Prevention of Helicobacter pylori–Induced Gastric Cancers in Gerbils by a DNA Demethylating Agent

Tohru Niwa¹, Takeshi Toyoda², Tetsuya Tsukamoto³, Akiko Mori¹, Masae Tatematsu⁴, and Toshikazu Ushijima¹

Abstract
Suppression of aberrant DNA methylation is a novel approach to cancer prevention, but, so far, the efficacy of the strategy has not been evaluated in cancers associated with chronic inflammation. Gastric cancers induced by Helicobacter pylori infection are known to involve aberrant DNA methylation and associated with severe chronic inflammation in their early stages. Here, we aimed to clarify whether suppression of aberrant DNA methylation can prevent H. pylori–induced gastric cancers using a Mongolian gerbil model. Administration of a DNA demethylating agent, 5-aza-2’-deoxycytidine (5-aza-dC), to gerbils (0.125 mg/kg for 50–55 weeks) decreased the incidence of gastric cancers induced by H. pylori and N-methyl-N-nitrosourea (MNU) treatment from 55.2% to 23.3% (P < 0.05). In gastric epithelial cells, DNA methylation levels of six CpG islands (HE6, HG2, SB1, SB5, SF12, and SH6) decreased to 46% to 68% (P < 0.05) of gerbils without 5-aza-dC treatment. Also, the global DNA methylation level decreased from 83.0% ± 4.5% to 80.3% ± 4.4% (mean ± SD) by 5-aza-dC treatment (P < 0.05). By 5-aza-dC treatment, Il1b and Nos2 were downregulated (42% and 58% of gerbils without, respectively) but Tnf was upregulated (187%), suggesting that 5-aza-dC treatment induced dysregulation of inflammatory responses. No obvious adverse effect of 5-aza-dC treatment was observed, besides testicular atrophy. These results showed that 5-aza-dC treatment can prevent H. pylori–induced gastric cancers and suggested that removal of induced DNA methylation and/or suppression of DNA methylation induction can become a target for prevention of chronic inflammation–associated cancers. Cancer Prev Res; 6(4); 263–70. ©2013 AACR.

Introduction
DNA methylation is an epigenetic mechanism for gene regulation. Methylation of promoter CpG islands (CGIs) consistently suppresses expression of their downstream genes (1), and physiologic methylation of retrotransposons is involved in their transcriptional repression (2). In cancers, tumor suppressor genes are frequently inactivated by aberrant methylation of their promoter CGIs (3, 4). Such aberrant methylation is present not only in cancers but also in noncancerous tissues exposed to chronic inflammation, such as colonic mucosae with ulcerative colitis, liver tissues exposed to hepatitis, and gastric mucosae exposed to chronic gastritis (5–10). In the case of the stomach, Helicobacter pylori infection is known to induce severe chronic inflammation (11–13) and aberrant methylation in gastric epithelial cells (GEC; ref. 14). Accumulation levels of aberrant methylation in gastric mucosae correlate with risk of gastric cancers (8–10). Suppression of aberrant methylation is considered as one of the novel targets for cancer chemoprevention (15, 16). Traditionally, chemoprevention has used substances based on 2 strategies: the anti-initiation and anti-promotion/progression strategies (17–19). In the former strategy, blockage of activity of carcinogens that induce genetic or epigenetic alterations and enhancement of repair systems have been targeted. In the latter strategy, suppression of proliferation of initiated cells and induction of their apoptosis have been targeted. However, neither of these strategies targeted removal of genetic or epigenetic alterations accumulated in the cells, which can be achieved by DNA demethylating agents, such as 5-aza-2’-deoxycytidine (5-aza-dC; refs. 20, 21).

