photoreaction decreased considerably; this well matched results obtained from fluorescence microscopy. When we calculated the rate, during first 6 s, of the fluorescence increase with 0.25 μM DCFH, it was 20 times slower than with 5 μM DCFH.

The photoreaction of DCFH in cells is not specific to DMSO-HL60 cells, which generate $O_2^{\cdot-}$ upon stimulation. We also observed DCFH photoreaction in undifferentiated HL60 cells that do not generate $O_2^{\cdot-}$ upon stimulation (data not shown). Setsukinai et al. [10] have also clearly shown that DCFH photoreaction occurs in HLE cells. Most reports of experiments using DCFH as an ROS indicator show that concentrations at 5–50 μM were used [1–4,10,11,16–20]. At such concentrations, however, DCFH photoreaction proceeds so rapidly that we consider that such concentrations of DCFH are especially unsuitable for fluorescence microscopic observation. We suggest that, according to the experimental conditions, before analytical observations, the DCFH concentration should be adjusted to a level low enough to minimize photoreaction to negligible levels. Conditions affecting DCFH photoreaction speed also include the microscope magnification setting. Cell type, intracellular materials [7], light source, and microscope lenses should be taken into account.

DCFH is also used for flowcytometric analysis of ROS in cells [1,2,4]. Because the fluorescence intensities of the excited cells (laser-irradiated cells) are measured immediately after irradiation, this technique may not be affected to the same extent by DCFH photoreactivity. Even so, for accurate evaluation, we consider it better to determine the optimal concentration of DCFH.

DCFH is a popular and widely used fluorescent probe for ROS. Our results show that DCFH is easily photo-activated, thus special care must be taken when using DCFH for evaluation. Here we have proposed ways to considerably suppress the photoreaction so that DCFH is useful for spectrofluorometric and fluorescence microscopic analyses.

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RESISTANT *HELICOBACTER PYLORI*

Helicobacter pylori-induced enlarged-fold gastritis is associated with increased mutagenicity of gastric juice, increased oxidative DNA damage, and an increased risk of gastric carcinoma

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Abstract

Background and Aim: The severe inflammation, increased cell proliferation and marked acid inhibition observed in subjects with *Helicobacter pylori*-associated enlarged-fold gastritis suggest that enlarged-fold gastritis may be a risk factor for gastric carcinoma. The purpose of the present study was to determine whether a relationship exists between enlarged-fold gastritis and gastric carcinoma.

Methods: One hundred and thirty-five *H. pylori*-positive patients with early gastric carcinoma and 141 age- and sex-matched *H. pylori*-positive controls without gastric carcinoma were involved in the study. The widths of gastric body folds were measured by double-contrast radiographs. The mutagenicity of gastric juice was assayed using the Ames test and *Salmonella typhimurium* TA-98 or TA-100 with S9-mix. Levels of 8-hydroxydeoxyguanosine (8-OHdG) in gastric mucosa were examined using high-performance liquid chromatographic-electrochemical detection.

Results: An upward shift in the distribution of gastric fold widths in *H. pylori*-positive patients with early gastric carcinoma was found. Enlarged-fold gastritis (fold width ≥5 mm) was observed in 81% of the patients with gastric carcinoma, compared with 46% of *H. pylori*-positive controls. The odds ratio for gastric carcinoma increased with increasing fold width to a maximum of 35.5 in persons with fold width ≥7 mm. The prevalence of diffuse-type early gastric carcinoma in the body region increased with increasing fold width. The mutagenicity of gastric juice from the patients with enlarged-fold gastritis was significantly higher than that in *H. pylori*-negative controls and *H. pylori*-positive patients without enlarged folds. Mucosal 8-OHdG levels in the body region of patients with enlarged-fold gastritis were significantly higher than in *H. pylori*-negative controls and *H. pylori*-positive patients without enlarged-fold gastritis. Eradication of *H. pylori* significantly decreased the mutagenicity of gastric juice and 8-OHdG levels in the gastric mucosa from patients with enlarged-fold gastritis.

Conclusion: A significant association is suggested between enlarged-fold gastritis and gastric carcinoma.

Key words: 8-hydroxydeoxyguanosine, enlarged-fold gastritis, *Helicobacter pylori*, mutagenicity, oxidative DNA damage.

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INTRODUCTION

Gastric body folds are generally considered to be enlarged when the widest fold is ≥5 mm, based on the Sydney system, as well as on previous studies.[1–5] Enlarged folds in the gastric body are often observed during radiographic or endoscopic examination of adults. Enlarged gastric folds are associated with a variety of diseases, including hypertrophic gastritis, Ménétrier's diseases, Zollinger–Ellison syndrome, primary gastrin cell hyperplasia, carcinoma, and lymphoma.[6,7] It has recently been suggested that *Helicobacter pylori*-induced gastritis may be a possible cause of enlarged gastric folds.[8,9] The authors have previously reported that the eradication of *H. pylori* improved the inflammation of the gastric mucosa and decreased fold width in *H. pylori*-positive subjects with enlarged folds, that is, enlarged-fold gastritis.[4] In agreement with the authors' findings, an improvement in gastric-fold thickening after eradication of *H. pylori* has been reported by Stolte *et al.*[10] and Avunduk *et al.*[11] Thus, *H. pylori*-associated enlarged-fold gastritis constitutes a subgroup of the population with *H. pylori* infections.

It is now a generally accepted fact that *H. pylori* is an important cause of gastric carcinoma. The International Agency for Research on Cancer classified *H. pylori* as a group 1 carcinogen.[12] A recent prospective study in Japan demonstrated that gastric carcinoma developed in persons infected with *H. pylori*, but not in uninfected persons.[13] Because the great majority of *H. pylori*-associated gastritis subjects never develop neoplasias, the logical next step would be to identify the factors that precisely determine risk among the *H. pylori*-infected population. A long-recognized critical factor in *H. pylori*-induced gastric carcinogenesis is gastric acid secretion. In subjects with gastritis confined to the antral region, gastric acid secretion is normal or increased. Such subjects have an increased risk of duodenal ulceration, but little association with an increased risk of gastric carcinoma has been found. In contrast, gastritis involving the body region leads to hypochlorhydria and an increased risk of gastric carcinogenesis. In earlier studies,[4,5,14,15] the authors have reported that enlarged-fold gastritis is accompanied by a massive infiltration of inflammatory cells, and by profound production of interleukin-1β and hepatocyte growth factor, which decrease gastric acid secretion and increase the rate of proliferation of the gastric epithelial cells. Thus, among an *H. pylori*-infected population, subjects with enlarged-fold gastritis may have a relatively increased risk of gastric carcinogenesis.

In the present study, the prevalence of enlarged gastric fold in patients with gastric carcinoma was examined. In an effort to obtain molecular evidence for an association between enlarged-fold gastritis and gastric carcinogenesis, the authors also examined the mutagenicity of gastric juice and the formation of oxidative DNA damage in gastric mucosa from the subjects with enlarged-fold gastritis.

METHODS

Measurement of width of gastric body fold

One hundred and thirty-five *H. pylori*-positive patients with early gastric carcinoma (125 men, 10 women; age range 29–71 years, mean 54 years) and 141 age- and sex-matched *H. pylori*-positive controls without gastric carcinoma (130 men, 11 women; age range 29–69 years, mean 53 years) were analyzed (Table 1). Early gastric carcinoma was pathologically diagnosed, as defined by the Japanese Gastroenterological Society, by the growth of a malignant tumor confined to the mucosa and submucosa of the stomach regardless of the presence or absence of metastatic disease in the perigastric lymph node. Advanced gastric carcinoma, in which carcinoma cells are deeply infiltrated beyond the submucosal layer, was not included in the present study, to avoid possible thickening of gastric folds as a result of the invasion of carcinoma cells. All patients and controls were members of health insurance organizations in Osaka, and participated in mass screening programs for gastric carcinoma using radiographs.

