RESEARCH COMMUNICATION

Lack of Carcinogenic Sensitivity of the Glandular Stomach in Heterozygous *p*53 Knockout Mice Given *N*-Methyl-*N*-Nitrosourea in their Drinking Water for 26 Weeks

Hisayoshi Takagi[1], Kunitoshi Mitsumori[1,2], Akiyoshi Nishikawa[1], Hiroshi Onodera[1], Fumio Furukawa[1], Ken-ichiro Kasahara[1], Masao Hirose[1]

Abstract

Gastric tumorigenic sensitivity to *N*-methyl-*N*-nitrosourea (MNU) was examined in heterozygous *p*53 knockout (*p*53(+/-)) CBA mice and their wild-type littermates (*p*53(+/+)). In Experiment 1, 37 male *p*53(+/-) or 38 male *p*53(+/+) CBA mice were given MNU in their drinking water at concentration of 50ppm (Group 1 or 4), 10ppm (group 2 or 5) or 0ppm (group3 or 6) for 26 weeks. In Experiment 2, *p*53(+/-) and *p*53(+/+) CBA mice of both sexes received water containing 50ppm MNU for 26 weeks. In Experiment 1, the incidences of hyperplasias in the glandular stomach observed in *p*53 (+/-) CBA mice treated with 50ppm and 10ppm MNU were significantly increased, as compared with the control group. No tumors were induced in the stomach of any treated groups. Some proliferative or non-neoplastic lesions were observed in some *p*53 (+/-) CBA mice, but there was no significant difference in their incidences between treated and control groups. In Experiment 2, the incidences of hyperplasias in the glandular stomach observed in *p*53 (+/-) CBA mice of both sexes treated with 50ppm MNU were not significantly increased, as compared with the treated *p*53(+/+) CBA group. One papilloma of the forestomach was observed only in a male *p*53(+/-) CBA mouse treated with 50ppm MNU. The present study suggests that *p*53 (+/-) CBA mice have low susceptibility to MNU-induced gastic carcinogenesis.

Key words: heterozygous p53 deficient CBA mouse - carcinogen sensitivity - glandular stomach - N-methyl-N-nitrosourea

Asian Pacific J Cancer Prev, **1**, 299-303

Introduction

In the past, for the safety assessment of carcinogenic potential of pharmaceuticals in the USA, European Union and Japan, data on long-term carcinogenicity studies using two rodent species, usually rats and mice, were required. However, the International Conference on Harmonization of Technical Requirements of Pharmaceuticals for Human Use (ICH) in the 4th meeting held in 1997 concluded that the carcinogenic potential of pharmaceuticals could be evaluated from the data of one long-term conventional carcinogenicity using one rodent species plus the findings of one other short- or medium-term carcinogenicity test system such as an initiation-promotion model, genetically-engendered animals such as transgenic (Tg) and knockout mice or newborn animals (D'Arcy et al., 1998). With regard to the models of genetically-engendered animals, one alternative under validation study is the heterozygous p53 knockout mouse [p53 (+/-) mouse] model, in which one p53 allele has been inactivated, and extensive short-term evaluation studies less than 6 months carried out on a global scale (Tennant et al., 1995, 1996). So far, there have been several reports that p53 (+/-) mice have high sensitivity to genotoxic carcinogens (Dunnick et al., 1997; Ozaki et al., 1998; Tennant et al., 1995). Such findings provide a useful basis for short-term carcinogenicity testings, but further analyses of other types of carcinogens are pivotal for drawing definite conclusions. Two different strains of p53 (+/-) mice,

[1] Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-Ku, Tokyo, Japan. [2] Laboratory of Veterinary Pathology, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo , Japan
Mailing address: Kunitoshi Mitsumori, Laboratory of Veterinary Pathology, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo , Japan TEL : 042-367-5771 FAX : 042-367-5771 E-mail : mitsumor@cc.tuat.ac.jp

