Original

Tongue Carcinogenic Susceptibility of p53 Deficient Mice to Methyl-n-amyl nitrosamine

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Abstract: Mutation of the p53 tumor suppressor gene is a common genetic alteration in human squamous cell carcinoma of the tongue. Mice deficient in p53 have recently attracted attention for their potential to identify chemical genotoxins. In this study we investigated the susceptibility of p53 nullizygous (–/–), heterozygous (+/–), and wild type (+/+) mice to methyl-n-amyl nitrosamine (MNAN) induced squamous cell carcinoma (SCC) of the tongue. The p53 (+/–), and (+/+) mice were treated with 5 p.p.m. MNAN in drinking water for 8 weeks then held without further treatment for an additional 7 or 17 weeks, and killed at 15 or 25 experimental weeks. A separate group of the p53 (–/–) mice were given 5 p.p.m. MNAN for 8 weeks and were killed at 15 weeks. At 15 weeks, SCCs and papillomas were observed in 5/12 (41.7%) and 2/12 (16.7%) of p53 (–/–) mice, respectively, but not in p53 (+/–) and (+/+) mice. At 25 weeks, carcinomas *in situ* (CIS) were detected in 1/16 (6.3%) of p53 (+/–) and 1/13 (7.7%) of p53 (+/+) mice, and a papilloma was observed in the p53 (+/–) mouse which had CIS. PCR-single strand conformation polymorphism analysis of exons 5–8 of the p53 gene demonstrated a missense mutation in the CIS from p53 (+/+) mouse. These results suggest that a lack of p53 gene function predisposes the tongue to the development of SCCs in mice treated with MNAN, and show that p53 (–/–) mouse was a useful model for demonstrating carcinogenicity of MNAN to tongue. (J Toxicol Pathol 2002; 15: 209–214)

Key words: p53 knockout, mice, tongue, squamous cell carcinoma, methyl-n-amyl nitrosamine

Introduction

The p53 tumor suppressor gene encodes a transcriptional regulator that prevents the propagation of genetically damaged cell¹ and alterations in p53 gene have been reported in a wide range of human cancers^{2,3}. The role of p53 includes contribution to G1 cell cycle arrest and induction of DNA repair genes in response to DNA damage as well as activation of genes promoting apoptosis^{1,4}. Mutation of the p53 gene is a common genetic alteration in human squamous cell carcinoma of the tongue^{5–7}. The frequencies of p53 mutations and consequent loss of wild-type p53 function are assumed to play a role in tongue SCC development. Mice with homozygous or heterozygous p53-deficient were susceptible to the early onset of spontaneous tumors⁸. These animals have been a model for assessing the

function of p53 in tumor development and for identifying carcinogens^{9–11}. Nullizygous p53-deficient mice are highly susceptible to early onset of spontaneous tumors. By the age of 4.5 months, approximately half of the nullizygotes developed tumors and by 10 months of age, all of the mice died or developed tumors¹². Most of these tumors consisted of lymphomas and sarcomas. Furthermore, great enhancement of malignant progression was reported in chemically induced skin tumors in the nullizygous p53-deficient mice¹³. In contrast, the heterozygotes were viable and showed a low background incidence of spontaneous tumors up to almost 12 months of age^{8,14}. The low background tumor incidence combined with the increased tumor susceptibility to chemically induced tumors make the p53 (+/–) mouse useful for short-term bioassay^{15,16}. MNAN, an N-nitroso compound, is known to induce esophageal cancer in rats¹⁷, and in a previous study administration of

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Abbreviations: KO, knockout; nullizygous, (–/–); heterozygous, (+/–); wild type (+/+); MNAN, methyl-n-amyl nitrosamine; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism; SCC, squamous cell carcinoma; CIS, carcinoma *in situ*.

