RESEARCH ARTICLE

Inflammation Enhanced X-irradiation-Induced Colonic Tumorigenesis in the Min mouse

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Abstract

Inflammation is potential risk factor of various human malignancies. Inflammatory bowel syndromes such as ulcerative colitis are well known as risk factors for colon cancer. Here, we examined enhancing effects of dextran sulfate sodium (DSS)-associated inflammation on X-irradiation induced colonic tumorigenesis in Min and wild-type (WT) mice. Animals were X-irradiated at 1.5 Gy at 5 weeks of age (at 0 experimental week) and 2% DSS in drinking water was administered at 5 or 11 experimental weeks. Mice were sacrificed at 16 weeks and incidence and multiplicity of colonic tumors were assessed. Incidence of colonic tumors in Min mouse was increased from 33.3% to 100% ($p<0.05$) with X-irradiation alone, whereas no tumors were developed in WT mice. In DSS-treated Min mice, X-irradiation increased the number of colonic tumors. Total number of colonic tumors was increased 1.57 times to 30.7±3.83 tumors/mouse with X-irradiation+DSS at 5 weeks comapared to 19.6±2.9 in corresponding DSS alone group ($p<0.05$). When the duration of inflammation was compared, longer period of DSS effect promoted more colonic tumorigenesis. Collectively, we conclude that X-irradiation and DSS-induced inflammation act synergistically for colonic tumorigenesis.

Keywords: Min mouse - X-irradiation - DSS - colon

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Introduction

Inflammation has been widely known as strong risk and promoting factor of carcinogenesis (Balkwill and Mantovani, 2001) in various types of human cancers (Ohshima et al., 2003). Among them, ulcerative colitis is in high risk condition in colonic carcinogensis (Munkholm, 2003). In the animal counterpart, dextran sulfate sodium (DSS) (Okayasu et al., 1990) showed powerful tumor promoting effect in murine colonic carcinogenesis models initiated with azoxymethane (AOM) (Tanaka et al., 2003), 1,2-dimethylhydrazine (DMH) (Kohno et al., 2005), and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Tanaka et al., 2005).

Familial adenomatous polyposis (FAP) is an inherited human disease characterized by numerous colorectal tumorigenesis (Kinzler and Vogelstein, 1996). FAP is caused by mutation in the adenomatous polyposis coli (APC) tumor suppressor gene (Powell et al., 1993). Min (multiple intestinal neoplasia) mouse is a murine model of human FAP (Moser et al., 1990), which has nonsense mutation at codon 850 in Apc gene (Su et al., 1992). The mouse develops multiple intestinal adenomas with inactivation of wild type allele. Min mouse have been revealed to be highly susceptible to carcinogenic agents. Subcarcinogenic low-dose *N*-ethyl-*N*-nitrosourea (ENU) increased the tumor incidence in the intestine and mammary gland in Min mice (Shoemaker et al., 1995). Other colonic carcinogens including PhIP (Steffensen et al., 1997) and AOM (Paulsen et al., 2003) also increased intestinal tumors. Besides chemical carcinogens, Min mice have been revealed to be susceptible to ionizing radiation (Luongo and Dove, 1996) in the age-dependent manner (Okamoto and Yonekawa, 2005). Inflammatory stimuli by DSS strongly induced colonic neoplasia in Min mice (Tanaka et al., 2006).

In this study, we investigated whether DSS-induced inflammation enhanced colorectal tumorigenesis initiated with low-dose X-irradiation in Min mice.

Materials and Methods

Animals and genotyping

Male C57BL/6J-ApcMin/J (Min) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Female wild type (WT) C57BL6/J were obtained from Clea Japan (Tokyo, Japan). They were housed in plastic cages with hardwood chips in an air-conditioned room

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Table 1. Incidence of Colon Tumors

		WT		Min		WT vs. Min
		Effective No. (male/female)	No. of mice with colon tumors (Incidence)	Effective No. (male/female)	No. of mice with colon tumors (Incidence)	
Groups	A	12 (6/6)	0 (0%)	12 (10/2)	4 (33.3%)	p=0.09
	B	14 (7/7)	3 (21.4%)	9 (4/5)	9 (100%)	p<0.002
	C	17 (8/9)	0 (0%)	11 (4/7)	11 (100%)**	p<0.0001
	D	9 (3/6)	3 (33.3%)*	8 (3/5)	8 (100%)	p<0.001
	E	15 (9/6)	0 (0%)	7 (3/4)	7 (100%)	p<0.0001
	F	10 (5/5)	2 (20.0%)	6 (5/1)	6 (100%)	p<0.001

*p<0.05 vs. WT-C, **p<0.002 vs. Min-A

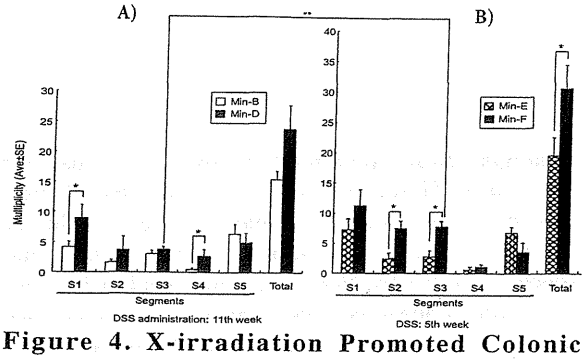

Figure 4. X-irradiation Promoted Colonic Tumorigenesis in DSS Treated Min mice. Group Min-B (Open bar), Min-D (Hatched bar), Min-E (Cross-hatched bar), and Min-F (Closed bar). *p<0.05 and **p<0.01

p<0.05) (Figure 3).

When 2% DSS was administered at 11 experimental week (Groups B and D), the number of colonic tumors were 8.88±2.20 (p<0.05) and 2.63±1.15 (p<0.05) in S1 and S4 in Group D, compared to 4.11±0.87 and 0.44±0.24, respectively, in Group B. It suggested enhancing effect of X-irradiation in Group D (Figure 4A).

If DSS was given at 5 week (Groups E and F), the numbers were 7.33±1.26 and 7.67±0.96 in S2 and S3, respectively, in Group F, compared with 2.43±0.87 and 2.71±1.04 in Group E, also indicating stimulating effect of X-irradiation in Group F (Figure 4B). Total numbers of colonic tumors was 30.67±3.83 and 19.57±2.9 in Groups F and E, respecively; the former was significantly higher than the latter (p<0.01).

When the two X-irradiation+DSS groups (Groups D and F in Figure 4 crossing left and right panels) were compared, earlier administration of DSS (7.67±0.96, Group F) was more effective in increment of colonic tumors in S3 compared to Group D (3.75±0.45) (p<0.05).

Multiplicity of colon tumors in WT mice

The number of total colonic tumors were 0.00±0.00, 0.50±0.27, 0.00±0.00, 0.78±0.46, 0.00±0.00, and 0.20±0.13/mouse in Groups A, B, C, D, E, and F, respecively, showing no significant differences among these groups.

Discussion

In the present study, we analyzed promoting effect of DSS-induced inflammation on X-irradiated colorectal carcinogenesis in WT and Min mice. Firstly, X-irradiation alone without DSS was assessed to confirm the effect of X-irradiation. In the Min mouse, incidence of colonic tumors was increased to 100% compared to non-irradiated group (33.3%). The number of colonic tumors in S2 was also increased with X-irradiation compared with non-irradiated Min mice. On the other hand, WT mice were not influenced with X-irradiation. It suggested that *Apc* locus might be more sensitive to loose normal APC function in Min mice although chromosome abberation might have occurred independent of their sequence (Rydberg, 1996). Okamoto and Yonekawa (Okamoto and Yonekawa, 2005) reported that 10 days old Min mice were more sensitive than other ages. In this study, since the mice were X-irradiated around 5 weeks old (35 days old), enhancing effect may have become unclear.

In the Min mice, DSS alone has known to enhance colonic tumorigenesis (Tanaka et al., 2006). X-irradiation was further added to assess if it may have enhanced colonic tumorigenesis. When Group F was compared with E, tumor multiplicity was increased in S2 and S3. Then, Group D vs. B, the number of tumors was increased upon X-irradiation in S1 and S4. Although localization of colonic tumors were different, X-irradiation was proved to exacerbate DSS-associated colonic tumorigenesis in Min mice. When the timing of DSS treatment was compared whether duration of inflammation could affect promoting effect, longer duration of inflammation had more enhancing effect (Group F) compared with shorter period (Group D). Similar phenomenon was observed in carcinogen induced murine colonic carcinogenesis model (Tanaka et al., 2003) and DSS alone induced model (Tanaka et al., 2006).

Considering WT mice, tumor incidence was increased in Groups D and F compared with non-treated or X-irradiation alone Groups, although enhancing effect was not as clear as that of Min mice. It was suggested that DSS treatment could bring latent genetic damage to apparent colonic tumors.

