Molecular Pathology on Chemical-induced Squamous Cell Carcinomas in

the Esophagus and Tongue of *p53*-deficient Mice

(*p53* 遺伝子欠損マウスの食道および舌における

化学物質誘発扁平上皮癌の分子病理学的研究)

学位論文：博士（獣医学）甲 132

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Contents

Abbreviations

CDK	Cyclin-dependent kinase
CIS	Carcinoma in situ
dNTP	Deoxyribonucleotide triphosphate
dCTP	Deoxycytidine triphosphate
EGFR	Epidermal growth factor receptor gene
GADD	Growth arrest and DNA damage gene
HNSCC	Sqamous cell carcinoma of the head and neck
IPAP	Image processor for analytical pathology
KO	Knockout
LA PCR	Long and accurate polymerase chain reaction
MNAN	Methyl-n-amylnitrosamine
PCR	Polymerase chain reaction
P450	Cytochrome P450
Rb	Retinoblastoma gene
SCC	Squamous cell carcinoma
SSCP	Single strand conformation polymorphism
XPA	Xeroderma pigmentosum group A gene
(+/-)	Heterozygotes
(-/-)	Nullizygotes
(+/+)	Wild type

Preface

Identifying potential cancer hazards is of great importance for the prevention of cancers in humans. It has been estimated that about 300 million tons of organic chemicals per year are released into the environment [41]. Over the past 30 years there has been a broad spectrum of chemicals reported to have the potential to cause cancer in humans based on two-year, long term rodent bioassays. The two-year bioassay using both sexes of two rodent species has essentially become a standardized protocol that is currently used to evaluate chemical carcinogenicity, and data from these studies are generally required by regulatory agencies for new drug applications. Evidence of a chemically induced increase in tumor incidence in multiple species is generally considered to constitute strong evidence of carcinogenic potential in humans. Conversely, extrapolation from responses in a single species to predict cancer risk in humans is problematic because of species-specific genetic influences [77]. However, a single two-year assay is expensive, requires large numbers of animals (250/sex), and normally takes up to 5 years for the evaluation to be completed. Furthermore, recent concerns over assay sensitivity and lack of correlations between mouse liver tumor data and response in humans have led to the need for an optimized approach. Therefore, in addition to the two-year carcinogenicity assessment, the development of currently available alternative

models has recently provided a different approach for identifying carcinogens in a shorter period of time that uses fewer animals and involves aspects of the mechanism of action. These models include genetically altered mouse assays, newborn rodent assays, and initiation-promotion assays. With new advances in biotechnology, there have been a number of transgenic mouse lines created that respond to carcinogen exposure in less than one year [12, 45, 67].

Through an international research program under the International Life Sciences Institute (ILSI), and Health and Environmental Science Institute (HESI), heterozygous *p53* (+/-) deficient mice, *ras*H2 mice, Tg.AC mice, and *XPA* (-/-) repair gene-deficient mice have been evaluated as readily available genetically altered mouse models for assessing the carcinogenic potential of chemicals [7] (Table 1).

The *p53* gene which encodes a transcriptional regulator that prevents the propagation of genetically damaged cells [46], is frequently mutated in a wide range of human cancers (including esophagus, head and neck, lung, breast, colon, liver, bladder, ovary and brain cancers) [29, 30]. When DNA is damaged, the level of the p53 is rapidly increased, and it binds to DNA and stimulates transcription of several genes that mediate G1 cell cycle arrest, DNA repair, and apoptosis. Cell cycle arrest in G1 and induction of DNA repair are mediated by up-regulation of the cyclin-dependent kinase inhibitor *p21*, and the

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GADD45 genes, respectively. If DNA damage cannot be successfully repaired, *p53*-induced activation of the *bax* gene leads cells to apoptosis. If the *p53* is deficient, neither cell cycle arrest nor DNA repair occur, and this subsequently allows genetically damaged cells to proliferate and become malignant [3, 43, 46] (Figure 1).

In addition to the frequency of somatic mutations of the *p53* gene in sporadic human cancers, humans who are heterozygous for the wild-type allele of *p53* gene develop a variety of early-onset mesenchymal and epithelial tumors at multiple sites with a striking frequency. Li-Fraumeni syndrome is such a familial autosomal dominant disease associated with germ-line mutations in the *p53* gene and persons with that syndrome have a 25-fold greater chance of developing a malignant tumor by age 50 than the general population [16].

Donehower *et al.* [12] established a mouse germ line with mutated *p53* alleles by transmitting a disrupted *p53* allele with a *Pol*II-neo expression cassette into the germ line of mice. Mice homozygous for the mutated gene are viable and appear developmentally normal. They are completely deficient in p53 protein, however, and susceptible to spontaneous tumor development by the age of 10 months, indicating that the loss of normal *p53* is sufficient to predispose the mice to tumor development. Most of the tumors that develop in the homozygous *p53* deficient mice are thymic lymphomas and sarcomas. Mice heterozygous for the mutated *p53* gene are also susceptible to tumors but

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at a significantly delayed rate compared to the nullizygotes. The tumor types in the *p53* heterozygous mice are predominantly lymphomas and sarcomas, with a small number of carcinomas.

Humans are exposed to preformed N-nitroso compounds in foods, beverages and cigarette smoke, and in certain industries as well as to N-nitroso compounds produced *in vivo*. A principal pathway for the formation of the N-nitroso compounds is the nitrosation of amines either naturally present in the diet or formed during ingestion, digestion, or metabolism [44]. Such nitrosamines may be significant inducers of human cancer in the mouth, tongue and esophagus [32, 52]. Methyl-n-amylnitrosamine (MNAN), an N-nitroso compound, is known to induce esophageal cancer in rats [51] and is considered a possible etiologic agent for esophageal and oral tumors in humans [23]. MNAN can be broken down by rat and human esophageal microsomes [51] to hydroxy-MNANs, aldehydes and alkylating agents. The alkylating agents, subsequently, easily bind to nucleotides in DNA, and the alkylation of DNA with small alkyl groups or large bulky adducts may lead to mutations resulting in cancer. It is suspected that the lack of *p53* function 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.

The etiology of oral and esophageal cancers in humans is well studied

and predominantly involves the use of tabacco. Tabacco-associated nitrosamines are possible carcinogenic agents. Mutations of the *p53* gene are etiologically associated with the development of oral and esophageal squamous cell carcinomas (SCCs). Taken together, direct exposure of some carcinogens to the oral cavity and esophagus, and consequent alterations in *p53* are considered to be crucial factors of the development of SCCs on these sites. Within oral tissues, the tongue is the most common site for oral SCCs in humans. Although heterozygous *p53* knockout (KO) mice have been demonstrated to be sensitive to various carcinogens at different sites (Table 2), there is paucity of reports on lingual and esophageal carcinogenesis in the heterozygous *p53* KO mice.

In the present studies, Chapter 1 discusses whether *p53*-deficient mice, including heterozygotes (+/-) and nullizygotes (-/-), are susceptible to methyl-n-amylnitrosamine (MNAN)-induced esophageal carcinogenesis, and the relevance of *p53* gene mutations in esophageal cancer development. Chapter 2 covers susceptibility of the tongue in *p53*-deficient mice to MNAN carcinogenicity, and finally draws some general conclusions.

Table 1. Carcinogenic responses of selected bioassays using genetically engineered mice

Chemical	2 years study		6-9 months study			
	Rats	Mice	p53+/-	RasH2#	TG.AC	XPA-/-
Genotoxic human carcinogens						
Cyclophosphamide	+	+	+	+	+	NE
Melphalan	+	+	+	Eq.	+	NE
Phenacetin	+	+	-	+	-	-
Immunosuppressant human carcinogens						
Cycolsporin A	-	Eq.	+	-	+	+
Hormonal human carcinogens						
Diethylstilbestrol	NE	+	+	+	+	+
Estradiol	+	+	Eq.	-	+	-
Nongenotoxic rodent carcinogens but						
putative human non-carcinogens						
Phenobarbital	+	+	-	-	-	-
Methapyrilene	+	-	-	-	-	NE
Reserpine	+	+	-	-	-	-
Clofibrate*	+	+	-	+	+	NE
Diethylhexylphthalate*	+	+	Eq.	+	-	-

+: positive, -: negative, Eq.: equivocal, NE: not examined
*: Peroxisome proliferators
#: Dermal and oral systems are combined.

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Table 2.   Sensitive responses to various carcinogens in *p53* (+/-) mice

| Tumor	Chemical	Reference
Subcutaneous sarcomas	Benzene	[19]
Bladder tumors	*p*-Cresidine	[19]
	N-Butyl-N-(4-hudroxybutyl)-nitrosamine (BBN)	[59]
Skin tumors	4-Vinyl-1-cyclohexene diepoxide (VCD)	[78]
Lymphomas	N-methylnitrosourea (NMU)	[56]
	Phenolphthalein	[14]
	Cyclophosphamide	[76]
	Cyclosporin A	[76]
	Melphalan	[76]
Vascular tumors	Urethane	[4]
Liver hemangiosarcomas	Dimethylnitrosamine	[26]
Uterine and lung tumors	N-ethyl-N-nitrosourea (ENU)	[53]
Mammary tumors	7,12-dimethyl[a]benzanthracene	[37]
Pituitary and testicular tumors	Diethylstilbestrol (DES)	[76]

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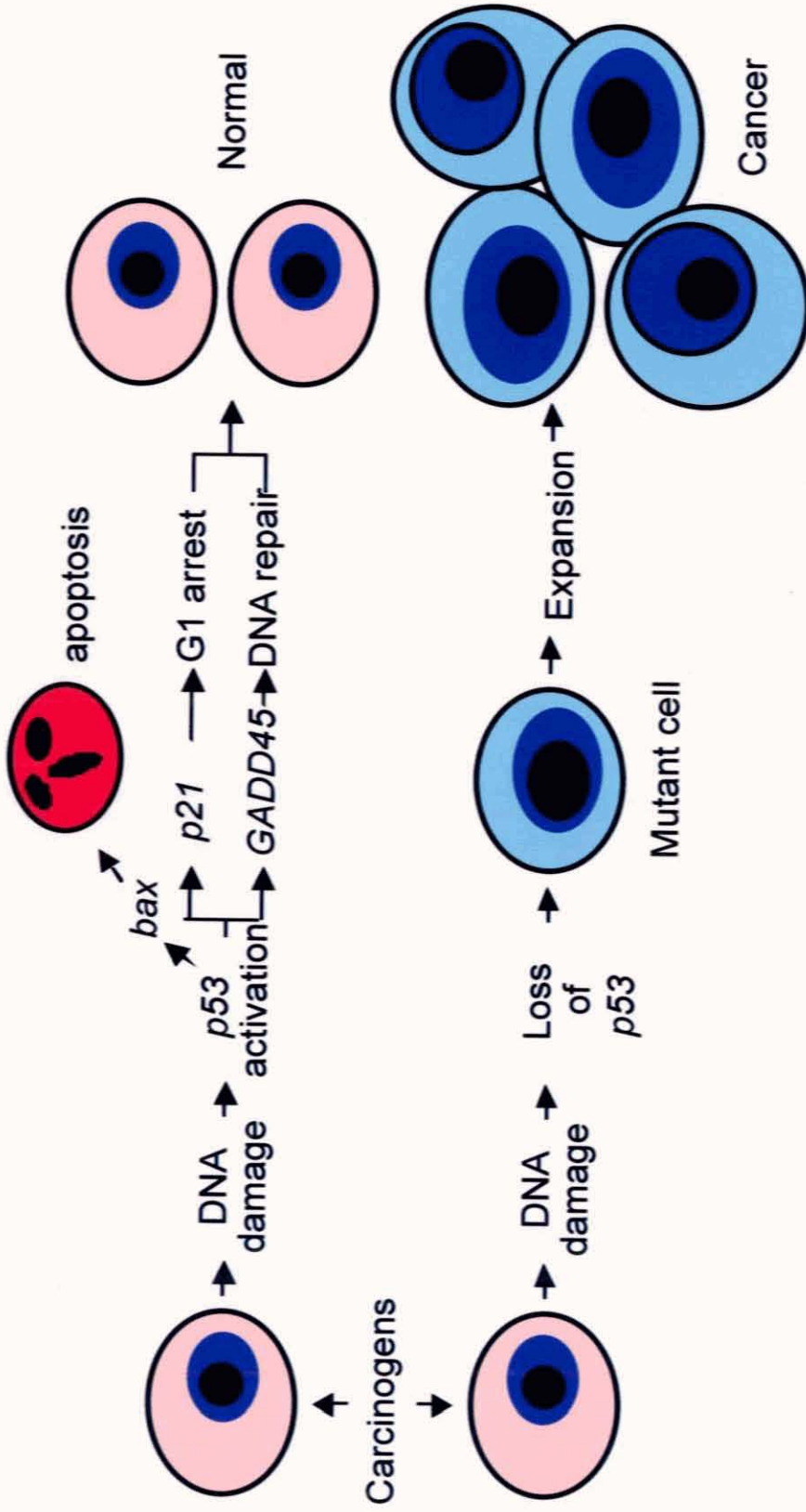
Figure 1. The role of *p53*

Chapter 1

Elevated susceptibility of methyl-n-amylnitrosamine carcinogenesis in the

esophagus of *p53* knockout mice

Abstract

Mutations of the *p53* tumor suppressor gene constitute one of the most frequent molecular changes in a wide variety of human cancers, including those in the esophagus. Mice deficient in *p53* have recently attracted attention for their potential to identify chemical genotoxins. In this study the susceptibility of *p53* nullizygous (-/-), heterozygous (+/-), and wild type (+/+) mice to methyl-n-amylnitrosamine (MNAN)-induced esophageal tumors, and the frequency of *p53* gene mutations in esophageal tumors were investigated.

The *p53* (+/-), and (+/+) mice were treated with 5 or 15 ppm MNAN in their drinking water for 8 weeks, then maintained without further treatment for an additional 7 or 17 weeks, being sacrificed at experimental weeks 15 or 25. An additional group of *p53* (-/-) mice were given 5 ppm MNAN for 8 weeks and sacrificed at week 15. At 15 weeks in the 5 ppm groups, squamous cell carcinomas (SCCs) were observed in 10/12 (83.3%) *p53* (-/-) and 1/15 (6.7%) *p53* (+/-) mice, but in none of the *p53* (+/+) mice. In the animals receiving 15 ppm, 2/14 (14.3%) *p53* (+/-) and 1/11 (9.1%) *p53* (+/+) mice developed SCCs. At 25 weeks, the incidences of SCCs were 7/16 (43.8%) and 8/14 (57.1%) in *p53* (+/-) mice and 1/13 (7.7%) and 2/10 (20.0%) in *p53* (+/+) mice at 5 and 15 ppm, respectively. Of the SCCs examined by PCR-single strand conformation polymorphism analysis, 61% (14/23) from *p53* (+/-) and 50% (6/12) from *p53*

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(+/+) mice demonstrated mutations in the *p53* gene (exons 5-8). These results indicate the order of susceptibility to MNAN-induced esophageal tumorigenesis to be as follows: nullizygotes (-/-) > heterozygotes (+/-) > wild type (+/+), and provide strong evidence of involvement of *p53* mutations in the development of esophageal SCCs.

Introduction

The *p53* gene which encodes a transcriptional regulator that prevents the propagation of genetically damaged cells [46], is frequently mutated in a wide range of human cancers [29, 30]. In addition to somatic mutations of *p53* in sporadic cancers, germ-line mutations in the *p53* gene are associated with Li-Fraumeni syndrome, a familial autosomal dominant disease characterized by a predisposition to the development of a variety of tumors [75]. The role of *p53* includes involvement in G1 cell cycle arrest and induction of DNA repair genes in response to DNA damage, as well as activation of genes promoting apoptosis [43, 46].

