducted as described previously.[11]

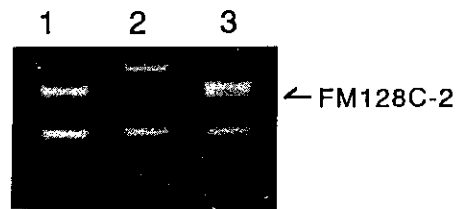


Fig. 1. SSCP of 1) K-*ras* 12 normal, 2) K-*ras* 12 mutant and 3) a sample from a tumor (FM128C). FM128C-2 is a mutated band. Samples were electrophoresed on MDE gel and stained with SYBR Green I.

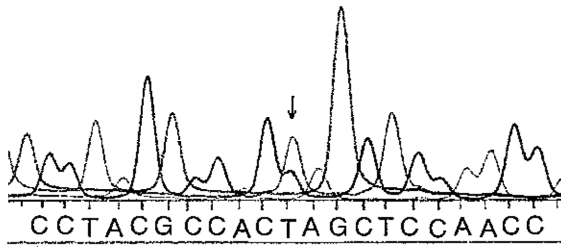
RESULTS

Direct sequencing of *ras* exons 1 and 2 of mouse stomach tumors No mutations were observed by direct sequencing in H-*ras* codon 1 of DNA from 14 specimens, H-*ras* codon 2 from 19 specimens, K-*ras* codon 1 from 23 specimens, K-*ras* codon 2 from 18 specimens, N-*ras* codon 1 from 11 specimens and N-*ras* codon 2 from 21 specimens. In total, 26 specimens were analyzed, comprising 10 adenocarcinomas, 10 adenomatous hyperplastic regions, 2 squamous cell carcinomas and 4 normal-like regions from 6 mice.

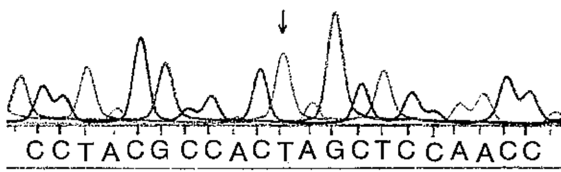
Direct sequencing of *p53* exons 5, 6, 7 and 8 of mouse stomach tumors No mutations were detected by direct sequencing in *p53* exon 5 of DNA from 17 specimens, exon 6 from 30 specimens, exon 7 from 29 specimens and exon 8 from 24 specimens. In total, 30 specimens were analyzed, comprising 13 adenocarcinomas, 8 adenomatous hyperplastic regions, 2 squamous cell carcinomas, 1 papilloma and 6 normal-like regions from 7 mice.

PCR-SSCP of K-*ras* codon 12 A mutation was found in an adenocarcinoma by PCR-SSCP (Fig. 1). The shifted position of the mutated band at K-*ras* codon 12 corresponds to mutation of GGT to AGT. This mutation was confirmed by sequencing of the mutated band (Fig. 2). The reamplified product was still a mixture of normal

a MNU-induced mouse stomach tumor FM128C-2



b K-*ras* 12 Ser (synthetic mutant)



c K-*ras* 12 Gly (normal)

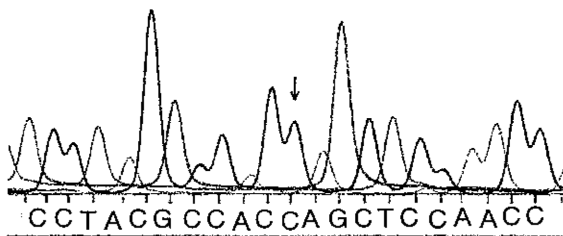


Fig. 2. DNA sequence patterns around K-*ras* codon 12. a, Shifted band from FM128C (FM128C-2). Codon 12 was (ACT/C) (antisense). b, Synthetic mutant (ACT). c, Normal (ACC).