In the serial research in Life Span Study cohort of atomic bomb surviviors (Ozasa et al., 2012), the additive radiation risk of solid cancers continues to increase throughout life with a linear dose-response relationship. The estimated lowest dose range with a significant excessive relative risk (ERR) for all solid cancer was 0 to 0.20 Gy indicating no threshold. The risk of cancer mortality increased significantly for most major sites, including colon, whereas rectum did not. In the current study, multiplicities of colonic tumors (S2-S4 regions) were significantly increased with X-irradiation in Min-C (S2), Min-D (S4), and Min-F (S2 and S3) groups. In the

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Gene expression analysis of a *Helicobacter pylori*-infected and high-salt diet-treated mouse gastric tumor model: identification of CD177 as a novel prognostic factor in patients with gastric cancer

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Abstract

Background: *Helicobacter pylori* (*H. pylori*) infection and excessive salt intake are known as important risk factors for stomach cancer in humans. However, interactions of these two factors with gene expression profiles during gastric carcinogenesis remain unclear. In the present study, we investigated the global gene expression associated with stomach carcinogenesis and prognosis of human gastric cancer using a mouse model.

Methods: To find candidate genes involved in stomach carcinogenesis, we firstly constructed a carcinogen-induced mouse gastric tumor model combined with *H. pylori* infection and high-salt diet. C57BL/6J mice were given *N*-methyl-*N*-nitrosourea in their drinking water and sacrificed after 40 weeks. Animals of a combination group were inoculated with *H. pylori* and fed a high-salt diet. Gene expression profiles in glandular stomach of the mice were investigated by oligonucleotide microarray. Second, we examined an availability of the candidate gene as prognostic factor for human patients. Immunohistochemical analysis of CD177, one of the up-regulated genes, was performed in human advanced gastric cancer specimens to evaluate the association with prognosis.

Results: The multiplicity of gastric tumor in carcinogen-treated mice was significantly increased by combination of *H. pylori* infection and high-salt diet. In the microarray analysis, 35 and 31 more than two-fold up-regulated and down-regulated genes, respectively, were detected in the *H. pylori*-infection and high-salt diet combined group compared with the other groups. Quantitative RT-PCR confirmed significant over-expression of two candidate genes including *Cd177* and *Reg3g*. On immunohistochemical analysis of CD177 in human advanced gastric cancer specimens, over-expression was evident in 33 (60.0%) of 55 cases, significantly correlating with a favorable prognosis ($P = 0.0294$). Multivariate analysis including clinicopathological factors as covariates revealed high expression of CD177 to be an independent prognostic factor for overall survival.

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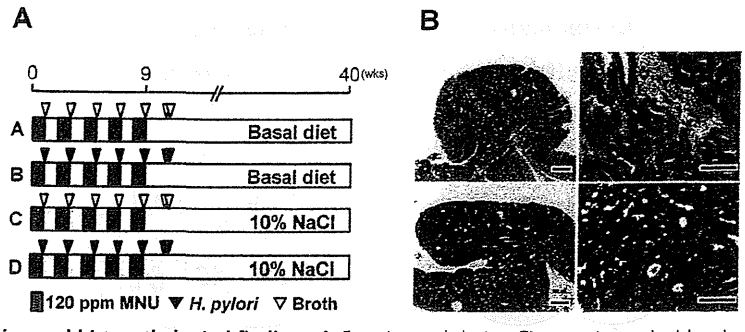

Figure 1 Experimental design and histopathological findings. A: Experimental design. Five- to six-week-old male C57BL/6J mice were inoculated with *H. pylori* SS1 strain (Groups B and D) or Brucella broth (Groups A and C). All animals were administered 120 ppm MNU in their drinking water on alternate weeks (total exposure, 5 weeks). Mice of Groups C and D were given basal diet (CE-2) containing 10% NaCl. **B**: Histopathological findings for MNU-induced mice gastric tumors. (a and b) Gastric adenoma in the pyloric region of an MNU-treated and *H. pylori*-infected mouse (Group B). (c and d) Gastric adenocarcinoma observed in Group B. Note the high cell density and cellular and structural atypia. Bar = 200 (a and c) or 100 μm (b and d).

MNU was freshly dissolved in distilled water three times per week. Mice of Groups C and D received CE-2 diets (basal sodium content of 0.36%; CLEA Japan) containing 10% NaCl. During the exposure period, one animal of Group B, one of Group C and six of Group D died or became moribund and they were excluded from the experiment. At 40 weeks, the remained animals were subjected to deep anesthesia and laparotomy with excision of the stomach.

Histological evaluation

For histological examination, the stomachs were fixed in 10% neutral-buffered formalin for 24-h, sliced along the longitudinal axis into strips of equal width, and embedded in paraffin. Four-μm thick sections were prepared and stained with hematoxylin and eosin (H&E) for histological observation. Tumors were classified into adenoma and adenocarcinoma based on cellular and morphological atypia and invasive growth to submucosa as we reported previously [21].

RNA preparation and oligonucleotide microarray analysis

Total RNA was extracted from the whole gastric mucosa including both tumor and peripheral tissue using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and its quality checked with a microchip electrophoresis system (i-chip SV1210; Hitachi Chemical, Tokyo, Japan). High-quality samples were selected, and pooled for each group to avoid individual difference for oligonucleotide microarray assessment (Group A, n = 3; B, n = 4; C, n = 6; D, n = 7). The CodeLink Mouse Whole Genome Bioarray (Applied Microarrays, Tempe, AZ, USA) containing 35,587 probe sets per chip was used to analyze gene expression profiles. Hybridization, processing, and scanning were performed by Filgen, Inc. (Nagoya, Japan), scan data images being analyzed using a software package (Microarray Data Analysis Tool, Filgen). Complete-linkage hierarchical clustering was also examined on the four groups using a qualified probe subset (Filgen).

Quantitative real-time RT-PCR of expression profiles in mice stomach

Relative quantitative real-time RT-PCR was performed using a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the mouse-specific glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene as an internal control. After DNase treatment, first strand cDNAs were synthesized from total RNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). The PCR was accomplished basically following the manufacturer's instructions using a QuantiTect SYBR Green PCR Kit (Qiagen). The primer sequences for each gene are listed in Table 1. Specificity of the PCR reactions was confirmed using a melt curve program provided with the StepOne software and electrophoresis of the PCR samples in 3% agarose gels. The expression levels of mRNAs were normalized to the mRNA level of *Gapdh* and compared with the control mice (Group A) by the ΔΔCT method.

Patients and tumor specimens

A total of 55 cases of primary advanced gastric cancer, surgically resected at Aichi Cancer Center Hospital (Nagoya, Japan) between 1995 and 2002, were investigated after obtaining informed consent. The study was approved by the ethics committee of Aichi Cancer Center. The patients were all male and the mean age and median follow-up period were 58.6 ± 10.2 years and 83 weeks, respectively. None had received preoperative chemotherapy or radiotherapy. Carcinomas with adjacent mucosa tissue were fixed and embedded in paraffin, and sectioned for staining

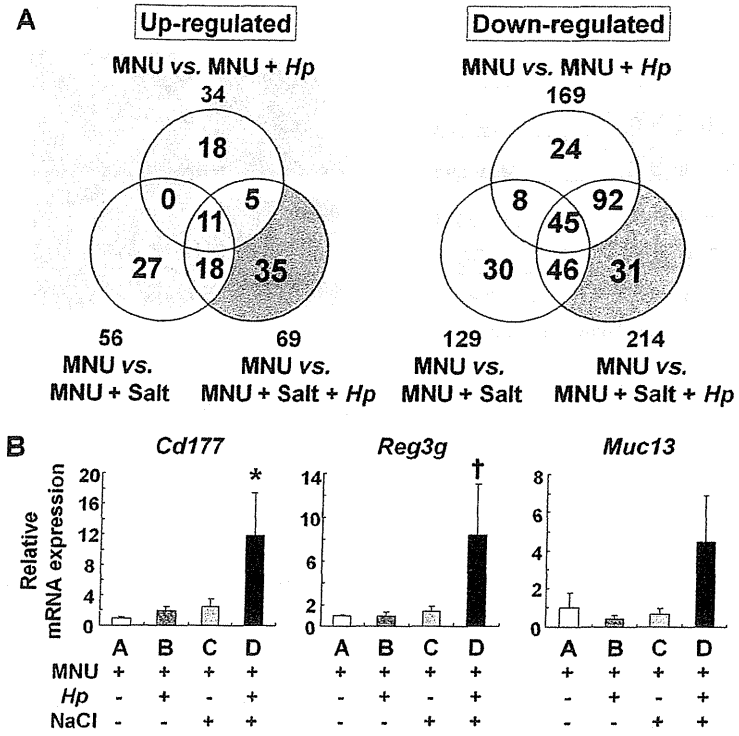

Figure 2 Global gene analysis in the glandular stomach of MNU-treated mice using oligonucleotide microarray. A: Number of genes up- or down-regulated more than two-fold in the stomach of MNU-treated mice. In Venn's diagram, the circles indicate up- (left) or down-regulated (right) genes in the stomach of MNU-treated mice with *H. pylori* infection, high-salt diet or their combination. The shaded area represents the up- or down-regulated genes more than two-fold only by the combination. **B**: Quantitative real-time RT-PCR analysis of three selected up-regulated genes (*Cd177*, *Reg3g*, and *Muc13*) in the stomachs of MNU-treated mice. Expression levels of the genes in each sample were normalized by *Gapdh* as internal control using ΔΔCT method. Relative expression levels were represented as the X-fold change relative to Group A (fixed as 1.0). Statistical analysis was performed by the Kruskal-Wallis test for general analysis and Tukey test for multiple comparison. Bars, SE; *, $P < 0.01$ *vs.* Group A and < 0.05 *vs.* Group C; †, $P < 0.01$ *vs.* Group C.