heterozygous TSG p53^R knockout mice [p53 (+/-) TSG mice] and p53 (+/-) CBA, have been used in Japan. p53 (+/-) TSG mice, in which exon 2 of the lateral p53 allele was inactivated, are maintained by Taconic Farms (Germantown, NY, USA), and were the offspring of the knockout mice derived from the mouse strain 129 back-crossed with the C57BL/6 lineage. Therefore, they were on a C57Bl/6 genetic background. p53 (+/-) CBA mice, in which exon 5 of the lateral p53 allele was inactivated (Tsukada et al., 1993), are maintained by Oriental Yeast Co. Ltd. (Tokyo, Japan). They were F1 offspring of heterozygous p53 deficient C57 BL/6J male mice back-crossed with CBA female mice. Therefore, they were on a CBA genetic background. It has been reported that the administration of a strong stomach carcinogen, N-methyl-N-nitrosourea (MNU), to BALB/c or C3H mice results in good yields of adenocarcinomas in the glandular stomach (Tatematsu et al., 1992, 1993; Yamachika et al., 1998). In addition, Ohgaki et al. (2000) recently reported that gastric adenocarcinomas were induced both in p53 (+/-) and p53 wild-type (+/+) mice of C57Bl/6 background at week 50 after the gastric intubation of 5 μg/g body weight MNU 3 times/week for 5 weeks, and the development of the adenocarcinomas invading into the submucosa occurred only in p53 (+/-) mice but not in p53 (+/+) mice. The results of their study suggest that p53 (+/-) C57BL/6 mice are more susceptible to intragastric application of MNU than in p53 (+/+) mice. However, it is not known whether the carcinogenic sensitivity to MNU in p53 (+/-) CBA mice is the same as that in p53 (+/-) TSG mice. In the present study, p53 (+/-) CBA mice were given water containing MNU to examine their carcinogenic sensitivity.

Materials and Methods

Animals and Chemicals

p53 (+/-) CBA mice and littermates (p53 (+/+) mice), kindly provided by Oriental Yeast Co. Ltd. (Tokyo, Japan), at 5-6 weeks of age, were housed at a maximum of 5 to a polycarbonated cage with white chips as bedding in an air-conditioned animal room (room temperature, 23+5°C; relative humidity, 55+5%; lighting cycle, 12 light /12 dark cycle). Mice without abnormal findings after a 1-week acclimatization period were selected for study. They were allowed free access to a diet (Oriental CRF-1) throughout. MNU, from Sigma Chemical Co., (St Louis, MO), was dissolved in distilled water and freshly prepared three times per week.

Protocols

In Experiment 1, 37 male p53 (+/-) and 38 male p53 (+/+) mice were divided into 3 groups. Fifteen, 15 and 7 p53 (+/-) and 15, 15 or 8 p53 (+/+) animals, respectively, were given MNU in their drinking water at a concentration of 50ppm (Groups 1,4),10ppm (Groups 2,5) or 0ppm (Groups 3,6) for 26 weeks. In Experiment 2, 7 male and 10 female p53 (+/-) CBA mice and 10 male and 10 female p53 (+/+) CBA mice were given MNU in their drinking water at a

Table 1. Incidences and Multiplicities of Gastric Proliferative Lesions in Experiments 1 and 2

Experiment 1		Incidence (%)	
Lesions		p53 (+/-)	p53 (+/+)
Hyperplasia in the glandular stomach			
	50ppm MNU	6/13 (46.1)*	2/14 (14.3)
	10ppm MNU	5/14 (35.7)*	3/15 (20.0)
	0ppm MNU	0/7 (0)	0/8 (0)
Papilloma in stomach			
	50ppm MNU	0/13 (0)	0/14 (0)
	10ppm MNU	0/14 (0)	0/15 (0)
	0ppm MNU	0/7 (0)	0/8 (0)
Experiment 2		Incidence (%)	
Lesions		p53 (+/-)	p53 (+/+)
Hyperplasia in the glandular stomach			
Male	50ppm MNU	2/7 (38.6)	2/10 (20.0)
Female	50ppm MNU	3/8 (37.5)	2/10 (20.0)
Papilloma in stomach			
Male	50ppm MNU	1/7 (14.3)	0/10 (0)
Female	50ppm MNU	0/8 (0)	0/10 (0)

* : Significantly different from the Oppm group at $p<0.05$

Fig. 1. Microscopic photographs of glandular stomach from p53 (+/-) CBA mice of the control group (A) and 50ppm MNU group (B). Diffuse hyperplasia of the fundic gland is evident in the 50 ppm MNU group. H-E stain x40

Table 2. Incidences of Proliferative or Non-Proliferative Lesions in p53 (+/-) and p53 (+/+) CBA mice of both sexes given 50ppm MNU and killed at week 26 (Experiment 1)