Since the first description of homozygous and heterozygous *p53*-deficient mice in which the early onset of spontaneous tumors was demonstrated [26, 27], they have attracted interest as a model for assessing the significance of the loss of *p53* in tumor development and as experimental animals for assays of carcinogenic potential [10, 11, 36, 77]. By the age of 4.5 months, approximately half of nullizygous *p53*-deficient (-/-) mice develop tumors, and by 10 months of age the incidence is 100%. Most of the tumors are lymphomas or sarcomas, rapidly causing mortality. In contrast, the heterozygotes (+/-) show only a low background incidence of spontaneous tumors until almost 12 months of age and have much longer life spans [13, 27].

This low background tumor incidence, combined with elevated susceptibility to chemical induction of tumors, make the *p53* (+/-) mouse useful for short-term bioassays [10, 78], as well as providing a model for the human Li-Fraumeni syndrome.

In most countries of the world, squamous cell carcinomas (SCCs) constitute the most common esophageal cancers in humans, and mutations in the *p53* gene are frequently detected in these SCCs [28, 64, 71, 72]. Thus *p53* alteration may be a crucial event in esophageal carcinogenesis. There are, however, paucity of reports on chemical-induced esophageal carcinogeneis in *p53*-deficient mice. The aims of the present study were to examine whether *p53* knockout (KO) mice, including heterozygotes (+/-) and nullizygotes (-/-), might be more susceptible to methyl-n-amylnitrosamine (MNAN)-induced esophageal tumorigenesis compared with wild type (+/+) mice, and to determine whether deficiency in *p53* plays a role in esophageal tumor development.

Materials and methods

Animals

p53 knockout mice on a C57BL/6 genetic background, produced by

Donehower *et al.* [12], were reproduced and maintained at the Animal Facility of

Aichi Cancer Center Research Institute (Chikusa, Nagoya, Japan). At six

weeks old, male mice were subjected to the experiment. They were housed

3-5/plastic cage with hardwood chips in an air-conditioned room at 20-24°C 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.*

For genotyping of each mouse, the following procedure was performed

as described previously [81]: DNA samples were extracted from the tail using

QIAamp tissue kit (QIAGEN, K.K., Tokyo, Japan). The 25 μl PCR reaction

mixture consisted of 1.25 units of Taq DNA polymerase (Takara Shuzo Co., Ltd.,

Shiga, Japan), 1x provided buffer, 200 μM dNTP, 200 nM each of 5'- and

3'-primers (10681, 10480, 10588, and 10930 as listed in Table 3), and 2.5 μl of

genomic DNA. PCR was performed as follows: 94°C 1 min x 1 cycle: 94°C 1

min-65°C 1 min-72°C 1 min x 35 cycles: 72°C 10 min using a Takara PCR

Thermal Cycler MP (Takara Shuzo Co., Ltd., Shiga, Japan). The amplification

products were visualized after electrophoresis on agarose gels under ultraviolet

illumination in the presence of ethidium bromide (Figure 2).

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 or 15 ppm 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 sacrificed at week 15 or 25. An additional group of *p53* (-/-) mice were also treated with 5 ppm MNAN for 8 weeks and sacrificed at week 15. All mice were sacrificed by exsanguinations under ether anesthesia. Three groups of control animals with *p53* (+/+), *p53* (+/-) or *p53* (-/-) received unsupplemented drinking water (Figure 3).

Histopathologic analysis

At necropsy, mice were sacrificed by exsanguinations via the posterior vena cava under ether anesthesia. Following an external examination, mice were dissected and the internal organs were removed for visual examination. The esophagus of each animal was resected, from the larynx to the stomach, and slit longitudinally along the midline of the dorsal wall. After fixation in 4% paraformaldehyde in phosphate buffered saline, and embedded in paraffin, each was sectioned and stained with hematoxylin and eosin for microscopic examination. The total numbers of tumors were counted and the total areas

and distances of each from the larynx were measured by computerized image analysis, using a microscope equipped with an image processor for analytical pathology (IPAP; Sumika Technos, Hyogo, Japan).

PCR-single strand conformation polymorphism analysis (SSCP)

Nineteen tumors from *p53* (+/+) mice and 35 from *p53* (+/-) mice were subjected to PCR-SSCP. Each tumor was identified by means of 1 to 19 and 101 to 135 sequential numbers, respectively. PCR-SSCP was conducted basically as described previously [58]. Briefly, genomic DNA was extracted from tumor areas in paraffin sections with DEXPAT (Takara Shuzo Co., Ltd., Shiga, Japan) [88] or from frozen tissues by treatment with proteinase-K and phenol as detailed elsewhere [69]. Four pairs of PCR primers for mouse *p53* exons 5-8 were designed based on the published sequence as listed in Table 3 [81]. The 5 µl PCR reaction mixture consisted of 0.025 µl of Ampli Taq Gold, 0.5 µl of Gene Amp dNTP MIX (Takara Shuzo Co., Ltd., Shiga, Japan), 0.5 µl of 10x provided buffer, 200 µM each of 5'- and 3'-primers for each exon, 167 µM^{32}P-dCTP and 1 µl of genomic DNA. PCR was performed with a Takara PCR Thermal Cycler MP (Takara Shuzo Co., Ltd., Shiga, Japan) as follows: 95°C 10 min x 1 cycle: 94°C 1 min-94°C 45 sec-54°C 30 sec-71°C 1 min x 50 cycles: 71°C 10 min. The amplification products were heat-denatured, then electrophoresed in 0.625 x MDE polyacrylamide gels (FMC, BioProducts,

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Rockland, ME, USA) with 5% glycerol. These were run at room temperature for 18h at 8 watts, dried, and applied to imaging plates, which were then analyzed with BAS 2500 (Fuji Film, Kanagawa, Japan).

Direct sequencing

Sequencing was performed using an AmpliCycle Sequencing Kit (Perkin Elmer, Roche Molecular Systems Inc., Branch burg, NJ, USA) as described previously [82]. Briefly, SSCP bands of interest in polyacrylamide gels were excised, and DNA was eluted in 50 µl of 10 mM Tris-HCl, pH 8.0 at 50°C for 30 min. PCR was performed with 10 µl of the eluted DNAs, purified with a QIAquick PCR purification kit (QIAGEN K.K., Tokyo, Japan), and used as templates for the following sequencing reaction. The sense primer for target exon 5, 6, 7 or 8 was end-labeled with $[\gamma^{32}P]$ ATP by T4 polynucleotide kinase (New England Biolab, Beverly, MA, USA) and the sequencing reaction was performed as follows: 95°C -2 min x 1, 95°C -1 min: 55°C -1 min: 72°C -1 min ×30 with PCR Thermal Cycler MP (Takara Shuzo Co., Ltd., Shiga, Japan). This was stopped with Stop Solution (provided by the manufacture), then the heat-denatured samples were electrophoresed in 6% Long Ranger gel solution (FMC BioProducts, Rockland, ME, USA) containing 7 M urea, dried, and exposed to imaging plates for analysis with the BAS 2500 (Fuji Film, Kanagawa, Japan).

LA PCR

For freshly collected and frozen samples with positive PCR-SSCP results from *p53* (+/-) mice, LA PCR was performed to specifically amplify the wild-type and mutant alleles with PCR primers 10681 or C/10930 in combination with the antisense primer for exon 8 using a TaKaRa LA PCR kit (Takara Shuzo Co., Ltd., Shiga, Japan) [81] (Table 3). The PCR conditions were as follows: 94°C for 1 min; 35 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 4 min; 72°C for 10 min. The products were subjected to direct sequencing.

Statistical analysis

Data for incidences of histopathological lesions were analyzed by the Fisher's exact test method. The numbers and areas of tumors were analyzed with the Mann-Whitney rank sum test. Survival curves were drawn by the Kaplan-Meier method and analyzed using the log rank test [65].

Results

Mortality of each genotype

Administration of 5 and 15 ppm MNAN in drinking water was well-tolerated by both *p53* (+/-) and *p53* (+/+) mice. Survival was not significantly different between *p53* (+/-), and *p53* (+/+) mice. However, *p53* (-/-) mice demonstrated a high mortality rate (Figure 4). Of the 8 dead *p53* (-/-) mice, four animals died of lymphomas and one animal died of soft tissue sarcoma. Causes of death were not determined in other mice. These deaths occurred at 8 weeks and thereafter.

Necropsy findings

Grossly, esophageal tumors appeared as pale, papillary or dome-shaped masses with variable sizes up to 1 cm in diameter on the mucosal surface. The masses were focally or multifocally distributed throughout the esophagus (Figure 5).

Histopathological analysis

Administration of MNAN induced a 100% incidence of esophageal diffuse hyperplasia characterized by thickening of the squamous epithelium in *p53* (-/-), *p53* (+/-), and *p53* (+/+) mice. This was often accompanied by

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subepithelial inflammatory infiltration (Figure 6-1B) with clear contrast to normal mucosa (Figure 6-1A).

Papillomas were characterized by nodular mucosal elevation of proliferating epithelial cells (Figure 6-2A). Squamous cell carcinomas (SCCs) showed invasive growth of atypical squamous epithelial cells (Figure 6-2B). Neither adenocarcinomas nor Barrett's esophagus, in which squamous epithelium is replaced by a columnar epithelium, were detected in any of the mice. There was no regional preference for tumor development with the esophageal mucosa (Figure 7).

In addition to esophageal lesions, occasional nodular thickenings of the mucosa were observed in the forestomach in mice of all three genotypes. However, no lesions were detected in the glandular stomach.

The major cause of death in $p53$ (-/-) mice was thymic malignant lymphoma, and the representative features are shown in Figure 8-1. One $p53$ (-/-) mouse which died had a subcutaneous sarcoma in the dorsal skin. This sarcoma was composed of large pleomorphic, poorly differentiated spindle cells with scant stroma. The tumor cells had large oval to round and vesicular nuclei with nucleoli. Multinucleated cells and bizarre mitoses were common (Figure 8-2).

Incidence, number and size of esophageal tumors

In the mice treated with 5 ppm MNAN, the incidence (Table 4) of SCCs was significantly higher ($P < 0.001$) in *p53* (-/-) (83.3%) than *p53* (+/-) (6.7%) and *p53* (+/+) (0%) mice at 15 weeks after starting treatment. At 25 weeks, the SCC incidence in *p53* (+/-) (43.8%) were significantly increased ($P < 0.05$) when compared to *p53* (+/+) (7.7%) mice. Only one SCC developed in a *p53* (+/+) mouse at 25 weeks. The total number of tumors per mouse (left box of Figure 9A) (mean±SD) was also higher in *p53* (-/-) (4.8±1.4) than *p53* (+/-) (1.5±1.2) and *p53* (+/+) (0.8±0.9) groups at 15 weeks ($P < 0.001$) and in *p53* (+/-) (4.4± 2.6) than in *p53* (+/+) (2.7±1.5) mice at 25 weeks ($P < 0.05$). The size of the tumors (left box of Figure 9B) (mean±SD) was larger in *p53* (-/-) (0.3±0.05 mm^2) than *p53* (+/-) (0.1±0.1 mm^2) and *p53* (+/+) (0.07±0.03 mm^2) mice at 15 weeks ($P < 0.005$). There were no significant differences in tumor size between *p53* (+/-) and *p53* (+/+) mice at 15 or 25 weeks.

In the mice receiving 15 ppm MNAN, there was a trend for greater SCC incidences in *p53* (+/-) as compared to *p53* (+/+) mice at 15 and 25 weeks; 14.3% and 9.1%, respectively, at 15 weeks, and 57.1% and 20.0%, at 25 weeks (Table 4). Average tumor size (right box of Figure 9B) (mean±SD) in the *p53* (+/-) case (0.8±2.1 mm^2) was significantly larger ($P < 0.05$) than in *p53* (+/+) (0.2±0.2 mm^2) mice at 25 weeks, although there was little difference in the number of the tumors (right box of Figure 9A).

PCR-SSCP analysis of the p53 gene in tumors

PCR-SSCP and sequencing analyses for exons 5-8 of *p53* gene were performed, representative results being illustrated in Figures 10-1 and 10-2. *p53* mutations were identified in 6 out of 12 SCCs (50%) and 14 out of 23 SCCs (61%) in *p53* (+/+) (Table 5) and *p53* (+/-) (Table 6) mice, respectively. There were 3 SCCs (tumor IDs: 109, 129 and 133) with more than one mutations in *p53* (+/-) mice. Only one of 19 papillomas examined (tumor ID: 10) had a mutation. DNA sequencing demonstrated 8 mutations in exon 5 (33%), 6 in exon 6 (25%), 8 in exon 7 (33%), and 2 in exon 8 (8%) of the total of 24 mutations identified in 20 SCCs. Of these mutations 2 (tumor IDs: 11 and 133) were silent and 1 (tumor ID: 4) was of nonsense type. All the others were missense mutations. There were 19 transitions (79%) and 5 transversions (21%). G:C to A:T transitions at non-CpG sites accounted for approximately half of all mutations. A total of 20 SCCs exhibiting *p53* mutation widely varied in size from 0.13 to 9.34 mm^2; 1.7±2.7 mm^2 (mean±SD) and *p53* mutation rate was comparable in smaller and larger carcinomas.

Frozen tissues were available from two tumors (tumor IDs: 101 and 126) from *p53* (+/-) mice in which *p53* mutations were observed. LA PCR-amplified DNAs from these samples exhibited missense mutations not in the mutant but rather in the wild-type allele, indicating loss of functional *p53* protein. The other tumors developing in *p53* (+/-) mice could not be subjected

to LA PCR analysis due to poor preservation of genomic DNA in paraffin blocks.

Discussion

The present study demonstrated that nullizygous and heterozygous *p53*

KO mice are more susceptible to esophageal tumorigenesis induced by MNAN,

a genotoxic carcinogen [52], than their wild-type counterparts, as indicated by

an increased incidence and tumor size of SCCs. Furthermore, PCR-SSCP

analysis revealed a high frequency of missense mutations in *p53* with evidence

of loss of functional *p53* protein in esophageal malignancies. These results

are consistent with the observation that *p53* (+/-) mice are generally susceptible

to genotoxic carcinogens [78]. In addition, accelerated tumor development

with chemical exposure in *p53* (+/-) mice has been reported with regard to

lymphomas [14], mesotheliomas [50], skin tumors [40, 79], vascular tumors [4],

urinary bladder tumors [59, 78], and lung tumors [18]. Taken together, the

results support the hypothesis that mutational inactivation of the retained

wild-type allele or loss of *p53* heterozygosity, with consequent loss of p53

function, eventually results in development of neoplasias as occurs with the

human Li-Fraumeni syndrome [16]. The lack of any increased susceptibility of

p53 (+/-) mice to hepatocarcinogenesis [8, 39], gastric carcinogenesis [88], and

mammary carcinogenesis [37] may reflect organ/tissue specific dependence in the requirement for the *p53* gene product in tumorigenesis. While the mechanism may involve an additional "hit" to inactivate the second normal allele, Venkatachalam *et al.* [84] have proposed that reduction of the *p53* gene products may be sufficient to promote tumorigenesis.

There is growing evidence that esophageal adenocarcinoma and Barrett's esophagus are related to the reflux of duodenal content in human [15, 21, 80]. Both of these conditions can be induced in the lower esophagus by reflux of duodenal content in rats [6]. Fein *et al.* [17] reported that total gastrectomy with esophagojejunostomy caused esophageal adenocarcinomas as well as dysplasia of the squamous epithelium in the operated *p53* (-/-) mice. However, in addition to no regional preference in squamous cell tumor development, no adenocarcinomas were detected in the current study, although a high percentage of *p53* (-/-) mice developed squamous cell carcinomas. These results are consistent with no evidence of reflux.

