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Original

Gastric Carcinogenesis and Intestinalization Induced by N-methyl-N-nitrosourea in the Senescence-Accelerated Mouse (SAMP3)

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Abstract: The relationship between gastric neoplasia and intestinal metaplasia, a process whose incidence increases with age, remains controversial. In the present experiment, we therefore investigated induction of both intestinal metaplasia and gastric cancers in the Senescence-accelerated mouse (SAMP3/Aicc) treated with N-methyl-N-nitrosourea (MNU). Seven-week-old animals of both sexes received the carcinogen in their drinking water at 120 or 60 ppm on alternative weeks for 10 weeks, or continuously at 30 ppm for the same period, then maintained without further treatment until sacrificed at week 50. The incidences of adenocarcinomas in the 120, 60, and 30 ppm MNU treated and control groups were 6/10 (60%), 5/16 (31.3%), 1/17 (5.9%), and 0/6 (0%) in males, and 2/7 (28.6%), 3/13 (23.1%), 1/6 (16.7%), and 0/9 (0%) in females, respectively. All neoplasms were of well-differentiated type, mainly consisting of gastric epithelial type cells. With immuno- and enzyme-histochemistry, intestinal alkaline phosphatase (I-ALP) positive intestinal absorptive cell-like elements were observed in regions of hyperplasia, adenomas, and adenocarcinomas of MNU treated mice, but no phenotypic expression of goblet or Paneth cells was found. In the control mice, gastric mucosa showed no intestinal epithelium phenotype. Since the degree of appearance of I-ALP positive cells in the lesions in SAMP3/Aicc mice was not different from that found for six other strains mice in our previous work, the results suggest that Senescence-acceleration may not influence intestinalization in the gastric mucosa or induction of gastric carcinomas. (*J Toxicol Pathol* 2003; 16: 33–39)

Key words: Senescence-Accelerated Mouse (SAM), MNU, gastric cancer, intestinal metaplasia, susceptibility

Introduction

Gastric adenocarcinomas display specific histological and biological characteristics and have been classified histologically into two types, intestinal and diffuse¹ or differentiated and undifferentiated². Furthermore, cytobiologically, cells of human stomach cancers have been classified into a gastric epithelial cell type (consisting of surface mucous and pyloric gland cells) and an intestinal epithelial cell type (intestinal absorptive and goblet cells) on the basis of their phenotypic expression^{3–5}. Intestinal

metaplasia is commonly seen in human gastric mucosa. While it has been reported to be a putative preneoplastic lesion, associated in particular with intestinal type gastric cancers^{6–8}, this conclusion is controversial since early gastric tumors are mainly composed of gastric epithelial cells and the phenotypic change to intestinal type occurs along with tumor progression^{9–11}.

For analysis of gastric carcinogenesis, experimental animal models are very useful. One rat model featuring N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) treatment¹², has been widely used which demonstrates the same intestinal phenotypic changes in the stomach in neoplasia as shown in the human case¹³. Adenocarcinomas induced by MNNG in the rat stomach are mainly of differentiated type and induction of undifferentiated type stomach cancers is relatively difficult. The mouse also has advantages, especially the recently genetically engineered strains. We

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have established mouse models for stomach carcinogenesis using N-methyl-N-nitrosourea (MNU), with particular similarities to human gastric cancers from the viewpoint of histopathological variation¹⁴, poorly differentiated adenocarcinomas as well as signet-ring cell carcinomas being induced. However, there are differences with regard to intestinal change in the stomach between the human and mouse cases. We have examined carcinogenesis with MNU in six strains of mice (BALB/cA, C57BL/6N, CBA/JN, C3H/HeN, DBA/2N, and CD-1) and the typical intestinal metaplasia and intestinal type gastric tumors which appear in the rat^{15,16} were not observed, despite the fact that the lesions positive for intestinal alkaline phosphatase (I-ALP), a marker of intestinal absorptive cells, could be detected with immuno- and enzyme-techniques¹⁷. Since intestinal metaplasia is commonly observed in the human stomach of old individuals, it could be considered as an age-related change. The observed differences between humans and mice might therefore be due to variation in life span and aging processes.