and B), the incidence was also increased by *H. pylori*-infection (Group A, 61.9% and Group B, 100%), albeit without statistical significance. The multiplicities of total tumors in both *H. pylori*-infected groups (Group B, 3.3 ± 1.0 tumors/mouse and Group D, 2.6 ± 1.1) were markedly higher than those in non-infected groups (Group A, 0.9 ± 0.8 and Group C, 1.0 ± 1.2) ($P < 0.05$). The multiplicity of gastric adenocarcinoma in Group D (2.1 ± 1.4) was slightly higher than that in Group B (1.8 ± 1.0) and significantly increased over the Group C value (0.8 ± 1.0) ($P < 0.05$). In contrast, the multiplicities of adenomas in Groups A and D (0.1 ± 0.4 and 0.4 ± 0.5, respectively) were significantly lower than in Group B (1.5 ± 0.6) ($P < 0.05$ and 0.01).

Gene expression profiling in the glandular stomachs by oligonucleotide microarray

With oligonucleotide microarrays, compared with the non-infected and basal diet-treated group (Group A), 34 genes were up-regulated and 169 were down-regulated more than two-fold in *H. pylori*-infected mice (Group B), 56 up-regulated and 129 down-regulated in high-salt diet-treated mice (Group C), and 69 up-regulated and 214 down-regulated in the combined group (Group D) (Figure 2A). Taken together, as shown in Table 3, we found that 35 genes were up-regulated and 31 genes were down-regulated more than two-fold only by the combination of *H. pylori* infection and high-salt diet. In addition, hierarchical clustering analysis was performed on the four groups with a total of 303 qualified probes using the complete-linkage clustering algorithm (Figure 3). Thirty-one probes including *Cd177*, *Reg3g* and *Muc13* were confirmed to be within a cluster of probes up-regulated only in Group D. Subsequent analysis in the present study was focused on these genes, because it was considered that the genes in which expression was altered only in the combined group might be associated with gastric carcinogenesis and progression in humans.

The entire results of this microarray analysis have been submitted and are readily retrievable from the

Table 3 Regulated genes by combination of *H. pylori* infection and high-salt diet in mouse gastric mucosa *(Continued)*

NM_146667	Olfr740	Olfactory receptor 740	0.44
NM_007550	Blm	Bloom syndrome homolog (human)	0.44
NM_011243	Rarb	Retinoic acid receptor, beta	0.44
NM_184052	Igf1	Insulin-like growth factor 1	0.45
NM_013893	Reg3d	Regenerating islet-derived 3 delta	0.46
NM_008645	Mug1	Murinoglobulin 1	0.46
NM_029550	Keg1	Kidney expressed gene 1	0.46
NM_019388	Cd86	CD86 antigen	0.46
NM_011316	Saa4	Serum amyloid A 4	0.47
NM_007811	Cyp26a1	Cytochrome P450, family 26, subfamily a, polypeptide 1	0.47
NM_011538	Tbx6	T-box 6	0.48
NM_011086	Pip5k3	Phosphatidylinositol-3-phospate/phosphatidylinositol 5-kinase, type III	0.48
NM_133723	Asph	Aspartate-beta-hydroxylase	0.48
NM_001081390	Palld	Palladin, cytoskeletal associated protein	0.48
NM_007858	Diap1	Diaphanous homolog 1 (Drosophila)	0.48
NM_053271	Rims2	Regulating synaptic membrane exocytosis 2	0.48
NM_153163	Cadps2	Ca2+–dependent activator protein for secretion 2	0.49
NM_007541	Bglap1	Bone gamma carboxyglutamate protein 1	0.49
NM_031871	Ghdc	GH3 domain containing	0.49
NM_025545	Aptx	Aprataxin	0.49
NM_177322	Agtr1a	Angiotensin II receptor, type 1a	0.49
NM_026872	Ubap2	Ubiquitin-associated protein 2	0.49
NM_028045	Erv3	Endogenous retroviral sequence 3	0.49
NM_011641	Trp63	Transformation related protein 63	0.49

public database NCBI Gene Expression Omnibus (GEO) with the accession number GSE29444 (sample number: GSM728857-60).

Quantitative real-time RT-PCR analysis of gene expression profiles in MNU-treated mouse stomachs

Relative quantitative real-time RT-PCR analysis of three selected up-regulated genes (*Cd177*, *Reg3g*, and *Muc13*) in *H. pylori*-infected and high-salt diet-treated mice confirmed increased expression of *Cd177* and *Reg3g*, as shown in Figure 2B, with significant differences. Although expression level of *Muc13* in Group D was higher than all other groups, there was no statistical significance among them ($P = 0.0712$ *vs.* Group C).

Immunohistochemical expression of CD177 in human advanced gastric cancers and correlation with clinicopathological factors

On immunohistochemical analysis of human gastric cancer tissues, CD177 was observed not only in the membranes and cytoplasms of infiltrated neutrophils, but also in gastric cancer cells of both well- and poorly-differentiated adenocarcinomas (Figure 4A). Cancer cells of signet-ring cell type (2 cases) were negative for CD177. Among 55 gastric cancer cases, moderate to strong expression of CD177 was observed in 33 (60.0%) (Table 4).

The follow-up period of the patients ranged from 9 to 606 weeks (median = 83 weeks). Five-year survival rates for CD177-positive and negative were 39.4% and 18.2%, respectively. From the Kaplan-Meier survival curve analysis, CD177-positive expression was associated with better overall survival ($P = 0.0294$, log-rank test) (Figure 4B). There was no statistically significant correlation of CD177 expression with age, histological classification, depth of invasion, and lymph node metastasis (Table 4).

Multivariate analysis for overall survival of human gastric cancer cases

Using the Cox proportional hazards model, multivariate analysis of clinicopathological variables, including the patient age, tumor histological classification, invasion depth, lymph node metastasis, and CD177 expression (Table 5), revealed the last to be an independent factor for overall survival ($P = 0.0323$). Patient age and low

Table 4 CD177 expression in gastric carcinomas and its correlation with clinicopathological factors

	Case no.	CD177 Over-expression				*P* value‡
		Positive		Negative		
		Strong	Moderate	Weak	None	
Gastric adenocarcinomas	55	18	15	17	5	
Age						
Years (means ± SD)		55.3 ± 10.4	60.2 ± 8.13	59.8 ± 11.0	60.4 ± 13.0	0.5039
Histological classification						
Well/moderately-differentiated type*	21	6	9	4	2	0.1904
Poorly-differentiated/Signet-ring cell type**	34	12	6	13	3	
Depth of invasion†						
T1-3	27	5	10	10	2	0.2011
T4	26	11	5	7	3	
Lymph node metastasis						
N0	6	1	2	2	1	0.7869
N1-3	49	17	13	15	4	

* Lauren's intestinal type, ** Lauren's diffuse type, † Case number was reduced to fifty-three because the depth of invasion was not classified in two cases, ‡ ANOVA and Chi-square test were performed for age and other factors, respectively.

neoplasms. A number of rodent models of gastric cancer have been developed under various conditions, including *H. pylori* or *H. felis* infection, exposure to chemical carcinogens, and genetic modification [21,30]. Since *H. pylori* is known as a most closely-associated risk factor in man, animal models with infection of the bacterium, such as that utilizing Mongolian gerbils, are considered to be particularly important to mimic the background of human gastric carcinogenesis. On the other hand, there is a consensus that gastric cancer is a multifactorial disease [31]. Epidemiological studies and animal experiments have demonstrated that development of stomach cancer is also associated with many other factors including salt intake, alcohol drinking and cigarette, containing a wide variety of chemical carcinogen. In the present study, we attempted to mimic the gastric environment of human high-risk group exposed to combination of *H. pylori* infection, salt intake, and carcinogen.