Mutations in the *p53* gene commonly occur at hot spots in human cancers [30], but the mutation database for laboratory animals is limited. Our results point to a high frequency of *p53* mutations in esophageal malignancies, the most common being missense, as reported for human cancer [2, 33, 63]. In contrast to the frequent *p53* mutations in SCCs even in small carcinomas, only one mutation was detected in 19 papillomas analyzed. This might simply

be a reflection of *p53* mutations occurring preferentially in malignant lesions. Similar findings have been reported in murine skin tumors induced with benzo[a]pyrene in which the majority of *p53* mutations were identified in squamous cell carcinomas; they were rare in papillomas [66]. Moreover, abnormal p53 protein is infrequently identified in human esophageal squamous cell papillomas [62] while even dysplastic lesions exhibit *p53* mutations [20, 49, 85]. It is worth noting that there might also have been normal cell contamination of the papilloma samples, although the proportion of normal cells derived from the interstitium and margin of a papilloma, for example, can be estimated at 50% of a sample at most. When 1 out of 2 alleles is mutated in the remaining 50%, the proportion of the mutant allele would correspond to 25% of the original sample. Theoretically, any mutant allele would be detected as a mobility shift by PCR-SSCP [58].

Regarding the location of *p53* mutations, they were randomly distributed through exons 5 to 8, with more than one mutation detected at codons 164 (8%, 2/25), 219 (4/25, 16%), 232 (2/25, 8%) and 250 (4/25, 16%). Approximately half of the mutations observed in our study were G:C to A:T transitions at non-CpG sites. Retrospective analyses of *p53* gene mutations in human esophageal cancers have also shown a predominance of G:C to A:T transitions [23, 51], indicating the advantage of our MNAN-induced esophageal carcinogenesis model to mimic the human diseases.

In conclusion, the present study demonstrated an increased susceptibility to esophageal tumorigenesis by a genotoxic agent in *p53* nullizygotes (-/-) and then *p53* heterozygotes (+/-) as compared with wild type (+/+) mice, providing strong evidence of involvement of *p53* mutations in the development of esophageal SCCs. Although consideration must be given to carcinogen and/or tissue specificity, *p53* KO mice provide a useful model for identification and understanding of human carcinogens.

Table 3. PCR primers for genotyping and for SSCP analysis of mouse *p53*

Target		Primer Sequence	Product length (bp)
Wild-type	10681	5'-GTGTTTCATTAGTTCCCCACCTTGAC-3'	320
	10480	5'-ATGGGAGGCTGCCAGTCCTAACCC-3'	
Mutant	10588	5'-GTGGGAGGGACAAAAGTTCGAGGCC-3'	150
	10930	5'-TTTACGGAGCCCTGGCGCTCGATGT-3'	
(LA PCR)	C/10930	5'-ACATCGAGCGCCAGGGCTCCGTAAA-3'	
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'-TCACCTGGATCCTGTGTATT-3'	170
	antisense	5'-CAGGCTAACCTAACCTACCA-3'	
Exon 8	sense	5'-ACTGCCTTGTGCTGGTCCTT-3'	279
	antisense	5'-GGAGAGGCGCTTGTGCAGGT-3'	

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Table 4. Incidences of esophageal lesions

Exp. week	MNAN (ppm)	p53 geno-type	No. animals	No. premature death	No.[a] examined	No. of mice with lesions(%) Papilloma	No. of mice with lesions(%) SCC[b]
15W	5	(+/+)	13	0	13	7 (53.8)	0
		(+/-)	17	2[f]	15	12 (80.0)	1 (6.7)
		(-/-)	20	8[g]	12	10 (83.3)	10 (83.3)[c,d]
	15	(+/+)	12	1[h]	11	10 (90.9)	1 (9.1)
		(+/-)	15	1[i]	14	13 (92.9)	2 (14.3)
	0	(+/+)	5	0	5	0	0
		(+/-)	5	0	5	0	0
		(-/-)	4	0	4	0	0
25W	5	(+/+)	14	1[j]	13	13 (100.0)	1 (7.7)
		(+/-)	16	0	16	15 (93.8)	7 (43.8)[e]
	15	(+/+)	11	1[k]	10	10 (100.0)	2 (20.0)
		(+/-)	17	3[l]	14	13 (92.9)	8 (57.1)
	0	(+/+)	5	0	5	0	0
		(+/-)	6	0	6	0	0

[a] Mice which died before the end of experiment were excluded.
[b] SCC: Squamous cell carcinoma
[c] Significantly different from p53 (+/+) using the Fisher's exact test, P<0.001
[d] Significantly different from p53 (+/-) using the Fisher's exact test, P<0.001
[e] Significantly different from p53 (+/+) using the Fisher's exact test, P<0.05
[f,h,i,j,k,and l] Cause of death were not determined.
[g] Eight deaths include 4 from lymphomas, 1 from a sarcoma, and 3 of unknown causes.

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Table 5. *p53* gene mutations identified in esophageal tumors in *p53* (+/+) mice

Tumor ID	Geno-type	Histology	Exon	Codon	Nucleotide change	Aminoacid change	Event
1	+/+	SCC	5	155	CGC→TGC	Arg→Cys	Transition
2	+/+	Papilloma	-				
3	+/+	Papilloma	-				
4	+/+	SCC	5	173	TGC→TGA	Cys→Stop	Transversion
5	+/+	SCC	-				
6	+/+	Papilloma	-				
7	+/+	SCC	7	250	ACC→ATC	Thr→Ile	Transition
8	+/+	Papilloma	-				
9	+/+	Papilloma	-				
10	+/+	Papilloma	5	164	CAG→AAG	Gln→Lys	Transversion
11	+/+	SCC	5	151	GGG→GGA	Gly→Gly	Transition
12	+/+	SCC	-				
13	+/+	Papilloma	-				
14	+/+	SCC	5	165	CAC→AAC	His→Asn	Transversion
15	+/+	SCC	8	299	GGG→TGG	Gly→Trp	Transversion
16	+/+	SCC	-				
17	+/+	SCC	-				
18	+/+	SCC	-				
19	+/+	SCC	-				

-: Mutation not detected in exons 5,6,7, and 8.
SCC: Squamous cell carcinoma
Arg: Arginine, Cys: Cysteine, Thr: Threonine, Ile: Isoleucine, Gln: Glutamine, Lys: Lysine, Gly: Glycine, His: Histidine, Asn: Asparagine, Trp: Tryptophan

Table 6. *p53* gene mutations identified in esophageal tumors in *p53* (+/-) mice

Tumor ID	Geno-type	Histology	Exon	Codon	Nucleotide change	Aminoacid change	Event
101	+/-	SCC	6	204	GAA→AAA	Glu→Lys	Transition
102	+/-	SCC	8	301	GCA→GTA	Ala→Val	Transition
103	+/-	SCC	-				
104	+/-	SCC	-				
105	+/-	Papilloma	-				
106	+/-	Papilloma	-				
107	+/-	Papilloma	-				
108	+/-	Papilloma	-				
109	+/-	SCC	5	164	CAG→AAG	Gln→Lys	Transversion
			7	250	ACC→ATC	Thr→Ile	Transition
110	+/-	Papilloma	-				
111	+/-	SCC	-				
112	+/-	SCC	6	219	CCA→TCA	Pro→Ser	Transition
113	+/-	SCC	-				
114	+/-	Papilloma	-				
115	+/-	Papilloma	-				
116	+/-	Papilloma	-				
117	+/-	Papilloma	-				
118	+/-	SCC	-				
119	+/-	SCC	6	219	CCA→TCA	Pro→Ser	Transition
120	+/-	SCC	-				
121	+/-	Papilloma	-				
122	+/-	Papilloma	-				
123	+/-	SCC	5	136	AAG→AGG	Lys→Arg	Transition
124	+/-	SCC	6	219	CCA→TCA	Pro→Ser	Transition
125	+/-	Papilloma	-				
126	+/-	SCC	6	219	CCA→TCA	Pro→Ser	Transition
127	+/-	SCC	-				
128	+/-	SCC	7	250	ACC→ATC	Thr→Ile	Transition
129	+/-	SCC	5	157	ATG→ACG	Met→Thr	Transition
			7	232	AAG→AGG	Lys→Arg	Transition
			7	251	ATC→GTC	Ile→Val	Transition
130	+/-	SCC	-				
131	+/-	SCC	-				
132	+/-	SCC	7	232	AAG→AGG	Lys→Arg	Transition
133	+/-	SCC	5	139	CCT→CCC	Pro→Pro	Transition
			7	241	GGG→GAG	Gly→Glu	Transition
134	+/-	SCC	6	192	ATC→CTC	Ile→Leu	Transversion
135	+/-	SCC	7	250	ACC→ATC	Thr→Ile	Transition

-: Mutation not detected in exons 5,6,7, and 8.
SCC: Squamous cell carcinoma
Glu: Glutamic acid, Lys: Lysine, Ala: Alanine, Val: Valine, Gln: Glutamine, Thr: Threonine
Ile: Isoleucine, Pro: Proline, Ser: Serine, Arg: Arginine, Met: Methionine, Gly: Glycine,
Leu: Leucine
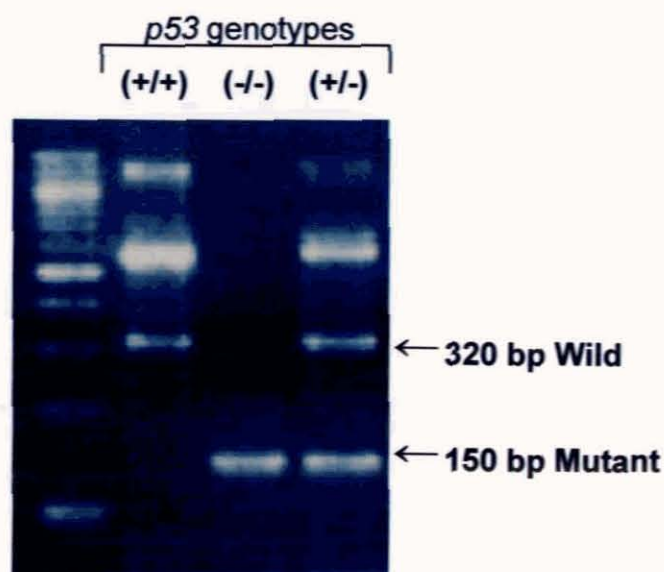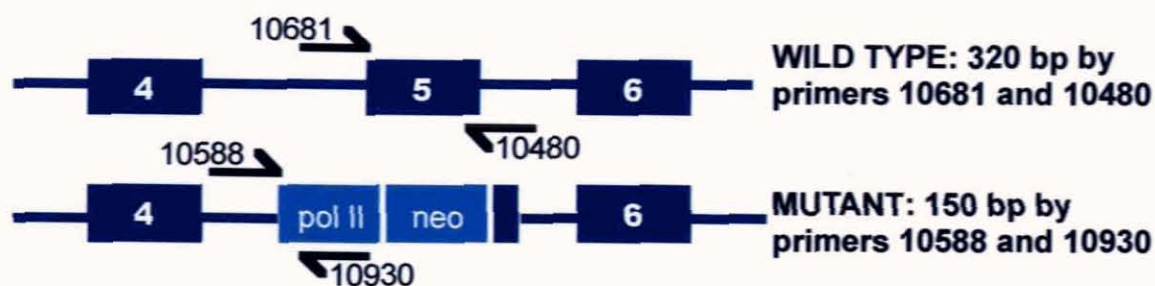
Figure 2. *p53* Knockout mice genotype analysis. Mutant allele possesses the *p53* gene interrupted in exon 5 by a *Pol* II-neo expression cassette missing a poly-adenylation signal. The insertion of the neo cassette is also accompanied by deletion of a 450-base-pair *p53* fragment containing 106 nucleotides of exon 5 and about 350 nucleotides of intron 4. The bottom panel shows the results of agarose gel electrophoresis. Lane (+/-) represents heterozygous for the mutant allele, (-/-) reveals homozygous for the mutant allele, and (+/+) represents a wild-type genotype.

Figure showing experimental design timeline.

p53 genotypes	MNAN		

6 weeks old

(weeks)

0   8   15   25

(+/+),(+/-),(-/-)	0 ppm
(+/+),(+/-),(-/-)	5 ppm
(+/+),(+/-)	15 ppm
(+/+),(+/-)	0 ppm
(+/+),(+/-)	5 ppm
(+/+),(+/-)	15 ppm

Animals : male *p53* knockout mice, 6 weeks old, C57BL/6 genetic background

MNAN : methyl-n-amylnitrosamine

$$CH_3\diagdown$$
$$N - N = O$$
$$CH_3\ CH_2\ CH_2\ CH_2\ CH_2 \diagup$$
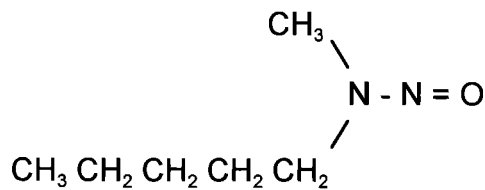
■ : Administration of MNAN at 15 ppm in drinking water

▨ : Administration of MNAN at 5 ppm in drinking water

☐ : Unsupplemented drinking water

S : sacrifice

Figure 3. Experimental design
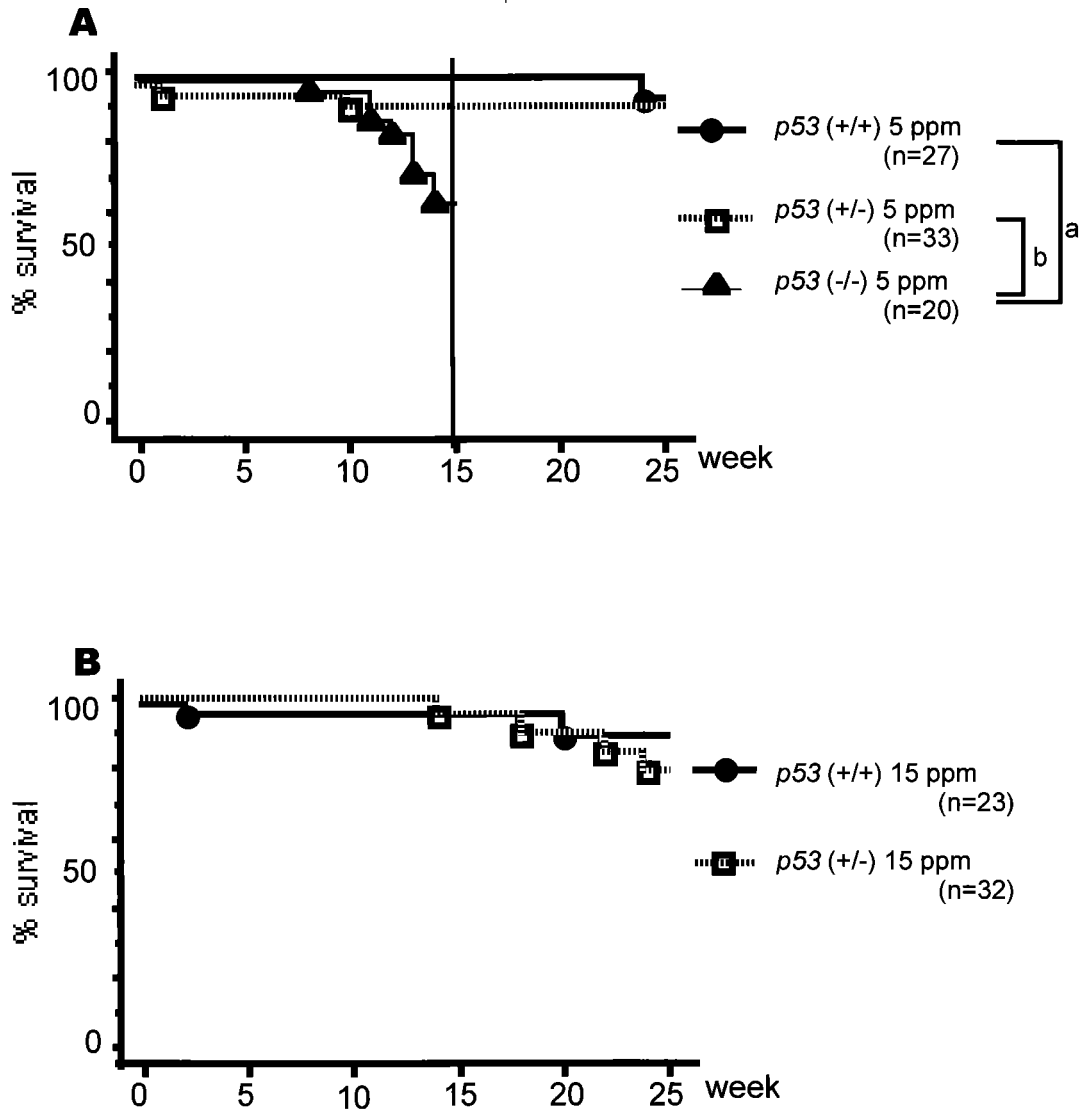
Figure 4. Survival curves of *p53* (+/+), (+/-) and (-/-) mice treated with 5 ppm MNAN (A) and those of *p53* (+/+) and (+/-) mice at 15 ppm (B). At week 15, all remaining *p53* (-/-) mice were sacrificed.