MNAN yielded cancers in esophagus of p53-deficient mice with higher incidence compared to wild-type counterparts¹⁸. Diffusion of MNAN into the esophagus is a possible factor in carcinogenesis. Considering that the tongue is directly exposed to MNAN in drinking water, cancers are likely to occur in tongue as well. In the present study we examined association of p53 deficiency with tongue cancer development in the p53 knockout mice following administration of MNAN.

Materials and Methods

Animals

p53 knockout mice on a C57BL/6 genetic background, produced by Donehower *et al.*¹⁹, were maintained at the Animal Facility of Aichi Cancer Center Research Institute. Six-week-old males were used in the experiment. They were housed in plastic cages with hardwood chips in an air-conditioned room with a 12 h light-12 h dark cycle and given basal diet (Oriental NMF, Oriental Yeast Co., Tokyo, Japan) and drinking water *ad libitum*. Genotyping of each mouse was performed as described earlier²⁰.

Carcinogen treatment

MNAN was purchased from Sakai Rikagaku Institute (Fukui, Japan) and dissolved in drinking water weekly to achieve the desired concentrations. Drinking water containing 5 p.p.m. of MNAN was filled into black bottles and provided *ad libitum* to p53 (+/+) and (+/–) mice for 8 weeks. Mice were then maintained without further treatment for an additional 7 or 17 weeks, and killed at week 15 or 25. A separate group of p53 (–/–) mice were also treated with 5 p.p.m. MNAN for 8 weeks and killed at week 15. These dose and duration of treatment had previously revealed significant increases in esophageal cancers in both p53 (–/–) and p53 (+/–) mice¹⁸. Three groups of control animals with p53 (+/+), p53 (+/–) or p53 (–/–) and additional two groups of control animals with p53 (+/+) or p53 (+/–) received unsupplemented drinking water and killed at week 15 and 25, respectively. All mice were killed by exsanguinations under ether anesthesia. This experimental protocol was approved by the Aichi Cancer Center Animal Committee.

Histopathological analysis

At necropsy, tongue tissues were resected, fixed in 4% paraformaldehyde in phosphate buffered saline, embedded in paraffin, sectioned and stained with hematoxylin and eosin for microscopic examination.

PCR-single strand conformation polymorphism analysis (SSCP)

Tumor samples from p53 (+/+) and p53 (+/–) mice were subjected to PCR-SSCP. PCR-SSCP was conducted basically as described previously²¹. Briefly, genomic DNA was extracted from tumor areas in paraffin sections with DEXPAT (Takara)²² as detailed elsewhere. Four pairs of PCR primers for mouse p53 exons 5–8 were designed based

Table 1. PCR Primers for SSCP Analysis of Mouse p53

Target		Primer Sequence	Product length (bp)
Exon 5	sense	5'-TCTCTTCCAGTACTCTCCTC-3'	214
	antisense	5'-AGGCGGTGTTGAGGGCTTAC-3'	
Exon 6	sense	5'-GGCTTCTGACTTATTCTTGC-3'	181
	antisense	5'-CAACTGTCTCTAAGACGCAC-3'	
Exon 7	sense	5'-TCACCTGGATCCTGTGTCTT-3'	170
	antisense	5'-CAGGCTAACCTAACCTACCA-3'	
Exon 8	sense	5'-ACTGCCTTGTGCTGGTCCTT-3'	279
	antisense	5'-GGAGAGGCGCTTGTGCAGGT-3'	

on the published sequence²² as listed in Table 1. PCR was performed with a Takara PCR Thermal Cycler MP (Takara) and products were electrophoresed in 0.625 × MDE polyacrylamide gels (FMC, Rockland, ME) with 5% glycerol. These were run at room temperature for 18 h at 8 W, dried, and applied to imaging plates, which were then analyzed with BAS 2500 (Fuji Film, Kanagawa, Japan).