Between 1990 and 2000, 135 *H. pylori*-positive patients with early gastric carcinoma were enrolled. *Helicobacter pylori* infection was identified by histological examination, a rapid urease test (CLO; Delta West, Bentley, Australia), and a serological evaluation. Patients in whom any of these assays were positive were considered to be *H. pylori*-positive. Two hundred and forty healthy subjects without gastric carcinoma were examined for *H. pylori* infection using HM-CAP anti-*H. pylori* immunoglobulin G EIA (Enteric Products, Westbury, NY, USA), and 141 subjects were positive; these subjects were age- and sex-matched with carcinoma patients. Informed consent for testing *H. pylori* infection was obtained from all subjects. In each person, the widths of the gastric body folds were measured by a computerized image analyzer on double-contrast radiographs of the appropriately distended stomach in the

Table 1 Clinical features of the 135 patients and 141 controls studied

	Patients	Controls
Age in years (range)	54 (29–71)	53 (29–69)
Sex		
Male	125	130
Female	10	11
Tumor site		
Antrum/prepylorus	44	
Body	90	
Fundus	1	
Tumor size in mm (range)	23 (5–85)	
Histological type		
Intestinal	66	
Diffuse	69	
Depth of invasion		
Mucosal	75	
Submucosal	60	

supine position; the carcinoma lesions were masked and the measurer had no knowledge of the disease status of any of the patients. The median value of randomly measured widths in 11 different locations was regarded as the gastric body fold width in each person. To obtain reproducible results, a standardized protocol was employed in all patients by using a fixed amount of effervescent powder and contrast medium: 150 mL of 180% barium sulfate with 5 g of effervescent powder.

Measurement of mutagenicity of gastric juice

Fifteen patients with enlarged-fold gastritis (13 men, two women; age range 30–60 years, mean 45 years), seven *H. pylori*-positive patients without enlarged folds (six men, one woman; age range 30–50 years, mean 43 years) and six *H. pylori*-negative patients with dyspeptic symptoms (four men, two women; age range 39–62 years, mean 46 years) were examined. Informed consent was obtained from all subjects and the investigation was approved by the Research Ethical Committee of Osaka University Medical School. Patients were divided into three groups after double-contrast radiographs and endoscopic examination as described above. The diagnosis of *H. pylori* infection was made based on a positive culture (Department of Chemotherapy, Pharmacological Research Laboratory, Fujisawa Pharmaceutical, Osaka, Japan) and/or by the result of a urease test (CLO; Delta West) using biopsy specimens from the antrum and the greater curvature of the upper portion of the gastric body.[16,17]

After overnight fasting and discarding the residual gastric juice in the stomach, new gastric juice was collected for 30 min using a sterile gastric tube. The gastric juice (0.1 mL) was assayed for mutagenicity using the Ames test and *Salmonella typhimurium* TA-98 or TA-100 with S9-mix. An equal volume of physiological saline was used as a control. The mutagenicity of the gastric juice was evaluated as the total number of revertant colonies obtained from the total amount of the gastric juices. Twelve patients with enlarged-fold gastritis received triple therapy of clarithromycin, 400 mg twice daily; amoxycillin 1500 mg three times daily; and lansoprazole 30 mg once daily for 2 weeks, and mutagenicity was re-examined in 12 patients 2 months after eradication of *H. pylori*.

Measurement of 8-hydroxydeoxyguanosine levels in the gastric mucosa

8-Hydroxydeoxyguanosine (8-OHdG), which causes DNA mutation *in vivo* and *in vitro*, represents a sensitive marker of oxidative DNA damage. The 8-OHdG levels in gastric mucosa were examined in 13 patients with enlarged-fold gastritis (10 men, three women; age range 23–58 years, mean 45 years), nine *H. pylori*-positive patients without enlarged folds (six men, three women; age range 25–68 years, mean 50 years) and 11 *H. pylori*-negative patients with dyspeptic symptoms (eight men, three women; age range 27–60 years, mean 43 years). Informed consent was obtained from all subjects and the investigation was approved by the Research Ethical Committee of Osaka University Medical School.

Patients were divided into three groups after double-contrast radiographs and endoscopic examination as described above. Four biopsy samples were taken from the antrum and the corpus at endoscopy in all subjects. The samples were immediately frozen in liquid nitrogen and kept at −80°C until the 8-OHdG level was measured. In nine patients with enlarged-fold gastritis, the samples were taken before and 2 months after eradication of *H. pylori*. DNA extraction and digestion were carried out inside an anaerobic incubator EAN-140 (Tabai Espec, Osaka, Japan) to prepare samples under oxygen-free conditions. DNA was extracted from the samples using proteinase K, lysis buffer, and 99.5% ethanol without phenol. The DNA was hydrolyzed to deoxynucleosides by digestion with nuclease P1, followed by alkaline phosphatase. The 8-OHdG levels were detected using high-performance liquid chromatographic-electrochemical detection and expressed as the ratio of 8-OHdG/10^5 deoxyguanosine.[18]

Histological evaluation of inflammation in the gastric mucosa

In addition to 8-OHdG levels, infiltration of mononuclear and polymorphonuclear cells was also estimated in 19 *H. pylori*-positive subjects. Biopsy specimens were fixed with 10% phosphate-buffered formalin. Thin sections of paraffin-embedded tissues were stained with hematoxylin and eosin for evaluation of mononuclear and polymorphonuclear infiltration. In the biopsy specimens, infiltration of mononuclear and polymorphonuclear cells were graded as follows: 0 = none or minimal, 1 = mild, 2 = moderate, and 3 = severe.[2] When the score of biopsy specimen was 0 or 1, the degree of polymorphonuclear infiltration and mononuclear infiltration was considered to be mild. When the score was 2 or 3, it was considered to be severe.

Statistical analysis

Statistical analyses were performed using the Mann–Whitney *U*-test and Student's *t*-test. Statistical significance was assigned for any *P*-value <0.05. Data were shown as mean ± SEM.

RESULTS

Comparison of gastric body fold width

Distribution of the gastric body fold widths is presented in Figure 1. A significant upward shift of width distribution in *H. pylori*-positive patients with early gastric carcinoma was found ($P < 0.01$). An enlarged gastric fold (fold width ≥ 5 mm) was observed in 81% of the patients with gastric carcinoma, compared with 46% of

H. pylori-positive controls. A fold width in excess of 7 mm was observed in 18% of patients with gastric carcinoma, compared with only 1.4% of *H. pylori*-positive controls. The odds ratio for gastric carcinoma increased with increasing fold width to a maximum of 35.5 in persons with a fold width of ≥7 mm (Table 2). In all cases of gastric carcinoma, invasion of carcinoma cells was restricted to within the submucosal layer, and the absence of fold thickening as a result of the massive invasion of carcinoma cells and/or fibrosis was histologically confirmed in the resected specimens. Furthermore, radiographs taken more than 1 year before the diagnosis of carcinoma were available in 97 of 135 cases with early gastric carcinoma, and no significant changes in fold widths were found (5.7 ± 0.1 mm compared with 5.8 ± 0.1 mm, previous and final fold widths, respectively, $P = 0.42$). Thus, an association between enlarged folds and gastric carcinoma is not merely a secondary phenomenon resulting from carcinoma development.

Histology and location of gastric carcinomas

Gastric carcinomas can be divided into two types according to Lauren's classification: intestinal and diffuse types. An association between the gastric-body-fold width and the histology of gastric carcinoma was found (Fig. 2). The prevalence of diffuse-type early gastric carcinoma increased from 31% to 71% of the total cases with increasing fold width. Mean fold widths were significantly different between intestinal- and diffuse-type gastric carcinomas (5.1 ± 0.1 mm compared with 5.9 ± 0.2 mm, respectively, $P < 0.01$). Thus, enlarged-fold gastritis may be a disorder that may precede the development of diffuse-type gastric carcinoma. A similar association between gastric-body-fold width and the location of the gastric carcinoma was found. Forty-four gastric carcinomas were located in the distal stomach (antrum/pylorus), 90 in the body and one in the gastric fundus. An increased fold width reduced the prevalence of gastric carcinomas in the distal stomach and increased those in the body (Fig. 2), which is well known to be a preferred location of diffuse-type gastric carcinoma. Thus, a correlation exists between the fold width and the incidence of diffuse-type carcinoma in the body (Fig. 3).