p53 (-/-) mice survival was less than in the (+/+) ([a]$P<0.01$) or (+/-) cases ([b]$P<0.05$). The total numbers of the mice are described in the parenthesis. The number of the animals at the beginning of the each experimental group ("No. animals") and at the scheduled sacrifice ("No. examined") are as listed in Table 4.
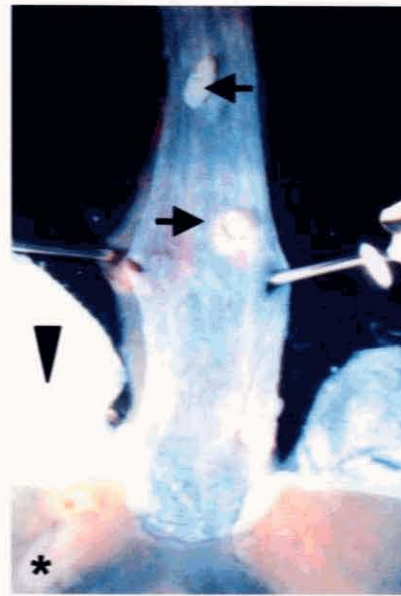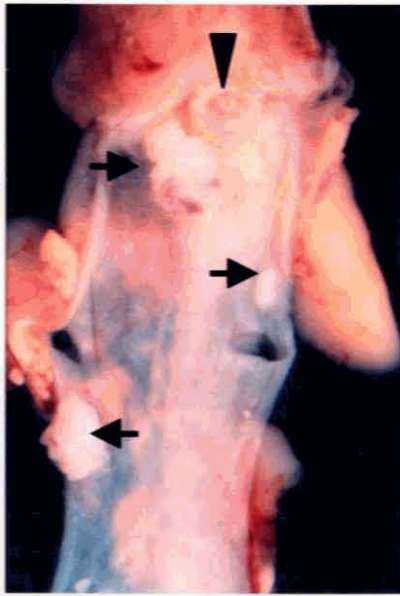
Figure 5. Representative macroscopic features of esophageal tumors.

Left: Upper esophageal region.
        Three neoplastic nodules are indicated by arrows.
        The arrowhead indicates the larynx.

Right: Lower esophageal region.
        Two neoplastic nodules are indicated by arrows.
        The arrowhead indicates the forestomach and the asterisk
        indicates glandular stomach.
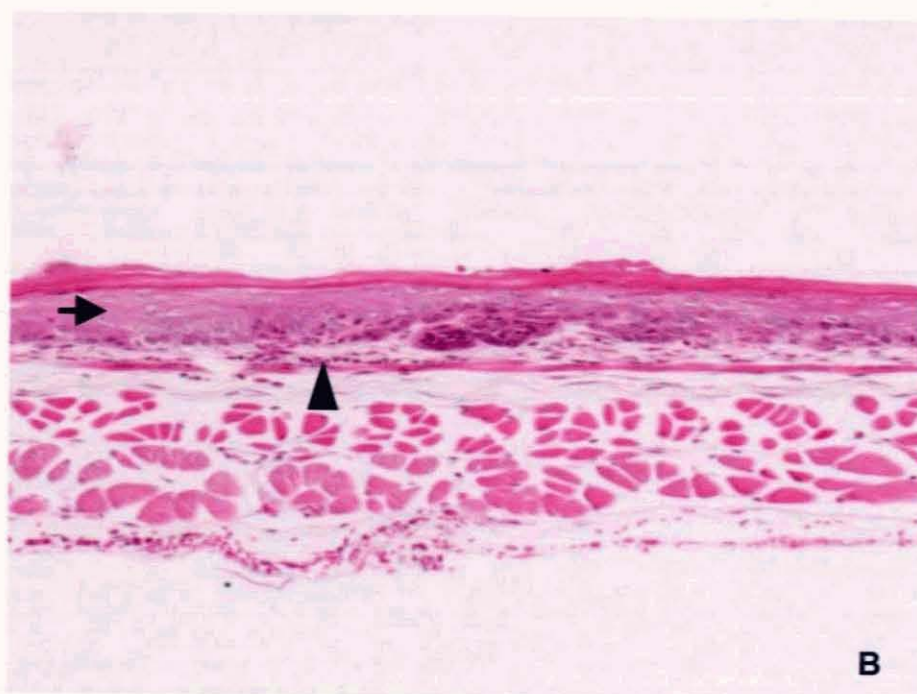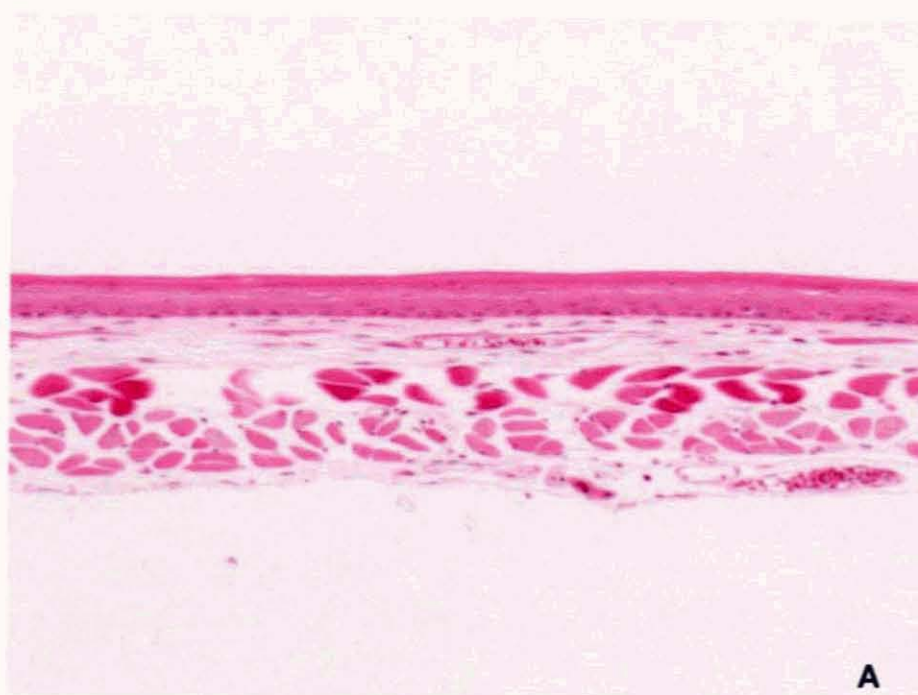
Figure 6-1. Photomicrographs of normal mucosa at 100X (A), and diffuse hyperplasia at 200X (B) in the esophagus of *p53* (+/-) mice treated with MNAN. Thickening of the squamous epithelium (arrow) and minimal subepithelial inflammatory infiltrates (arrowhead).
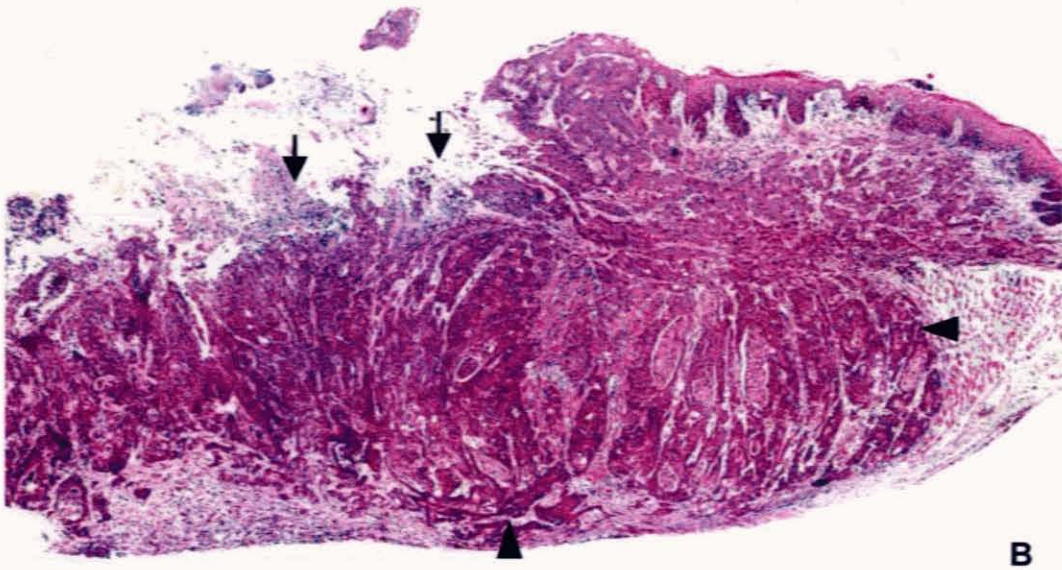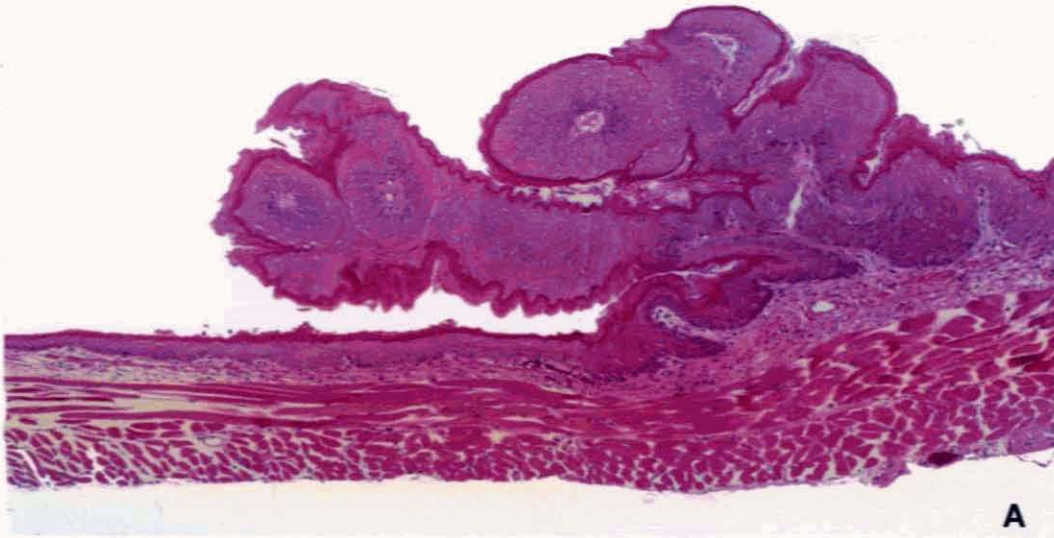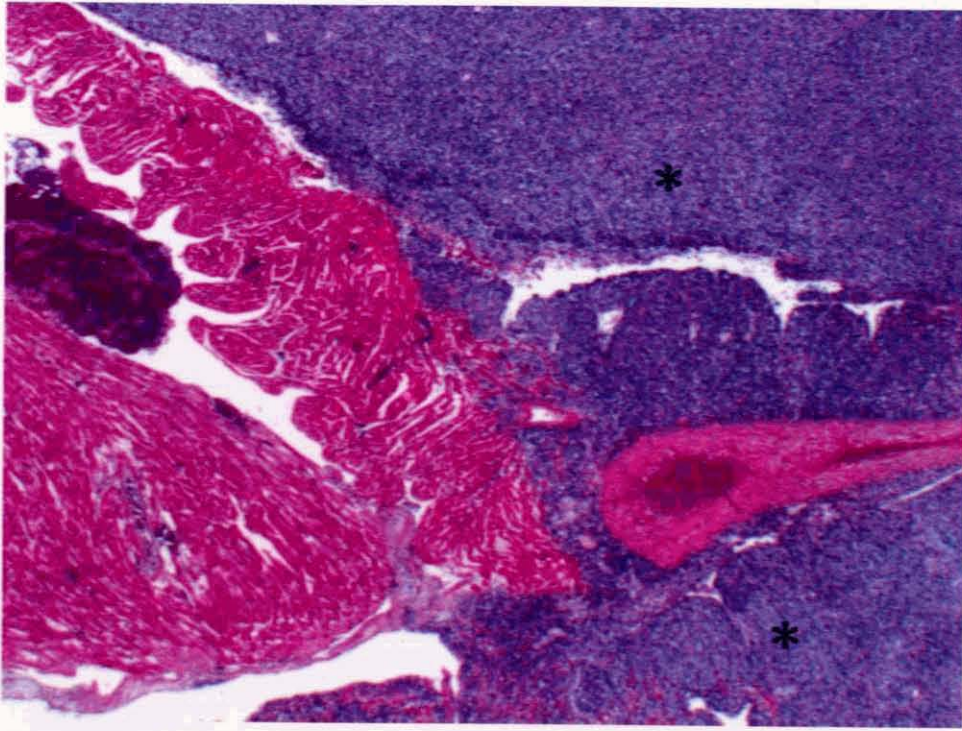
Figure 6-2. Photomicrographs of papilloma at 25X (A), and squamous cell carcinoma at 10X (B) in the esophagus of *p53* (+/-) mice treated with MNAN. Squamous cell carcinoma is invasive into muscularis (arrowheads). Arrows indicate ulceration.
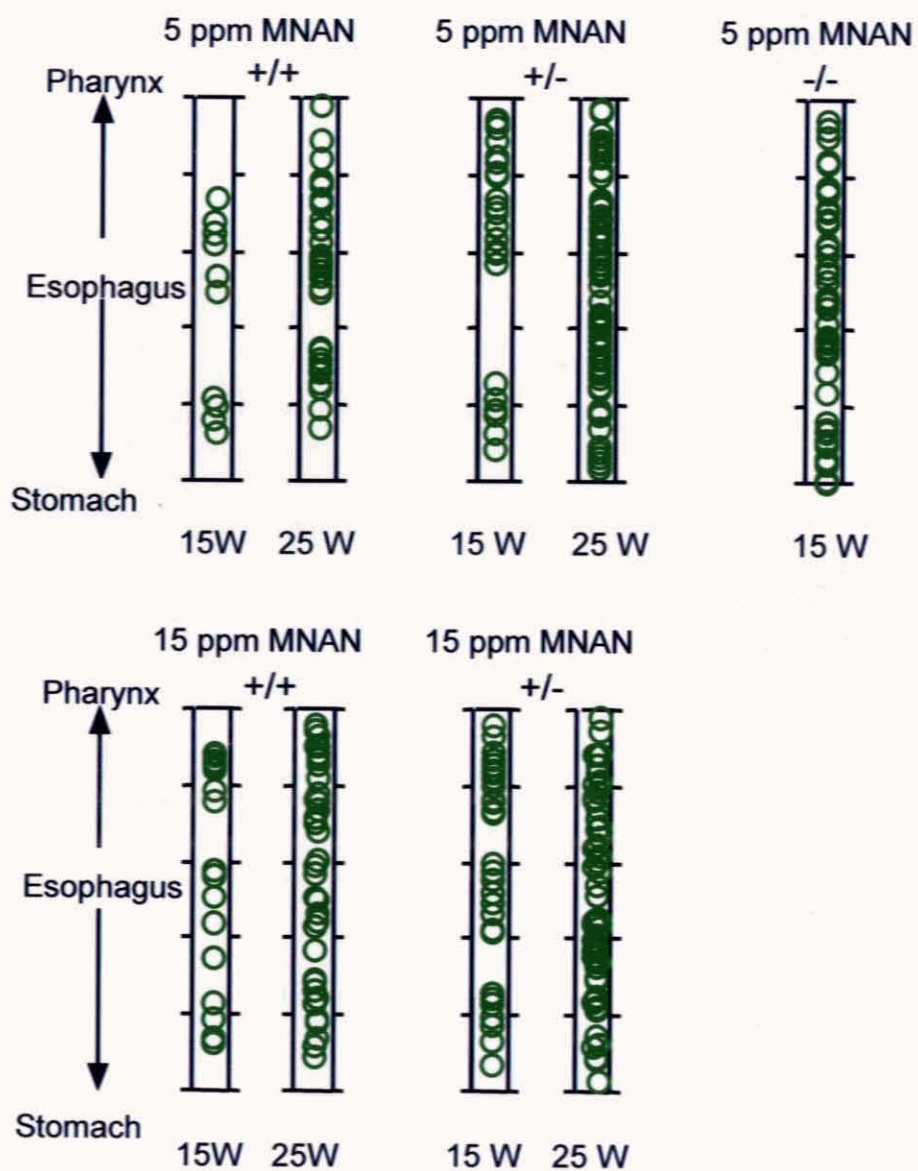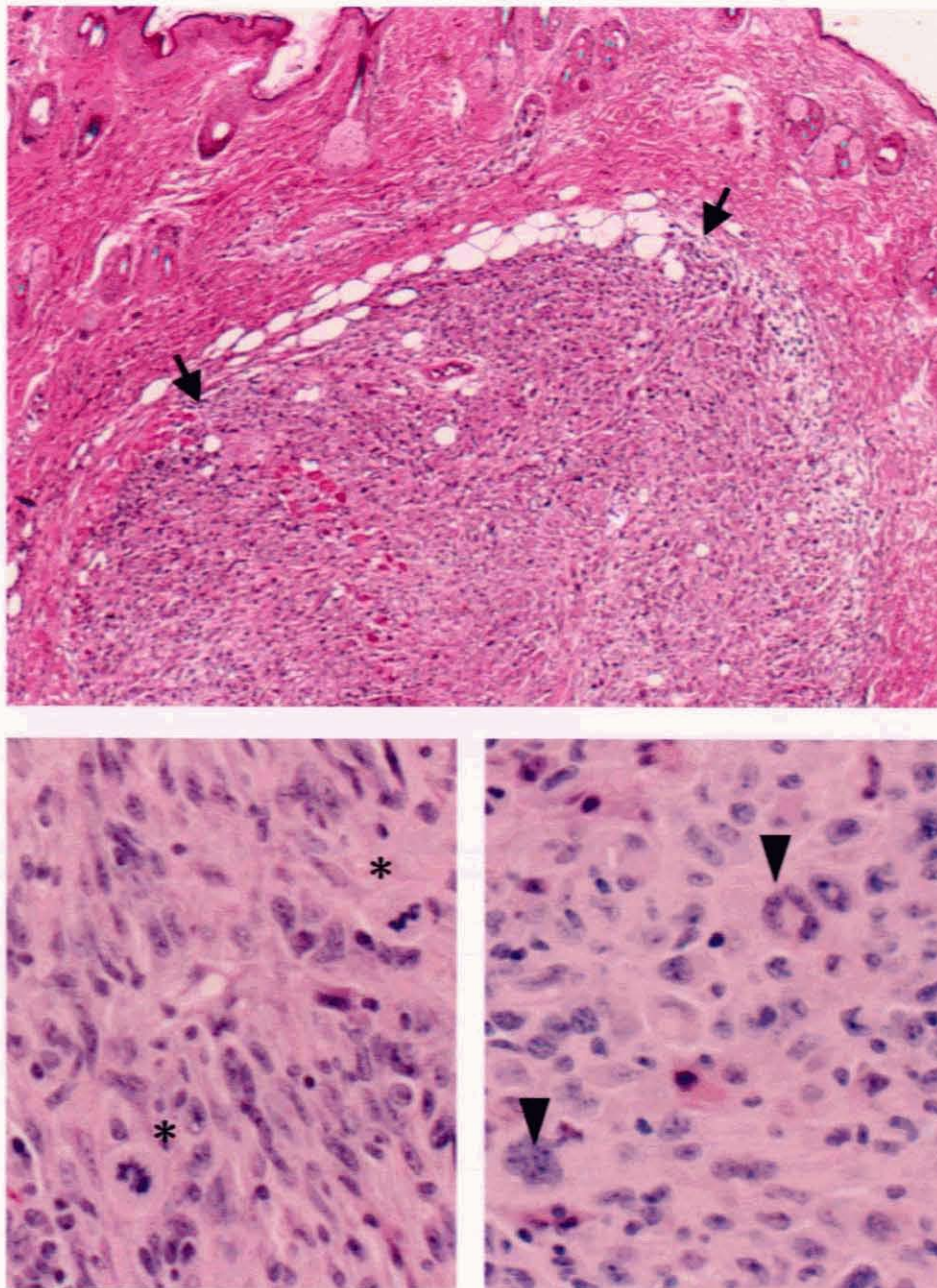
Figure 7. Distribution of tumors throughout esophageal mucosa. A green circle represents a tumor.
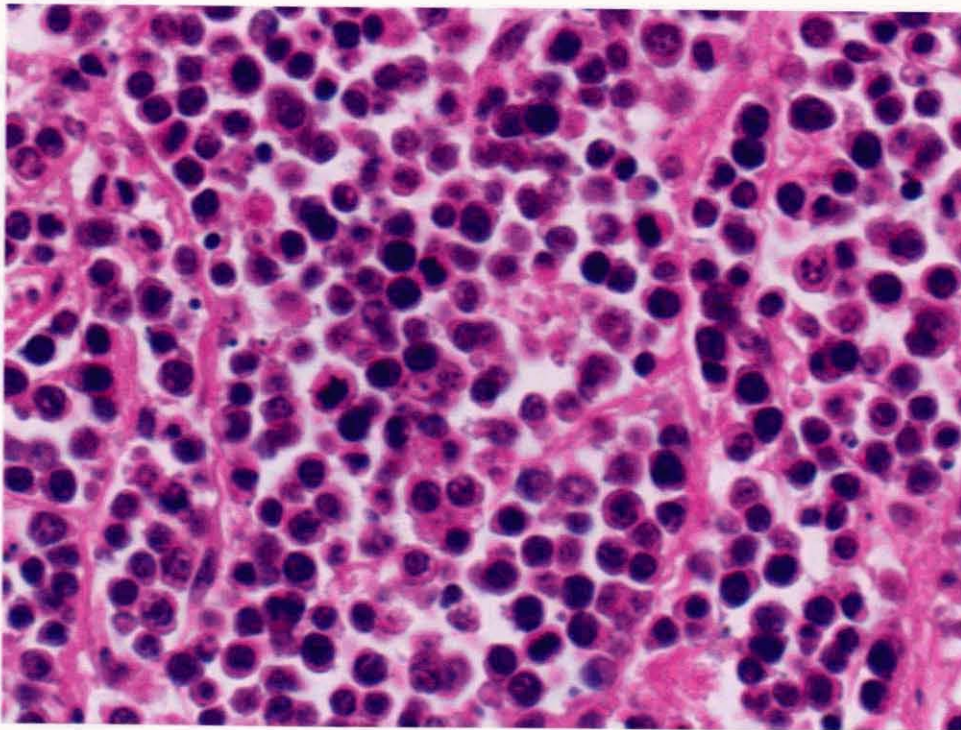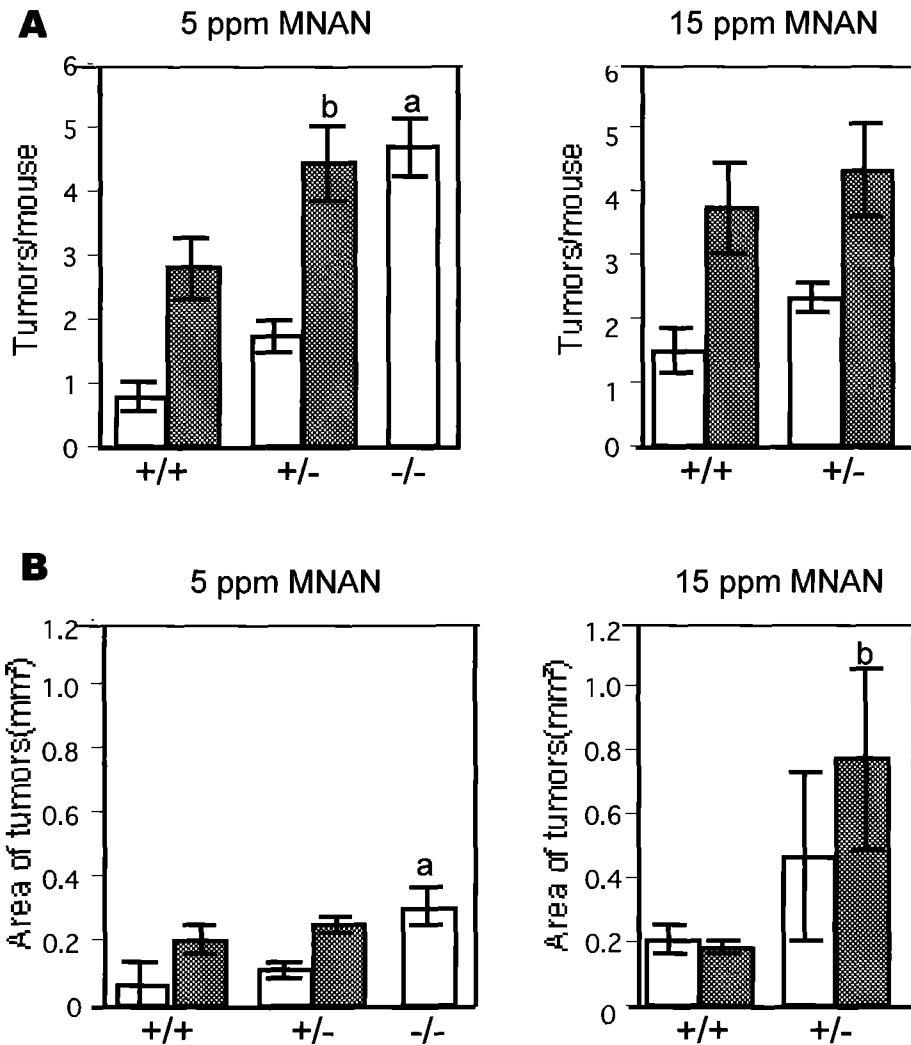
Figure 8-1. A thymic malignant lymphoma (asterisks) involving the heart in a *p53* (-/-) mouse. Upper: 20X. Lower: 400X.