Direct sequencing

Sequencing was performed with ABI PRISM 3100 using a BigDye Terminator v3.0 Cycle Sequencing Ready Kit (Applied Biosystems, Forester City, CA). Sequence data were analyzed with DNASIS software (Hitachi Software Engineering, Yokohama, Japan).

Statistical analysis

Data for incidences of histopathological lesions were analyzed by the Fisher's exact test method. Survival of each genotype mice was analyzed using the log rank test²³.

Results

Mortality of each genotype

Administration of 5 p.p.m. MNAN in drinking water was well-tolerated by both p53 (+/–) and p53 (+/+) mice. Survival was not significantly different between p53 (+/–), and p53 (+/+) mice. However, eight p53 (–/–) mice were found dead until week 15. These included 4 lymphomas, 1 subcutaneous sarcoma, and 3 unknown cause of death. The causes of death for two p53 (+/–) mice and one p53 (+/+) mouse were not determined because of cannibalism.

Histopathological analysis

The incidences of MNAN induced papillomas and squamous cell carcinomas of tongue are summarized in Table 2. Papillomas were characterized by the exophytic masses with fronds of proliferating epithelial cells (Fig. 1A). Squamous cell carcinomas were locally invasive tumors that sometimes extended deep into the lingual skeletal musculature (Fig. 1B). These tumors occurred at the dorsal, lateral or ventral surface from the middle through posterior tongue. At week 15, although no microscopic changes were observed in both p53 (+/+) and (+/–) mice, SCCs were found

Table 2. Incidences of Lingual Neoplasms in p53 Knockout Mice Treated with MNAN

Exp. week	MNAN (p.p.m.)	p53 geno-type	No. animals	No. ^a examined	No. of mice with lesions (%)		
					Papilloma	SCC ^b	CIS ^c
15 W	5	(+/+)	13	13	0	0	0
		(+/-)	17	15	0	0	0
		(-/-)	20	12	2	5 ^d	0
	0	(+/+)	5	5	0	0	0
		(+/-)	5	5	0	0	0
		(-/-)	4	4	0	0	0
25 W	5	(+/+)	14	13	0	0	1
		(+/-)	16	16	1	0	1
	0	(+/+)	5	5	0	0	0
		(+/-)	6	6	0	0	0

a: Mice which died before the end of experiment were excluded, b: SCC: Squamous cell carcinoma, c: CIS: Carcinoma *in situ*, d: Significantly different from p53 (+/+); P<0.01, and from p53 (+/-); P<0.05, using the Fisher's exact test.

Fig. 1. Photomicrograph of papilloma at 40× (A), and squamous cell carcinoma at 100× (B).

in 5/12 (41.7%) p53 (–/–) mice and the incidence was significantly higher than that of p53 (+/+) (P<0.05) and p53 (+/–) (P<0.01). Additionally, 1 of 5 p53 (–/–) mice that had SCCs and another p53 (–/–) mouse had papilloma. At week 25, carcinoma *in situ* (CIS) was observed in one of each p53 (+/+) and (+/–) mice, and a papilloma was found in the p53 (+/–) mouse which had CIS. CIS was characterized by intraepithelial growth of neoplastic cells with loss of polarity.

PCR-SSCP analysis of the p53 gene in tumors

PCR-SSCP and sequencing analyses for exons 5–8 of p53 gene were performed on 2 CIS samples obtained from one of each p53 (+/+) and (+/–) mice, and on a papilloma obtained from a p53 (+/–) mouse. p53 mutations were identified in a CIS of p53 (+/+) mouse, and in a papilloma of p53 (+/–) mouse (Table 3). DNA sequencing for the CIS from p53 (+/+) mouse revealed CAC→TAC transition at codon 211 in exon 6 resulting in the replacement of His by Tyr, a missense mutation (Fig. 2A). A papilloma from p53

Table 3. p53 Gene Mutations Identified in Lingual Tumors in Mice Treated with MNAN

Geno- type	Histology	Exon	Codon	Nucleotide change	Aminoacid change	Event
+/+	CIS	6	211	CAC→TAC	His→Tyr	Transition
+/-	CIS	–	–	–	–	–
+/-	Papilloma	7	254	CTG→CTA	Leu→Leu	Transition

–: Mutation not detected in exons 5,6,7, and 8.