The usefulness of DNA demethylating agents in cancer chemoprevention has been shown in several animal models, including intestinal tumors in Apcmin/+ mice (22, 23), prostate tumors in transgenic mice harboring probasin promoter-driven SV40 antigen (24), 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone–induced mouse lung tumors (25), and 4-nitroquinoline 1-oxide–induced mouse liver tumors (26). DNA demethylating agents, such as 5-aza-dC, have been shown to prevent incidence of tumors (21). The demethylation property of 5-aza-dC is well documented (27). The present study investigated the effects of a DNA demethylating agent, 5-aza-dC, on the prevention of carcinogenesis in Mongolian gerbils infected with H. pylori.
oral tumors (26). Genetic suppression of a maintenance DNA methyltransferase (Dnmt1) also suppressed tumor development in some of these models (22, 27, 28). However, so far, the efficacy of suppression of aberrant DNA methylation was not evaluated in chronic inflammation–associated cancers, in which aberrant DNA methylation is heavily involved (29, 30). From this aspect, gastric cancers induced by *H. pylori* infection of Mongolian gerbils (*Meriones unguiculatus*) have several advantages. In gerbils, *H. pylori* infection induces severe chronic inflammation, as in humans, and promotes gastric cancers initiated by *N*-methyl-N-nitrosourea (MNU; refs. 31, 32). Also, 10 CGIs have already been established as markers that can be methylated by *H. pylori* infection, and a critical role of inflammation triggered by *H. pylori* infection, not a direct effect of *H. pylori*, in methylation induction has been shown (14). In contrast, few markers for methylation induction have been isolated in *H. pylori* or *Helicobacter felis*–infected mice, except Tff2 promoter (33).

In this study, using the gerbil model, we aimed to clarify whether 5-aza-dC treatment can prevent chronic inflammation–induced gastric cancers and evaluate its effects on methylation induction and inflammation triggered by *H. pylori* infection.

Materials and Methods

Animals and sample preparation

Male Mongolian gerbils (MGS/Sea) were purchased from Kyudo and divided into 10 groups (G1–6 in Fig. 1A and G7–10 in Fig. 2A). Gerbils were inoculated with *H. pylori* [4 × 10⁸ colony-forming units (CFU)/gerbil, ATCC 43504; American Type Culture Collection] at 5 weeks of age (34). In a carcinogenicity experiment, 10 ppm of MNU (Sigma-Aldrich) was given in drinking water to gerbils. 5-Aza-dC (125 μg/kg body weight in sterilized PBS; Sigma-Aldrich) was administered to gerbils intraperitoneally twice per week. The dose was selected from the 3 doses (125, 250, and 500 μg/kg) tested in a preliminary experiment for lack of toxicity. Timing and duration of the treatments are shown in Figs. 1A and 2A.

The stomach was resected and cut along the greater curvature. In a carcinogenicity experiment (G1–6), the antral region was fixed in formalin for histologic analysis. From the body region, GECs were isolated by the gland isolation technique (35) and stored in 100% ethanol at −80°C until DNA extraction. The testes, small intestine, liver, and kidneys were resected, and half parts were fixed in formalin. The other halves were snap-frozen for RNA extraction. In an experiment to induce *H. pylori*–triggered gastritis (G7–10), the antral region was cut into 2 pieces—one was snap-frozen for RNA extraction and the other half was fixed in formalin.

In both experiments, samples (tissues or GECs) were digested with proteinase K, and gDNA was extracted by the standard phenol/chloroform method. RNA of gastric tissue was isolated using ISOGEN (Nippon Gene). Whole blood was obtained from the inferior vena cava, and gDNA was extracted by a QuickGene DNA Whole Blood Kit (Fujifilm). All the animal experiments were approved by the Committee for Ethics in Animal Experimentation at the National Cancer Center.

Histological analysis

Formalin-fixed tissues were sliced along the longitudinal axis into strips of 5 mm width and embedded in paraffin. Sections of 3 μm thickness were prepared and stained with hematoxylin and eosin. Neoplastic lesions in the stomach were diagnosed as previously described (36). The size of a gastric cancer was determined as the maximum diameter of the gastric cancer in the neighboring sections. The degree of infiltration of mononuclear and polymorphonuclear cells was graded on a 4-point scale (0–3; 0, no or faint; 1, mild; 2, moderate; 3, marked) as described (37).