Figure 8-2. A subcutaneous sarcoma (arrows) arisen from subcutis of dorsal skin in a *p53* (-/-) mouse. Anisokaryosis, bizarre mitoses (asterisks), and multinucleated cells (arrowheads).
Upper: 20X. Lower: 400X.

Figure 9. (A) Average number of esophageal tumors in *p53* (+/+), (+/-) and (-/-) mice treated with 5 (left) or 15 (right) ppm MNAN for 8 weeks then maintained without further treatment for an additional 7 or 15 weeks. [a]P<0.001 vs. (+/+) and (+/-), [b]P<0.05 vs. (+/+). (B) Sizes of esophageal tumors in *p53* (+/+), (+/-) and (-/-) mice of 5 (left) or 15 (right) ppm MNAN treatment groups. [a]P<0.005 vs. (+/+) and (+/-), [b]P<0.05 vs. (+/+). Vertical bars indicate standard errors of the mean. Open columns, 15 weeks after starting treatment; closed columns, 25 weeks after starting treatment.

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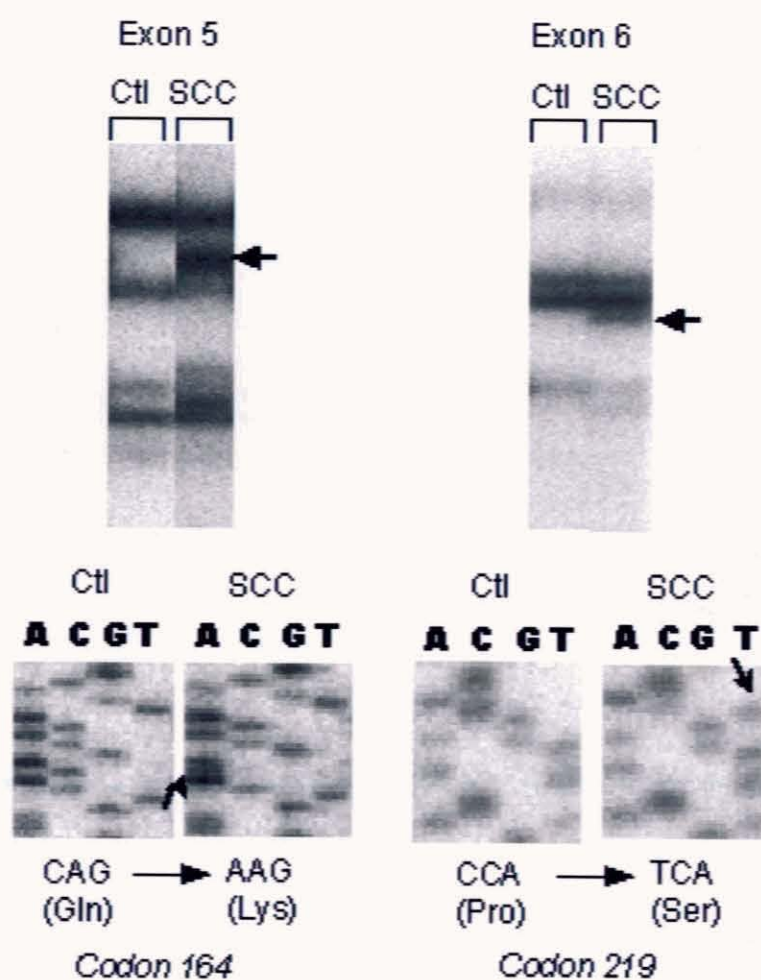
Figure 10-1. Representative results of PCR-SSCP analysis and DNA sequencing of *p53* exons 5 and 6. Arrows indicate shifted bands on SSCP analysis and mutations of the *p53* gene at exons 5 and 6 on DNA sequencing. Ctl: Control. SCC: Squamous cell carcinoma.
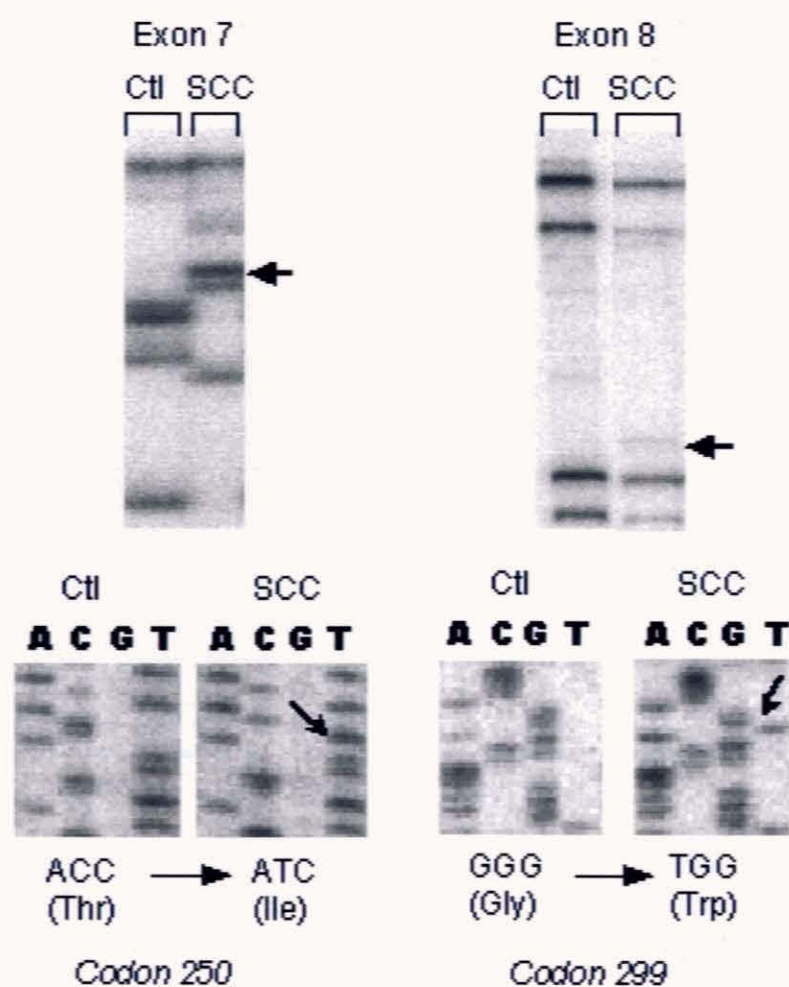
Figure 10-2. Representative results of PCR-SSCP analysis and DNA sequencing of *p53* exons 7 and 8. Arrows indicate shifted bands on SSCP analysis and mutations of the *p53* gene at exons 7 and 8 on DNA sequencing. Ctl: Control. SCC: Squamous cell carcinoma.