Production of mutagens in the stomach

Because enlarged-fold gastritis is characterized by a severe inflammation in the body region, endogenous inflammation-related mutagens may be the missing

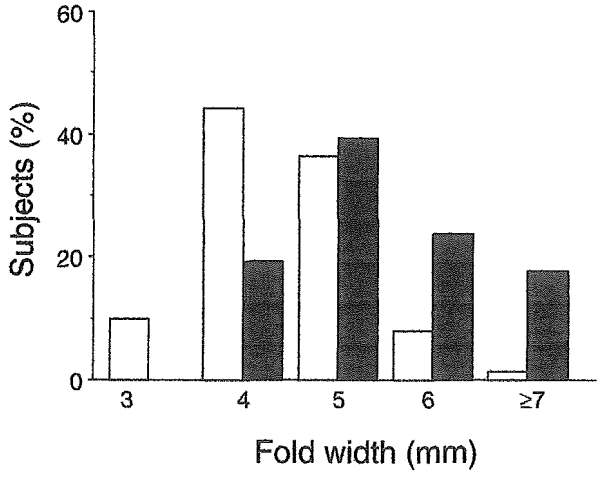

Figure 1 Distribution of gastric fold width among *Helicobacter pylori*-positive subjects (■) with or (□) without early gastric carcinoma. The data are expressed as a percentage of subjects in each fold width.

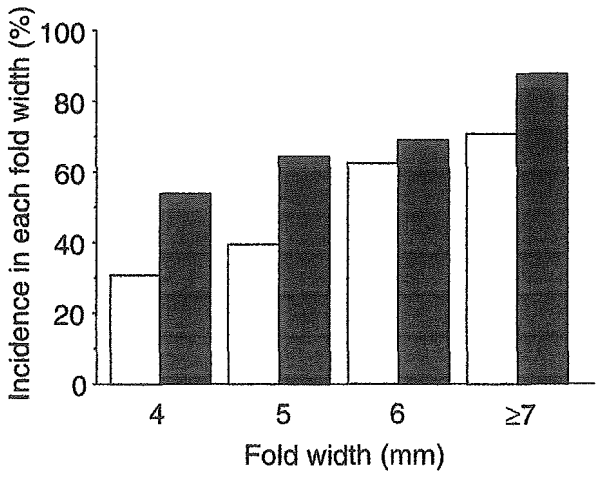

Figure 2 Prevalence of (□) diffuse-type carcinoma and (■) body carcinoma as a function of gastric fold width.

Table 2 Odds ratio for gastric carcinoma as a function of fold width

Fold width (mm)	No. of cases	No. of controls	Odds ratio (95% confidence interval)
≤4	26	77	1.0
5	53	51	3.1 (1.7–5.5)
6	32	11	8.6 (3.8–19.6)
≥7	24	2	35.5 (7.9–160.8)

link between enlarged-fold gastritis and carcinogenesis. The authors estimated and compared the mutagenicity of gastric juice in controls and patients with or without enlarged-fold gastritis. As shown in Figure 4, the mutagenicity determined by the Ames test using TA-98 or TA-100 in patients with enlarged-fold gastritis was significantly higher than that in *H. pylori*-negative controls and *H. pylori*-positive patients without enlarged folds. Furthermore, the mutagenicity was significantly decreased after the eradication of *H. pylori* (Fig. 5).

Oxidative DNA damage in the gastric mucosa

As an indication of oxidative DNA damage, 8-OHdG levels in the gastric mucosa were examined in the gastric antral and body regions. In patients with enlarged-fold gastritis, the 8-OHdG levels in the antral mucosa were significantly higher than in *H. pylori*-negative controls (Fig. 6). However, no significant difference in antral 8-OHdG levels was found between non-enlarged- and enlarged-fold gastritis. In contrast, the 8-OHdG levels in the body mucosa from patients with enlarged-fold gastritis were significantly higher than in *H. pylori*-negative controls and *H. pylori*-positive patients without

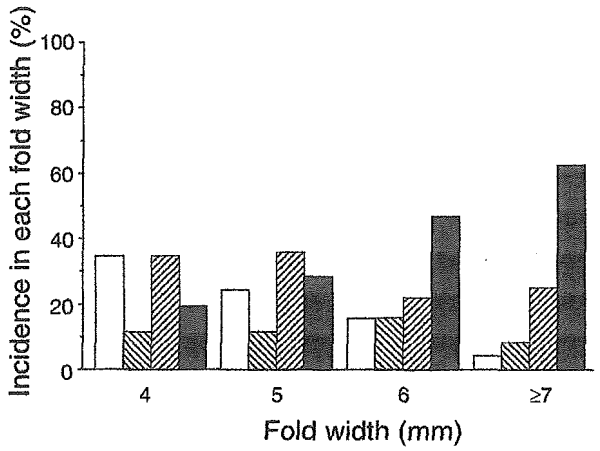

Figure 3 Histological type and location of early gastric carcinoma as a function of fold width. (□) Intestinal type in the distal stomach, (▨) diffuse type in the distal stomach, (▧) intestinal type in the body, (■) diffuse type in the body.

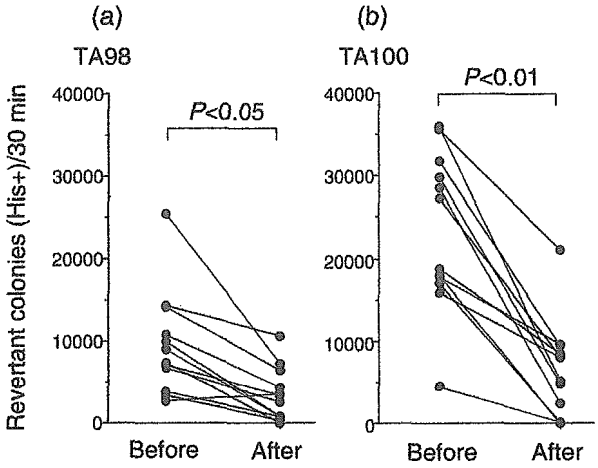

Figure 5 Changes in the mutagenicity of gastric juice from patients with enlarged-fold gastritis 2 months after eradication of *Helicobacter pylori*.

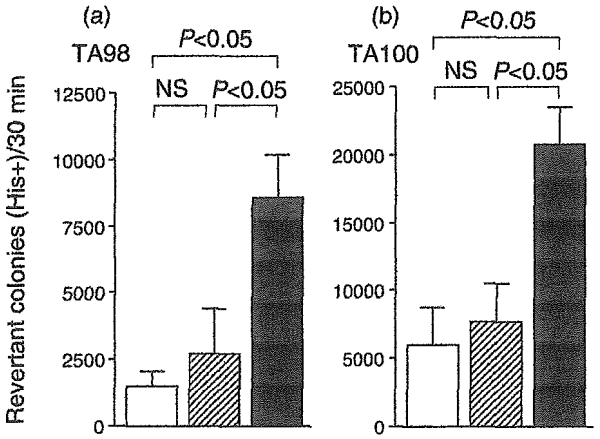

Figure 4 Mutagenicity of gastric juice assayed using Ames test and *Salmonella typhimurium* strain (a) TA-98 or (b) TA-100. The data are expressed as mean ± SEM. NS, not significant. (□) *Helicobacter pylori*-negative controls ($n = 6$), (▨) *H. pylori*-positive without enlarged fold ($n = 7$), (■) enlarged-fold gastritis ($n = 15$).

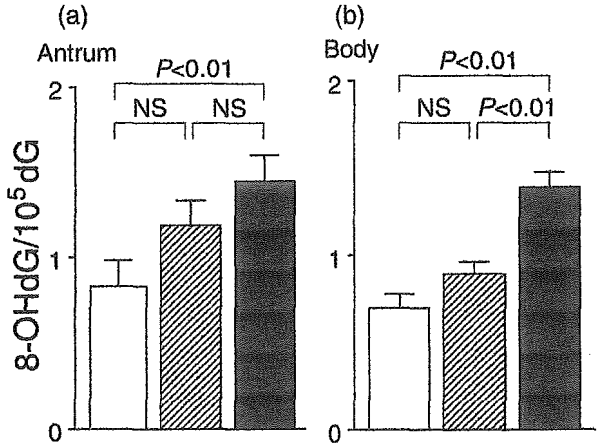

Figure 6 8-Hydroxydeoxyguanosine (8-OHdG) content of mucosal DNA samples from gastric antrum or body. The data are expressed as mean ± SEM. NS, not significant. (□) *Helicobacter pylori*-negative controls ($n = 11$), (▨) *H. pylori*-positive without enlarged fold ($n = 9$), (■) enlarged-fold gastritis ($n = 13$).

enlarged-fold gastritis. Eradication of *H. pylori* significantly decreased the 8-OHdG levels in both the antral and the body mucosa of patients with enlarged-fold gastritis (Fig. 7).

