RESEARCH COMMUNICATION

Intestinal Phenotypes of Stomach Cancers Arising after *Helicobacter pylori* Eradication in Carcinogen-treated Mongolian Gerbils

Tsutomu Mizoshita[1,2], Tetsuya Tsukamoto[1,*], Takeshi Toyoda[1], Hisayo Ban[1], Koji Nozaki[1], Masae Tatematsu[1]

Abstract

Aims: We have previously demonstrated the importance of gastric and intestinal phenotypic expression for the histogenesis of stomach cancer. However, the phenotypes of stomach cancers arising after *Helicobacter pylori* (Hp) eradication have hitherto remained unclear. We therefore examined a series of lesions occurring after Hp eradication in the Mongolian gerbil (MG) model. Methods: Totals of 6 and 20 advanced glandular stomach cancers were evaluated in Hp–eradicated and Hp–infected MGs treated with N-methyl-N-nitrosourea (MNU-MGs), using several gastrointestinal epithelial phenotypic markers. The lesions were divided phenotypically into gastric (G type), gastric-and-intestinal mixed (GI type), intestinal (I type), and null (N type) phenotypes. Results: All 4 differentiated type lesions in Hp-eradicated MNU-MGs were classified as G type, while both of the undifferentiated lesions exhibit the GI type. In Hp-infected MNU-MGs, the lesions were classified as 10 G, 8 GI, and 2 I types, with undifferentiated type lesions having more intestinal phenotypic expression than their differentiated counterparts ($P < 0.01$). Conclusions: Our data suggest that the differentiated stomach cancers exhibit the G type in Hp-eradicated MNU-MGs, suggesting that a kind of non-neoplastic G type gland may be precancerous. Intestinalization may still occur, especially in undifferentiated stomach cancers, even if Hp eradication is successful.

Key Words: Stomach cancer - *Helicobacter pylori* - Mongolian gerbil - eradication - phenotype

Asian Pacific J Cancer Prev, **8**, 267-271

Introduction

The effects of *Helicobacter pylori* (Hp) treatment on prevention of stomach cancer development in patients with chronic gastritis remain a topic of discussion (Tatematsu et al., 2005). Uemura et al (1997) previously described the possibility that Hp eradication may prevent stomach cancer development in cases whose primary cancerous lesion was removed by endoscopic resection. In addition, Hp eradication appeared to reduce the risk of developing stomach cancer in patients with gastric ulcers (Take et al., 2005). On the other hand, the incidence of stomach cancer development at the population level in China was found to be similar in participants receiving Hp eradication treatment and in those receiving placebo over a period of 7.5 years (Wong et al., 2004). Importantly, however, eradication did significantly decrease the risk in the subgroup of Hp carriers without precancerous lesions. In other words, those in which neoplasia has progressed beyond a certain point may continue to be at risk. Regarding this 'point of no return', analyses of stomach cancers occurring after Hp eradication are important for elucidation of the histogenesis.

The Hp-infected Mongolian gerbil (MG) has been established as an appropriate animal model for study of stomach cancer development, with induction of adenocarcinomas by N-methyl-N-nitrosourea (MNU) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as carcinogens (Hirayama et al., 1996; Sugiyama et al., 1998; Tatematsu et al., 1998; Shimizu et al., 1999). Using this animal model, Shimizu et al. provided direct evidence that Hp eradication may be useful as a prevention approach against stomach cancer (Shimizu et al., 2000). Eradication of infection results in curtailment of enhancing effects, particularly in early stages of associated inflammation (Nozaki et al., 2003; Cao et al., 2004). However, in some MGs, stomach cancer development was observed after successful Hp eradication, similar to the human case (Nozaki et al., 2003). Regarding the histogenesis of human stomach cancer, we have previously demonstrated the importance of gastric phenotypic expression, using gastric and intestinal epithelial cell markers such as MUC5AC, MUC6, MUC2, and villin (Mizoshita et al., 2003; Tatematsu et al., 1990; Tatematsu et al., 2003; Tsukamoto et al., 2005). We also

[1] *Division of Oncological Pathology, Aichi Cancer Center Research Institute,1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan*
[2] *Department of Internal Medicine and Bioregulation, Nagoya City University Medical School, Nagoya, Japan *For correspondence: Phone: +81-52-762-6111 x7062 / Fax: +81-52-764-2972 E-mail: ttsukamt@aichi-cc.jp*

have provided direct evidence that most advanced adenocarcinomas retain a gastric cellular phenotype in the glandular MG stomach (Mizoshita et al., 2006). However, there have hitherto been no data on the phenotypic classification of the glandular stomach cancers occurring after Hp eradication in MGs. Since evaluation of the phenotype is very important with reference to histogenesis, the present study of glandular stomach carcinomas in Hp-eradicated and Hp-infected MGs treated with MNU (MNU-MGs) was conducted using several gastrointestinal epithelial phenotypic markers.

