Tumor Induction by Azoxymethane (AOM) and 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in F344 Rat Gastric Mucosa Featuring Intestinal Metaplasia Caused by X-irradiation

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Male F344 5-week-old rats were X-irradiated, and 16 weeks after the first dose, azoxymethane (AOM) was injected or 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) was given by intragastric intubation. Tumors in the pylorus of the glandular stomach were observed in 4 out of the 29 animals receiving X-rays + AOM and in 4 out of the 25 animals receiving X-rays + PhIP, 12 months after administration. No such lesions were found in the chemical or X-ray alone groups. Intestinal metaplasia and some induced tumors were positive for CDX2. It was concluded that the presence of intestinal metaplasia may increase sensitivity to the induction of gastric tumors by colon carcinogens.

Key Words: Gastric tumor, Azoxymethane, 2-Amino-1-methyl-6-phenylimidazo [4,5-b]pyridine, F344 rats, Intestinal metaplasia

Based on investigations in humans, intestinal metaplastic changes in the stomach have been considered precancerous lesions or a predisposing condition for differentiated gastric carcinoma development (1-7). However, we experimentally investigated an inverse relationship between quantity of intestinal metaplasia, with or without Paneth cells, and gastric tumor development, and established that its presence does not exert a positive influence on induction of gastric neoplasia by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or N-methylnitrosoure (MNU) in rats (8,9). The situation is complex, however, because Nakagawa et al. have indicated that colorectal mucosa implanted into the glandular stomach, like the intrinsic large intestine, is sensitive to tumorigenesis caused by the colon carcinogen, 1,2-dimethylhydrazine (DMH), in contrast to normal gastric mucosa (10). Furthermore, we reported that induction of intestinal metaplastic mucosa in the glandular stomach is associated with susceptibility to tumorigenesis due to DMH (11,12).

The present study was designed to further examine whether intestinal metaplasia might be a target for azoxymethane (AOM) or 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)-induction of malignant tumors in the glandular stomach.

Materials and Methods

Animals. Male F344/DuCrj rats, 5 weeks of age at the commencement, were purchased from Charles River and housed five to a polycarbonate cage under constant conditions of temperature (24 ± 2°C) and relative humidity (55 ± 10%), with a 12:12-hour light-dark cycle. The animals were maintained according to the "Guide for the Care and Use of Laboratory Animals" established by Hiroshima University. All rats were provided with a commercial diet (MF: Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*.

The animals were X-irradiated according to the method described previously (8,9,13), with two X-ray doses of 10 Gy each at a three-day interval (total dose, 20 Gy).

Four months after the first irradiation, initiation of azoxymethane (AOM, Sigma Chemical Co., St. Louis, MO) (14,15) or 2-amino-1-methyl-6-pheny-

limidazo [4,5-b]pyridine (PhIP, NARD, Amagasaki, Japan), both of which induce colon cancer (16-19), was commenced. AOM was given in weekly subcutaneous injections of 15 mg/kg body wt for 3 weeks, while PhIP was administered every 2 days, 3 times per week for a total of 10 doses of 75 mg/kg body wt by intragastric intubation.

Experimental Procedure. A total of 169 rats was divided into 6 groups. The animals in Groups 1 to 3 were X-ray irradiated. Those in Groups 1 and 4 were given PhIP, while Groups 2 and 5 received AOM. All were fed a normal MF diet throughout the experimental period. The animals were killed and autopsied when they became moribund and all remaining rats were killed by ether anesthesia 12 months after the initial chemical carcinogen treatment. The stomach, and the small and large intestinal tracts were removed, opened and extended on cardboard for inspection. The location of individual tumors was recorded by measuring the distance from the pyloric ring in the small intestine and from the anus in the large intestine. The numbers and sizes of individual tumors were also noted. Whole tissues were fixed in 10% neutral formalin. Alkaline phosphatase (ALP)-positive foci in the gastric mucosa were detected by the naphthol-AS-MX-phosphate-fast blue RR staining method (20) and the numbers of ALP-positive foci in the whole gastric mucosa per rat were counted under a dissection microscope with a double-blind protocol. Sections of paraffin-embedded tissue were routinely stained with hematoxylin and eosin, and for clarification, when necessary, with periodic acid Schiff-Alcian-blue (AB-PAS). Other organs were removed, fixed in 10% neutral formalin and stained with HE.