Fig. 2. (A) DNA sequencing of p53 exon 6 for the SCC from p53 (+/+) mouse. (Upper) Normal sequences; (lower) a CAC→TAC mutation at codon 211 from a tumor. (B) DNA sequencing of p53 exon 7 for the papilloma from p53 (+/–) mouse. (Upper) Normal sequences; (lower) a CTG→CTA mutation at codon 254 from a tumor sample.

(+/-) mouse exhibited CTG→CTA (Leu→Leu) transition at codon 254 in exon 7, a silent mutation (Fig. 2B). Both mutations were G:C to A:T transitions. No mutation was detected in a CIS from p53 (+/-) mouse.

Discussion

In the present study, administration of MNAN, a genotoxic carcinogen²⁴, to nullizygous p53 KO mice clearly demonstrated its carcinogenicity to tongue by an increased incidence of SCCs while heterozygous p53 KO mice and their wild-type counterparts were less susceptible to tongue carcinogenesis.

Although the number of nullizygous p53 KO mice without MNAN treatment was small in the present study, no spontaneous tongue neoplasms developed in any untreated nullizygous p53 KO mice. Furthermore, no spontaneous tongue lesions have been reported in nullizygous p53 KO mice¹⁹. These imply that the lack of p53 function itself does not produce tongue neoplasms but results in an amplification of genetic alterations following DNA damage and consequent cancer development as supported by the fact that p53 regulates cell cycle arrest, nucleotide excision repair, and apoptosis^{1,4}. Further study will be necessary to elucidate other genes implicated with tongue carcinogenesis in mice.

There was missense mutation of p53 gene in a CIS from wild-type mice treated with MNAN. The presence of p53 gene mutations have been reported in chemically induced tongue SCCs in xeroderma pigmentosum group A gene-deficient mice²⁵ and in hamsters²⁶ as well as tongue SCCs in human^{5,7}. There seems to be involvement of p53 gene alterations for tongue carcinogenesis in a wide range of species. Although no p53 mutation was detected in a CIS from heterozygous p53 KO mice, it may have gone undetected in this study as a consequence of restricting the analysis to exons⁵⁻⁸.

The type of p53 mutation found in our study was G:C to A:T transitions. These patterns of mutations are the most prevalent types of p53 mutations in human SCC in oral cavity including tongue^{7,27}. G:C to A:T transitions are also the most common mutations detected in hamster buccal pouch SCCs induced by N-methyl-N-benzyl nitrosamine, being a potent alkylating carcinogen²⁸.

The rare occurrence of tongue neoplasms in wild-type and heterozygous p53 KO mice in the present study might be a reflection of murine resistibility to tongue carcinogenesis. While 4-nitroquinoline-1-oxide (4NQO) is known to produce tongue carcinoma in rodents with variable susceptibility among species²⁹, Ide *et al.*²⁵ indicated that mice were resistant to administration of 4NQO at low concentrations (10 p.p.m.) via drinking water based on the fact that no neoplasms were induced in control mice treated with 4NQO via drinking water up to 2 years.

In contrast with the study using xeroderma pigmentosum group A gene-deficient mice treated with 4NQO in which first tongue tumor was detected in experimental week 32²⁵, tongue cancers developed earlier in

nullizygous p53 KO mice given MNAN. Although consideration must be given to carcinogen specificity, the nullizygous p53 KO mice can be a useful model for identification and understanding of tongue carcinogens.

In conclusion, this study showed that nullizygous p53 deficiency enhanced tongue carcinogenesis in mice by the administration of MNAN and suggested that a lack of p53 gene function predisposed tongue to the development of SCC.

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