Materials and Methods

Experimental design

MGs received MNU in their drinking water at a concentration of 30 ppm during alternate weeks, for a total of 5 weeks exposure. At experimental week 10, Hp was inoculated into MGs. Animals then underwent eradication of Hp with lansoprazol, amoxicillin, and clarithromycin at weeks 35 or 55, or were maintained without eradication. The precise experimental design was as previously described (Nozaki et al., 2003). In the present study, 20 lesions were examined in the Hp–infected MNU-MGs that experienced no eradication, "Hp-infected MNU-MGs". Six lesions were evaluated in MNU-MGs that underwent eradication of Hp, "Hp-eradicated MNU-MGs" (Figure 1 and Table 1). No stomach cancers were observed in Hp-infected animals not exposed to MNU (Nozaki et al., 2003; Tatematsu et al., 2005).

Samples and Tissue Collection

Totals of 6 and 20 advanced glandular stomach cancers in Hp–eradicated and Hp–infected MNU-MGs (Nozaki et al., 2003) were histopathologically classified according to the Japanese Classification of Gastric Carcinomas (Japanese Gastric Cancer Association, 1998). Tumor tissues and adjacent non-neoplastic mucosa were fixed in 4% paraformaldehyde in phosphate-buffered saline

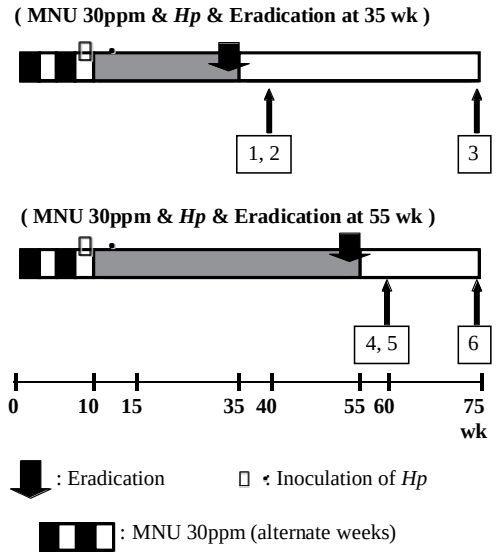

Figure 1. Experimental Design for Hp-eradicated MGs Treated with MNU. The numbers (1-6) reflect those for stomach cancers in Table 1.

Table 1. Six Glandular Stomach Cancers in Hp-eradicated MGs treated with MNU

Number	Group	Detected	Histology	Phenotype
1	MNU+Hp+Erad at 35wk	40wk	well	G
2	MNU+Hp+Erad at 35wk	40wk	sig	GI
3	MNU+Hp+Erad at 35wk	75wk	well	G
4	MNU+Hp+Erad at 55wk	60wk	well	G
5	MNU+Hp+Erad at 55wk	60wk	well	G
6	MNU+Hp+Erad at 55wk	75wk	por	GI

Hp, Helicobacter pylori; MG, Mongolian gerbil; MNU, N-methyl-N-nitrosourea;well, well differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet-ring cell carcinoma; G, gastric phenotype; GI, gastric-and-intestinal mixed phenotype

(PBS) (pH 7.2), Bouin's solution, 10% buffered formalin, or 95% ethanol containing 1% acetic acid (Cao et al., 2002; Cao et al., 2004; Nozaki et al., 2002a), sectioned at 5 mm, and stained with hematoxylin and eosin (H&E) for histological examination. The analyzed cancers all demonstrated invasion into the muscularis propria (mp), the subserosa (ss), or the serosa and the peritoneal cavity (se), sometimes with involvement of adjacent organs (si).