Intestinal metaplasias were categorized using the following histological criteria (21,22): type A, gastric mucosa with goblet cells which were positive for AB-PAS: type B, intestinal-type crypts without Paneth cells or type C, intestinal metaplasia with Paneth cells (alkaline phosphatase-positive foci). Using these criteria, the numbers of metaplastic crypts were counted separately for 2 sections through the lesser curvature (pylorus) and 4 through the greater curvature (fundus) in a double-blind fashion. Tumors in the stomach, small intestine and large intestine were classified into two types, adenoma, especially stomach, atypical hyperplasia (ATP, shown in Fig.1) and adenocarcinomas invading the musclaris mucosa or further, and also into two histological types, the well-differentiated (Fig.2) and poorly-differentiated types (Fig.3), the latter including both mucinous and signet ring cell forms.

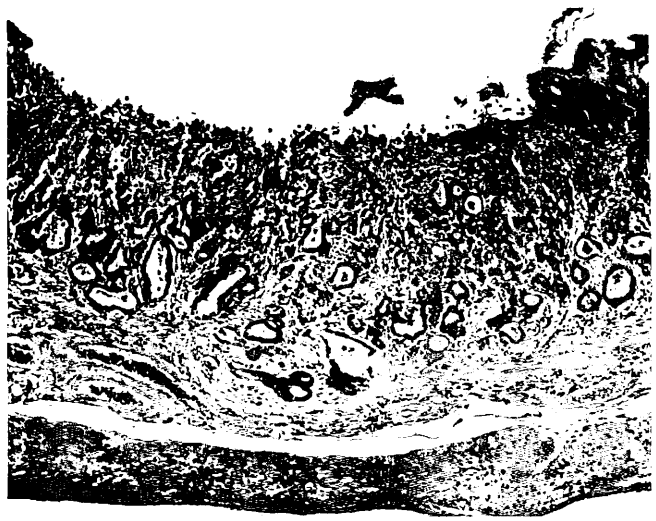

Fig. 1 - Atypical hyperplasia shown proliferation of atypical glands in mucosa, x100, HE staining.

Fig. 2 - Well differentiated adenocarcinoma shown atypical glands invaded all the layer of the gastric wall, x100, HE staining.

Immunohistochemistry. Paraffin-embedded sections were deparaffinized in xylene, and rehydrated through graded alcohols. A 0.05 M PBS buffer was used to prepare solutions and for washes between the various steps. Incubations were performed in a humidified chamber. Three-μm-thick sections were treated for 30 min at room temperature with 2% BSA and incubated with primary antibodies against CDX2 (diluted 1:50; Biogenex CDX2-88) (23) for 1 hour at

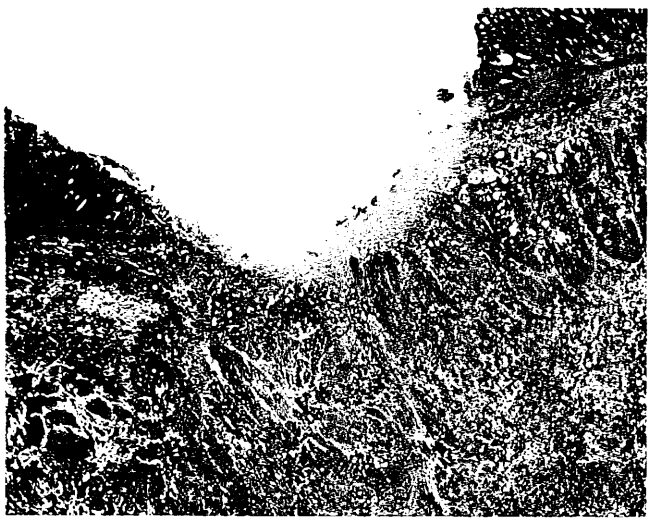

Fig. 3 - Signet ring carcinoma, x40, HE staining.

room temperature. For each case, negative controls were performed on serial sections whereby incubation with the primary antibody was omitted. All slides were then exposed to the secondary antibody, biotinylated horse anti-universal-monkey IgG (Vectastain Universal Quick Kit, Vector Laboratories, Ca., Catalog No. PK-8800) and peroxidase-conjugated streptavidin complexes. Peroxidase activity was visualized by treatment with H_2O_2 and diaminobenzidine for 5 min. At the last step, the sections were counterstained with hematoxylin for 1 min. CDX2-positive cells were observed.