Senescence accelerated mice (SAM) have been derived from AKR/J mice by Takeda and co-workers¹⁸ and SAMP1, P2, P3, P6, P7, P8, P9, and P10 strains (P for prone) have now been established¹⁹. They have a normal development but exhibit accelerated senescence. They thus show moderate to severe loss of activity, deterioration of hair quality and fallout, skin coarseness, periophthalmic lesions, increased lordokyphosis of the spine, and a shortened life span. Each SAMP strain has a strain-specific pathological phenotype, for example, deficits in learning and memory being found in SAMP8 and P10, senile amyloidosis in SAMP1, P2, P7, and degenerative joint disease of temporomandibular joints in SAMP3. SAMP3 has a life span of approximately 16 months, while the other strains live on average for 10–13 months. Since induction of intestinal

metaplasia and gastric tumors generally requires a relatively long experimental period the SAMP3/Aicc (subline of SAMP3 at Aichi Cancer Center) strain was selected for the present investigation of the relationship between intestinal change and aging in gastric mucosa as well as gastric tumorigenesis due to MNU.

Materials and Methods

Experimental design

The SAMP3 strain, kindly provided by Dr. Toshio Takeda, Kyoto University, Kyoto, Japan, was maintained in the Animal Facility of Aichi Cancer Center Research Institute with brother-sister mating for more than 15 generations. The substrain is named SAMP3/Aicc. All animals were housed in plastic cages with hard wood chips in an air-conditioned room with a 12 h light-12 h dark cycle and received basal diet (Oriental NMF, Oriental Yeast Co., Tokyo, Japan) *ad libitum*. With administration of the gastric carcinogen, MNU (Sigma Chemical Co., St Louis, MO), at 120 ppm in the drinking water continuously for 18 weeks, six other strains of mice (BALB/cA, C57BL/6N, CBA/JN, C3H/HeN, DBA/2N, and CD-1) survived for one year in our previous study¹⁷. However, a preliminary experiment revealed that the same administration of MNU caused early mortality of SAMP3/Aicc mice. Thus MNU was given on alternate weeks at 120 and 60 ppm or continuously at 30 ppm, as shown in Fig. 1. The carcinogen solutions were freshly prepared three times per week and administered in light-shielded bottles. Controls received tap water. The animals were sacrificed at week 50 and necropsies were also performed on all those that died or were killed upon becoming moribund. The excised stomachs were fixed in 1% acetic acid in 95% ethanol, cut into about 6 strips, and embedded in paraffin.

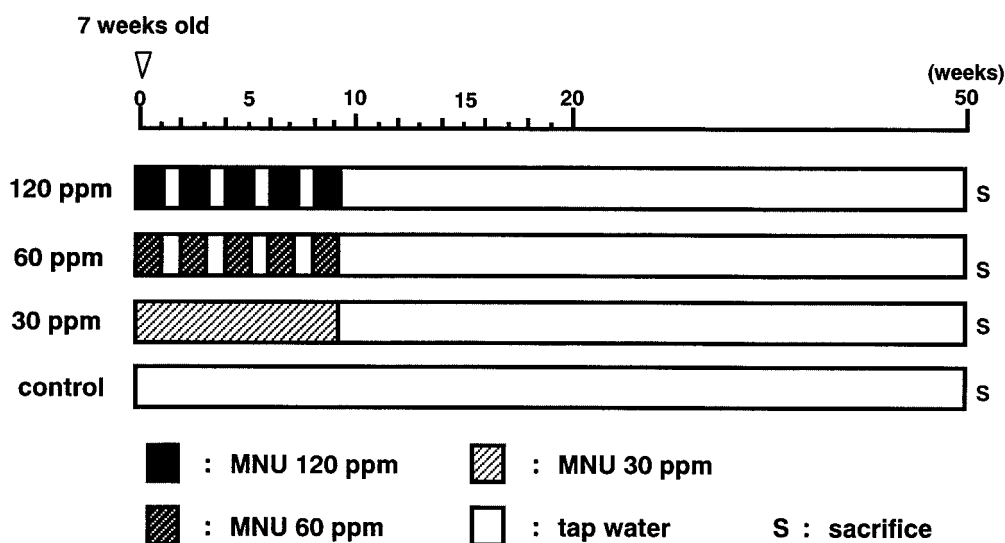


Fig. 1. Experimental design. Seven-week-old male mice were administered MNU at 120 ppm (black), 60 ppm (bold hatching) or 30 ppm (light hatching) following the indicated schedules or water (open). S; sacrifice.