Chapter 2

Tumor susceptibility to methyl-n-amylnitrosamine

in the tongue of *p53* deficient mice

Abstract

Mutation of the *p53* tumor suppressor gene is a common genetic alteration in human squamous cell carcinoma (SCC) of the tongue as well as esophagus. In this study the carcinogenic susceptibility was evaluated in the tongue of *p53* nullizygous (-/-), heterozygous (+/-), and wild type (+/+) mice treated with methyl-*n*-amylnitrosamine (MNAN). The *p53* (+/-), and (+/+) mice were given 5 ppm MNAN in drinking water for 8 weeks, then held without further treatment for an additional 7 or 17 weeks, and sacrificed at 15 or 25 experimental weeks. A separate group of the *p53* (-/-) mice were given 5 ppm MNAN for 8 weeks and were sacrificed 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 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.

Introduction

Many studies have discussed "head and neck cancer" as a group, sometimes including the lip, mouth, maxillary sinuses, pharynx, larynx, salivary glands and skin as disease sites, and head and neck cancer is the fifth most common human cancer in the world [61]. Of these disease sites, the tongue is the most common site for squamous cell carcinoma (SCC) of the head and neck (HNSCC) in humans [22]. 4-nitroquinoline 1-oxide (4NQO) is known to produce lingual SCC in rodents [57], and a study using 4NQO showed that the frequency of lingual SCC was significantly higher in xeroderma pigmentosum group A gene-deficient ($XPA^{-/-}$) mice [35]. In addition to the XPA, a nucleotide excision repair gene, $p53$ is considered to act as a defensive factor against lingual carcinogenesis. Actually, mutation of the $p53$ gene is one of the most frequent genetic alterations in the SCCs of the tongue [1, 34, 42, 68, 74] as well as those of the esophagus in humans. In a previous study of $p53$-deficient mice it was shown that $p53$ (-/-) and (+/-) mice were more susceptible than $p53$ (+/+) mice to MNAN esophageal carcinogenesis, and the $p53$ deficiency contributes to the development of esophageal SCCs [73]. Diffusion of nitrosamines that are derived from MNAN into the esophagus is a possible factor in esophageal carcinogenesis [24]. Considering that both tongue and esophagus are directly exposed to MNAN in drinking water, and the nature of

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their overlying epithelia are identical, SCCs are likely to occur in the tongue as well. In the present study, we examined the relationship between *p53* deficiency and lingual cancer development in *p53* knockout mice following administration of MNAN.

Materials and methods

Tissue samples

Tongues collected from the *p53* (+/+), (+/-) and (-/-) mice that were given MNAN in drinking water for 8 weeks at a concentration of 5 ppm (see Chapter 1) were used in this study. Tongues from untreated mice of each genotype were served as controls. The numbers of animals examined for each genotype are shown in Table 8. The specimens selected were based on results from the previous study for esophageal carcinogenesis in which administration of 5 ppm MNAN resulted in significant increases in esophageal cancer development in both p53 (-/-) and (+/-) mice [73].

Histopathological analysis

Tongue tissues were resected, fixed in 4% paraformaldehyde in phosphate buffered saline, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE) 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 previously [58]. Briefly, genomic DNA was extracted from tumor areas in paraffin sections with

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DEXPAT (Takara Shuzo Co., Ltd., Shiga, Japan) as detailed elsewhere [88].

Four pairs of PCR primers for mouse *p53* exons 5-8 were designed based on

the published sequence as listed in Table 7 [88]. PCR was performed with a

Takara PCR Thermal Cycler MP (Takara Shuzo Co., Ltd., Shiga, Japan) and

products were electrophoresed in 0.625 x MDE polyacrylamide gels (FMC,

Rockland, ME, USA) with 5% glycerol. These were run at room temperature

for 18h at 8W, dried, and applied to imaging plates, which were then analyzed

with a BAS 2500 (Fuji Film, Kanagawa, Japan).

Direct sequencing

PCR products showing a band shift on polyacrylamide gels were

sequenced. Sequence analysis was carried out with ABI PRISM 3100 using a

BigDye Terminator v3.0 Cycle Sequencing Ready Kit (Applied Biosystems,

Forester City, CA, USA). 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 were analyzed

using the log rank test [65].

Results

Histopathological analysis

The incidences of MNAN induced papillomas, squamous cell carcinomas (SCCs) and carcinomas in situ (CIS) of the tongue are summarized in Table 8. Papillomas were characterized by the exophytic masses with fronds of proliferating epithelial cells (Figure 11A). Squamous cell carcinomas were locally invasive tumors that sometimes extended deep into the lingual skeletal musculature (Figures 12A and 12B). CIS was characterized by intraepithelial growth of neoplastic cells with disorganized polarity (Figure 11B). These tumors occurred at dorsal, lateral or ventral surface from middle through the posterior tongue. At week 15, although no microscopic changes were observed in both *p53* (+/+) and (+/-) mice, SCCs were found 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 a papilloma. At week 25, carcinomas in situ (CIS) were observed in one of each *p53* (+/+) and (+/-) mice, and a papilloma was found in the *p53* (+/-) mouse which had CIS.

PCR-SSCP analysis of the p53 gene in tumors

PCR-SSCP and sequencing analyses for exons 5-8 of *p53* gene were

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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 9). 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 (Figure 13A). A papilloma from *p53* (+/-) mouse exhibited CTG→CTA (Leu→Leu) transition at codon 254 in exon 7, a silent mutation (Figure 13B). 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 carcinogen [52], to nullizygous p53 KO mice clearly demonstrated its carcinogenicity to the tongue by an increased incidence of SCCs while heterozygous p53 KO mice and their wild-type counterparts were less susceptible to lingual carcinogenesis.

Although the number of nullizygous p53 KO mice without MNAN treatment was small in the present study, no spontaneous lingual neoplasms developed in any untreated nullizygous p53 KO mice. Furthermore, no spontaneous tongue lesions have been reported in the tongue of nullizygous p53 KO mice [12, 26]. 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 [43, 46]. Further study will be necessary to elucidate other genes implicated with lingual carcinogenesis in mice. The nullizygous p53 KO mice can be a useful model for inducing lingual cancers and identifying genes involved in carcinogenesis.

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 chemical-induced lingual SCCs in xeroderma pigmentosum group A

gene-deficient mice [35] and in hamsters [70] as well as lingual SCCs in humans [1]. There seems to be involvement of *p53* gene alterations for lingual carcinogenesis in a wide range of species. The type of *p53* mutation found in this 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 the tongue [32, 51]. G:C to A:T transitions are also the most common mutations detected in hamster buccal pouch SCCs induced by N-methyl-N-benzylnitrosamine, being a potent alkylating carcinogen [5].

The rare occurrence of neoplasms in the tongue of wild-type and heterozygous *p53* KO mice in the present study might be a reflection of murine resistibility to lingual carcinogenesis. While 4-nitroquinoline-1-oxide (4NQO) is known to produce lingual carcinoma in rodents with variable susceptibility among species [5], Ide *et al.* [35] indicated that mice were resistant to administration of 4NQO based on the fact that no neoplasms were induced in control mice treated with 4NQO at concentration of 10 ppm 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 lingual tumor was detected at experimental week 32 [35], lingual 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 lingual carcinogens.

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

Table 7. PCR primers for SSCP analysis of mouse *p53*

Target		Primer Sequence	Product length (bp)
Exon 5	sense	5'-TCTCTTCCAGTACTCTCCTC-3'	214
	antisense	5'-AGGCGGGTGTTGAGGGCTTAC-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'-GGAGAGGGCGCTTGTGTGCAGGT-3'	

Table 8.   Incidences of lingual neoplasms in *p53* knockout mice treated with MNAN

Exp. week	MNAN (ppm)	p53 geno-type	No. animals	No.[a] examined	No. of mice with lesions(%)		
					Papilloma	SCC[b]	CIS[c]
15W	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
25W	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.

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Table 9. *p53* gene mutations identified in lingual tumors in mice treated with MNAN

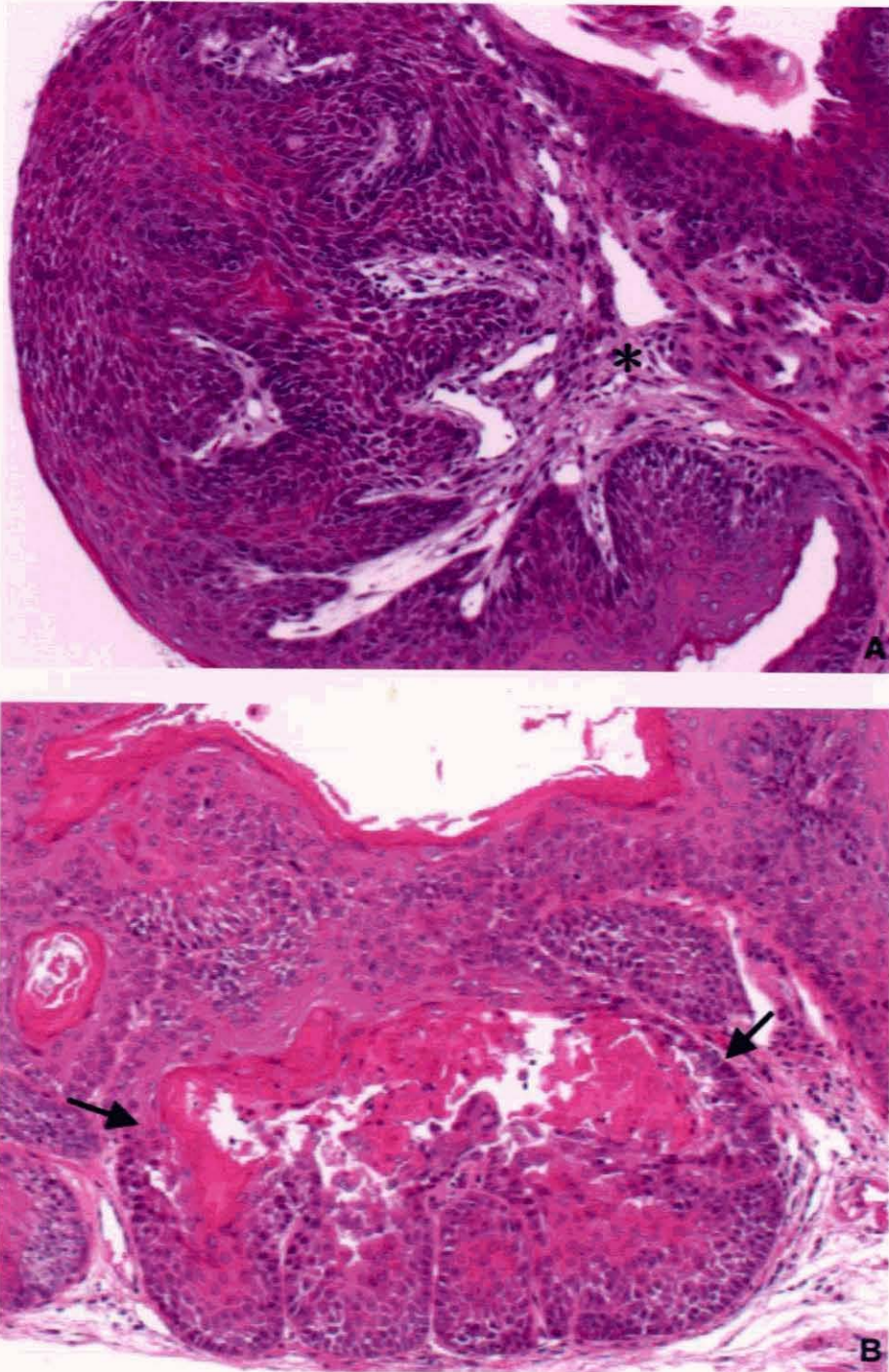| Geno-type	Histology	Exon	Codon	Nucleotide change	Aminoacid change	Event
+/+	CIS	6	211	CAC→TAC	His→Tyr	Transition
+/-	CIS	-	-	-	-	-
+/-	Papilloma	7	254	CTG→CTA	Leu→Leu	Transition