Statistical analysis. The significance of differences in numerical data was evaluated by using the chi-squared test and Student's t test.

Results

Mean survival did not significantly differ among the groups. Body weights in the chemical carcinogen treatment groups were significantly decreased as compared to those in the control group (Table I). Heart weights were lower in the X-ray + PhIP and X-ray and PhIP groups and liver in the AOM, kidneys in the PhIP and AOM, testes in the AOM and spleens in the X-ray + PhIP and X-ray groups were significantly smaller than those of the controls. On the other hand, spleen weights significantly increased. Relative liver, kidney, and testis weights (relative weight; organ weight/body weight x 1,000) in the X-ray + PhIP group and relative spleen weights in the AOM group were significantly enlarged, while in the X-ray goup they were smaller than those in the controls (Table II).

The incidence and number of areas of intestinal metaplasias in the X-ray irradiated groups were significantly increased as compared to those in the non-irradiated groups (Tables III and IV). Incidences in

Table I - Body and organ weights

Group	Mean survival	Body Weight (g)	Organ weight (g)					
			Heart	Liver	Kidney	Adrenal	Testis	Spleen
X-ray+PhIP	356±32	386±50**	1.12±0.09**	11.6±1.5	2.47±0.35	0.052±0.006	3.24±0.18	0.60±0.13**
X-ray+AOM	337±50	415±37**	1.19±0.11	12.0±3.0	2.36±0.21	0.084±0.101	3.36±0.34	0.80±0.42
X-ray	365±11	433±35	1.14±0.13**	11.7±1.1	2.37±0.19	0.056±0.014	3.35±0.27	0.63±0.13**
PhIP	364	425±26**	1.14±0.10**	11.3±1.0	2.29±0.23*	0.063±0.012	3.21±0.30	0.79±0.10
AOM	359±10	427±23*	1.21±0.11	11.2±1.1*	2.29±0.14*	0.055±0.010	3.14±0.35**	1.09±0.19**
Control	364	452±33	1.26±0.12	12.2±1.3	2.43±0.20	0.065±0.015	3.39±0.29	0.86±0.10

: Significantly difference from Control value (P<0.05)* - *: Significantly difference from Control value (P<0.01)*

Table II - Relative weight*

Group	Heart	Liver	Kidney	Adrenal	Testis	Spleen
X-ray+PhIP	2.96±0.59	30.7±5.9**	6.56±1.52**	0.139±0.031	8.61±1.92**	1.59±0.43
X-ray+AOM	2.88±0.26	29.1±8.2	5.69±0.38	0.199±0.226	8.16±1.04	1.96±1.14
X-ray	2.66±0.33	27.1±2.4	5.49±0.43	0.131±0.037	7.76±0.61	1.47±0.42**
PhIP	2.68±0.18	26.7±1.9	5.41±0.72	0.149±0.031	7.58±0.76	1.88±0.30
AOM	2.83±0.23	26.1±1.8	5.37±0.31	0.128±0.021	7.35±0.84	2.55±0.46**
Control	2.78±0.24	26.9±1.7	5.39±0.37	0.144±0.031	7.52±0.68	1.91±0.20

*:*Organ weight/Body weight x 1,000* - **: *Significantly difference from Control value (P<0.01).*

Table III - Incidence of Intestinal metaplasia (%)

Group	ALP	Pylorus				Pylorus+Fundus			
		A	B	C	Total	A	B	C	Total
X-ray+PhIP	81	32	91	59	95	36	95	50	95
X-ray+AOM	66	26	78	65	82	26	87	74	87
X-ray	66	0	94	47	94	0	94	50	94
PhIP	0	8	0	0	8	8	8	0	25
AOM	0	17	8	0	25	17	25	0	33
Control	0	0	0	0	0	0	11	0	11

ALP: Alkaline phosphatase positive intestinal metaolasia - A: Goblet cells with the gastric musosa
B: Intestinal type crypt without Paneth cells - C: Intestinal type crypt with Paneth cells.

the X-ray groups were 84-95%, and in the non-irradiated groups were only 11-33%. The number of type B metaplasias and totals in the X-ray groups were significantly increased, and type C lesions were also more common in the X-ray + AOM and X-ray alone groups than in the controls (Table IV).