Histopathological analyses

All tissue sections were stained with hematoxylin and eosin (H&E). Neoplastic lesions of glandular stomach were classified into adenomas and adenocarcinomas according to the criteria previously reported¹⁴. Tissue samples without good preservation or without sufficient amounts were not further analyzed.

Immunohistochemical, enzyme histochemical and mucin histochemical analyses

Anti-rat Pg1²⁰ and anti-rat I-ALP antibodies were prepared¹⁶ for immunohistochemical analyses. The avidin-biotin complex method with a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA) was used for detection. I-ALP enzyme activity was demonstrated using nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoxyl phosphate (NBT/BCIP) (Roche Diagnostics, Tokyo, Japan) in the reaction mixture containing 1 mM of levamisole. For mucin histochemistry, Alcian blue (pH 2.5) periodic acid Schiff (AB-PAS) and paradoxical concanavalin A staining (PCS) methods²¹ were employed.

When a lesion contained I-ALP positive epithelial cells, it was judged as IAP-positive. Because the cells in the normal pyloric mucosa just adjacent to the duodenum exhibited a mixture of gastric and intestinal phenotypes, this area was excluded from the analysis.

Statistical analysis

The incidences of stomach tumors and I-ALP positive lesions were analyzed using the one-tailed Fisher's exact probability test.

Results

Histopathological findings for tumors

Stomach cancers induced by MNU were all well-differentiated adenocarcinomas. The degree of invasion was up to the submucosa, with no involvement of the subserosa or serosa. Fig. 2 shows a well-differentiated adenocarcinoma invading the submucosa. The incidences of hyperplasias, adenomas, and adenocarcinomas in the glandular stomach are summarized in Table 1. Most of the lesions developed in the pyloric mucosa. Hyperplasias were observed in most animals treated with MNU, whereas incidences of adenomas and adenocarcinomas were dose-dependent. There were no significant differences in incidence between males and females.

Cellular phenotypes of neoplastic lesions

In the normal pyloric mucosa, Pg1 and PCS were clearly positive in pyloric gland cells. In all hyperplasias, Pg1 staining became weak or negative, while PCS staining was still positive. In tumors, Pg1 was very low or negative but PCS expression varied from region to region. Staining of Pg1 and PCS was decreased along with the degree of histological evidence of malignancy. Pg1 decreased first followed by PCS.

No typical intestinal metaplastic lesions, showing goblet or complete differentiated intestinal absorptive cell types were observed. Immunohistochemical and enzyme histochemical analyses revealed the existence of I-ALP positive areas with incomplete intestinal absorptive cells in hyperplasias (Fig. 3) and tumors. I-ALP was found in the covering epithelium associated with a change of mucin type from PAS positive to negative or weakly Alcian blue positive. The incidences of I-ALP positive lesions in the MNU treated and in control groups are summarized in Table 2. Adenocarcinomas tended to have lower I-ALP positivity than adenomas. In control animals, the stomach mucosa did not exhibit any intestinal features.

Discussion

It is clear that SAMP3/Aicc mice have higher susceptibility to MNU carcinogenicity than other commercially available mice and administration of 120 ppm continuously for 18 weeks caused tumors in multiple organs before stomach lesions developed. Thus milder treatment schedules were selected here. We earlier reported that the proportion of undifferentiated adenocarcinomas in the glandular stomach induced by MNU depends on the concentration of the carcinogen²² and in this study, all adenocarcinomas were of well-differentiated type.