Immunohistochemistry and mucin histochemistry

Immunohistochemical staining was carried out with antibodies against the following antigens: human gastric mucin (HGM) (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK); small intestinal mucinous antigen (SIMA) (Novocastra); intestinal type alkaline phosphatase (I-ALP) (kindly provided by Dr. Kazuyuki Hirano, Department of Pharmaceutics, Gifu Pharmaceutical University, Gifu, Japan); and CD10 (Novocastra) (Mizoshita et al., 2006; Tsukamoto et al., 2006) The precise procedures for immunohistochemical demonstration were as previously described. (Mizoshita et al., 2006; Tatematsu et al., 2003; Tatematsu et al., 2005; Tsukamoto et al., 2006). With regard to gastric and intestinal phenotypic markers, we used normal gastric mucosa and normal ileum as respective positive controls. Briefly, 4 mm-thick consecutive sections were deparaffinized and hydrated through a graded series of alcohols. After inhibition of endogenous peroxidase activity by immersion in 3% H2O2 / methanol solution, antigen retrieval was achieved by heating in 10 mM citrate buffer (pH 6.0) in a microwave oven for 10 minutes at 98˚C. Then, sections were incubated with primary antibodies. After thorough washing in phosphate-buffered saline (PBS), they were next incubated with biotinylated secondary antibodies, and then with avidin-biotin horseradish peroxidase complexes (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA). Finally, immune complexes were visualized by incubation with 0.01% H2O2 and 0.05% 3,3'- diaminobenzidine tetrachloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin.

For mucin histochemistry, we adopted paradoxical concanavalin A staining (PCS) for identifying class III mucins in mucous neck and pyloric gland cells (Katsuyama and Spicer, 1978; Katsuyama et al., 1985). We also performed Alcian blue-periodic acid Schiff (AB-PAS) for identifying gastric surface mucous cells with mucin stained

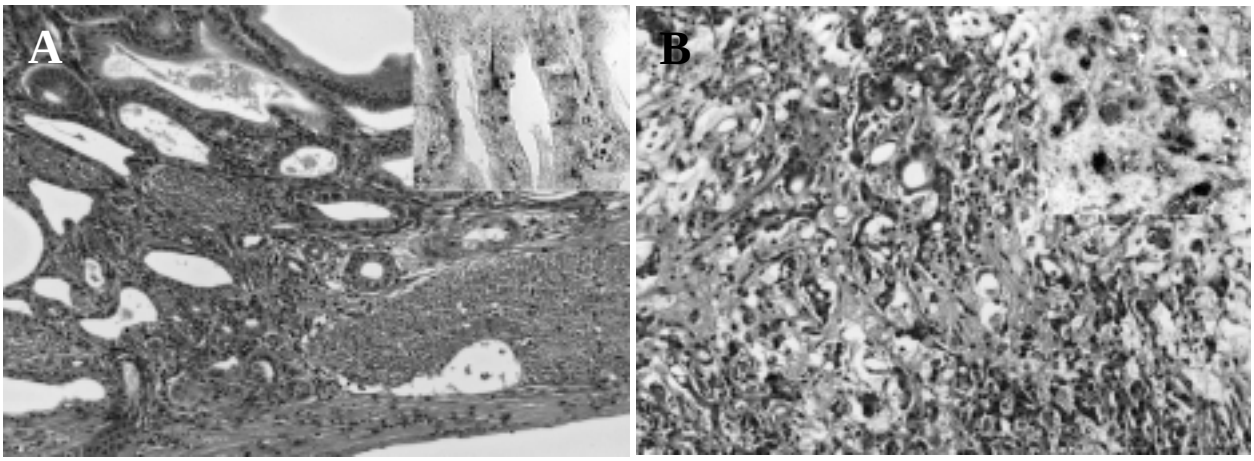

Figure 2. Histopathological and Immunohistochemical Analysis of Adenocarcinomas. (A) Well differentiated adenocarcinoma developing after eradication. H&E x125. Inset: HMG is positive and the lesions is judged as a G type, x200. (B) Poorly differentiated adenocarcinoma in a non-eradicated animal. H&E x125. Inset: SIMA antigen is present and the lesion is considered as I type x250X

red and goblet cells stained blue (Nozaki et al., 2002b; Tsukamoto et al., 2004).

Two independent pathologists (T.M. and T.T.) judged the histology and immunohistochemical and mucin histochemical staining of the phenotypic markers. With regard to the phenotypic markers, the results of immunohistochemical and mucin histochemical staining were evaluated in terms of the percentage of positively stained cancer cells, with 10% and above considered positive, as previously described (Mizoshita et al., 2006; Tatematsu et al., 2003; Tatematsu et al., 2005).

Classification of cancers

Tumors were classified phenotypically with reference to the expression patterns of a battery of phenotypic markers (Mizoshita et al., 2006). Glandular stomach cancers in which more than 10% of the section area consisted of at least one gastric or intestinal epithelial cell phenotype were classified as gastric (G type) or intestinal (I type) phenotype cancers, respectively. Those which showed both gastric and intestinal phenotypes were classified as gastric-and-intestinal mixed phenotype (GI type) cancers, while those showing neither gastric nor intestinal phenotype expression were grouped as null (N type).