The first tumor appeared at 204 days, in an X-ray + AOM animal. Total tumors in the AOM groups were significantly more numerous than in the PhIP groups. Gastric tumors in the glandular stomach were observed in four out of the 25 (17%, three ATP, one adenocarcinoma) X-ray + PhIP animals, and four out of the 29 (10%, one ATP and three adenocarcinomas) X-rays + AOM animals. One signet ring cell carcinoma was found in this group. All other tumors in the glandular stomach were well-differentiated without goblet cells or mucin and were located in the middle or upper portion. Nuclei of

Table IV - Mean number of intestinal metaplasia

Group	ALP	Pylorus				Pylorus+Fundus			
Group		A	B	C	Total	A	B	C	Total
X-ray+PhIP	19.1±26.4	0.6±1.1	6.9±5.5*	1.4±1.8	8.9±7.3*	0.6±1.1	7.4±6.2*	1.5±1.9	9.5±8.2**
X-ray+AOM	25.0±42.2*	0.4±0.8	8.4±7.7**	2.0±2.2*	10.9±9.3**	0.4±0.8	9.1±7.5**	2.4±2.2*	11.9±8.7**
X-ray	11.4±18.9	0	9.8±10.0**	1.8±3.4	11.2±11.4**	0	10.7±10.9**	2.3±3.7*	12.7±12.6**
PhIP	0	0.1±0.3	0.8±2.6	0	0.8±2.6	0.1±03	1.1±2.8	0	1.2±2.8
AOM	0	0.5±1.4	0.2±0.6	0	0.7±0.6	0.5±1.4	0.4±0.8	0	0.9±1.5
Control	0	0	0	0	0	0	0.1±0.3	0	0.1±0.3

**: Significantly difference from Control value (P<0.05) - **: Significantly difference from Control value (P<0.01)*

intestinal metaplasia (Fig.4), and the signet ring cell carcinoma (Fig.5) were positive for CDX2 by immunohistochemistry. On the other hand, the cytoplasm of a well-differentiated adenocarcinoma was positive (Fig.6). No gastric tumors were observed in the other groups (Table V).

The incidence of small intestinal tumors was 28% and 3% in the X-ray+AOM and AOM groups, respectively. The more frequent colon tumors were the multiple and the papillary and polypoid types. The incidence of colon tumors was 8%, 12%, 79% and 72% in the X-ray + PhIP, PhIP, X-ray + AOM and AOM groups, respectively, and the number of tumors was 0.08 ± 0.28, 0.12 ± 0.3, 1.31 ± 0.97 and 1.17 ± 1.04, respectively (Table V). The incidences of signet ring cell carcinomas were 2 (7%) in the X-ray + AOM and 5 (19%) in the AOM groups. Aberrant crypt foci were observed in all of the chemical carcinogen-treated groups (data not shown).

Pancreas (17-38%) and skin tumors (6-28%) also developed after X-ray treatments. Three kidney tumors were found in the X-ray + PhIP group, three

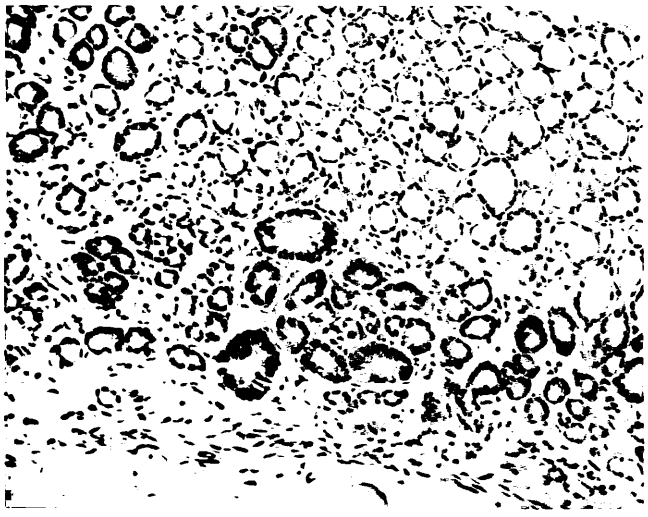

Fig. 4 - CDX-2 positive nuclei were observed in glands of intestinal metaplasia, x100, CDX-2 antibody staining.