We have described intestinalization observed in gastric carcinogenesis in six strains of mice (BALB/cA, C57BL/6N, CBA/JN, C3H/HeN, DBA/2N, and CD-1) induced by MNU to be limited to ectopic I-ALP expression without histological change¹⁷. The percentage of I-ALP positive cells in areas of hyperplasia increased from 10 to 52 weeks in all six strains. At 50 weeks in our SAMP3 strain, the values were not different from those at 52 weeks in the other strains (63.6–95.8%), so that senescence does not appear to accelerate intestinalization in the gastric mucosa.

All lesions observed here were heterogeneous for I-ALP expression, and none consisted only of I-ALP positive cells, in line with our findings for the other six mice strains¹⁷. Since adenomas and carcinomas are thought to be monoclonal lesions, this random expression must be the result of heterozygosity. In man, gastric and intestinal mixed types in single crypts are observed in intestinal metaplasia²³. Whether due to alteration in differentiation pathways or mutations, the heterogeneity indicates that intestinalization is not necessary for carcinogenesis in the mouse gastric mucosa.

Recently, not only aging but *Helicobacter pylori* (*H. pylori*) infection has been thought to be a major risk factor associated with intestinal metaplasia in man^{24,25}. In the immunocompetent mouse, infection with *H. pylori* is difficult to establish and histological gastritis has not been shown²⁶. However, *Helicobacter felis* infection in mice causes chronic atrophic gastritis and slight intestinal metaplasia, featuring Alcian blue positive mucous cells but no clear intestinal type goblet cells^{27,28}. Mongolian gerbils are also sensitive to *H. pylori*²⁹, with induction of chronic

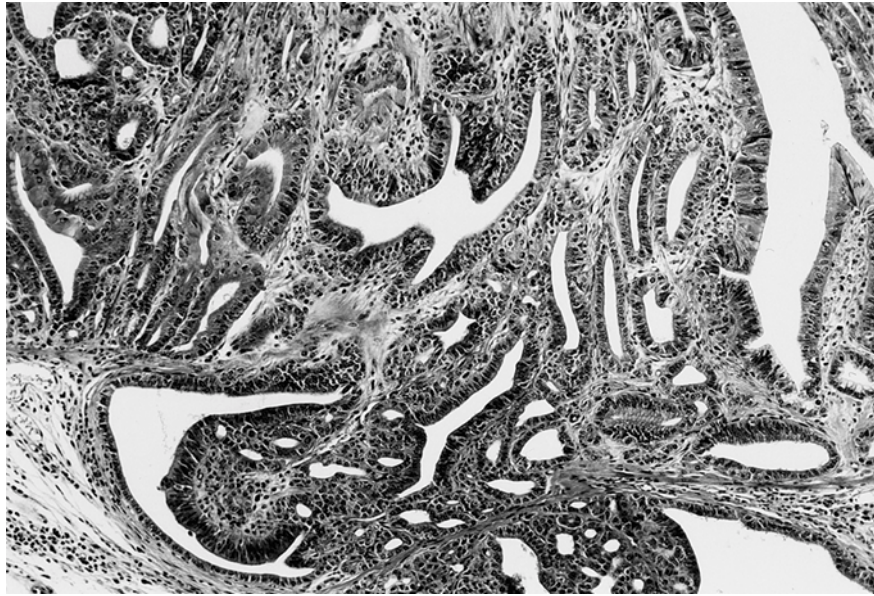


Fig. 2. A well-differentiated adenocarcinoma in a 120 ppm MNU-treated SAMP3/Aic mouse, invading the submucosa. H&E staining. $\times 30$.

Table 1. Incidences of Neoplastic Lesions in SAMP3 Mice Treated with MNU (%)

	MNU (ppm)	No. of mice	Histology		
			Hyperplasia (%)	Adenoma (%)	Adenocarcinoma (%)
Male	120	11	11 (100) ^{a)}	10 (90.9) ^{a)}	6 (54.5) ^{b), c)}
	60	16	15 (93.8) ^{a)}	13 (81.3) ^{a)}	5 (31.3)
	30	17	17 (100)	10 (58.8) ^{b)}	1 (5.9)
	0	6	0	0	0
Female	120	7	7 (100) ^{a)}	6 (85.7) ^{a)}	2 (28.6)
	60	13	12 (92.3) ^{a)}	9 (69.2) ^{a)}	3 (23.1)
	30	6	5 (83.3) ^{a)}	2 (33.3)	1 (16.7)
	0	9	0	0	0

with Fisher's exact test vs 0 ppm a): $p < 0.01$ b): $p < 0.05$, vs 30 ppm c): $p < 0.01$.