As might be expected, there are both advantages and disadvantages of *Helicobacter*-infected mouse models. Instability of *cag* pathogenicity islands (PAI), a particularly important virulence factor of *H. pylori*, has been reported in the mouse model using SS1 strain [32]. Multiplicity of gastric tumors is difficult to examine in the gerbil model, because almost all of the stomach tumors in gerbils show invasive growth into the lamina propria or muscle layer. In the present study, our results demonstrated that *H. pylori* infection increased not only incidence but also multiplicity of gastric tumors in MNU-treated mice. Thus, the mouse model presented here has advantages in respect to investigate the multiplicity and tissue sampling for gene expression analysis.

In this study, we focused on the genes in which the expression was regulated only in *H. pylori*-infection and high-salt diet combined mice, which are expected to reflect the background of human high-risk group, to explore examples which might be associated with tumor progression. The two up-regulated genes selected, *Cd177* and *Reg3g* could be confirmed to exhibit significant over-expression by relative quantitative RT-PCR. Expression level of *Muc13* showed a tendency for increase with combination of *H. pylori* and salt, although this was not statistically significant. *Muc13* is a recently identified gene encoding transmembrane mucin that is expressed in the stomach to large intestine [33]. Shimamura et al. have reported that overexpression of *Muc13* is associated with differentiation towards the intestinal (differentiated) type of human gastric cancer [34]. In addition, the combined expression of

Table 5 Multivariate analysis of prognostic factos in patients with gastric cancer using Cox proportional hazard model

Factors	Hazard ratio	95% CI	*P* value
CD177 expression (negative)	2.07	1.063-4.021	0.0323
Age (year)	1.04	1.001-1.071	0.0439
Histological type (poorly-differentiated)	4.06	1.695-9.742	0.0017
Depth of invasion (high grade)	1.64	0.790-3.410	0.1838
Lymph node metastasis (positive)	3.40	0.773-14.92	0.1055

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REVIEW

Helicobacter pylori infection and gastric carcinogenesis in rodent models

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Abstract *Helicobacter pylori* infection is an important factor for gastric carcinogenesis in human. In carcinogen-treated Mongolian gerbils, *H. pylori* infection enhances stomach carcinogenesis, while infection alone induced severe hyperplasia called heterotopic proliferative glands. A high-salt diet or early acquisition of the bacteria exacerbates inflammation and carcinogenesis. Oxygen radical scavengers or anti-inflammatory chemicals as well as eradication of *H. pylori* are effective to prevent carcinogenesis. *H. pylori*-associated inflammation induces intestinal metaplasia and intestinalization of stomach cancers independently. It is necessary to control cancer development not only in *H. pylori*-positive cases but also in *H. pylori*-negative metaplastic gastritis.

Keywords *Helicobacter pylori* · Mongolian gerbil · Intestinal metaplasia · Gastric adenocarcinoma · Chemoprevention

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Introduction

Gastric cancer is in decreasing trend nowadays [119], however, it remains the fourth most common cancer and second leading cause of cancer-related death worldwide [76]. It is still an important medical problem and its prevention is one of the most important aspects of cancer control programs. Many pathological and biological analyses of gastric carcinomas, including precancerous lesions, have been performed with experimental animals as well as human samples. Major model animals susceptible to induction of gastric adenocarcinomas include rats, mice, and Mongolian gerbils, the latter two offering powerful tools for analysis of *Helicobacter pylori*-associated gastric disorders. In this article, we introduce major animal models while comparing with human lesions, concentrating special attention on pathological and biological findings.

H. pylori infection and the development of gastric cancer: epidemiological findings

H. pylori has been discovered from patients of chronic gastritis as gram-negative, flagellate, and microaerophilic

bacilli [52, 127] and has been revealed as a major causative factor for gastric disorders. Strong epidemiological evidence has been accumulated indicating a significant relationship with active chronic gastritis, peptic ulcers, atrophic gastritis, intestinal metaplasia, and malignant lymphoma or cancer development [3, 15, 18, 23, 28, 29, 42, 67, 77, 78, 114]. Prospective study confirmed that gastric cancers developed in 2.9 % of the *H. pylori*-infected group but none of the uninfected patients [126]. Based on the epidemiological findings, *H. pylori* was defined as a "definite biological carcinogen" by World Health Organization/International Agency for Research on Cancer (WHO/IARC) in 1994. However, regarding the relationship between *H. pylori* infection and development of gastric cancers, the lack of evidence of induction in experimental animals was one point, which received stress in the WHO/IARC report [30].

Establishment of the animal models

To identify pathological and molecular biochemical mechanisms, various experimental animal models have been established in rats and mice with chemical carcinogens including *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and *N*-methyl-*N*-nitrosourea (MNU). Since *H. pylori* is one of the most important factors for human stomach disorders, the *H. pylori*-infected and carcinogen-treated Mongolian gerbil has proven very useful for analyses of underlying processes [123].

Rat models

In some of the earliest studies, researchers attempted to induce gastric cancers in animal models using chemical carcinogens such as benzo[a]pyrene [82] or 3-methylcholanthrene [90] by direct injection into the stomach or the gastric wall. Others tried oral administration, a more natural administrative method, using 2-acethylaminofluorene [129]. However, the resultant incidences of lesions were low. In 1967, however, Sugimura and Fujimura [92] utilized *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in drinking water and were able to report good yields of adenocarcinomas in the glandular stomachs of rats. Upon exposure to MNNG, erosive lesions occurred and subsequent disordering of glandular structures and proliferation of epithelial cells in the pyloric mucosa were observed. After appearance of atypical glands, finally both differentiated and undifferentiated adenocarcinomas were induced in this model, mimicking the situation in humans. Oral administration of 4-nitroquinoline 1-oxide (4-NQO) and 4-hydroxyaminoquinoline 1-oxide (4-HAQO) similarly induces carcinomas in the stomach as well as the various other tissues [61, 62]. The presence of surfactants, such as alkylbenzenesulfonate, was found to enhance the effects of 4-NQO in the stomach of animals [96, 99]. Another carcinogen, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine [43, 94], could be shown to cause gastric cancers not only in rats but also in dogs.

Mouse models

Administration of MNNG in the drinking water to BRSUNT/NJms mice over the life span only resulted in adenomatous hyperplasia of the gastric epithelium, suggesting resistance to this carcinogen [93]. However, oral administration of 4-NQO and 4-HAQO did induce carcinomas in the stomach as well as various other tissues [61, 62]. Finally, MNU was successfully introduced for good yields of adenocarcinomas in the glandular stomach of BALB/c mice [110]. Comparable yields were obtained in the C_3H [111] and other strains [133]. In the mouse models, both differentiated and undifferentiated types of adenocarcinomas typically develop, showing more significant cellular atypia compared with lesions in MNNG-treated rats. Taking advantage of this characteristic, intramucosal neoplasms became recognizable. The establishment of mouse models further opened up new approaches using transgenic and knockout animals. Yamamoto et al. utilized p53 knockout mice and revealed that nullizygous p53 knockout mice showed higher susceptibility to carcinogen in contrast to heterozygous animals [132].

Helicobacter-infected mouse models

H. pylori infection has been tried on many animals to study the pathogenetic background, but none of the early models proved sufficiently similar to the human situation [41, 80]. In 1990, Lee et al. [46] isolated *Helicobacter felis* from the cat stomach and inoculated germ-free mice with the bacteria, which colonized in the stomach like as *H. pylori* and induced acute and chronic inflammation. p53 wild-type and hemizygous mice were inoculated with *H. felis* and the latter showed higher proliferative index in the gastric foveolar epithelium [19]. *H. pylori* isolated from human clinical specimens were also utilized to inoculated nude and euthymic mice causing chronic active gastritis [34, 35, 51]. *H. pylori*, named Sydney strain (SS1), with higher colonizing ability were established by screening of fresh clinical isolates in long-term mouse adaptation and is currently widely used worldwide for mice experiments [47].

Oshima et al. constructed transgenic mice (K19-C2mE transgenic mouse) simultaneously expressing cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 under keratin 19 promoter in the gastric epithelial cells and revealed the importance of these pathways in gastric tumorigenesis especially with *Helicobacter* infection [72]. Additional expression of Wnt1 converted more dysplastic gastric tumors [73]. MNU treatment and *H. pylori* (Sydney strain, SS1) infection of K19-C2mE mice induced adenocarcinomas

not only in pyloric mucosa but also in fundic glands, serving a better model for increasing proximal gastric cancers [101].