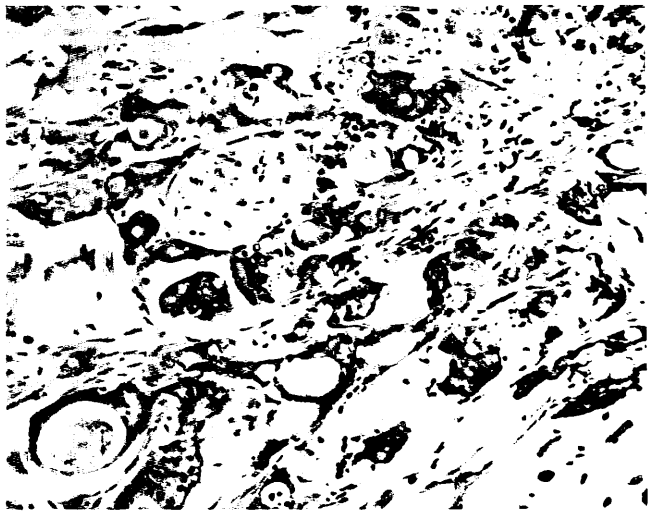

Fig. 5 - CDX-2 positive nuclei were observed in signet ring cell carcinoma, x 200, CDX-2 antibody staining.

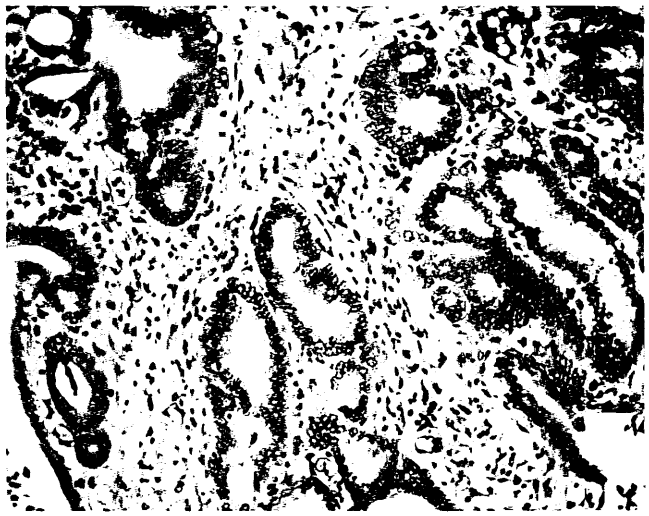

Fig. 6 - CDX-2 positive cytoplasm was observed in well differentiated adenocarcinoma x 200, CDX-2 antibody staining.

ear duct tumors, one bone and two liver tumors in the X-ray + AOM group, and one tail papilloma in the PhIP group (Table V).

Discussion

In the present experiment, induction of intestinal metaplastic mucosa in the glandular stomach was associated with susceptibility to tumorigenesis induced by PhIP and AOM, in contrast to non-susceptible normal gastric mucosa. In earlier studies, regression analysis of gastric tumors per rat against the frequency of intestinal metaplasia, with or without Paneth cells, yielded a significant inverse relationship, suggesting that the development of intestinal metaplasia and gastric tumors might be independent (8,9). Previously, we reported that intestinal metaplasia is not susceptible to gastric tumor induction by MNNG and MNU (8,9) and that colonic mucosa transplanted into gastric mucosa lacks susceptibility to these carcinogens when given orally (24,25). Intestinal metaplasia or colorectal mucosa implants into the glandular stomach are sensitive to DMH carcinogenicity, whereas the normal gastric mucosa is not (10). Thus, it would appear that areas of intestinal metaplasia induced by X-irradiation might be susceptible to damage due to carcinogens targeting the large intestine. In the present experiment, CDX-2 appeared in intestinal metaplasia and in some of the gastric tumors. CDX-2 is not expressed in the normal stomach but is highly expressed in the normal intestine and intestinal metaplasia (23,27) and carcinoma of the stomach, indicating its involvement in these lesions. So, it is considered that some of the gastric tumors in this experiment might have been caused by intestinal metaplasia and/or circumstances