Inflammatory cell infiltration

The authors have previously reported that mononuclear and polymorphonuclear infiltrates in the body mucosa, as well as in the antral mucosa, of patients with enlarged-fold gastritis were significantly more severe than those in *H. pylori*-negative subjects. Inflammatory cell infiltration was more severe in the body mucosa of patients with enlarged-fold gastritis than in *H. pylori*-positive patients without enlarged folds, although these data were not statistically significant.[14] In the present study, the relationship between the degree of inflammatory cell infiltration and the 8-OHdG content of gastric mucosal DNA among *H. pylori*-infected patients was examined. As shown in Figure 8, the degree of polymorphonuclear infiltration was significantly correlated with mucosal 8-OHdG levels in both the antral and the body regions. While severe infiltration of mononuclear cells seems to increase mucosal 8-OHdG content, these findings were not statistically significant (Fig. 9).

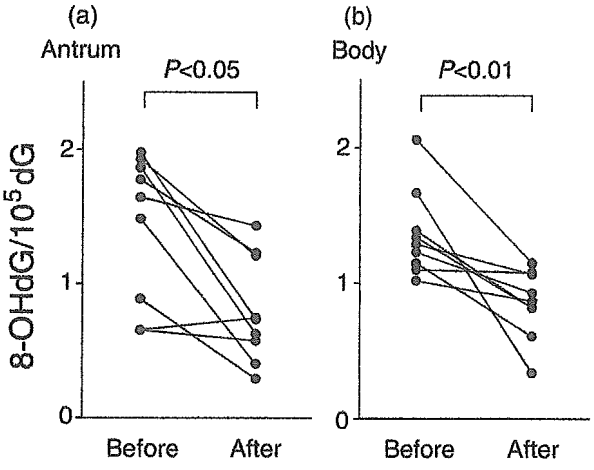

Figure 7 Changes in 8-hydroxydeoxyguanosine (8-OHdG) levels in patients with enlarged-fold gastritis 2 months after the eradication of *Helicobacter pylori*.

DISCUSSION

The present study has shown that patients with early gastric carcinoma showed an increased prevalence of enlarged-fold gastritis. This suggests that enlarged-fold gastritis is associated with an increased risk of gastric carcinoma and that a careful examination should be made to detect gastric carcinoma.

Gastric carcinomas can be divided into two types according to Lauren's classification: intestinal and diffuse types. The epidemiology of gastric carcinoma suggests that these two types develop through distinct causal pathways. Patients with intestinal-type carcinoma are typically characterized by old age and tumor location in the lower third of the stomach, with a male predominance, whereas patients with diffuse-type carcinoma are younger, with the tumor location in the middle third of the stomach. Intestinal-type carcinomas frequently show atrophy and intestinal metaplasia of the

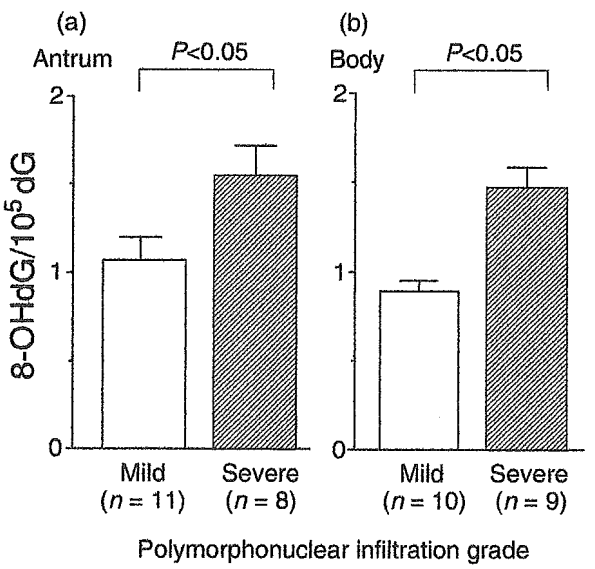

Figure 8 Relationship between polymorphonuclear infiltration and 8-hydroxydeoxyguanosine (8-OHdG) content of gastric mucosal DNA in *Helicobacter pylori*-infected patients. The data are expressed as mean ± SEM.

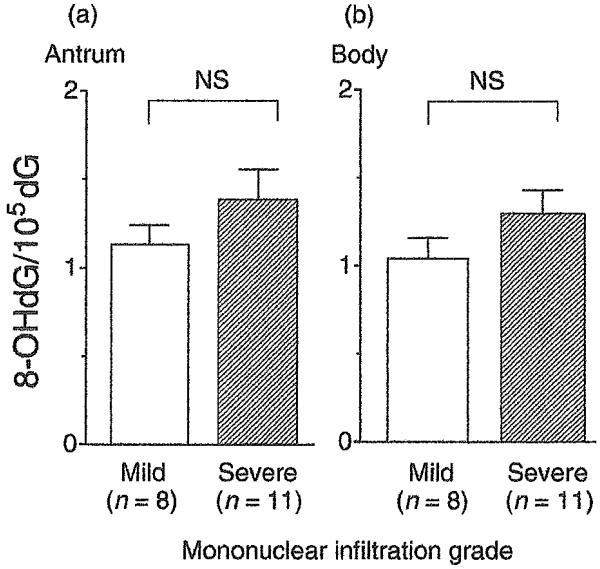

Figure 9 Relationship between mononuclear infiltration and 8-hydroxydeoxyguanosine (8-OHdG) content of gastric mucosal DNA in *Helicobacter pylori*-infected patients. The data are expressed as mean ± SEM. NS, not significant.

surrounding non-neoplastic gastric mucosa. In contrast, diffuse-type carcinomas display such lesions less frequently. Thus intestinal- and diffuse-type gastric carcinoma may be epidemiologically distinct. Interestingly, in the present study, the thicker the body folds, the higher was the incidence of diffuse-type carcinoma in the gastric body. If the histogenesis of diffuse-type carcinoma is still somewhat obscure, the intestinal-type confirms the sequence: atrophic gastritis → intestinal metaplasia → dysplasia → neoplasia. In patients with enlarged-fold gastritis, mucosal atrophy and intestinal metaplasia were less frequent in the body mucosa, while this was not true for the antral mucosa.[14] This condition may result in a preference for the development of diffuse-type carcinoma in the gastric body during the progression of mucosal atrophy in patients with enlarged-fold gastritis.

A plausible hypothesis for the missing link between *H. pylori* infection and gastric carcinogenesis involves endogenous inflammation-related mutagens, such as oxygen free radicals. *Helicobacter pylori*-related gastritis is accompanied by an increased oxygen free radical formation and peroxidative damage.[19–21] The authors have previously reported that patients with enlarged-fold gastritis had extensive polymorphonuclear and mononuclear cells, particularly macrophages, with *H. pylori* colonization in their gastric body mucosa. Polymorphonuclear cells and macrophages produce oxygen free radicals that could cause DNA damage to the adjacent cells.[22] Accumulation of oxidative DNA damage could lead to gene modifications of gastric epithelial cells that are mutagenic or carcinogenic. Gastric carcinoma patients showed significantly higher levels of 8-OHdG, the main DNA modifying agent produced by oxygen free radicals, in their tumor-adjacent tissues and tumor tissues than in normal tissues.[23] The present study showed that mutagenicity of the gastric juice of patients with enlarged-fold gastritis was significantly increased compared with *H. pylori*-negative controls and *H. pylori*-positive patients without enlarged folds. The authors further demonstrated an increase in the 8-OHdG content of mucosal DNA in patients with enlarged-fold gastritis. These results support the view that enlarged-fold gastritis is a risk factor for gastric carcinoma.