H. pylori-infected Mongolian gerbil models: promoting effects of *H. pylori*

Besides the mouse models, Mongolian gerbil (*Meriones unguiculatus*) model was successfully established to mimic human severe *H. pylori* infection and inflammation, with the bacteria detectable throughout the study period up to 1 year [25]. Gerbils can be readily infected with *H. pylori*, and the resultant chronic active gastritis, peptic ulcers, and intestinal metaplasia resemble lesions apparent in man (Fig. 1a). Later in 1998, stomach carcinogenesis model was established using Mongolian gerbils with MNU and MNNG as the carcinogens (Fig. 1b) [112]. *H. pylori* infection was subsequently found to increase the incidence of both MNU- and MNNG- induced adenocarcinomas of all histological types including differentiated and undifferentiated adenocarcinomas and signet-ring cell carcinoma in the gerbils' glandular stomach (Fig. 1c, d) [85, 86, 95]. Several studies based on detailed histopathological assessment showed no carcinomas in animals treated only with *H. pylori* infection [85–87, 95, 112]. Thus, we consider that *H. pylori* is a strong promoter of gastric carcinogenesis. The *H. pylori*-infected and chemical carcinogen-treated Mongolian gerbils have thus proved very useful for the analysis of gastric carcinogenesis.

Besides adenocarcinomas, neuroendocrine tumors (NET) (endocrine cell hyperplasia/dysplasia and carcinoid tumors) were frequently induced in *H. pylori*-infected Mongolian gerbil model in association with serum gastrin level. Eradication of *H. pylori* prevents occurrence of NET lesions in the glandular stomach, this being strongly linked with reduction in serum gastrin levels [9]. Proton pump inhibitors (PPIs), routinely used for control of upper gastrointestinal disorders, have been some concern about the long-term safety and the possibility of cancer induction and development of NET in the stomach. When PPI was administered to *H. pylori*-infected Mongolian gerbils, PPI at high dose increased NET development with higher serum gastrin; in contrast, PPI at low dose had no influence on development of carcinomas and NETs in the *H. pylori*-infected and uninfected gerbils' glandular stomach [124].

Modifying factors of stomach carcinogenesis

Host risk factors for gastric cancer: age of infection of *H. pylori*

Childhood infection with *H. pylori* is a major concern in the pediatrics field. To compare severity of inflammation and susceptibility to induction of cancer in childhood and the adult, *H. pylori* inoculation and subsequent MNU administration were started at different time points in the gerbil's lifespan. Early acquisition of *H. pylori* significantly increased gastric carcinogenesis in carcinogen-treated gerbils, as compared to the case with later infection, possibly because of differences in host gastric mucosal and immunologic factors, as well as age-dependent sensitivity to chemical carcinogens (Fig. 1e, f) [5]. This would imply that childhood *H. pylori* infection must not be overlooked in

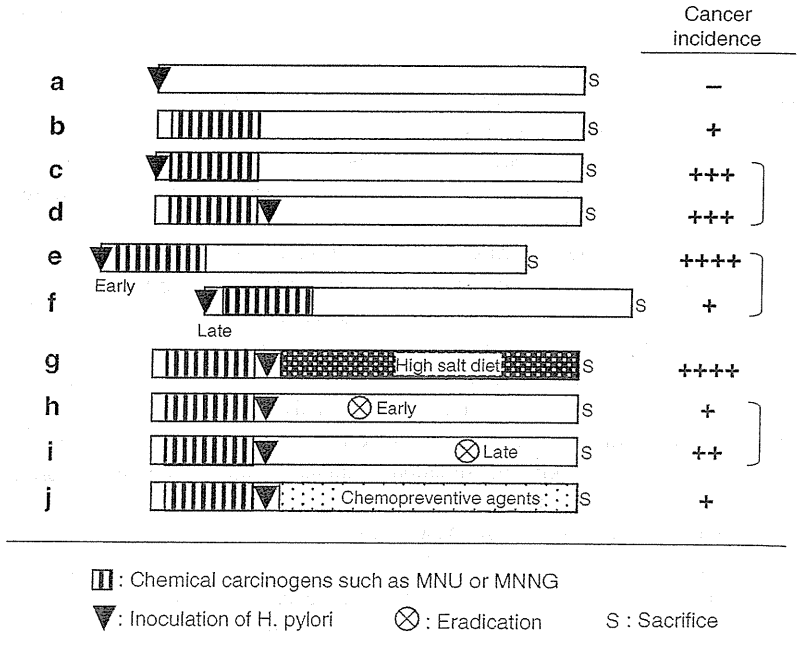

Fig. 1 Modifying factors for *H. pylori*-associated stomach carcinogenesis in Mongolian gerbils. **a** *H. pylori* infection alone is not carcinogenic. **b** Drinking water containing chemical carcinogens including MNU or MNNG induces stomach cancers. **c**, **d** When combined, *H. pylori* become a strong promoter. **e**, **f** Earlier infection increases the risk than later event. **g** A high-salt diet exacerbates inflammation and increases the incidence of *H. pylori*-associated cancer. **h**, **i** Earlier eradication of *H. pylori* reduces risk of stomach cancers. **j** Various natural products and pure chemicals appear to have chemopreventive potential

approaches to the prevention of gastric cancer later in adult life [14, 50].

Synergistic effects of *H. pylori* and high-salt diet

Among various food ingredients, salt and salted foods are probable risk factors for gastric cancer, based on evidence from a large number of case–control and ecological studies [33, 39, 97, 118]. In an experimental study, sodium chloride (NaCl) was found to enhance the carcinogenic effects of MNNG and 4-NQO in the rat glandular stomach [103], possibly partly due to decrease in the viscosity of the gastric mucus and so reduction in the protective mucous barrier. When given alone, NaCl has no apparent carcinogenicity in rats but, when administered with MNNG or 4-NQO, it promotes gastric carcinogenesis in the rat glandular stomach [103], in a dose-dependent fashion [100]. A high concentration causes initial tissue damage and consequent regenerative cell proliferation and this is also in line with accelerated lesion development [21].

Furthermore, in 2002, Nozaki et al. demonstrated a high-salt diet to enhance the effects of *H. pylori* infection on gastric carcinogenesis, with these two factors acting synergistically to promote the development of stomach cancers in the Mongolian gerbil model, although high-salt intake alone has a minor influence compared to *H. pylori* [68]. Further, to examine the dose dependence and the mechanisms underlying enhancing effects, Mongolian gerbils were treated with MNU, *H. pylori*, and food containing various concentrations of salt, and were sacrificed after 50 weeks. Among gerbils treated with MNU and *H. pylori*, the incidences of glandular stomach cancers were 15 % in the normal diet group and 33, 36, and 63 % in the 2.5, 5, and 10 % NaCl diet groups, showing dose-dependent increase. Intermittent intragastric injection of saturated NaCl solution, in contrast, did not promote gastric carcinogenesis. In gerbils infected with *H. pylori*, a high-salt diet was associated with elevation of anti-*H. pylori* antibody titers, serum gastrin levels, and inflammatory cell infiltration in a dose-dependent fashion (Fig. 1g). Ten percent NaCl diet upregulated the amount of surface mucous cell mucin, suitable for *H. pylori* colonization, despite no increment of MUC5AC mRNA, while *H. pylori* infection itself had an opposing effect, stimulating transcription of MUC6 and increasing the amount of gland mucous cell mucin. High-salt diet, in turn, decreased the amount of gland mucous cell mucin, which acts against *H. pylori* infection by inhibiting the bacterial cell wall component [38]. Reduction of salt intake could thus be one of the most important chemopreventive methods for human gastric carcinogenesis [36].

Besides the salt itself, *H. pylori* infection and high intake of various traditional salt-preserved foods are regarded as risk factors for human gastric cancer. Indole compounds, such as indole-3-acetonitrile is contained in Chinese cabbage and converted to a mutagen, 1-nitrosoindole-3-acetonitrile (NIAN), with nitrite under acidic condition in the stomach. Administration of NIAN to Mongolian gerbils induced well to moderately differentiated adenocarcinomas under *H. pylori* infection. Such lesions were not induced in gerbils given NIAN alone or infection with *H. pylori* alone. Thus, endogenous carcinogens formed from nitrosation of indole compounds could be critical risk factors for human gastric cancer development under the influence of *H. pylori* infection. [53]

Prevention of gastric cancer by eradication of *H. pylori*

To clarify the effects of eradication of *H. pylori* on prevention of gastric cancer development in patients with chronic gastritis, Uemura et al. [125] conducted a nonrandomized *H. pylori* eradication trial in cases whose gastric cancer was removed by endoscopic resection, and suggested that *H. pylori* eradication might improve neutrophil infiltration and intestinal metaplasia in the gastric mucosa and inhibit the development of new carcinomas. A randomized controlled trial conducted in China revealed that the incidence of gastric cancer development at the population level was similar between participants receiving *H. pylori* eradication treatment and those receiving placebo over a period of 7.5 years [130]. However, in the subgroup of *H. pylori* carriers without precancerous lesions, eradication of *H. pylori* significantly decreased the development of gastric cancer [130]. In *H. pylori*-infected Mongolian gerbils treated with MNU, Shimizu et al. have provided direct evidence that *H. pylori* eradication may be useful as a prevention approach against gastric cancer [87]. The incidences of gastric cancers after curative treatment for *H. pylori* were thus significantly lower than without *H. pylori* eradication. For further evaluation, an experimental model with eradication in the early, middle, and late periods was studied using *H. pylori*-infected and MNU-treated Mongolian gerbils [70]. *H. pylori* infection was found to strongly enhance gastric carcinogenesis initiated with the chemical carcinogen, and following eradication at an early period, this effect was effectively reduced (Fig. 1h, i).