Table V - Incidence and number of colon tumors

	No	Total (%)	Gatsric (%)	Small intestine (%)	Colon tumor Incidence (%)	Colon tumor Number per rat	Pancreas (%)	Skin (%)	Other (%)
X-ray+PhIP	25	16(64)	4(17)	0	2(8)	0.08±0.28	8(32)	7(28)	3(12) Kidney 3
X-ray+AOM	29	27(93)	4(10)	8(28)	23(79)	1.31±0.97	5(17)	3(10)	6(21) Ear duct 3 Liver 2 Bone 1
X-ray	32	15(47)	0	0	0	0	12(38)	2(6)	2(6) Squamous cell carcinoma 1 Papilloma 1
PhIP	25	4(16)	0	0	3(12)	0.12±0.33	0	0	1 Tail papilloma1
AOM	28	23(79)	0	1(3)	21(72)	1.17±1.04	0	0	2(9) Liver 1 Testis 1
Control	30	0	0	0	0	0	0	0	0

**:Significantly difference from X-ray group (P<0.05) - a: Significantly difference (P<0.05) - b: Significantly difference (P<0.01)*

of intestinal metaplasia.

We must consider the alternative possibility that the effects of irradiation and DMH and other colon carcinogens on glandular stomach epithelial cells are additive or synergistic. Tatemichi et al. reported that the cytochrome P450 monooxygenase 1A1 expressed in intestinal metaplasia, and carcinogen activation by 1A1 enzymes expressed in the gastric mucosa, may contribute to carcinogenesis of the stomach (28). It appears likely that intestinal mucosal stem cells are susceptible to colon carcinogenesis, independently of the administration route or their location. Thus, the intestinal mucosal phenotype appears to be the most important determinant of response to colon carcinogens, rather than the intestinal macro-environment itself.

In summary, the presence of intestinal metaplasia, with or without Paneth cells, may increase the sensitivity of the stomach to the induction of tumors by carcinogens like DMH, AOM or PhIP, but not by MNNG or MNU. The protocol used in the present experiment may provide a new approach for distinguishing between developmental events associated with intestinal metaplasia and gastric tumors.