		MNU (ppm)		
		0	10	50
p53 (+/-)				
No. of mice examined		7	14	13
Lung	Hyperplasia	1 (14)	1 (7)	2 (15)
	Adenoma	0	0	2 (15)
Liver	Hepatocellular Foci	1 (14)	4 (31)	2 (15)
	Hepatocellular Adenoma	0	1 (7)	1 (8)
	Cytoplasmic vacuolization of hepatocytes, centrilobular	4 (57)	8 (57)	7 (54)
Kidney	Vacuolization, tubular epithelia	7 (100)	14 (100)	13 (100)
	Basophilic change, tubular epithelia	2 (29)	7 (50)	6 (46)
p53 (+/+) mice				
No. of mice examined		8	15	15
Lung	Hyperplasia	0	0	0
	Adenoma	0	0	0)
Liver	Hepatocellular Foci	0	1 (7)	1 (7)
	Hepatocellular adenoma	0	1 (7)	1 (7)
	Cytoplasmic vacuolization of hepatocytes, centrilobular	2 (25)	3 (20)	3 (20)
Kidney	Vacuolization, tubular epithelia	6 (75)	10 (67)	12 (80)
	Basophilic change, tubular epithelia	3 (38)	5 (33)	4 (27)

Table 3. Incidences of Proliferative or Non-Proliferative Lesions in p53 (+/-) and p53 (+/+) CBA mice of both sexes given 50ppm MNU and killed at week 26 (Experiment 2)

Lesions		p53 (+/-)	p53 (+/+)
Males given 50ppm MNU			
No. of mice examined		7	8
Lung	Hyperplasia	1 (14)	2 (25)
	Adenoma	2 (29)	3 (38)
Liver	Hepatocellular Foci	4 (57)	3 (30)
	Hepatocellular adenoma	0	0
	Cytoplasmic vacuolization of centrilobularhepatocytes	4 (57)	5 (63)
Kidney	Vacuolization, tubular epithelia	14 (100)	13 (100)
	Basophilic change, tubular epithelia	7 (50)	6 (46)
Female given 50ppm MNU			
No. of mice examined		10	10
Lung	Hyperplasia	1 (10)	0
	Adenoma	1 (10)	1 (10)
Liver	Hepatocellular Foci	0	0
	Hepatocellular adenoma	0	0
	Cytoplasmic vacuolization of centrilobular hepatocytes	6 (60)	5 (50)
Kidney	Vacuolization, tubular epithelia	10 (67)	12 (80)
	Basophilic change, tubular epithelia	5 (33)	4 (27)

concentration of 50 ppm for 26 weeks, to examine whether there is any sex difference in the MNU carcinogenicity. At the end of the 26 week experimental periods, animals in Experiments 1 and 2 were subjected to a full autopsy and extensive organs and tissues were dissected out, fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin, sectioned at 4-5 μm, and stained with hematoxylin and eosin (H-E) for microscopic examination.

Results

In Experiment 1, the incidences of hyperplasias in the glandular stomach observed in p53 (+/-) CBA mice treated with 50ppm and 10ppm MNU were significantly increased, as compared with the control group (Table.1). No significant difference in the incidences of these hyperplasias was observed in treated p53 (+/+) CBA mice as compared with

the control. These hyperplasias were observed in the fundic gland (Fig.1). No tumors were induced in the stomach of any treated groups. Foci, hepatocellular adenomas, lung hyperplasias, lung adenomas, and vacuolization and/or basophilic change of renal proximal tubular epithelia were occasionally observed in some p53 (+/-) CBA mice treated with MNU, but there was no significant difference in there incidences between treated and control groups (Table 2).

In Experiment.2, the incidences of hyperplasias in the glandular stomach observed in p53 (+/-) CBA mice of both sexes treated with 50ppm were not significantly increased, as compared with treated p53(+/+) CBA group. One papilloma of the forestomach was observed only in a male p53(+/-) CBA mouse treated with 50ppm (Table 1). Foci, hepatocellular adenomas, lung hyperplasias, lung adenomas, and vacuolization and/or basophilic change of renal proximal tubular epithelia were observed in some p53 (+/-) CBA mice of both sexes treated with 50ppm MNU, but there was no significant difference in their incidences between treated p53 (+/-) and p53 (+/+) CBA mice (Table 3).