Results

Phenotypic classification of glandular stomach cancers in Hp–eradicated and Hp–infected MNU-MGs

We evaluated the totals of 14 differentiated and 12 undifferentiated glandular stomach cancers phenotypically using several epithelial phenotypic markers (Figure 2 and Table 2). In 6 Hp-eradicated MNU-MGs, the lesions were divided phenotypically into 4 G, 2 GI, and 0 I types. All 4 differentiated type lesions were classified as G type, while both undifferentiated type lesions exhibited the GI type. In 20 Hp-infected MNU-MGs, the lesions were divided phenotypically into 10 G, 8 GI, and 2 I types. Of 10 differentiated type cancers, all lesions (100%) had gastric phenotypic expression, while 1 (10%) also harbored intestinal elements. Of 10 undifferentiated type cancers, 8 (G+GI types, 80%) had gastric phenotypic expression, while 9 (GI+I types, 90%) harbored intestinal elements. Seven of 10 lesions (70%) were classified as GI type. The undifferentiated type lesions had more intestinal phenotypic expression compared with those of differentiated type in the Hp-infected MNU-MGs (Table 2, $P < 0.01$). No N type lesions were observed.

Discussion

Our data provide clear evidence, for the first time, that all of the differentiated glandular stomach cancers arising after Hp eradication exhibit a G phenotype in MNU-MGs. We have previously argued that non-neoplastic G type glands may be precancerous because stomach cancers at early stages consist mainly of gastric epithelial phenotypic cancer cells in man (Mizoshita et al., 2004; Tatematsu et al., 1990; Tatematsu et al., 2003), mice (Tatematsu et al., 1994), rats (Tatematsu et al., 1980; Tatematsu et al., 2005), and MGs (Mizoshita et al., 2006). In humans, to our knowledge, there are no reports of phenotypic expression of stomach cancers occurring after Hp eradication. We therefore here focused on the origin of stomach cancers after Hp-eradication in MGs.

In the present study, the undifferentiated carcinomas exhibited a GI type in the glandular stomach of Hp–eradicated MNU-MGs, in line with our earlier report of lesions in MGs (Mizoshita et al., 2006). Hp infection may trigger intestinalization of stomach cancers and non-neoplastic mucosa (Mizoshita et al., 2006) and we and others have also reported that a phenotypic shift from G

Table 2. Histologic and Phenotypic Classification for the Tumours

		Histologic		Phenotypic		
		G	GI	I	N	Total
(+)	Differentiated	4	0	0	0	4
	Undifferentiated	0	2	0	0	2
	Subtotal	4	2	0	0	6
(-)	Differentiated	9	1	0	0	10
	Undifferentiated	1	7	2	0	10
	Subtotal	10	8	2	0	20

G, gastric phenotype; GI, gastric-and-intestinal mixed phenotype; I, intestinal phenotype; N, null phenotype.

through GI to the I type occurs in accordance with increasing depth of invasion in stomach cancers in humans and in animal models (Bamba et al., 2001; Tatematsu et al., 2003). In Hp-infected MGs, this phenotypic shift also appears in heterotopic proliferative glands (HPGs) of the glandular stomach (Nozaki et al., 2002b). Taking into account the previous reports and our present data, we consider that the shift from G through GI to I type may occur with progression to undifferentiated stomach cancers in MGs, even after successful Hp eradication.

Hp eradication may reduce the risk of developing stomach cancer in patients with Hp infection (Take et al., 2005; Uemura et al., 1997; Wong et al., 2004) as well as in the MG model (Shimizu et al., 2000). Eradication at early stage of inflammation might be particularly effective for preventing Hp-related stomach carcinogenesis (Nozaki et al., 2003), the frequency of gastritis, erosion, and intestinal metaplasia in eradication therapy groups being markedly reduced as compared with the control and vehicle groups (Hirayama et al., 2002; Nozaki et al., 2002b). We thus consider that the Hp-eradicated MG model is useful for analysis of the so-called"point of no return".

In conclusion, our present data suggest that differentiated adenocarcinomas exhibit a G type in the glandular stomach of Hp-eradicated MNU-MGs, suggesting a derivation from non-neoplastic G type. Intestinalization may occur with progression to undifferentiated stomach cancers, even after successful Hp eradication.

Acknowledgments

The authors thank Dr. Malcolm A. Moore for revision of the scientific English language. This study was supported in part by a Grant-in-Aid for the Third-term Comprehensive 10-year Strategy for Cancer Control, a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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