Baik *et al.* measured the 8-OHdG content in gastric mucosa from pediatric patients with or without *H. pylori* infection,[24] and showed that increased levels of 8-OHdG were observed in patients with *H. pylori* infection, compared with *H. pylori*-negative controls. The 8-OHdG content of gastric mucosal DNA is correlated with the disease activity of gastritis,[25] and is decreased after the eradication of *H. pylori*.[26,27] In the present study, 8-OHdG levels in the body mucosa from patients with enlarged-fold gastritis were significantly higher than in *H. pylori*-negative controls and *H. pylori*-positive patients without enlarged-fold gastritis. Increased levels of mucosal 8-OHdG were found to correlate with the degree of infiltration of polymorphonuclear cells. While 8-OHdG levels in the antral mucosa of *H. pylori*-infected patients were significantly higher than in *H. pylori*-negative controls, no significant difference in antral 8-OHdG levels between non-enlarged- and enlarged-fold gastritis was found. These findings suggest that the body mucosa of patients with enlarged-fold gastritis has an increased risk for gastric carcinoma among *H. pylori*-infected subjects. This may explain the increased prevalence of gastric body carcinoma with increasing fold width.

In conclusion, the present study has demonstrated that enlarged-fold gastritis may be a major risk factor for gastric carcinoma among *H. pylori*-infected persons. Enlarged folds, severe inflammation, marked acid inhibition, increased mutagen production, and increased 8-OHdG content observed in patients with enlarged-fold gastritis are all reversible after the eradication of *H. pylori*. The authors propose that *H. pylori*-infected persons with enlarged-fold gastritis are a potential population for the prevention of gastric carcinoma via the use of antibiotics.

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Frequent retention of heterozygosity for point mutations in *p53* and *Ikaros* in *N*-ethyl-*N*-nitrosourea-induced mouse thymic lymphomas

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Abstract

In agreement with Knudson's two-hit theory, recent findings indicate that the inactivation of tumor suppressor genes is not only mediated by the loss of function but also by the dominant-negative or gain-of-function activity. The former generally accompanies loss of a wild-type allele whereas in the latter a wild-type allele is retained. *N*-Ethyl-*N*-nitrosourea (ENU), which efficiently induces point mutations, reportedly leads to the development of tumors by activating *ras* oncogenes. Little is known about how ENU affects tumor suppressor genes and, therefore, we examined ENU-induced mutations of *p53* and *Ikaros* in thymic lymphomas and compared these with mutations of K*ras*. In addition, loss of heterozygosity was examined for chromosome 11 to which both *p53* and *Ikaros* were mapped. The frequency of point mutations in *p53* and *Ikaros* was 30% (8/27) and 19% (5/27), respectively, comparable to that observed in K*ras* (33%: 9/27). In total, 14 of the 27 thymic lymphomas examined (52%) harbored mutations in at least one of these genes. One *Ikaros* mutation was located at the splice donor site, generating a novel splice isoform lacking zinc finger 3, *Ik (F3del)*. Interestingly, 90% (10/11) of the tumors with point mutations retained wild-type alleles of *p53* and *Ikaros*. Sequence analysis revealed that the most common nucleic acid substitutions were T > A (4/8) in *p53*, T > C (4/5) in *Ikaros* and G > A/T (8/9) in K*ras*, suggesting that the spectrum of mutations was gene dependent. These results suggest that point mutations in tumor suppressor genes without loss of the wild-type allele play an important role in ENU-induced lymphomagenesis.

Keywords: ENU; Heterozygous mutation; *p53*; *Ikaros*

1. Introduction

Tumor suppressor gene function can be inactivated either by point mutations or by deletion of both the alleles, known as Knudson's "two-hit" hypothesis [1,2]. In

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an examination of colon cancers, Vogelstein and colleagues found that the most prevalent pattern of *p53* mutations was a point mutation in one allele concomitant with the loss of the other allele [3]. On the other hand, several lines of evidence suggest that given types of p53 act as oncogenes in a dominant manner in the presence of a wild-type allele [3,4]. Since p53 functions as a tetrameric transcription factor, mutant p53 lacking DNA-binding ability is thought to inhibit the function of wild-type p53 [5,6]. These lines of evidence indicate that negative dominancy adds a new perspective to our understanding of tumor suppressors. However, little data are available on oncogenic changes in other tumor suppressor genes.

Ikaros is a tumor suppressor gene for T and B cell leukemogenesis in both the mice and humans [7–10]. Ikaros is a Kruppel-type zinc finger transcription factor involved in lineage commitment and differentiation in the lymphoid cells. Heterozygous mice expressing a short Ikaros isoform lacking the DNA-binding zinc fingers show aberrant T cell differentiation and develop thymic lymphomas within 3 months after birth [11]. Biochemical analysis has revealed that Ikaros forms homodimers or heterodimers with other Ikaros family members such as Aiolos or Helios [12,13]. Thus, short Ikaros isoforms lacking DNA-binding domains function in a dominant-negative fashion against wild-type Ikaros. Similar to p53, one might predict that Ikaros mutants containing lesions in the DNA-binding domain would affect wild-type Ikaros in a dominant-negative manner. We previously demonstrated that short Ikaros isoforms lacking the DNA-binding domain are expressed in radiation-induced mouse thymic lymphomas [14,15]. These lymphomas frequently retain a wild-type allele, suggesting dominant-negative interactions. Point mutations of *Ikaros* were observed in other lymphomas that had lost a wild-type allele, consistent with Knudson's two-hit theory [14–17].

N-Ethyl-*N*-nitrosourea (ENU) is a hyper-mutable alkylating agent that efficiently induces point mutations [18,19]. Treatment of mice with ENU leads to a high incidence of thymic lymphoma. Mutation of K*ras* genes is more frequently observed in ENU-induced lymphomas than in radiation-induced lymphomas, suggesting that oncogene activation plays an important role in ENU-induced lymphomagenesis [20–23]. In contrast, a genome-wide survey of loss of heterozygosity (LOH) showed a significantly lower LOH rate in ENU-induced lymphomas than in radiation-induced lymphomas [14]. ENU also induces point mutations within the *Aprt* locus but these lesions do not occur by deletion/recombination [24,25]. Therefore, it is less likely that inactivation of tumor suppressor genes contributes to ENU-induced lymphomagenesis. As mentioned above, however, point mutations of *p53* without accompanying LOH have occasionally been reported [3].

The aim of this study was to examine the rate and spectrum of mutations in *Ikaros* and *p53* tumor suppressor genes associated with LOH and to determine the contribution of tumor suppressor genes to ENU-induced tumorigenesis. We found that the mutation rates of *Ikaros* and *p53* are comparable to that of K*ras* and that, in contrast to radiation-induced lymphomas, ENU-induced lymphomas retain a wild-type allele of these genes. Thus, we conclude that heterozygous point mutations of tumor suppressor genes significantly contribute to ENU-induced lymphomagenesis.

2. Materials and methods

2.1. Mice and tumor induction

B6C3F1 mice were purchased from Charles River, Kanagawa, Japan. Induction of thymic lymphomas by ENU (Nakali, Japan) was as described previously [26]. Briefly, thymic lymphomas were induced in female mice that were a cross between thymic lymphoma-susceptible C57BL/6 and thymic lymphoma-resistant C3H strains. Five-week-old mice received 400 ppm ENU in their drinking water for 8 weeks. Mice were observed daily until moribund after which they were sacrificed under ether anesthesia and autopsied. Thymic tissue was then removed, weighed and prepared for serological and molecular analyses [15]. All the animal experiments were conducted in compliance with the institutional guidelines for the care of laboratory animals.

2.2. Antibodies and immunofluorescence analysis

FITC-conjugated anti-CD8, anti-cKit, anti-CD44, and anti-TCRαβ, and PE-conjugated anti-CD4, anti-IL2, anti-CD3, and anti-TCRγδ were purchased from Pharmingen (San Diego, CA). After dispersing

the thymus until single cells were obtained, 10^6 cells were resuspended in 50 μl PBS supplemented with 1% FBS and FITC and/or PE-conjugated antibodies at an optimal dilution for 30 min on ice. Control levels of fluorescence were determined after staining with the appropriate isotype-specific Igs. The relative fluorescence intensity of 10,000 cells in each sample was measured using FACScan and was analyzed with Cell Quest software (Becton Dickinson, Mountain View, CA).