Discussion

MNU is a multipotent carcinogen and induces tumors in various tissues of experimental animals depending on the route of administration (IARC, 1978). It has been reported that glandular stomach adenocarcinomas developed in a dose-dependent manner from weeks 31 to 54 in C3H mice given MNU in their drinking water at a concentration of 120, 60, 30 or 0 ppm for 30 weeks (Tatematsu et al., 1993). Another experimental study, in which male BALB/c mice were given MNU in their drinking water at a concentration of 240 ppm on alternate weeks for 10 weeks, 120 ppm on alternate weeks for 20 weeks, 60 ppm for 20 weeks or 30ppm for 40 weeks and were killed at 50 weeks, demonstrated that glandular stomach carcinomas were more common in mice treated with a high concentration of MNU for a short period than with a low concentration of MNU for a long period (Yamachika et al., 1998).

In addition, Ohgaki et al. recently reported that glandular stomach adenocarcinomas were induced in 2 of 8 homozygous p53 C57BL/6 mice (p53 (-/-) C57BL/6 mice that were subjected to gastric intubation of 5 μg/g body weight MNU 3 times/week for 5 weeks and were killed *in extremis* before week 50 (IARC, 1978). In their study, such adenocarcinomas were induced both in p53 (+/-) and p53 wild-type (+/+) mice of C57BL/6 background, that were subjected to the same treatment and were reared without any further exposure up to 50 weeks, and the development of the adenocarcinomas invading into the submucosa occurred only in p53 (+/-) C57BL/6 mice, not in p53 (+/+) C57BL/6 mice. The results of their study suggest that p53 (-/-) and p53 (+/-) C57BL/6 mice are more susceptible to the intragastric application of MNU than p53 (+/+) mice. In their other study of p53 (-/-) and p53 (+/-) C57BL/6 mice, in which these animals were given water containing 30 ppm MNU for 10 weeks and killed at week 25, adenomas and adenocarcinomas of the grandular stomach were induced in both these knockout mice and the incidences of them in p53 (-/-) C57BL/6 mice were significantly increased as compared to p53 C57Bl/6 (+/-) mice (Tatematsu et al., personal observation).

On the other hand, in the present study, hyperplasias of the glandular stomach were induced both in p53 (+/-) CBA and p53 (+/+) CBA mice given MNU for 26 weeks, but no tumors of the glandular stomach were induced in them except for only one forestomach papilloma in a male p53 (+/-) CBA mice receiving 50ppm MNU. The results of the present study strongly suggest that p53 (+/-) CBA mice have low susceptibility to MNU-induced gastric carcinogenesis, as compared to p53 (+/-) C57BL/6 mice.

Other p53 (+/-) C57BL/6 mice, called p53 (+/-) TSG mice, are maintained by Taconic Farms (USA). This mouse strain is highly sensitive to genotoxic carcinogens, such as N-butyl-N-(4-hydroxybutyl)nitrosamine (Ozaki et al., 1998) or *p*-cresidine (Tennant et al., 1995), and skin carcinogenesis with 4-vinyl-1-cyclohexene diepoxide (Tennant et al., 1995). In addition, in further evaluation studies performed in the U.S. National Toxicology Program, in which an additional 11 chemicals have been tested, it was found that there was high concordance between the predicted and observed outcomes of the p53 bioassay in response to a variety of chemicals and pharmaceuticals, such as melphalan, 2,4-diaminotoluene, diethylstilbestrol, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (Easin et al., 1998). In our previous study in which female p53 (+/-) and p53 (+/+) CBA mice were given an intraperitoneal injection of 120 mg/kg body weight of N-ethyl-N-nitrosourea (ENU) and were maintained without any other treatment for a further 26 weeks, uterine tumors (endometrial polyps as well as stromal sarcomas) and lung adenomas were induced in both p53 (+/-) and p53 (+/+) mice (Mitsumori et al., 2000). The incidences of uterine tumors (94%) and lung adenomas (81%) in p53 (+/-) mice significantly increased as compared to those (37 and 42 %, respectively) in p53 (+/+) CBA mice. Based on these results, it has been suggested that female p53 (+/-) CBA mice are also very susceptible to ENU-induced carcinogenesis, as compared to p53 (+/+) CBA mice.