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References

1. Jarvi O.H., Lauren P.: On the role of heterotopias of the intestinal epithelium in the pathogenesis of gastric cancer. Acta Pathol. Microbiol. Scand. (Suppl) 29: 26-43, 1951.
2. Kawachi T., Kogure K., Tanaka N., et al.: Studies of intestinal metaplasia in the gastric mucosa by detection of disaccharidases with "Test-Tape". J. Natl. Cancer Inst. 53: 19-30, 1974.
3. Lev R.: The mucin histochemistry of normal and neoplastic gastric mucosa. Lab. Invest. 14: 2080-2100, 1965.
4. Mangus HA.: Observations on the presence of intestinal metaplasia with gastric mucosa. J. Pathol. Bacteriol. 44: 389-398, 1937.
5. Morson BC.: Carcinoma arising from areas of intestinal metaplasia in the gastric mucosa. Br. J. Cancer 9: 377-388, 1955.
6. Nakamura K., Sugano H., Takagi K.: Carcinoma of the stomach in the incipient phase: its histogenesis and histological appearance. Gann 59: 251-258, 1968.
7. Tahara E.: Molecular mechanisms of stomach carcinogenesis. J. Cancer Res. Clin. Oncol. 119: 265-272, 1992.
8. Watanabe H., Ito A.: Relationship between gastric tumorigenesis and intestinal metaplasia in rats given X-radiation and/or N-methyl-N'-nitro-N-nitrosoguanidine. J. Natl. Cancer Inst. 76: 865-870, 1986.
9. Watanabe H., Ando Y., Yamada K., Okamoto T., Ito A.: Lack of any positive effect of intestinal metaplasia on induction of gastric tumors in Wistar rats treated with N-methyl-N-nitrosourea in their drinking water. Jpn. J. Cancer Res. 85: 892-896, 1994.
10. Nakagawa Y., Watanabe H., Takahashi T., Ito A., Dohi K.: Carcinogenicity of 1,2-dimethylhydrazine in colon tissue heterotopically transplanted into the glandular stomach of rats. Jpn. J. Cancer Res. 83: 24-30, 1992.
11. Watanabe H., Uesaka T., Kido S., et al.: Gastric tumor induction by 1,2-dimethylhydrazine in Wistar rats with intestinal metaplasia caused by X-irradiation. Jpn. J. Cancer Res. 90: 1207-1211, 1999.
12. Ando Y., Watanabe H., Tatematsu M., et al.: Gastric tumorigenicity of 1,2-dimethylhydrazine on the background of gastric intestinal metaplasia induced by X-irradiation in CD(SD) rats. Jpn. J. Cancer Res. 87: 433-436, 1996.
13. Watanabe H.: Experimentally induced intestinal metaplasia in Wistar rats by X-ray irradiation. Gastroenterology 75: 796-799, 1978.
14. Wattenberg L.W., Lam L.K., Fladmoe A.V., Borchert P.: Inhibition of colon carcinogenesis. Cancer 40 (5 Suppl): 2432-2435, 1977.
15. Hirose Y., Kuno T., Yamada Y., et al.: Azoxymethane-induced beta-catenin-accumulated crypts in colonic mucosa of rodents as an intermediate biomarker for colon carcinogenesis. Carcinogenesis 24: 107-111, 2003.
16. Hasegawa R., Sano M., Tamano S., et al.: Dose-dependence of 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP) carcinogenicity in rats. Carcinogenesis 14: 2553-2557, 1993.
17. Hirose M., Yamaguchi T., Lin C., et al.: Effects of arctiin on PhIP-induced mammary, colon and pancreatic carcinogenesis in female Sprague-Dawley rats and MeIQx-induced hepatocarcinogenesis in male F344 rats. Cancer Lett. 155: 79-88, 2000.
18. Nakagama H., Ochiai M., Ubagai T., et al.: A rat colon cancer model induced by 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine, PhIP. Mutat. Res. 506-507: 137-144, 2002.
19. Shirai T., Kato K., Futakuchi M., et al.: Organ differences in the enhancing potential of 2-amino-1-methyl-6- phenylimidazo[4,5-b]pyridine on carcinogenicity in the prostate, colon and pancreas. Mutat. Res. 506-507: 129-136, 2002.
20. Nakahara K.: Special features of intestinal metaplasia and its relation to early gastric carcinoma in man; observation by a method in which leucine aminopeptidase activity is used. J. Natl. Cancer Inst. 61: 693-702, 1978.
21. Watanabe H., Kamikawa M., Nakagawa Y., Takahashi T., Ito A.: The effects of ranitidine and cysteamine on intestinal metaplasia induced by X-irradiation in rats. Acta Pathol. Jpn. 38: 1285-1290, 1988.
22. Watanabe H., Okamoto T., Fudaba Y., Ogundigie P.O., Ito A.:

Influence of gastric pH modifiers on development of intestinal metaplasia induced by X-irradiation in rats. Jpn. J. Cancer Res. 84: 1037-1042, 1993.
23. Werling R.W., Yaziji H., Bacchi C.E., Gown A.M.: CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: An immunohistochemical survey of 476 primary and metastatic carcinomas. Am. J. Surg. Pathol. 27: 303-310, 2003.
24. Nakagawa Y., Ando Y., Fujimoto N., et al.: Colon tissue implanted into the glandular stomach in rats lack susceptibility to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) carcinogenesis. Oncol. Rep. 4: 517-519, 1997.
25. Ando Y., Uesaka T., Kido S., et al.: No susceptibility of colon tissue implanted into glandular stomach of rats to N-methyl-N-nitrosourea (MNU) carcinogenesis. Oncol. Rep. 5: 1373-1376, 1998.
26. Bai Y.Q., Yamamoto H., Akiyama Y., et al.: Ectopic expression of homeodomain protein CDX2 in intestinal metaplasia and carcinomas of the stomach. Cancer Lett. 176: 47-55, 2002.
27. Yuasa Y.: Control of gut differentiation and intestinal-type gastric carcinogenesis. Nat. Rev. Cancer 3: 592-600, 2003.
28. Tatemichi M., Nomura S., Ogura T., Sone H., Nagata H., Esumi H.: Mutagenic activation of environmental carcinogens by microsomes of gastric mucosa with intestinal metaplasia. Cancer Res. 59: 3893-3898, 1999.

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