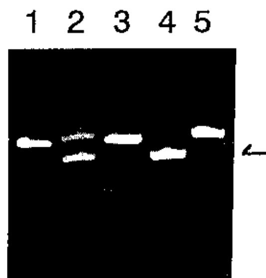


Fig. 3. Electrophoresis of reamplified mutant and normal K-*ras* codon 12 bands after *Bfa* I digestion. 1) FM128C-2 undigested. 2) FM128C-2 digested. The 88 bp band (arrow) was produced by *Bfa* I digestion. 3) Mutant undigested. 4) Mutant (AGT) digested. The 88 bp band was produced by *Bfa* I digestion. The restriction site is C↓TAG. 5) Normal (GGT) digested.

and mutated DNA. The mutation of GGT to AGT at K-*ras* codon 12 was also confirmed by electrophoresis after enzymatic digestion with *Bfa* I (Fig. 3). This mutation was found in only one specimen from a total of 19 specimens analyzed, comprising 5 adenocarcinomas, 9 adenomatous hyperplastic regions, 1 squamous cell carcinoma and 4 normal-like regions from 4 mice.

DISCUSSION

No mutations were detected in H-, K- and N-*ras* oncogenes exons 1 (codons 12 and 13) and 2 (codon 61) by direct sequencing in a total of 26 specimens (14 H-*ras* codon 1, 19 H-*ras* codon 2, 23 K-*ras* codon 1, 18 K-*ras* codon 2, 11 N-*ras* codon 1 and 21 N-*ras* codon 2) representing different stages of mouse stomach carcinogenesis induced by MNU. Similarly no mutations were detected in *p53* oncogene exons 5, 6, 7 and 8 by direct sequencing in a total of 30 specimens (17 codon 5, 30 codon 6, 29 codon 7 and 24 codon 8). Although the numbers of H-*ras* codon 1 and N-*ras* codon 1 analyses are not large, the results suggest that the *ras* and *p53* oncogenes were not mutated clonally from the early stage of MNU stomach carcinogenesis and that these two oncogenes do not play a role in mouse stomach carcinogenesis induced by MNU. Using PCR-SSCP, we further searched for any mutation of K-*ras* codon 12, and found a GGT-to-AGT mutation in one adenocarcinoma from a total of 19 specimens comprising 5 adenocarcinomas, 9 adenomatous hyperplastic regions, 1 squamous cell carcinoma and 4 normal-like regions from 4 mice. This result also suggests that K-*ras* oncogene mutations do not play a role in MNU-induced mouse stomach carcinogenesis. Further investigation is necessary to elucidate the molecular mechanisms in rodent stomach chemical carcinogenesis.

In previous reports, *ras* oncogene mutations were different in various tumors induced by MNU. Frequent activation of the K-*ras* oncogene at codon 12 in rat prostate adenocarcinoma and neurogenic sarcomas was noted.[17] Rare occurrence of *p53* and *ras* gene mutations was reported in preneoplastic and neoplastic mouse endometrial lesions induced by MNU and 17β-estradiol.[3] In rat bladder carcinogenesis induced by *N*-[4-(5-nitro-2-furyl)-2-thiazoyl]formamide, *N*-(4-hydroxybutyl)nitrosamine and MNU, *p53* mutation was found to be infrequent.[5] The present results suggest that *ras* and *p53* gene mutations are rare or non-existent in MNU-induced mouse stomach carcinogenesis. However, frequent *p53* gene mutation was reported in mouse urinary bladder carcinomas induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine,[18, 19] and in mouse skin carcinogenesis induced by UVB radiation.[20] Mutations in *ras* and *p53* were diverse, depending on carcinogen, species and organs.

In human stomach cancers, *ras* oncogene mutations are rare.[21,22] In studies of *p53* oncogene alterations in human stomach cancers, deletion was frequent while point mutation was moderate (20%) in well-differentiated adenocarcinomas.[23] Microsatellite instability was considerable (>30%) in human gastric cancers.[24] Amplification of c-*erb*B-2 occurred frequently (40%) in tubular adenocarcinoma of human stomach.[25] These results suggest the existence of a range of genetic alterations in human stomach carcinogenesis.

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