2.3. Western blot analysis of p53 and Ikaros in thymocytes

Western blotting was performed as described previously [15] with the following minor modifications: after transfer of proteins from the gel, the membrane was blocked and probed with polyclonal antisera against p53 (1:5000; Santa Cruz Biotechnology Inc., Santa Cruz, CA) or Ikaros (1:5000). Blots were washed and then incubated with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:50,000; Amersham Pharmacia Biotech Inc., Piscataway, NJ). Ikaros proteins were detected using Super Signal Dura Chemiluminescent Substrate (Pierce, Rockford, IL) and were analyzed using a Kodak Digital analyzer (Kodak, Rochester, NY).

2.4. LOH analysis

Genomic DNA was extracted from the tumors according to the standard techniques. The LOH region on chromosome 11 was defined using the following 10 polymorphic loci: *D11Mit71*, *D11Mit62*, *D11Mit2*, *D11Mit204*, *D11Mit150*, *D11Mit77*, *D11Mit20*, *Acrb*, *D11Mit14* and *D11Mit203*. Single-strand length polymorphism (SSLP) analysis was performed as previously described [14].

2.5. Mutation analysis of Ikaros, p53 and Kras

Total RNA extracted from lymphoma cells was reverse-transcribed to obtain cDNA as described previously [15]. Expression status of *Ikaros* was determined using the primers *Ikaros* 2F and 7R and *Gapd* (sense and antisense) as a control. Mutations in the genes *p53*, *Ikaros* and K*ras* were assessed in all thymic lymphomas. The primer sets for the amplification of the entire *Ikaros* cDNA (exons 1–7) were *Ikaros* 1F and 7R(3) [15]. Sequencing to determine mutations in the splicing junction between exons 3 and 4, or exons 4 and 5 was performed using PCR fragments amplified from genomic DNA using LA Taq (Takara Shuzo Co., Shiga, Japan). The primer set for the junction between exons 3 and 4 was sense 3F(2), 5′-AGG CAT TCG ACT TCC TAA CG-3′ and antisense 4R, 5′-GGT TGC ACT GGA AAG GCC GT-3′. The primer set for the junction between exons 4 and 5 was sense 4F, 5′-GGT GAA CGG CCT TTC CAG TGC-3′ and antisense 5R, 5′-TGT TTA TAG CTC CGG CCA CAA T-3′. PCR products were purified using Quantum Prep Freeze N Squeeze DNA extraction spin columns (Bio-Rad, Hercules, CA), concentrated by ethanol precipitation and suspended in distilled water prior to sequencing. The primer set for the amplification of *p53* cDNA (between exons 5 and 9 containing the DNA-binding domain) was sense *p53* F(1), 5′-CCC TGT CAT CTT TTG TCC CTT-3′ and antisense *p53* R(1), 5′-CGC GGA TCT TGA GGG TGA AAT-3′. For the *ras* gene, the first, second and third exons of K*ras* were analyzed by single-strand conformation polymorphism (SSCP) analysis of genomic DNA followed by TA-cloning sequencing as described previously [27].

Sequencing was performed using the BigDye Terminator Cycle Sequencing FS Ready Reaction kit for both the direct and TA-cloning sequencing (PE Applied Biosystems, Foster City, CA) and the products were analyzed on an ABI PRISM 310 DNA Sequencer (PE Applied Biosystems). For direct sequencing, at a minimum, both forward and reverse sequences were determined for each sample. For TA-cloning, at least five clones from each sample were analyzed. Any mutations detected in cDNA were confirmed by analysis of the corresponding genomic DNA to exclude the possibility of false positives resulting from the errors during PCR amplification.

3. Results

3.1. Development of thymic lymphomas and phenotypic staging

Five-week-old mice were treated with ENU for 8 weeks. Thymic lymphomas developed in 75% (45/60) of the mice. The average latency period was 7 ± 4 weeks after the end of the treatment and the lifes-

pan of treated mice was 20 ± 4 weeks. These values are much shorter than those observed for lymphomas induced by X-irradiation (latency, 16 ± 3 weeks after treatment; lifespan, 22 ± 3 weeks) ($p < 0.01$) [14]. The mass of ENU-induced thymic lymphomas was 520 ± 34 mg compared with 486 ± 36 mg for X-ray-induced tumors (not significantly different; $p = 0.12$).

Expression of the surface antigens CD4, CD8, CD3, CD125 (IL2Rα), CD44, c-Kit, TCRαβ and TCRγδ was examined to classify the thymic lymphomas into stages of differentiation. Sixteen of the 19 thymic lymphomas (84%) were $CD4^+CD8^+$, 9 of which were immature $CD3^-$ with the remaining 7 being mature $CD3^+$. One thymic lymphoma was $CD4^+CD8^{\pm}CD3^+$, reflecting the mature differentiated stage of the $CD4^+$ single positive. The remaining two lymphomas were $CD4^{\pm}CD8^+CD3^-$ and $CD4^{\pm}CD8^-CD3^-$, both of which were at an immature stage. Expression of c-Kit and TCRγδ were negative in all the thymic lymphomas examined (data not shown). These results are consistent with the previous studies [21,28].

3.2. LOH status in chromosome 11

p53 and *Ikaros* are located on chromosome 11 in the telomeric (39.0 cM) and centromeric regions (6.0 cM), respectively. Ten microsatellite markers on chromosome 11 were used for allotyping in 27 thymic lymphomas. *p53* has been mapped close to *Acrb* and *Ikaros* has been mapped between *D11Mit62* and *D11Mit2* [14]. LOH was detected in 2/27 lymphomas (7%). These two tumors (S1603 and S1610) exhibited LOH at all loci examined, indicating non-disjunction as a mechanism of LOH. Thus, the frequency of LOH was low in ENU-induced thymic lymphomas compared with the LOH rates observed in radiation- (50%) or 1,3-butadiene-induced lymphomas (26%) [14,29].

3.3. Expression of Ikaros

We previously demonstrated that 25% of the radiation-induced thymic lymphomas from B6C3F1 mice have altered *Ikaros* expression (i.e., silencing or alternative splicing) [15]. The *Ikaros* expression pattern in ENU-induced lymphomas was essentially identical to that in the normal thymocytes (Fig. 1a), with concomitant expression of 750 and 490-bp products corresponding to *Ik1* and *Ik2*, respectively [30]. One

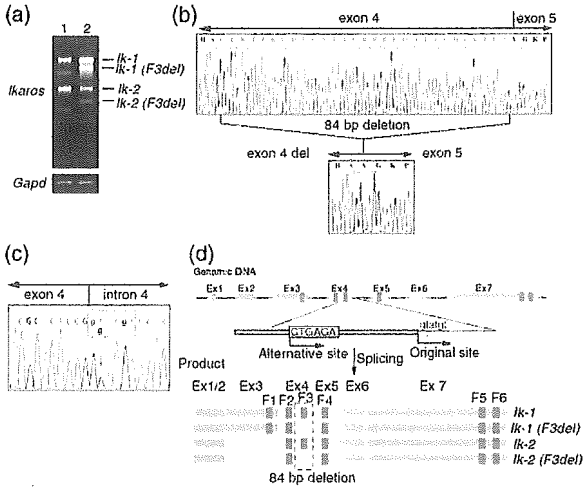

Fig. 1. Expression and sequence analysis of the shortened *Ikaros* isoform in ENU-induced thymic lymphoma S1624. (a) RT-PCR analysis of *Ikaros* expression in normal thymocytes from 6-week-old mice (lane 1) or in the thymic lymphoma S1624 (lane 2). Each cDNA sample was amplified with the primers for *Ikaros* (2F/7R) and *Gapd* (control). (b) Sequence analysis of *Ikaros* expressed in S1624. The upper panel depicts the sequence between exons 4 and 5 in *Ik1* or *Ik2* and the lower panel shows the sequence of the corresponding shortened fragment in S1624. Codons and amino acid sequences are shown above each sequence panel. An 84-bp deletion was found at the 3′ end of exon 4. (c) Heterozygous (t > t/g) point mutation in a splice donor site in intron 4 of S1624, as determined by direct sequencing. (d) Schematic presentation of genomic mutations and alternative splicing of *Ikaros* in S1624. A heterozygous mutation in intron 4 resulted in the creation of the mutant Ikaros isoforms *Ik-1 (F3del)* and *Ik-2 (F3del)* in addition to *Ik1* and *Ik2*.

lymphoma, S1624, expressed two additional isoforms of slightly reduced size relative to *Ik1* and *Ik2* (Fig. 1a). These results were confirmed by Western blotting (data not shown). Sequence analysis demonstrated that the shorter isoforms had an 84-bp deletion in the latter half of exon 4, resulting in the deletion of zinc-finger 3 within the N-terminal DNA-binding domain (Fig. 1b and d). We designated these internally deleted genes as *Ik1 (F3del)* and *Ik2 (F3del)*. No such *Ikaros* mutants have been reported previously. Cobb et al. [31] reported a deletion construct of *Ikaros dF3*, which is identical to *Ik (F3del)*, and showed loss of both DNA-binding activity and pericentromeric targeting ability in 3T3 fibroblasts.