Luminometric methylation assay

gDNA from whole blood was amplified by an illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare) and used as fully unmethylated DNA. The unmethylated DNA was methylated by Sss1 methylase (New England Biolabs) and used as fully methylated DNA. A series of standard DNA was prepared by serial mixing of the unmethylated DNA and the methylated DNA.

Luminometric methylation assay (LUMA) was conducted as described (38) with slight modifications. Briefly, 3 μg of DNA was digested with 2 pairs of restriction enzymes (*Hpa*II and *Eco*RI or *Msp*I and *Eco*RI) in independent tubes (all restriction enzymes were purchased from Toyobo). The DNA was purified with a DNA Clean & Concentrator Kit (Zymo Research) and eluted in 40 μL of an annealing buffer (2 mmol/L magnesium acetate and 20 mmol/L Tris-acetate, pH7.6). Using the PSQ 96 Pyrosequencing System (Qiagen), 5′-CG overhang produced by *Hpa*II (or *Msp*I) and 5′-AATT overhang produced by *Eco*RI was sequenced, and an *Hpa*II/*Eco*RI (or *Msp*I/*Eco*RI) signal ratio was determined. An *Hpa*II/*Msp*I value was obtained as (*Hpa*II/*Eco*RI)/(*Msp*I/*Eco*RI) in each sample. The *Hpa*II/*Msp*I value was compared with those of the standard DNA series, and the global methylation level (GML), which is equivalent to the percentage of methylated DNA in the standard DNA, was determined.

Quantitative methylation-specific PCR

DNA digested with *Bam*HI was treated with sodium bisulfite as described (39) and used as a template for real-time PCR. With primer sets specific to methylated CGIs (HE6, HG2, SA9, SB1, SB5, SC3, SD2, SE3, SF12, and SH6; Supplementary Fig. S1) and a B2 repeat sequence, real-time PCR was carried out as described (14). On the basis of the copy number of sequences measured by real-time PCR, the methylation level was calculated as a percentage of the methylated reference (PMR), which was obtained as ([number of methylated fragments of a target CGI in sample]/(number of the B2 repeat in sample))/([number of
methylated fragments of a target CGI in SssI-treated DNA) / (number of the B2 repeat in SssI-treated DNA) \times 100.

Gene expression analyses
The number of cDNA molecules was quantified by quantitative reverse transcriptase-PCR (qRT-PCR) using gene-specific primers (Il1b, Nos2, and Tnf) as described (14). The number of cDNA molecules of a target gene was normalized to that of Gapdh.

Genomic PCR and sequencing
A forward primer (5'-AGATTCCTTGATGCCTGGGTGTC-3') was designed in a region of the mouse Tnf promoter highly conserved with the human corresponding region. A reverse primer (5'-AGATTCCTTGATGCCTGGGTGTC-3') was designed on the gerbil Tnf mRNA sequence (AB177841). The gerbil Tnf promoter was amplified using these primers, and the PCR product was directly sequenced with the same primers. The obtained sequence was
Suppression of H. pylori/MNU-induced gastric carcinogenesis by 5-aza-dC

To evaluate cancer prevention effects of 5-aza-dC, a carcinogenicity experiment was carried out (Fig. 1A). Among the gerbils with MNU treatment and H. pylori infection (G5 and G6), 5-aza-dC treatment decreased incidence of gastric cancers from 55.2% (G5) to 23.3% (G6, \( P < 0.05 \); Table 1). The incidence in G6 was similar to that in MNU-treated gerbils without H. pylori infection (G2, 20.7%). There were no significant differences in the tumor multiplicity and size among the groups. These results clearly showed that 5-aza-dC treatment suppressed H. pylori/MNU-induced gastric carcinogenesis in gerbils and suggested that it might have completely abrogated the cancer promotion effects of H. pylori infection.