However, the results of the present study do not unequivocally support the above conclusion, and suggest that there is a strain difference in carcinogen sensitivity between p53 (+/-) CBA and p53 (+/-) C57BL/6 mice with regard to genotoxic carcinogens. In conclusion, when p53 (+/-) mice are planned to be used for the evaluation of carcinogenic potential of newly-developed chemicals, this strain difference for p53 (+/-) mice should be taken into account.

Acknowledgments

This work was supported in part by a Grant-in-aid from the Ministry of health and Welfare.

References

D'Arcy PF, Harron DWG (Eds) (1998). Proceedings of the Fourth International Conference on Harmonization. Queen's University of Belfast, N. Ireland.

Dunnick JK, Hardisty JF, Herbert RA, et al (1997). Phenolphthalein induces thymic lymphomas accompanied by loss of the *p*53 wild type allele in heterozygous p53-deficient (+/-) mice. *Toxicol Pathol*, **25**, 533-40.

Easin WC, Haseman JK, Mahler JF, Buche, JR (1998). The National toxicology Program evaluation of generally altered mice as predictive model for identifing carcinogens. *Toxicol Pathol*, **26**, 461-73.

International Agency for Research on Cancer Monographs on the Evaluation of Carcinogenic Risk on Humans. (1978) Vol. 17, Some *N*-Nitroso Compounds. IARC, Lyon.

Mitsumori K, Onodera H, Shimo T, et al (2000). Rapid induction of uterine tumors with *p*53 point mutations in heterozygous *p*53 deficient CBA mice given a single intraperitoneal administration of *N*-ethyl-*N*-nitrosourea. *Carcinogenesis*, **21**, 1039-42.

Ohgaki H, Fukuda M, Tohma Y, et al (2000). Effect of intragastric application of *N*-methylnitrosourea (MNU) in *p*53 knockout mice. *Molec Carcinogenesis*, in press.

Ozaki K, Sukata T, Yamamoto S, et al (1998). High susceptibility of *p*53 (+/-) knockout mice in *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine urinary bladder carcinogenesis and lack of frequent mutation in the residual allele. *Cancer Res*, **58** (Suppl. 1), 3808-11.

Tatematsu M, Ogawa K, Shichino Y, *et al* (1992). Initiation of adenocarcinomas in the glandular stomach of BALB/C mice treated with *N*-methyl-*N*-nitrosourea. *Jpn J Cancer Res*, **83**, 915-8

Tatematsu M, Yamamoto M, Iwata H, et al (1993). Induction of glandular stomach cancers in C3H mice treated with N-methyl-N-nitrosourea in the drinking water. *Jpn J Cancer Res*, **84**, 1258-64

Tennant RW, French JE, Spalding JW (1995). Identifying chemical carcinogens and assessing potential risk in short-term bioassays using transgenic mouse models. *Environ Health Perspect*, **103**, 942-50.

Tennant RW, Spalding J, French JE (1996). Evaluation of transgenic mouse bioassays for identifying carcinogens and noncarcinogens. *Mutation Res*, **356**, 119-27.

Tsukada T, Tomooka Y, Takai S, et al (1993). Enhanced proliferative potential in culture of cells from p53-deficient mice. *Oncogene*, **8**, 3313-22.

Yamachika T, Nakanishi H, Inada K, et al (1998). *N*-Methyl-*N*-nitrosourea concentration-development, rather than total intake-development, induction of adenocarcinomas in the glandular stomach of BALB/c mice. *Jpn J Cancer Res*, **89**, 385-91

Personal Profile: Kunitoshi Mitsumori

Dr. Kunitoshi Mitsumori graduated from the Department of Veterinary Medicine, University of Osaka Prefecture in 1974 and received his Ph.D. degree from the same University in 1982. From 1974 to 1990, he worked as a toxicologic

pathologist in the Institute of Environmental Toxicology and for 2 years, as Expert Pathologist, at the Chemical Pathology Branch, National Institute of Environmental Health Sciences (NIEHS), USA. From 1991 to 2000, he joined the National Institute of Health Sciences (NIHS) as Section Chief of the Division of Pathology. From October, 2000, he moved to the Tokyo University of Agriculture and Technology, where he is currently Professor of the Laboratory of Veterinary Pathology.