We subsequently analyzed the genomic DNA sequence of the S1624 lymphoma between exons 4 and 5 of the *Ikaros* gene and identified a point mutation in

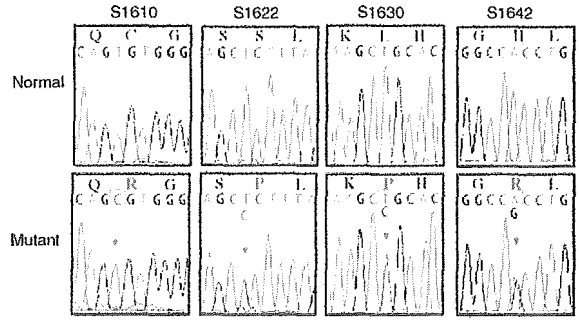

Fig. 2. Point mutations of *Ikaros* in ENU-induced thymic lymphomas. Each panel indicates codons and amino acid sequence for *Ikaros* (GenBank accession no. L03547 with insertion of exon 3) or mutants thereof. Arrows indicate the positions of base substitutions. The point mutation of S1610 (column 1) is homozygous while those of S1622 (column 2), S1630 (column 3) or S1642 (column 4) are heterozygous.

the splicing donor site within intron 4 (gtatgt > ggtatgt, Fig. 1c) that could potentially compromise donor function during pre-mRNA splicing. Consequently, the sequence GTGAGA in exon 4, located 84-bp upstream of the original splice donor site, was used as an altered (and novel) splice site. Because the point mutation was heterozygous, the wild-type allele underwent normal splicing as well. As a result, the S1624 lymphoma expressed the four alternative mRNAs *Ik1*, *Ik2*, *Ik1 (F3del)* and *Ik2 (F3del)* (Fig. 1d). This contrasts with the previous data in which no splice site mutations were found in the mouse thymic lymphomas and human leukemic cells expressing Ikaros isoforms *Ik4*, *Ik4 (del)*, *Ik6*, *Ik8* and *Ik8 (del)* [9,15].

3.4. Point mutations of Ikaros

Point mutations of *Ikaros* have also been described in radiation- and 1,2-butadiene-induced thymic lymphomas [15,17,29]. Therefore, we examined *Ikaros* mutations in 26 ENU-induced thymic lymphomas. Sequence analysis of the entire *Ikaros* cDNA (exons 1–7) and the corresponding genomic DNA revealed point mutations in four thymic lymphomas (S1610, S1622, S1630, S1642; Fig. 2; Table 1). Three of them (S1622, S1630, S1643) were heterozygous at the mutation site. All four were the missense mutations in the N-terminal zinc finger DNA-binding domain. Three of the four mutations (S1610, S1630, S1642) were located in the C_2H_2 zinc-finger structure important for interaction with zinc and one (S1622) was located at a serine residue next to leucine, which is a conserved hydrophobic residue within an α-helix involved in zinc finger secondary structure. The distribution of the point mutations was distinct from that seen in radiation-induced lymphomas where mutations were more sparsely distributed and were accompanied by LOH [15]. All point mutations were T > C transitions.

3.5. Analysis of p53 mutations

Next, we analyzed thymic lymphoma samples for the mutations in *p53*. Eight tumors (S1594, S1607, S1610, S1633, S1634, S1641, S1642 and S1643) harbored missense mutations in the DNA-binding region (Table 1). Two tumors (S1607 and S1610) did not express p53 protein while the other six (S1594, S1633, S1634, S1641, S1642 and S1643) expressed high levels of p53 (data not shown). These results are consistent with a previous report that mutant p53 has an increased half-life and accumulates in the nucleus [32]. As was the case for *Ikaros*, the majority (88%, 7/8) of lymphomas with *p53* point mutations retained a wild-type *p53* allele except for S1610.

3.6. Point mutations of Kras

Frequent mutations of the K*ras* oncogene have been reported in nitroso compound-induced tumors [21,33]. Seven tumors (S1601, S1607, S1622, S1630, S1631, S1634 and S1644) had K*ras* gene mutations, five of which (S1607, S1622, S1630, S1631, and S1644) were in codon 12, one (S1634) in codon 59 and one (S1601) in codon 61. All K*ras* mutations were heterozygous and retained a wild-type allele. Two tumors (S1607 and S1622) harbored two mutations concomitantly, displaying two additional bands in SSCP analysis (Fig. 3a), that resulted in nine point mutations in total. Sequence analysis after TA-cloning of the K*ras* gene revealed that these lymphomas contained three types of sequence in codon 12, namely GGT (wild-type), GAT and TGT, at a ratio of 3:2:5 (Fig. 3b).

3.7. Mutation spectrum in ENU-induced thymic lymphomas

The mutation and LOH status for each ENU-induced thymic lymphoma is summarized in Table 1.

Table 1
Alteration of *p53*, *Ikaros* and K*ras* and LOH status of chromosome 11 in ENU-induced thymic lymphomas (TL)

Sample	Latency (days)	TL size (mg)	*Ikaros*				*P53*				*Kras*	
			Codon	Nucleotide (aa)	Location	LOH[a]	Codon	Nucleotide (aa)	Protein[b]	LOH[a]	Codon	Nucleotide (aa)
S1594	110	743				–	160	TAC > CAC (Y > H)	+	–		
S1601	117	ND[c]				–				–	61	CAA > CTA (Q > L)
S1607	121	345				–	210	CGC > TGC (R > C)	–	–	12	GGT > TGT (G > C)
												GGT > GAT (G > D)
S1610	122	431	150	TGT > CGT (C > R)	F2[d]	B	213	GTG > TTG (V > L)	–	B		
S1622	130	864	215	TCT > CCT (S > P)	F4[e]	–				–	12	GGT > TGT (G > C)
											12	GGT > GAT (G > D)
S1624	130	615	int4 + 2	gt > gg (del 169–196)	F3del[f]	–				–		
S1630	132	994	166	CTG > CCG (L > P)	F2[d]	–				–	12	GGT > TGT (G > C)
S1631	136	309				–				–	12	GGT > GAT (G > D)
S1633	136	595				–	213	GTG > ATG (V > M)	+	–		
S1634	139	577				–	243	ATG > AAG (M > K)	+	–	59	GCA > GAA (A > E)
S1641	143	818				–	243	ATG > AAG (M > K)	+	–		
S1642	145	711	191	CAC > CGC (H > R)	F3[d]	–	190	CAT > CTT (H > L)	+	–		
S1643	145	892				–	194	GTG > GAG (V > E)	+	–		
S1644	145	877				–				–	12	GGT > GAT (G > D)

[a] Lost allele: B, B6; (–), heterozygous.
[b] Expression status of p53 protein: (+), over-expression; (–), no expression (normal type).
[c] ND: not determined.
[d] Mutation was located in the C_2H_2 zinc-finger structure for interaction with zinc.
[e] Mutation was located in an α-helix structure in the zinc-finger protein.
[f] Described in Fig. 1.

Fig. 3. Detection of *Kras* mutations in ENU-induced thymic lymphomas. (a) PCR-SSCP analysis. Lane 1 shows the *Kras* pattern from normal thymocytes, and lanes 2, 3, 4, 7 and 8 represent thymocytes from thymic lymphomas without K*ras* mutations. Lanes 5 (S1631), 6 (S1630) and 9 (S1607) show altered patterns. (b) Sequence analysis after TA-cloning of S1607 (lane 9 in panel a). Three types of sequence, GGT, GAT and TGT, were found at codon 12 (panel b).