Regardless of 5-aza-dC treatment, gerbils with MNU treatment (G2, G3, G5, and G6) showed low body weight than the gerbils without MNU treatment (G1 and G4), showing that the body weight loss was dependent upon MNU treatment, not upon 5-aza-dC treatment (Supplementary Fig. S2). Survival rates started to decrease from 25 weeks of age, and the decrease was dependent upon MNU treatment, not upon 5-aza-dC treatment (Supplementary Fig. S3). This showed that the dose of 5-aza-dC used in this study (125 \( \mu \)g/kg body weight) had no obvious effects on body weight and survival of gerbils.

Reduction of DNA methylation levels in GECs by 5-aza-dC

To confirm the demethylating effects of 5-aza-dC in vivo, methylation analyses were conducted in GECs. First, the global DNA methylation level was measured by LUMA, in which global CCGG methylation was measured by using

Table 1. Suppression of gastric cancers by 5-aza-dC

<table>
<thead>
<tr>
<th>Group</th>
<th>Effective number</th>
<th>Well-differentiated</th>
<th>Poorly differentiating</th>
<th>Multiplicity (mean ± SD)</th>
<th>Diameter (mean ± SD), mm</th>
<th>Incidence (%)</th>
<th>Adenoma</th>
<th>Sarcoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1. HP(−) + DW</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>3.3 ± 3.3</td>
<td>2/29 (70.7)</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G2. HP(−) + MNU + PBS</td>
<td>29</td>
<td>3</td>
<td>3</td>
<td>1.0 ± 0</td>
<td>0/8 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G3. HP(−) + MNU + 5-aza-dC</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>3.3 ± 3.3</td>
<td>2/33 (6.1)</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G4. HP(−) + DW</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>3.3 ± 3.3</td>
<td>0/8 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G5. HP(−) + MNU + PBS</td>
<td>29</td>
<td>15</td>
<td>1</td>
<td>1.1 ± 0.3</td>
<td>10/29 (35.2)*</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>G6. HP(−) + MNU + 5-aza-dC</td>
<td>30</td>
<td>4</td>
<td>3</td>
<td>1.0 ± 0</td>
<td>4.8 ± 1.7</td>
<td>7/30 (23.3)*</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

NOTE: 5-Aza-dC treatment decreased incidence of gastric cancers (adenocarcinomas) from 55.2% (G5) to 23.3% (G6).

*\( P < 0.05 \) compared with G3.

\( ^{\pm} P < 0.05 \) compared with G5.
to 187% of G9 (a possibility that upregulation of dysregulation, of inflammation-related genes. As there was dC treatment caused up- and downregulation, namely considered that the upregulation of can consistently silence their downstream genes (42), we Fig. S4). Because methylation of only promoters with CGIs region but found that there was no CGI (Supplementary methylation of its promoter CGI, we sequenced its promoter was previously shown to induce aberrant methylation quantitative methylation-specific PCR (qMSP). 5-Aza-dC treatment reduced methylation levels in G6 to 46% to 80% of those in G5 for 6 CGIs (HE6, HG2, SB1, SB5, SF12, and SH6; P < 0.05; Fig. 1C). These results showed that 5-aza-dC treatment suppressed methylation induction by H. pylori infection and MNU treatment in GECs. The methylation levels in G5 were higher than those in G4 whereas those in G2 were not elevated compared with those in G1, indicating that MNU treatment had an augmenting effect on H. pylori–induced aberrant methylation.

Dysregulation of inflammation-related genes by 5-aza-dC
Among 10 inflammation-related genes whose expression was examined in the stomach, expression of 3 genes (Il1b, Nos2, and Tnf) has been shown to be associated with induction of methylation in GECs (14, 41). Therefore, we examined whether 5-aza-dC treatment affected expression of these 3 genes in the stomach after H. pylori infection using H. pylori–infected and -uninfected gerbils without MNU treatment (Fig. 2A). In the H. pylori–infected gerbils with 5-aza-dC treatment (G10), expression levels of Il1b and Nos2 decreased to 42% and 58%, respectively (P < 0.01, respectively), of those in H. pylori–infected gerbils without 5-aza-dC treatment (G9; Fig. 2B). In contrast, Tnf was upregulated to 187% of G9 (P < 0.01). These results indicated that 5-aza-dC treatment caused up- and downregulation, namely dysregulation, of inflammation-related genes. As there was a possibility that upregulation of Tnf was due to demethylation of its promoter CGI, we sequenced its promoter region but found that there was no CGI [Supplementary Fig. S4]. Because methylation of only promoters with CGIs can consistently silence their downstream genes (42), we considered that the upregulation of Tnf was unlikely to be due to demethylation by 5-aza-dC.