Table 2. Incidences of I-ALP Positive Lesions in the Glandular Stomach in SAMP3 Mice Treated with MNU

MNU (ppm)	No. of mice ^{a)}	Hyperplasia (%)		Adenoma (%)		Adenocarcinoma (%)	
		Total no. of lesions ^{a)}	I-ALP positive ^{b)}	Total no. of lesions ^{a)}	I-ALP positive ^{b)}	Total no. of lesions ^{a)}	I-ALP positive ^{b)}
Male	120	8	6 (75.0)	14	7 (50.0)	5	0 (0)
	60	15	10 (71.4) ^{c)}	17	12 (70.6) ^{c)}	5	3 (60.0)
	30	13	8 (61.5)	13	7 (53.8)	1	0 (0)
	0	4	0	0	0	0	0
Female	120	7	6 (85.7) ^{c)}	9	7 (77.8) ^{c)}	1	0 (0)
	60	10	6 (66.7) ^{c)}	8	5 (62.5)	2	1 (50.0)
	30	6	5 (80.0) ^{c)}	3	2 (66.7)	0	0 (0)
	0	5	4	0	0	0	0

a): Total No. of mice/lesions examined for I-ALP analysis, b): Numbers (%) of lesions containing I-ALP positive cell. with Fisher's exact test vs 0 ppm c): $p < 0.05$.