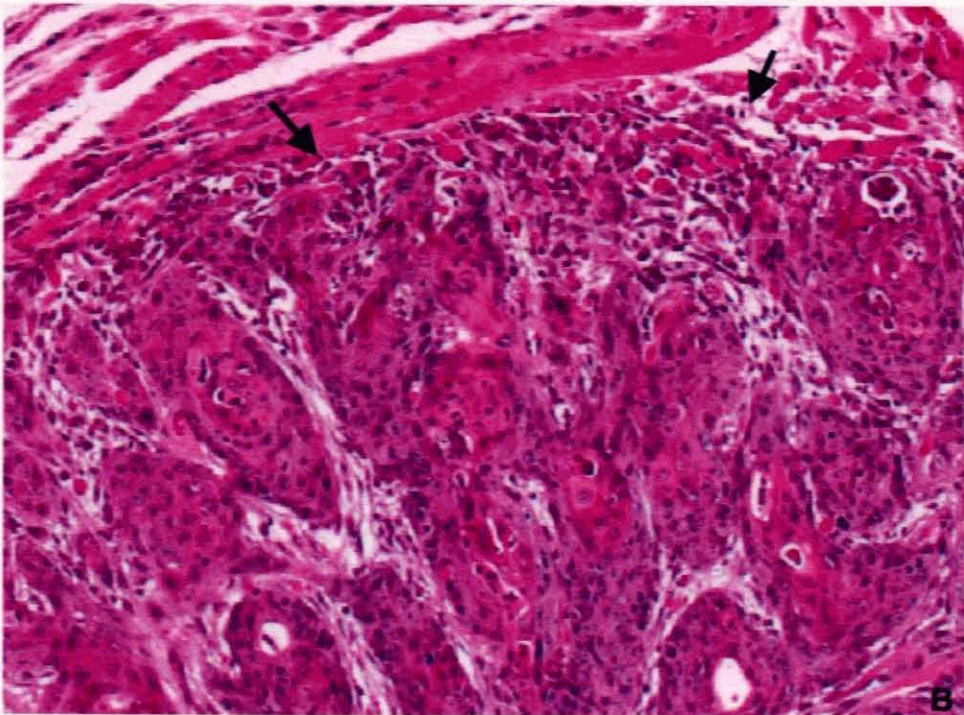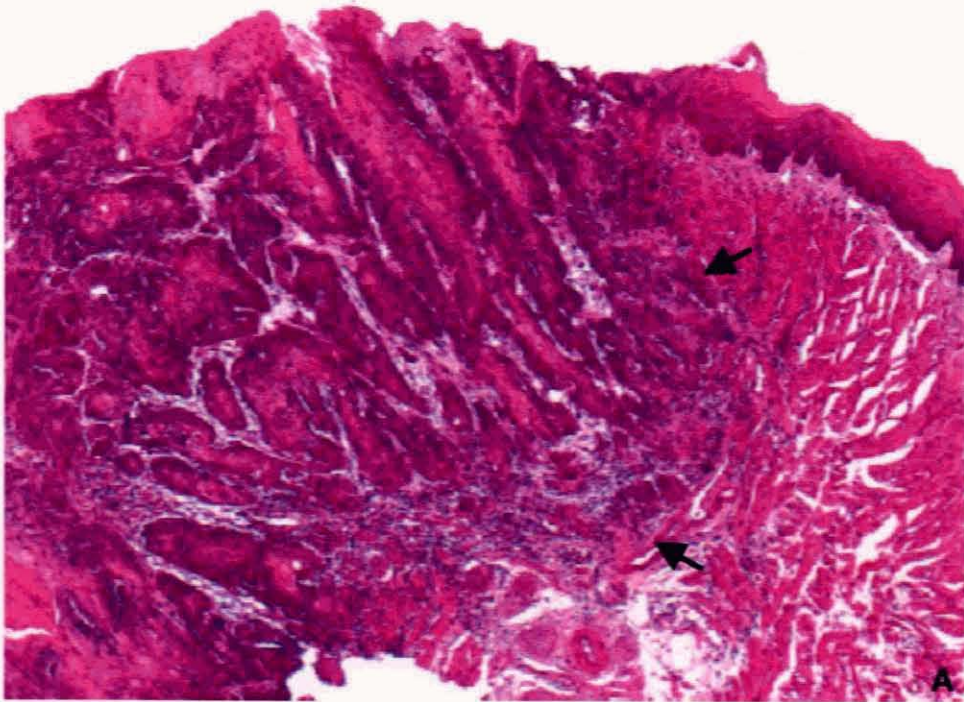-: Mutation not detected in exons 5,6,7, and 8.
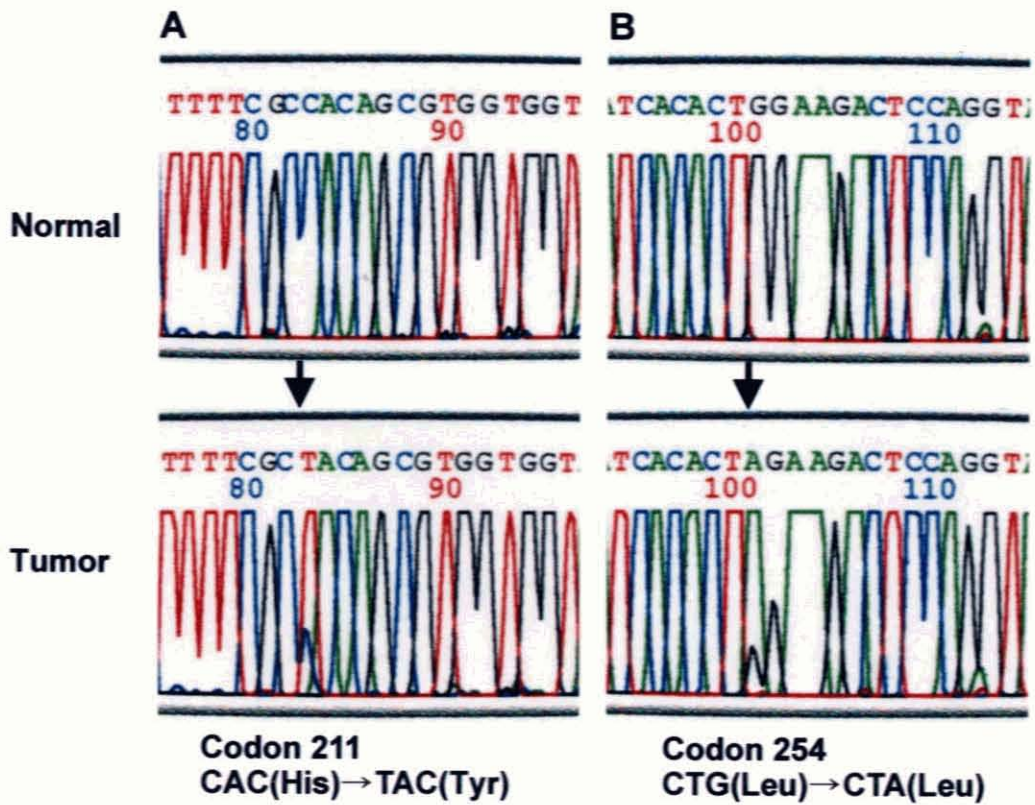CIS: Carcinoma in situ
His: Histidine
Tyr: Tyrosine
Leu: Leucine

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Figure 11. (A) A papilloma in a *p53* (+/-) mouse at 40X. Asterisk indicates a branching connective tissue core containing capillaries within papillary frond. (B) A carcinoma in situ (arrows) in a *p53* (+/-) mouse at 100X.

Figure 12. A squamous cell carcinoma invasing lingual musculature (arrows) in a *p53* (-/-) mouse at 40X (A) and 100X (B).

Figure 13. Sequencing of *p53* PCR products from the shifted bands by SSCP. (A) Exon 6; (B) Exon 7; (upper) Normal sequences; (lower left) a CAC→TAC mutation at codon 211 in a carcinoma in situ from a *p53* (+/+) mouse; (lower right) a CTG→CTA mutation at codon 254 in a papilloma from a *p53* (+/-) mouse.

General discussion

In this study the susceptibility of p53-deficient mice to MNAN-induced esophageal and lingual tumors were investigated. Mice were given MNAN in their drinking water for 8 weeks and sacrificed 7 or 17 weeks later, and esophageal and lingual neoplasms were evaluated both histologically and for the presence of p53 mutations.

Mice that had one allele of the p53 tumor suppressor gene knocked out were significantly more likely to develop esophageal papillomas and squamous cell carcinomas than p53 wild-type mice. On the other hand, lingual tumor development was infrequent in both heterozygous p53-deficient and wild-type mice. MNAN can readily diffuse into the esophageal mucosa, and be activated by cytochromes P450 to give hydroxy-nitrosamines, which decomposed to give aldehydes and agents that can alkylate critical sites in DNA in the affected organs [51]. Hence, the ability to diffuse into the tissue and/or tissue-specificity of P450 isozymes distribution might have contributed to the differences in tumorigenesis in these two sites. Saliva is another possible factor in the differences. Protective effect on saliva against chemically induced oral cancer has been shown in rats compared to desalivated rats [9] in addition to inactivation of mutagenicity of carcinogens by human saliva with complex mechanisms including biochemical reactions with enzymes and/or adsorption with high molecular weight substances in saliva such as proteins, mucous material, etc [55]. The difference in incidence of SCCs between the

esophagus and tongue in homozygous *p53* mice can reflect the different susceptibilities of these sites to MNAN.

Although *p53* mutations were uncommon in esophageal papillomas, at least half of the esophageal SCCs, even small carcinomas and carcinoma *in situ* of the tongue, had mutations in exons 5-8 of the *p53* gene. LA PCR-amplified DNA from the esophageal SCCs of heterozygous *p53*-deficient mice exhibited missense mutations, not in the mutant but rather in the wild-type allele, indicating loss of functional p53 protein. These findings suggest that mutated *p53* can lead to the loss of functional p53 protein, and is involved in malignant transformation of squamous cell carcinogenesis. In humans, *p53* gene alterations have been reported even in early esophageal precancerous lesions, such as carcinoma in situ and dysplasia [73, 85]. Carcinomas bearing *p53* mutations may have arisen from MNAN-initiated mucosa independent of intermediate papilloma formation.

Mice with both *p53* alleles knocked out were much more susceptible to MNAN-induced esophageal tumorigenesis, and were also likely to develop lingual carcinomas. Mechanisms other than *p53* are thought to be the basis for the induction of those tumors, which rarely develop spontaneously in mice. Other genetic changes may accumulate as the lesions progress under a variety of disturbances in growth control, DNA repair, and apoptosis by the lack of *p53* functions. Other possible mechanisms include deregulation of cell cycle

control by the cyclin-dependent kinase-RB pathway cell cycle control (inactivation of *p16* which is CDK inhibitor, amplification of *cyclin D1*, alterations of *RB*), activation of oncogenes causing deregulation of signal transduction (e.g., *EGFR, c-myc*) [38, 48, 49, 54, 87]. The *p53* nullizygous deficient mice have been regarded as no useful for potential carcinogen detection because of a high incidence of predominantly hematopoietic tumors within the first 6 months of life [79]. The nullizygotes, however, revealed the potential lingual carcinogenicity of MNAN. Similarly, N-methyl-N-nitrosourea (MNU) gastric carcinogenesis was clearly enhanced in the nullizygotes, while the incidences of gastric tumors were comparable in heterozygous *p53*-deficient and wild-type mice in a previous study [88]. Induction of squamous cell carcinomas in the skin after ultraviolet irradiation has also been demonstrated in the nullizygous *p53*-deficient mice [47]. Despite their limitations, nullizygotes can provide a powerful tool for cancer research in the short term.

More than half of the mutations observed in esophageal SCCs were G:C→A:T transitions , and all mutations detected in lingual carcinoma in situ and papillomas were also G:C→A:T transitions. Mutations of the *p53* gene are the most common genetic alterations in human cancers, and may serve as a marker in studies on molecular cancer epidemiology [25]. It has been reported that specific etiological agents can cause specific mutations in humans, *e.g.*, a typical G → A transversion mutation at codon 249 of the *p53* gene in

hepatocellular carcinomas in aflatoxin B1 contaminated areas [60], or a high prevalence of *p53* transition mutations in SCC in nitrosamine contaminated areas [86]. It is known that chemicals and their carcinogenic metabolites cause mutations by forming covalent adducts with the nucleotides in DNA [83]. Some small carcinogen-DNA adducts, such as O^6-methylguanine resulting from alkylating agents, may cause DNA polymerase to misread the base pairing. The most common mutations caused by alkylating agents are G:C$\rightarrow$A:T transitions [31]. The mutational patterns observed in the esophagus and tongue of mice treated with MNAN are consistent with the mutational spectrum caused by alkylating agents.

In conclusion, the current study demonstrated an increased susceptibility to esophageal tumorigenesis by a genotoxic agent, MNAN, in *p53* nullizygotes, and then *p53* heterozygotes as compared with wild-type mice, providing strong evidence of *p53* mutations in the development of esophageal SCCs. Lingual carcinogenicity of MNAN was also clearly apparent in the *p53* nullizygotes. The *p53* KO mice are one of several genetically engineered mice whose use may increase the sensitivity and decrease the time and cost of rodent carcinogenicity bioassays. Although consideration must be given to carcinogen and/or tissue specificity and background strain variability on tumorigenesis, *p53* KO mice provide a powerful tool for identification and understanding of human carcinogenesis.

Acknowledgements

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Donehower, L. A., Yoshida, K. and Tatematsu, M. (2000) *p53* knockout mice (-/-) are more susceptible than (+/-) or (+/+) mice to N-methyl-N-nitrosourea stomach carcinogenesis. *Carcinogenesis*, **21**, 1891-1897.

Conclusions

Over the past 30 years potential carcinogenic risk to humans from various chemicals has been evaluated by a two-year bioassay using two rodent species. However, it has been revealed that there is a lack of correlation between some chemical-induced tumors in rodents and risk assessment in humans. Furthermore, a single two-year assay is expensive, and it requires a lot of time and a large numbers of animals. Recently, the use of genetically engineered mice that can function as an alternative model for identifying carcinogens in a shorter period of time has drawn increasing interest. The heterozygous $p53$-deficient (+/-) mouse is one of those genetically engineered mice. The $p53$ gene is a tumor suppressor gene that is frequently mutated or deficient in a wide range of cancers in humans.

Mutations of the $p53$ gene are the most common genetic alterations in esophageal and lingual squamous cell carcinomas (SCCs) in humans. The etiology of these SCCs has been well studied and predominantly involves the use of tabacco. Tabacco-associated N-nitroso compounds are considered to be the etiologic agents.

In the present study, the susceptibility of nullizygous (-/-) and heterozygous (+/-) $p53$-deficient mice to methyl-n-amylnitrosamine (MNAN) carcinogenesis in the esophagus and tongue was evaluated to assess the

usefulness of this test system for identifying chemicals that cause cancers in the esophagus and tongue.

The relationship of changes in *p53* to esophageal and lingual tumorigenesis was also evaluated by analyzing *p53* mutations in the tumor cells.

The *p53* (-/-), (+/-) and wild type *p53* (+/+) mice were treated with 5 or 15 ppm MNAN in their drinking water for 8 weeks then maintained without further treatment for an additional 7 or 17 weeks and sacrificed at experimental weeks 15 or 25. Esophagi were examined histopathologically, and the types and incidences of tumors were evaluated in each genotype. At 15 weeks in the 5 ppm groups, esophageal SCCs were observed in 1/15 (6.7%) *p53* (+/-) mice, but in none of the *p53* (+/+) mice. The *p53* (-/-) mice exhibited a significantly increased incidence (10/12, 83.3%) in SCCs. In the animals receiving 15 ppm, 2/14 (14.3%) of the *p53* (+/-) mice and 1/11 (9.1%) of the *p53* (+/+) mice developed SCCs. At 25 weeks, the incidences of SCCs were significantly increased in *p53* (+/-) mice (7/16, 43.8%) compared to *p53* (+/+) mice (1/13, 7.7%) at 5 ppm of MNAN. In the animals receiving 15 ppm, there was a trend for increased incidence of SCCs in *p53* (+/-) mice (8/14, 57.1%) when compared to that of *p53* (+/+) mice (2/10, 20.0%). These results indicate the order of susceptibility to MNAN-induced esophageal carcinogenesis is as follows: nullizygotes (-/-) > heterozygotes (+/-) > wild type (+/+).

Of the SCCs examined by PCR-single strand conformation polymorphism (PCR-SSCP) analysis, 61% (14/23) from *p53* (+/-) and 50% (6/12) from *p53* (+/+) mice demonstrated mutations in the *p53* gene (exons 5-8). These results provide strong evidence of involvement of *p53* mutations in the development of esophageal SCCs. The patterns of *p53* mutations were predominantly G:C→A:T transitions that are most frequent mutations detected in human esophageal cancers, and this indicates the usefulness of the MNAN-induced esophageal carcinogenesis model in mimicking this disease in humans.

Histopathological assessment of the tongue of *p53* (-/-) mice given 5 ppm MNAN revealed lingual SCCs (5/12, 41.7%) and papillomas (2/12, 16.7%) at 15 weeks. There were no neoplastic lesions detected 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-SSCP analysis of exons 5-8 of the *p53* gene demonstrated point mutations in the CIS (C→T transition) and the papilloma (G → A transition) lesions. These patterns of mutations are consistent with the findings observed in the esophagus and considered to be the mutation spectrum related to MNAN administration. Mutation in the *p53* gene is one of the most common alterations in human cancers. Therefore, it is suggested that analysis of *p53* mutational spectra provides a unique opportunity

to investigate etiology.

In conclusion, this study revealed strong evidence of involvement of *p53* mutations in the development of SCCs in the esophagus and tongue, and indicated that *p53*-deficient mice provide a useful model for identification and understanding of human carcinogens, although consideration must be given to carcinogen and/or tissue specificity.