Reversibility of heterotopic proliferative glands induced by *H. pylori* infection

As WHO/IARC has mentioned in 1994 [30], it had to be clarified whether or not *H. pylori* itself was a carcinogen. Several studies based on histopathology showed no carcinomas in animals treated only with *H. pylori* infection [85–87, 95, 112]. However, two reports concluded that *H. pylori* infection alone can induce well-differentiated adenocarcinomas at very high incidences in the glandular stomach

of Mongolian gerbils [27, 128], while another study resulted in only one poorly differentiated adenocarcinoma [26]. The incidences and histological patterns of the lesions differed greatly in these three reports. After *H. pylori* infection, glands in the stomach of gerbils start to proliferate into the submucosa, disrupting the lamina muscularis mucosa. Resultant lesions, termed heterotopic proliferative glands (HPGs), frequently develop with *H. pylori* infection in the glandular stomach of infected Mongolian gerbils, with minimal dysplastic change of constituent cells [69]. HPGs often resemble differentiated or mucinous adenocarcinomas showing structural abnormality, but lacked obvious cellular atypia. Their characteristics are: (1) organized polarity of their component cells; (2) differentiation from gastric phenotype HPGs into intestinal phenotype HPGs with mature Paneth cells; (3) formation of large cystic dilatations containing mucin, often with calcification; (4) shedding of epithelial cells and necrosis at the tips of lesions; (5) high-grade inflammation with infiltration of inflammatory cells (neutrophils in acute phase and mononuclear cells in chronic phase); and (6) organized polarity of proliferating zones (Table 1 and Fig. 2) [69]. These features are quite different from those of well-differentiated adenocarcinomas, which are characterized by obvious cellular atypia. After eradication, HPGs are obviously reduced, and gastric lesions in mucosa also disappear with little evidence persisting of the former injury. Reversible HPGs are induced solely by *H. pylori* infection in this species, and our studies have shown they are related to severe gastritis, rather than being malignant in character. Eradication of *H. pylori* induces apoptosis and suppresses proliferation in HPGs of infected Mongolian gerbils, these lesions thus being apparently reversible through regulation of cell kinetics [6]. Thus, distinguishing reversible lesions from true neoplasms is necessary in investigating the relationship of *H. pylori* infection with gastric carcinogenesis in the Mongolian gerbil model [69]. Taking into account all the available data, we conclude that *H. pylori* is a strong promoter of gastric carcinogenesis rather than an initiator.

Chemoprevention of gastric cancer

COX-2 inhibitor

Overexpression of COX-2 has been shown to be associated with several cancers, including gastric and colorectal adenocarcinomas in mice models [48, 72, 73, 79, 101] (Fig. 1j). Furthermore, COX-2 selective inhibitors such as etodolac and celecoxib may have chemopreventive effects [22, 49] not only suppressing inflammation, but also causing regression of early-stage tumors [10, 81]. Therefore, there is a possibility that COX-2 inhibitors could be useful drugs for regression of remaining precancerous lesion and prevention of gastric cancer occurrence after *H. pylori* eradication.

Considering the human situation, eradication of *H. pylori* significantly lowers the development of metachronous gastric cancer [20]. However, there has not been any approach to prevent gastric carcinogenesis in extensive metaplastic gastritis, an *H. pylori*-negative precancerous lesion. Forty-seven patients with extensive metaplastic gastritis were selected based on endoscopic findings and serum pepsinogen test-positive and *H. pylori* antibody-negative conditions. Nonrandomized etodolac treatment (300 mg/day) was administered to 26 patients, while the remaining 21 were untreated up to 5 years. Five cancers developed in non-treated group significantly more than one case in the etodolac group. Long-term etodolac treatment effectively reduced metachronous cancer development in patients with extensive metaplastic gastritis, while it did not influence the extent of metaplastic gastritis. Regulation of COX-2 could be an effective chemoprevention of gastric cancer in the metaplastic gastritis. [134]

Table 1 Histological difference of heterotopic proliferative glands and well-differentiated adenocarcinoma

	Heterotopic proliferative glands (HPGS)	Well-differentiated adenocarcinoma
Distribution of glands	Dispersed	Compactly proliferated
Shape of glands	Large and cystic	Relatively small
Intracystic material	Eosinophilic, sometimes with calcification	Usually transparent
Lining epithelium	Shedding of epithelial cells with necrosis at the tip of the cyst	Fully lined with atypical cells.
Intestinalization	Frequent, sometimes with Paneth cells	Relatively infrequent
Stroma	Severe inflammatory cell infiltrates. Neutrophils in acute phase. Lymphocytes and plasma cell in chronic phase with lymphoid follicle formation.	Desmoplastic reaction
Cellular component	No atypia. Organized polarity. Partly degenerated.	Enlarged nuclei with increased chromatin. Loss of polarity.

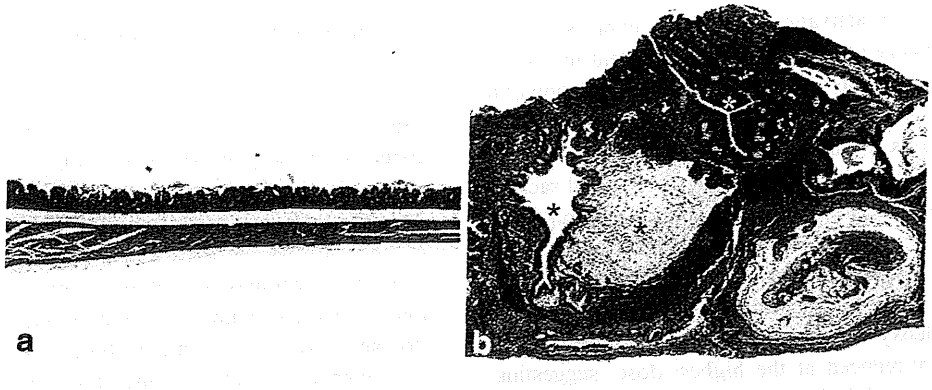

Fig. 2 Severe inflammatory response caused by long-term *H. pylori* infection in a Mongolian gerbil. **a** Normal gastric mucosa without *H. pylori* infection. **b** Heterotopic proliferative glands (HPGs) in the glandular stomach infected with *H. pylori* for 75 weeks. Proliferation and dilatation of gastric type gland (*red asterisk*) and intestinal metaplastic glands (*yellow asterisk*) with lymphoid follicle formation (*green asterisk*). Mucous lakes (*blue asterisks*) are developed with necrotic material (*blue asterisk on the right*). Hematoxylin and eosin staining

Oxygen radical scavenger

Recent epidemiological and experimental studies have demonstrated that consumption of certain natural products can lower gastric cancer risk in humans and animal models [120]. It has been also suggested that oxidative stress associated with inflammation plays an important role in gastric carcinogenesis as a mediator of DNA damage and carcinogenic compound formation [64]. Since the major determinant factor of gastric carcinogenesis is the severity of *H. pylori*-induced inflammation [7], the inhibition of *H. pylori*-induced inflammation and subsequent oxidative stress is a reasonable approach to prevent gastric cancer development.

To assess this hypothesis in the gerbil model, 4-vinyl-2,6-dimethoxyphenol (canolol), one of the most potent antioxidative compounds obtained from crude canola oil, was chosen to examine preventive effect of *H. pylori*-induced gastritis and gastric carcinogenesis [8]. The gerbils were subjected to *H. pylori*+MNU administration and were fed for 44 weeks with or without 0.1 % canolol. *H. pylori*-induced gastritis, expression of COX-2 and inducible nitric oxide synthase (iNOS), and increase in serum 8-hydroxy-2′-deoxyguanosine (8-OHdG) level were all attenuated in the canolol-treated groups. In addition, the incidence of gastric adenocarcinomas was markedly reduced. These data indicate that oxygen radical scavengers may suppress gastric inflammation and carcinogenesis in *H. pylori*-infected gerbils. Interestingly, the viable *H. pylori* count was not changed by the canolol-containing diet. Thus, the data point to the level of inflammation due to *H. pylori* rather than the existence of the bacteria as the determining factor is used.