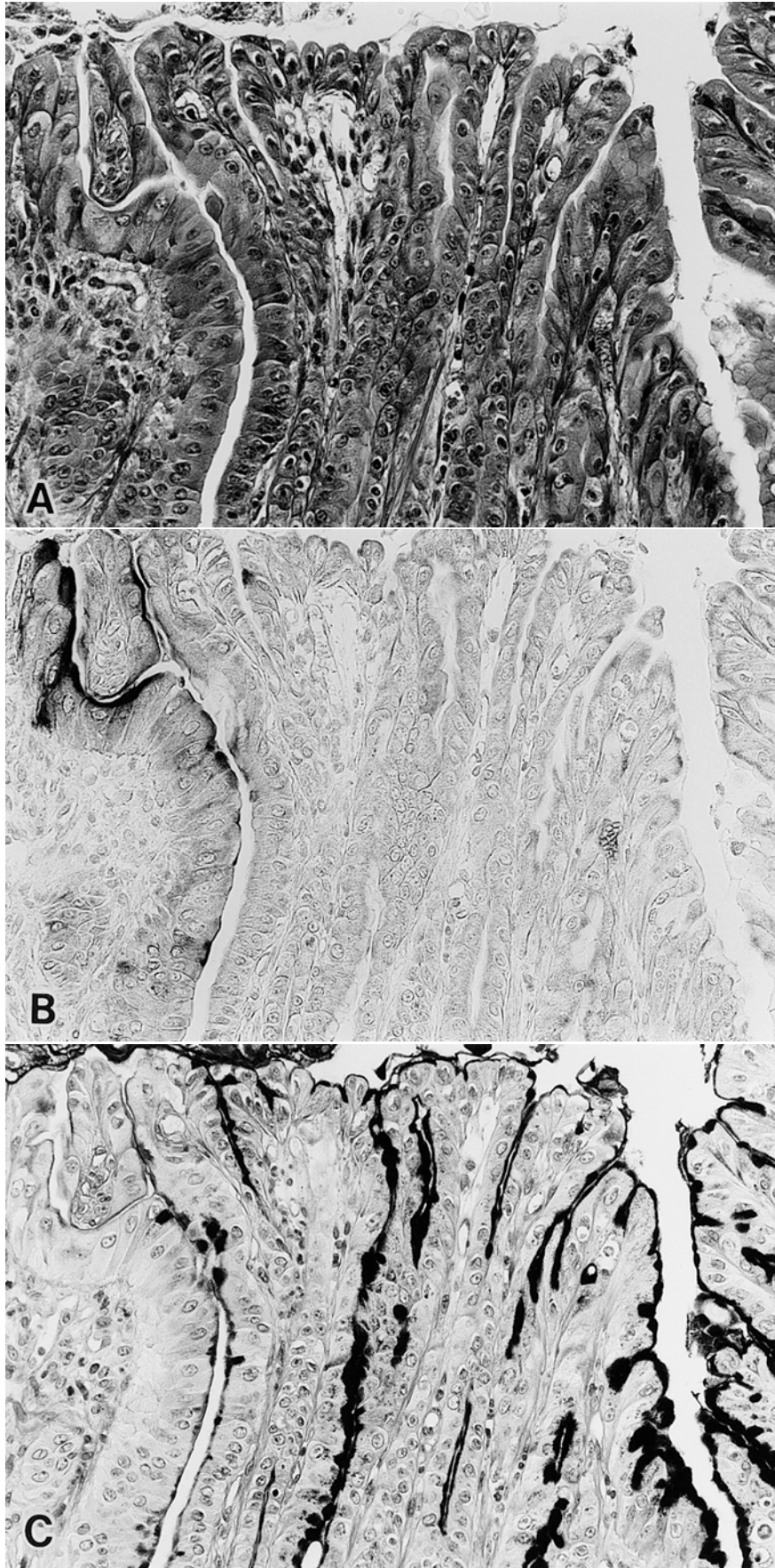


Fig. 3. An area of hyperplasia in a 120 ppm MNU-treated SAMP3/Aic mouse. $\times 80$ (A) H&E. (B) I-ALP immunostaining. (C) AB-PAS.

active gastritis from the fundic to the pyloric glands of the stomach. The carcinogens MNU and MNNG do not induce intestinal metaplasia that could be recognized by phenotype, but a combination of carcinogen and *H. pylori* or *H. pylori* alone was found to result in intestinal metaplasia with Alcian blue positive clear goblet cells in the gerbil glandular stomach^{30,31}. Chronic gastritis induced by germs may have influence on intestinal metaplasia of gastric epithelium. Using SAM mouse, there is one experiment, enteric inflammation develops in SAMP1/Yit, a subline of the SAMP1 mouse, under conventional but not in germ free conditions³². In our experiment, SAMP3 under conventional condition have no significant histopathological change.

Watanabe *et al.*³³ showed that gastric pH influences intestinal metaplasia in X-irradiated rats. They reported that the numbers of intestinal metaplastic lesions, enzyme histochemically ALP positive foci, and areas of type B metaplasia (featuring intestinal crypts without Paneth cells), were decreased in the low pH groups with induction of gastric acid secretion. It is possible that pH similarly affects intestinal metaplasia in the Mongolian gerbil or mouse infected with *Helicobacter*. In our mouse model of gastric tumors with MNU, however, apparent intestinal metaplasia consisting of goblet cells or Paneth cells were not induced probably because stomach pH might not be affected so much since fundic gland cells were well preserved as the lesions occur mainly in areas occupied by pyloric glands.

Regarding controls not given carcinogen, there was no remarkable difference between SAMP3/Aicc and other commercially available mice in terms of I-ALP expression. Thus MNU rather than accelerated aging influenced intestinal metaplasia in gastric mucosa, I-ALP positive areas only being observed in the carcinogen treated groups. However, they were distributed apparently at random, with no spatial relationship to glandular stomach cancers. There was no relation between intestinal metaplasia as shown with I-ALP expression and carcinogenesis. However, chemical, physical, or inflammatory injury such as X-irradiation, carcinogen exposure, or bacterial infection and subsequent regeneration of mucosa may influence the intestinalization.

In conclusion the present study revealed that whereas SAMP3/Aicc mice have a high sensitivity to MNU, their Senescence-acceleration does not appear to influence intestinalization of the gastric mucosa and there was no clear relationship between intestinalization and gastric tumors.

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