要　旨

　過去 30 年余り，2種のげっ歯類を用いた2年間の長期癌原性試験によって，化学

物質のヒトにおる癌原性が評価されてきた。しかし，現在，化学物質によって，げっ歯

類に誘発される腫瘍のいくつかは，ヒトへのリスクアセスメントにおいて明らかに関係

のないことが示されている。併せて，長期癌原性試験は，費用および時間がかかりす

ぎることや，使用される動物数が莫大であることから，近年，代替法となり得る，より

短期の試験系として遺伝子改変動物が注目されている。*p53*(+/−)欠損マウスはその

一つである。*p53*は，ヒトの多数の癌において変異あるいは欠失していることが知られ

ている癌抑制遺伝子である。

　ヒトの食道および舌における扁平上皮癌は，*p53* 変異が多い腫瘍である。また，そ

の病因の一つにタバコが挙げられており、その原因物質として N−ニトロソ化合物が考

えられている。

　本研究では，N−ニトロソ化合物であるmethyl-n-amylnitrosamine（MNAN)に対する

p53(−/−)および(+/−)欠損マウスの食道および舌における発癌感受性を調べ，これら

マウスの発癌性試験の試験系としての有用性を検討した。さらに，発生した腫瘍にお

ける *p53* 遺伝子の変異を解析し，*p53* 遺伝子異常と発癌の関連性を検討した。

　MNAN を 5 または 15 ppm の濃度で飲水に混入して *p53*(−/−)および(+/−)欠損マ

ウスならびに野生型(*p53*+/+)マウスに 8 週間連日投与した。実験開始 15 週後および

25 週後に動物を屠殺して食道を病理組織学的に検索し，腫瘍のタイプおよび発生頻

度を *p53*(−/−),(+/−),(+/+) の各遺伝子型について比較検討した。その結果，15 週後

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ではMNAN 5 ppm投与で扁平上皮癌が(+/−)に1/15例(6.7%)みられたが, (+/+)では認められなかった。(−/−)では扁平上皮癌は10/12例(83.3%)と発生頻度が有意に増加した。MNAN 15 ppm 投与では扁平上皮癌が(+/−)で 2/14 例(14.3%), (+/+)で 1/11 例(9.1%)認められた。25 週後では MNAN 5 ppm 投与で扁平上皮癌が(+/−)に 7/16 例(43.8%), (+/+)に 1/13 例(7.7%)認められ, (+/−)で有意に高い発生率を示した。また, MNAN 15 ppm 投与群における扁平上皮癌の発生頻度は(+/−)で 8/14 例(57.1%), (+/+)で 2/10 例(20.0%)であり, (+/−)では(+/+)に比べ発生率が高い傾向を示した。

以上, *p53*(−/−)では, (+/−), (+/+)に比べて明らかに短期間, 高頻度で食道扁平上皮癌が誘発された。また, *p53*(+/−)は(+/+)に比べ MNAN による食道の発癌感受性が高いことが示された。

次に, 誘発した腫瘍組織から DNA を抽出し, single strand conformation polymorphism (SSCP)法によって*p53*遺伝子の exon 5-8 における遺伝子変異を検索した。食道癌の癌細胞において, *p53*(+/+)では 6/12(50%), (+/−)では 14/23(61%)に *p53*遺伝子の変異が認められ, MNAN による *p53*遺伝子の異常と食道発癌との因果関係が示唆された。また, 検出された変異は大半が G:C→A:T の塩基転移(transition)であり, ヒトの食道癌における点変異の種類とよく符合した。以上のことから, MNAN誘発食道扁平上皮癌がヒト食道癌のモデルとして有用であることが示された。
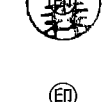
同様に, MNAN 5 ppm を飲水投与した *p53*(−/−), (+/−), (+/+) の各遺伝子型マウスの舌発癌感受性を検索した結果, 15 週後では(−/−)において舌扁平上皮癌が 5/12例(41.7%), 乳頭腫が2/12例(16.7%)に認められた。一方, (+/−)および(+/+)では増殖性病変の発生はなく, (−/−)では, (+/−), (+/+)に比べ明らかに短期間, 高頻度で舌扁平

上皮癌が誘発された。25 週後では，上皮内癌(carcinoma in situ)が(+/−)の 1/16 例 (6.3%)および (+/+)の 1/13 例(7.7%)，乳頭腫が(+/−)の 1/16 例(6.3%)に誘発された。*p53* (+/−), (+/+)に発生した carcinoma in situ および乳頭腫について SSCP 法により遺伝子検索した結果，C→T の塩基転移が carcinoma in situ で， G→A の塩基転移が乳頭腫でそれぞれ検出された。これらの塩基転移は食道扁平上皮癌における変異の種類と同一のものであり，MNAN による DNA 損傷の可能性が考えられた。

　本研究により，*p53*遺伝子欠損マウスが食道および舌の発癌性試験の試験系として有用であること，*p53* 遺伝子異常がこれら臓器における扁平上皮癌の発生と関連することが示された。さらに，*p53* 遺伝子変異の種類の分析により，原因物質が解明し得る可能性が示唆された。

学 位 論 文 審 査 結 果 の 要 旨

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審 査 委 員

題　　　目　　Molecular Pathology on Chemical-induced Squamous Cell Carcinomas in the Esophagus and Tongue of *p53*-deficient Mice

(p53 遺伝子欠損マウスの食道および舌における化学物質誘発扁平上皮癌の分子病理学的研究)

審 査 結 果 の 要 旨

　申請者は，ファイザー製薬株式会社・中央研究所において新規化合物の毒性評価に従事している。同者は，近年，化学物質の癌原性評価に際し，これまでの長期癌原性試験に替わりうる遺伝子改変マウスを用いた短期試験法の応用について世界規模で研究が進められていることに着目し，化学物質のヒトに対するリスク評価における *p53* 遺伝子欠損マウスの有用性を検討すべく，*p53* 遺伝子欠損マウスの食道および舌における発癌感受性の検索，さらに遺伝子変異を調べることによるメカニズム解析を行った。

1.　*p53* 欠損マウスの食道における methyl-n-amylnitrosamine による発癌感受性および発癌と遺伝子変異の関連についての研究(*Carcinogenesis*)

　　　p53 欠損マウスの化学物質による食道発癌の感受性および発癌過程における *p53* 遺伝子変異の関わりについて検討した。Methyl-n-amylnitrosamine(MNAN)を 5 または 15 ppm の濃度で飲水に混入して *p53-/-* および *p53+/-* 欠損マウスならびに野生型(*p53+/+*)マウスに 8 週間連日投与した。実験開始 15 週後に MNAN を 5 ppm 投与した *p53 -/-* で扁平上皮癌が

高頻度(10/12 例;83.3%)で認められた。*p53+/-*では扁平上皮癌が 1/15 例(6.7%)にみられたが，*p53+/+*での発生はなかった。15 ppm 投与による扁平上皮癌の発生頻度は *p53+/-*で 2/14 例(14.3%)，*p53 +/+*で 1/11 例(9.1%)であった。25 週後において，5 ppm 投与による扁平上皮癌の発生が *p53+/-*では 7/16 例(43.8%)であったのに対して *p53+/+*では 1/13 例(7.7%)に過ぎず，*p53+/-*で有意に高い発生率を示した。また，15 ppm 投与における発生頻度は *p53+/-*で 8/14 例(57.1%)，*p53+/+*で 2/10 例(20.0%)であり，*p53+/-*では *p53+/+*に比べ感受性が高い傾向を示した。

次に，誘発した腫瘍組織から DNA を抽出し，single strand conformation polymorphism(SSCP)法によって *p53* 遺伝子の exon 5-8 における遺伝子変異を検索した。食道癌において，*p53+/+*では 6/12(50%)，*p53+/-*では 14/23(61%)と，高頻度で *p53* 遺伝子の変異が検出され，*p53* 遺伝子の異常と食道発癌との因果関係が示唆された。また，検出された変異は大半が G:C→A:T の塩基転移であり，ヒトの食道癌でよく検出される点変異の種類と符合した。

以上，*p53-/-*では，*p53+/-*および *p53+/+*に比べて短期間かつ高頻度で食道扁平上皮癌が誘発された。また，*p53+/-*は *p53+/+*に比べ食道の発癌感受性が高いことが示された。さらに，腫瘍組織における *p53* 遺伝子変異のパターンはヒトと相同していたことから，化学物質の食道発癌性評価に，*p53* 遺伝子欠損マウスが有用であることが裏づけられた。

2. *p53* 欠損マウスの舌における methyl-n-amylnitrosamine による発癌感受性および発癌と遺伝子変異の関連についての研究(*Journal of Toxicologic Pathology*)

Methyl-n-amylnitrosamine(MNAN)を *p53-/-*, *p53+/-*, *p53+/+*の各遺伝子型マウスに 5 ppm の濃度で 8 週間連日飲水投与し，舌発癌感受性を検索した。実験開始 15 週後において *p53-/-*で扁平上皮癌が 5/12 例(41.7%)，乳頭腫が 2/12 例(16.7%)に認められた。一方，*p53+/-*および *p53+/+*では増殖性病変の発生はなかった。実験開始 25 週後では，上皮内癌(carcinoma *in situ*)が *p53+/-*で 1/16 例(6.3%)および *p53+/+*で 1/13 例(7.7%)，乳頭腫が *p53+/-*で 1/16 例(6.3%)に誘発された。さらに，*p53+/-*, *p53+/+*に発生した carcinoma *in situ* および乳頭腫について single strand conformation polymorphism(SSCP)法により *p53* 遺伝子の exon 5-8 における遺伝子変異を検索した結果，C→T の塩基転移が carcinoma *in situ* で，G→A の塩基転移が乳頭腫でそれぞれ検出された。

以上のことから，*p53-/-*は，*p53+/-*および *p53+/+*に比べて短期間かつ高頻度で舌扁平上皮癌が誘発され，舌発癌感受性が高いことが示された。また，検出された *p53* 遺伝子変異のパターンは，食道扁平上皮癌で最もよく認められた塩基転移と同じであったことから，この塩基転移は，MNAN による DNA 損傷の特徴である可能性が示唆された。

本研究により，*p53* 欠損マウスは MNAN に対する食道および舌の発癌感受性が高いことが判明した。さらに，分子病理学的手法を用いて *p53* 遺伝子変異を検索することにより発癌メカニズム解析を行い，*p53* 欠損マウスとヒトの扁平上皮癌との相同性を明らかにした。これらは，化学物質の安全性研究およびヒトの上部消化管における癌研究の分野において

多大な貢献を示すものと考えられた。

　以上について，審査委員全員一致で本論文が岐阜大学大学院連合獣医学研究科の学位論文として十分価値があると認めた。

基礎となる学術論文

1) 題　　目　：　Elevated susceptibility of the *p53* knockout mouse esophagus to methyl-*N*-amylnitrosamine carcinogenesis
   著 者 名　：　SHIRAI, Norimitsu　TSUKAMOTO, Tetsuya　YAMAMOTO, Masami　IIDAKA, Takeshi　SAKAI, Hiroki　YANAI, Tokuma　MASEGI, Toshiaki　DONEHOWER, Lawrence A. and TATEMATSU, Masae
   学術雑誌名　：　Carcinogenesis
   巻・号・頁・発行年：23(9)：1541～1547, 2002

2) 題　　目　：　Tongue carcinogenic susceptibility of *p53* deficient mice to methyl-*N*-amylnitrosamine
   著 者 名　：　SHIRAI, Norimitsu　TSUKAMOTO, Tetsuya　YAMAMOTO, Masami　IIDAKA, Takeshi　SAKAI, Hiroki　YANAI, Tokuma　MASEGI, Toshiaki　DONEHOWER, Lawrence A. and TATEMATSU, Masae
   学術雑誌名　：　Journal of Toxicologic Pathology
   巻・号・頁・発行年：15(4): 209～214, 2002

既発表学術論文

1) 題　　目　：　Nontraumatic osteonecrosis: MR perfusion imaging evaluation in an experimental model
   著 者 名　：　KAWAMOTO, Satomi　SHIRAI, Norimitsu　STRANDBERG, John D., BOXERMAN, Jerroid L. and BLUEMKE, David A.
   学術雑誌名　：　Academic Radiology
   巻・号・頁・発行年：7(2): 83～93, 2000

2) 題　　目　：　*p53* knockout mice (-/-) are more susceptible than (+/-) or (+/+) mice to *N*-methyl-*N*-nitrosourea stomach carcinogenesis
   著 者 名　：　YAMAMOTO, Masami　TSUKAMOTO, Tetsuya　SAKAI, Hiroki　SHIRAI, Norimitsu　OHGAKI, Hiroko　FURIHATA, Chie　DONEHOWER, Lawrence A.　YOSHIDA, Kenji and TATEMATSU, Masae
   学術雑誌名　：　Carcinogenesis
   巻・号・頁・発行年：21(10): 1891～1897, 2000

3) 題　　目　：　Expression of cyclin kinase inhibitor p27 (kip 1) in skin tumors of dogs
   著 者 名　：　SAKAI, Hiroki　YAMANE, Tomoko　YANAI, Tokuma　SHIRAI, Norimitsu and MASEGI, Toshiaki
   学術雑誌名　：　Journal of Comparative Pathology
   巻・号・頁・発行年：125(2～3): 153～158, 2001

4) 題　　目　：　Differential effects of partial hepatectomy and carbon tetrachloride administration on induction of liver cell foci in a model for detection of initiation activity

著 者 名 ： SAKAI, Hiroki　TSUKAMOTO, Tetsuya　YAMAMOTO, Masami　SHIRAI, Norimitsu　IIDAKA, Takeshi　YANAI, Tokuma　MASEGI, Toshiaki and TATEMATSU, Masae

学術雑誌名 ： Japanese Journal of Cancer Research

巻・号・頁・発行年 ： 92(10): 1018～1025, 2001

5) 題　　目 ： Summation of initiation activities in the liver after partial hepatectomy

著 者 名 ： SAKAI, Hiroki　TSUKAMOTO, Tetsuya　YAMAMOTO, Masami　HIRATA, Akihiro　INAGAMI, Atsushi　SHIRAI, Norimitsu　IIDAKA, Takeshi　YANAI, Tokuma　MASEGI, Toshiaki and TATEMATSU, Masae

学術雑誌名 ： Cancer Letters

巻・号・頁・発行年 ： 176(1): 1～5, 2002

6) 題　　目 ： The effects of D-galactosamine- or carbon tetrachloride-induced regeneration on induction of rat liver cell foci in a model for detection of initiation activities of chemicals

著 者 名 ： SAKAI, Hiroki　INAGAMI, Atsushi　HIRATA, Akihiro　TSUKAMOTO, Tetsuya　KOBAYASHI, Kiyoshi　DEGAWA, Masakuni　SHIRAI, Norimitsu　IIDAKA, Takeshi　YANAI, Tokuma　MASEGI, Toshiaki and TATEMATSU, Masae

学術雑誌名 ： Journal of Toxicologic Pathology

巻・号・頁・発行年 ： 15(1): 13～18, 2002

7) 題　　目 ： The effects of allyl alcohol-induced cell proliferation for detection of initiation activities of chemicals in rat liver

著 者 名 ： OKAMURA, Miwa　SAKAI, Hiroki　TAKAHASHI, Naofumi　INAGAMI, Atsushi　TSUKAMOTO, Tetsuya　YAMAMOTO, Masami　SHIRAI, Norimitsu　IIDAKA, Takeshi　YANAI, Tokuma　MASEGI, Toshiaki and TATEMATSU, Masae

学術雑誌名 ： Journal of Toxicologic Pathology

巻・号・頁・発行年 ： 15(2): 95～102, 2002

8) 題　　目 ： Proliferative activity of canine mast cell tumours evaluated by bromodeoxyuridine incorporation and Ki-67 expression

著 者 名 ： SAKAI, Hiroki　NODA, Ayako　SHIRAI, Norimitsu　IIDAKA, Takeshi　YANAI, Tokuma and MASEGI, Toshiaki

学術雑誌名 ： Journal of Comparative Pathology

巻・号・頁・発行年 ： 127(4): 233～238, 2002