Lignan, a plant-derived chemical

Another example of a chemopreventive agent is plant-derived lignan, a major group of plant compounds classified as phytoestrogens, which have attracted interest in recent years [1]. (+)-Syringarecinol, one of lignans contained in Japanese apricot, inhibited >90 % of the *H. pylori* motility at a concentration of 500 μg/mL and the IC50 value was 50 μg/mL *in vitro* [55]. Concentrated Japanese apricot significantly alleviated *H. pylori*-induced inflammation in Mongolian gerbils [75].

Since other lignans including arctiin, arctigenin, and nordihydroguaiaretic acid (NDGA) also inhibited proliferation and motility of *H. pylori in vitro* dose dependently, *in vivo* analyses were performed to assess preventive effect on *H. pylori*-associated gastritis and gastric cancer development in Mongolian gerbils using NDGA and arctigenin [115]. NDGA significantly decreased the incidence of gastric adenocarcinomas, the formation of intestinal metaplasia, and serum 8-OHdG levels. There were no differences in the titers of anti-*H. pylori* IgG or the expression of the *H. pylori*-specific *urease A* gene among all *H. pylori*-infected groups. These results suggest that NDGA might have suppressive effects on gastric carcinogenesis, with inhibitory effects on progression of gastritis and antioxidative activity rather than direct antimicrobial influence as the major mechanisms. Arctigenin, in contrast, failed to attenuate neoplasia in gerbils in spite of its potent suppressive effect on *H. pylori in vitro*. These results suggest the importance of *in vivo* animal experiments as well as *in vitro* analyses.

Nuclear factor-κB inhibitor

Nuclear factor-κB (NF-κB) plays a major role in host inflammatory responses and carcinogenesis and as such is an important drug target for adjuvant therapy. One of NF-κB inhibitors, caffeic acid phenethyl ester (CAPE), was analyzed on *H. pylori*-induced NF-κB activation in cell culture and chronic gastritis in Mongolian gerbils. In AGS human gastric cancer cells, CAPE significantly inhibited *H. pylori*-

stimulated NF-κB activation and mRNA expression of several inflammatory factors in a dose-dependent manner, and prevented degradation of IκB-α and phosphorylation of p65 subunit. To evaluate the effects of CAPE on *H. pylori*-induced gastritis, Mongolian gerbils were inoculated with *H. pylori*, fed diets containing 0.1 % CAPE, and sacrificed after 12 weeks. Infiltration of neutrophils and mononuclear cells and expression of NF-κB p50 subunit and phospho-IκB-α were significantly suppressed by CAPE treatment in the antrum of *H. pylori*-infected gerbils. Labeling indices for 5′-bromo-2′-deoxyuridine both in the antrum and corpus were markedly reduced at the highest dose, suggesting a preventive effect of CAPE on epithelial proliferation. Furthermore, in the pyloric mucosa, mRNA expression of inflammatory mediators including tumor necrosis factor-α (TNFα), interferon-γ, interleukin (IL)-2, IL-6, KC (IL-8 homologue), and iNOS was significantly reduced. These results suggest that CAPE has inhibitory effects on *H. pylori*-induced gastritis in Mongolian gerbils through the suppression of NF-κB activation, and may thus have potential for prevention and therapy of *H. pylori*-associated gastric disorders [117].

Statin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor

Statins are commonly used lipid-lowering drugs that reduce the risk of cardiovascular morbidity and mortality. Although recent studies have pointed to chemopreventive effects of statins against various cancers, their efficacy for gastric cancer is unclear. Thus, pitavastatin, a lipophilic statin, was examined on *H. pylori*-associated stomach carcinogenesis and gastritis using Mongolian gerbil and mouse models. The incidences of *H. pylori*-associated gastric adenocarcinomas and degrees of chronic gastritis were not decreased by pitavastatin compared with those of control values. Expression of IL-1β and TNFα mRNAs in the pyloric mucosa was markedly upregulated in pitavastatin-treated animals. Furthermore, in the *H. pylori*-infected groups, serum total cholesterol, triglyceride, and low-density lipoprotein levels were significantly increased by pitavastatin treatment, contrary to expectation. In the short-term study, *H. pylori*-infected gerbils and mice also showed significant upregulation of serum triglyceride levels by pitavastatin, whereas total cholesterol was markedly reduced and low-density lipoprotein exhibited a tendency for decrease in non-infected animals. These findings indicate pitavastatin to be ineffective for suppressing gastritis and chemoprevention of gastric carcinogenesis in *H. pylori*-infected gerbils. Our serologic results also suggest that the *H. pylori* infection and consequent severe chronic gastritis interfere with the cholesterol-lowering effects of pitavastatin [116].

Intestinal metaplasia and intestinalization of gastric cancer

Human gastric-and-intestinal-mixed-type intestinal metaplasia: aberrant expression of transcription factors

H. pylori plays a causative role in the development of chronic atrophic gastritis and intestinal metaplasia as well as stomach neoplasms. Although intestinal metaplasia has long attracted attention as a putative preneoplastic lesion for stomach cancers, its clinicopathologic significance has yet to be clarified in detail. Using gastric and intestinal epithelial cell markers, intestinal metaplasia was here divided into two major types: a gastric-and-intestinal-mixed type and a solely intestinal type [31, 121]. In the former, gastric and intestinal phenotypic markers appeared not only within the glandular but also at the same cellular level [66]. Furthermore, neuroendocrine cells also showed intestinalization along with their exocrine counterparts [74]. The molecular mechanisms of intestinal metaplasia include the ectopic expression of CDX1/CDX2 [2, 56, 88], OCT-1 [32], and members of the Erk pathway. Suppression of the expression of gastric transcription factors such as SOX2 [121], genes that are involved in the Sonic hedgehog pathway, and RUNX3 [71], a tumor suppressor gene, could be additional relevant alterations. The expression of PDX1 may also be associated with pseudopyloric gland metaplasia and intestinal metaplasia [84]. Detailed analysis of gene regulation may shed light on the molecular bases of gastric lesions, leading to strategies for chemoprevention [122].

Intestinal metaplasia in experimental animals

Experimentally, a phenotypic shift from gastric-and-intestinal-mixed-type intestinal metaplasia to solely intestinal type could be clearly observed on sequential observation of rat stomach treated with X-rays [138]. In Mongolian gerbil model, gastric-and-intestinal-mixed-type intestinal metaplasia was found to appear first, followed by the solely intestinal type with appearance of Paneth cells during the overall course of *H. pylori* infection in the HPGs [69].

Summarizing these data, it was suggested that intestinal metaplasia might be caused by the gradual intestinalization of stem cells from the gastric-and-intestinal-mixed type to the solely intestinal type.

Intestinalization of adenocarcinoma

Human gastric adenocarcinomas have been classified by Lauren into two major groups, the "intestinal" and "diffuse" types [45], which respectively nearly correspond to the "differentiated" and "undifferentiated" types [65, 91]. However, the above-mentioned classifications are inadequate for

studies of histogenesis of gastric carcinomas and phenotype expression at the cellular level because they confuse intestinal phenotypic cancer cells with a "diffuse" structure and gastric phenotypic ones with the "intestinal" type of Lauren [113]. The phenotypic expression of gastric cancer cells of each histological type can be clearly classified into gastric and intestinal epithelial cell types by immunohistochemistry using gastric and intestinal epithelial cell markers such as MUC5AC, MUC6, MUC2, and villin, independent of the histological type (Table 2) [16, 37, 40, 57–59, 98, 113, 135]. Gastric cancers comprising epithelial elements presenting only gastric or intestinal phenotypic expression are classified as of gastric, or intestinal phenotypes, respectively. Those with both gastric type cells and intestinal type cells have a gastric-and-intestinal-mixed phenotype, while the remainder exhibiting neither are grouped as a null type [37, 57, 98, 108, 135].

It has been suggested that "intestinal" type carcinomas arise in intestinalized mucosa, whereas the "diffuse" type develops from the gastric mucosa proper [11, 13, 45, 65] and a number of authors have proposed that intestinal metaplasia is a precancerous lesion for differentiated type gastric cancers [11, 12, 63, 89, 136, 137]. However, this hypothesis is based on morphological similarities between cancers and intestinal metaplasia in the surrounding mucosa and previous studies on phenotypic expression of each intestinal metaplastic or stomach cancer cells have pointed to several contradictions [16, 24, 37, 44, 57, 59, 83, 105–108]. In both experimental animals and humans, gastric cancers at early stages, independent of the histological type, mainly consist of gastric type cancer cells, and a phenotypic shift from gastric to intestinal phenotypic expression is clearly observed with progression [4, 104–106, 109, 113, 131]. When the phenotypic classification is compared in early and advance stomach cancers, shift from gastric toward intestinal, and then null phenotypes was observed [57, 59] (Fig. 4a). Regarding the histogenesis of gastric cancers, it would be logical if those originating from intestinal metaplasia should be of the intestinal type. Even if the phenotypic expression of intestinal type gastric cancer cells is unstable, the incidence of intestinal type cancer cells in small gastric cancers should then be higher than in large gastric cancers, the opposite from the actual case, and expression in fact appears to be stable [106]. In addition, on analysis of microsatellite instability, Tamura et al. [102] found that the majority of differentiated adenocarcinomas of the stomach may develop through a de novo pathway from the viewpoint of the microsatellite alterations. Endoh et al. [17] also clarified that genetic backgrounds of differentiated type tumors were quite different among cellular phenotypes. Thus, it has been proposed that intestinal metaplasia is important not as a precancerous lesion but as a paracancerous phenomenon [16, 54, 108]. Therefore, many questions remain regarding its pathogenesis as well as the actual relationship to gastric cancers.