In *Ikaros*, four of the five mutations (80%) were T > C. In *p53*, four of the eight mutations (50%) were T > A and two were G > A (25%). In K*ras*, four of the nine mutations were G > A (44%) and another four were G > T (44%). These results are in good agreement with the evidence that ENU produces significant levels of alkylation at oxygen atoms such as the O^6 position of guanine and the O^4 position of thymine in DNA [18]. The results also indicate, however, that the spectrum of base substitutions is different among these genes.

It is also noteworthy that 43% (6/14) of the lymphomas harbored double mutations. S1610 and S1642 harbored mutations in both *Ikaros* and *p53*. S1607 and S1634 had mutations in *p53* and K*ras*. S1622 and S1630 had mutations in *Ikaros* and K*ras*. In all, 14 of the 27 lymphomas (52%) had mutations in at least one of the three genes examined and most of these lymphomas also retained a wild-type allele of the *p53*, *Ikaros* and K*ras* genes.

4. Discussion

This study revealed point mutations in the tumor suppressor genes *p53* and *Ikaros* in 8 (30%) and 5 (19%) of the 27 ENU-induced thymic lymphomas, respectively. This was often accompanied by the retention of a wild-type allele (in 7/8 [88%] of *p53* mutations and 4/5 [80%] of *Ikaros* mutations). One of the *Ikaros* mutations was located at the splice donor site, generating a novel splice isoform lacking zinc finger 3, *Ik (F3del)*. Together with 7 lymphomas with K*ras* oncogene mutations (23%), 14 of the 27 (52%) thymic lymphomas harbored point mutations in at least 1 of the 3 genes examined.

Tumor suppressor genes can be inactivated by point mutations or deletions according to Knudson's "two-hit" hypothesis [1]. However, some tumor suppressor genes with particular types of mutations act as oncogenes in a dominant manner, probably by forming oligomeric complexes with wild-type or other proteins that results in the loss of wild-type protein function [5,34]. Recently, mice were produced having targeted point mutations of *p53* [35,36]. Mice heterozygous for a mutation in codon 172, R172H differed from p53+/− mice in tumor spectrum and metastatic frequency. Interestingly, loss of a wild-type *p53* allele was rarely observed in the tumors [35]. Cells from two other mice heterozygous for *p53* point mutations, R270H and P275S, exhibited delayed transcriptional activation of *p53* downstream target genes upon exposure to gamma-rays. They also showed severe defects in p53-dependent apoptosis, supporting negative dominance over wild-type p53 [36].

A *Plastic* mouse strain was recently developed that has the same point mutation as *Ikaros*, H191R, found in lymphoma S1642 (Fig. 2). It has been demonstrated that mice heterozygous for H191R, $Ikaros^{Plastic/+}$, develop leukemia/lymphomas frequently within 4 months after birth [37]. Although the lymphomas developed in $Ikaros^{Plastic/+}$ have not been examined for the loss of wild-type alleles, the S1642 lymphoma in the present study showed retention of a wild-type *Ikaros* allele. These data suggest negative dominance of this *Ikaros* point mutation in vivo. However, functional studies are required to confirm that this is indeed the case. There are other reports of tumor suppressor genes that are exceptions to the classical version of Knudson's two-hit theory [38,39]. These include *Caveolin-1* and *Smad4*. A point mutation, P132L, in codon 132 of *Caveolin-1* has been reported in 16% of the primary human breast cancers but no loss of heterozygosity of the wild-type *Caveolin-1* gene was observed [40]. A point mutation in *Smad4* has been frequently observed in pancreatic and colon cancers. Expression of Smad4 carrying an R497H mutation antagonized TGF-β signaling in a dominant-negative manner [41], consistent with the clinical observations that only one *Smad4* allele is affected in some cancers [42]. It seems likely that the cancers harboring heterozygous point mutations of Caveolin-1 and Smad4 are inactive for wild-type cave-

olin and Smad4 function because both Caveolin-1 and Smad4 form homo- and heteromeric complexes, respectively.

One cannot exclude the possibility, however, that inactivating one *p53* allele through the mutation results in a cellular phenotype caused by gene dosage reduction, a phenomenon previously described by others in p53+/− knockout mice [43]. Indeed, LOH at both *p53* and *Ikaros* loci without an additional point mutation in the other allele was found in lymphoma S1603. In addition, the p53 mutations found in the ENU-induced lymphomas are not the well known hot-spot mutations frequently found in the other tumor types that were previously shown to display dominant-negative activity [36,44]. These lines of evidence indicate that carcinogenesis occurs despite the production of wild-type tumor suppressor proteins in contrast to the standard definition of tumor suppressor genes.

In addition to the point mutations, alternative use of transcriptional initiation sites or alternative splicing that results in a lack of DNA-binding or transactivation activity may be another mechanism(s) to generate dominant-negative tumor suppressor proteins. N-terminally truncated p73 and several Ikaros isoforms (Ik4, Ik6 and Ik8) lacking DNA-binding domains have been reported in some tumors [12,45]. We previously reported that lymphomas expressing Ikaros splice variants (Ik4, Ik6 and Ik8) retain a wild-type *Ikaros* allele [15]. In the present study, lymphoma S1624 expressed a novel Ik (F3del) isoform caused by a point mutation at the splice site but retained a wild-type allele.

Knudson's two-hit model of tumor suppressor genes supposes biallelic disruption which frequently involves loss of a wild-type allele via deletion and/or mitotic recombination. LOH has previously been useful for predicting tumor suppressor loci. However, a growing number of reports have described candidate tumor suppressors that do not conform to this standard definition. These include loci that exhibit negative dominancy and haploinsufficiency. Indeed, we demonstrated here that lymphomas induced by ENU, which is effective at inducing point mutations but not in deletion and/or recombination events, frequently harbored mutations of tumor suppressor genes such as *p53* and *Ikaros* without accompanying LOH. Therefore, to obtain a more complete picture of carcinogenesis, it will be important to examine mutations/alterations of other tumor suppressor genes whose loci do not demonstrate LOH.

In summary, our data suggest that ENU facilitates T cell lymphomagenesis by dominant-negative inactivation of tumor suppressor gene function as well as by oncogene (*ras*) activation.

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Enhancement of Cisplatin-Induced Apoptosis and Caspase 3 Activation by Depletion of Mitochondrial DNA in a Human Osteosarcoma Cell Line

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ABSTRACT: **Cisplatin is an anticancer drug that can induce apoptosis. In this study, we investigated the effect of mitochondrial DNA (mtDNA) depletion on cisplatin-induced cell death using a human osteosarcoma cell line (143B) and mtDNA-depleted 143B cells (143B-ρ^0). Results showed that cisplatin decreased cell survival in 143B-ρ^0 cells. Moreover, cisplatin induced a greater extent of apoptosis-associated DNA fragmentation and caspase 3 activation in 143B-ρ^0 cells. The release of mitochondrial cytochrome *c* into cytosol by cisplatin was enhanced more obviously in 143B cells than in 143B-ρ^0 cells; however, in the control group of 143B-ρ^0 cells, it was already dramatically greater. Depletion of mtDNA may increase sensitivity of cells to cisplatin-induced apoptosis by enhancing caspase 3 activation via both cytochrome *c*–dependent and –independent pathways.**

KEYWORDS: **cisplatin; mitochondrial DNA; apoptosis; caspase 3; cytochrome *c***

INTRODUCTION

Cisplatin (cis-diamminedichloroplatinum [II]; CDDP) is a chemotherapeutic agent that is known to inhibit DNA replication by cross-linking DNA.[1] It has been shown that reactive oxygen species could be important in CDDP-induced apoptosis.[2] It has also been indicated that suppression of apoptosis was important in CDDP resistance in cancer cells,[3] and DNA platination or p53 status did not necessarily determine the sensitivity of cancer cells to CDDP-induced apoptosis.[4] In the mechanisms leading to apoptosis, activation of caspase 3 can result from different upstream caspases or release of the Smac/DIABLO protein from mitochondria independent of other caspases.[5] It has been indicated that CDDP induced apoptosis via release of mitochondrial cytochrome *c* into cytosol with subsequent activation of

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