Gastric and intestinal phenotypic expression in stomach cancers in carcinogen-treated and *H. pylori*-infected Mongolian gerbils

The *H. pylori*-infected *Mongolian gerbil* has been established as an appropriate animal model for studies of stomach cancer development. However, there have hitherto been no data on the phenotypic classification of glandular stomach cancers in *H. pylori*-infected and non-infected Mongolian gerbils. Thus, the phenotypes of 50 and six advanced glandular stomach cancers in *H. pylori*-infected and non-infected gerbils, respectively, were analyzed using several gastrointestinal epithelial phenotypic markers. The lesions were divided phenotypically into 21 gastric, 24 gastric-and-intestinal mixed, four intestinal and one null types, with 90.0 % of the lesions harboring gastric elements and

Table 2 The phenotypic markers for gastrointestinal epithelial cells

Tissue types	Cell types	Markers for human tissues	Markers for Mongolian gerbils
Gastric	Foveolar	MUC5AC	Human gastric mucin (HGM)
		Periodic acid-Schiff staining (PAS)	PAS
	Pyloric	MUC6	PCS
		Paradoxical concanavalin A staining (PCS)	
Intestinal	Goblet	MUC2	Small intestinal mucinous antigen (SIMA)
		Alcian blue	Alcian blue
		CDX2	
	Absorptive	Villin	Intestinal type alkaline phoshate (I-ALP)
		CD 10	CD 10
		CDX2	

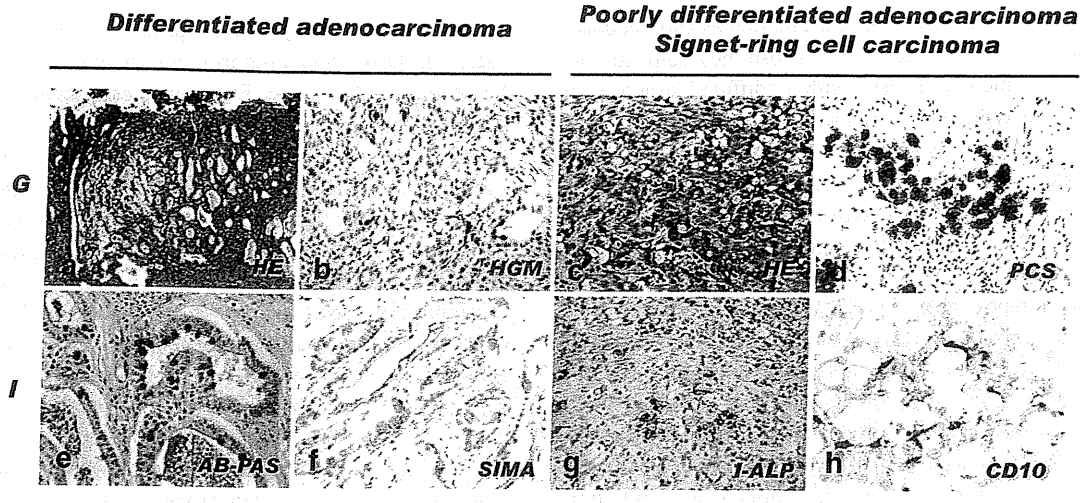

Fig. 3 Adenocarcinomas with gastric or intestinal phenotypic expression in MNU-induced *H. pylori*-infected Mongolian gerbils. **a**, **b**, **e**, **f** Differentiated adenocarcinoma. **c**, **d**, **g**, **h** Poorly differentiated adenocarcinoma/signet-ring cell carcinoma. **a**, **b**, **c**, **d** Gastric type (*G, colored red*). **e**, **f**, **g**, **h** Intestinal type (*I, colored blue*). **a**, **c** HE staining. **d** Paradoxical concanavalin A staining (*PCS*). **e** Alcian blue-periodic acid-Schiff (*AB-PAS*) staining. **b**, **f**, **g**, **h** Immunohistochemistry with antibodies against human gastric mucin (*HGM*) (**b**), small intestinal mucinous antigen (*SIMA*) (**f**), and intestinal-alkaline phosphatase (*I-ALP*) (**g**), and CD10 (**h**) (Table 2). Reproduced from Ref. [60]with permission

56.0 % demonstrating intestinal phenotypic expression in *H. pylori*-infected Mongolian gerbils. All six lesions were classified as gastric type in non-infected gerbils. There was no clear correlation with the presence of intestinal metaplasia in surrounding mucosa. Most of the advanced adenocarcinomas retain a gastric cellular phenotype, suggesting intestinal metaplasia as a paracancerous phenomenon rather than a premalignant condition. On the other hand, more than half of the cancers harbor intestinal phenotypes only in *H. pylori*-infected group compared with none in uninfected cancers with statistical significance. *H. pylori* infection was considered to trigger intestinalization of both stomach cancers and non-neoplastic mucosa (Figures 3 and 4b, Table 2) [60].

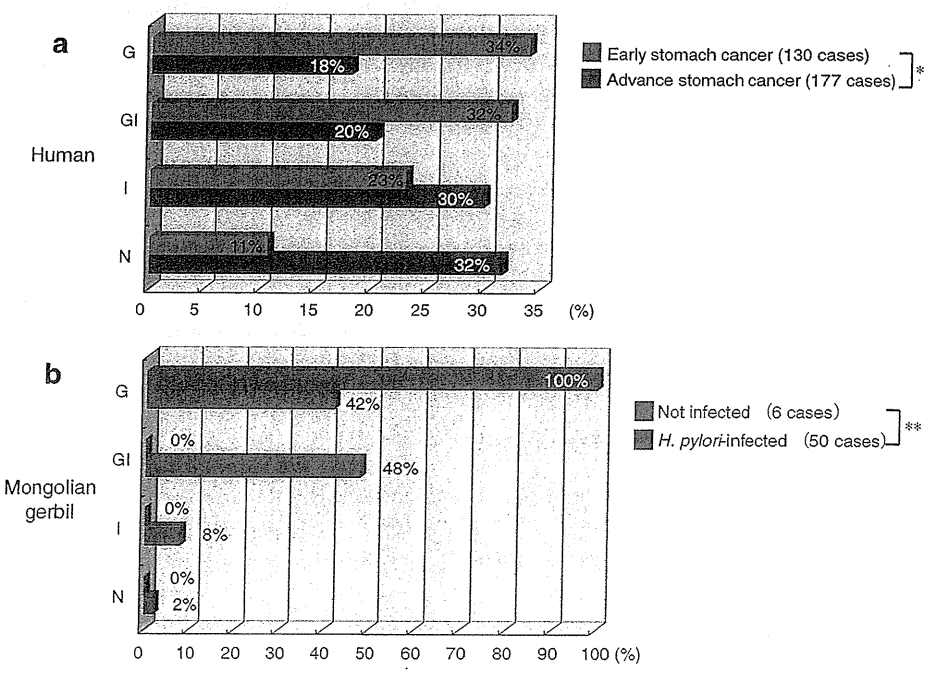

Fig. 4 Intestinalization of stomach cancers with *H. pylori* infection: **a** Phenotypic classification of human stomach cancers in early [59] (*orange bars*) and advanced cases [57] (*Burgundy bars*). Transition is apparent from gastric (*G*), gastric-and-intestinal mixed (*GI*), intestinal (*I*), and toward null (*N*) phenotypes in advanced cancers compared to early cases. *$P<0.0001$, χ^2 test for trend. **b** Phenotypic classification of stomach cancers in Mongolian gerbils. *H. pylori* infection (*red bars*) induced intestinalization of stomach cancers, whereas non-infected animals developed those with only gastric phenotype (*blue bars*)[59]. **$P<0.02$, χ^2 test for trend,

Conclusions

H. pylori infection is a very important factor for gastric carcinogenesis in human stomach. Since the discovery of *H. pylori*, the Mongolian gerbil has become one of the most important model animal for analysis of stomach carcinogenesis and trials of chemoprevention. As revealed by the experimental models described above, it was clarified that *H. pylori* itself only causes chronic inflammation and acts as promoter in stomach carcinogenesis. Further analyses need be conducted to determine mechanisms of carcinogenesis and contribute to chemopreventive methods.

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