We also analyzed infiltration of inflammatory cells in the stomach. In gerbils with H. pylori infection (G9 and G10), 5-aza-dC treatment did not affect infiltration of mononuclear cells and polymorphonuclear cells (Fig. 2C). In gerbils without H. pylori infection (G7 and G8), there was little mononuclear cell and polymorphonuclear cell infiltration, and no effect of 5-aza-dC treatment was observed. These results showed that 5-aza-dC treatment did not affect infiltration of inflammatory cells.

Extra-gastric effects of 5-aza-dC treatment
To evaluate possible adverse effects of 5-aza-dC treatment, we first conducted macroscopic analysis. Although most organs did not show any abnormality, the testes were prominently atrophic in 5-aza-dC–treated gerbils [0.47 ± 0.05 (mean ± SD) g/2 testes in G3 and 0.50 ± 0.05 g/2 testes in G6] compared with gerbils in other groups (1.10 ± 0.11 g/2 testes in G2 and 1.09 ± 0.13 g/2 testes in G5). We then conducted analysis of histopathologic abnormalities and global DNA methylation levels in the testes, small intestine, liver, and kidneys. In the testes of 5-aza-dC–treated gerbils, the numbers of spermatozoa and spermatids were markedly decreased regardless of H. pylori infection and MNU treatment and decreased global methylation levels in GECs.

Next, methylation of 10 CGIs, where H. pylori infection was previously shown to induce aberrant methylation (Supplementary Fig. S1; refs. 14, 41), was analyzed by quantitative methylation-specific PCR (qMSP). 5-Aza-dC treatment decreased the global methylation level from 84.4% ± 4.5% (mean ± SD)% (G5) to 80.3% ± 4.4% (G6, P < 0.05; Fig. 1B). Among the MNU–treated gerbils without H. pylori infection (G2 and G3), the methylation level was decreased by 5-aza-dC treatment (from 84.4% ± 2.3% in G2 to 82.2% ± 2.4% in G3, P < 0.05). No significant influence of H. pylori infection or MNU treatment was observed. These results indicated that 5-aza-dC worked as a DNA demethylating agent in vivo and decreased global methylation levels in GECs.

A, tissue sections of the testes and small intestine. Bottom, is a magnified view of the region in the black rectangle in the top. Numbers of spermatozoa and spermatids were markedly decreased in the testes of 5-aza-dC–treated gerbils. B, global methylation levels in the testes and small intestine. Five gerbils in each group were randomly selected, and the methylation levels were measured by LUMA. Mean and SD are shown. *P < 0.05. By 5-aza-dC-treatment, the global methylation level did not decrease in the testes but did in the small intestine.
infection status (Fig. 3A). Despite the presence of hyper-
permethogenesis, there was no significant decrease of the
global methylation level (Fig. 3B). In contrast, in the small
intestine, the global methylation level was reduced by 10.4%
and 5.6% (G3 and G6, respectively Fig. 3B). However, no
histologic changes compared with the untreated gerbils were
observed (Fig. 3A). As for the liver and kidneys, there were no
histologic abnormalities or reduction of global methylation
levels in G3 and G6 (Supplementary Fig. S5).

Discussion

Our study using a gerbil model showed that 5-aza-dC
treatment suppressed H. pylori/MNU-induced gastric can-
cers. This study showed for the first time that chemopre-
vention using a DNA demethylating agent is effective for
chronic inflammation–associated cancers. As chronic
inflammation contributes to about 25% of all cancer cases
(43), and aberrant DNA methylation is frequently observed
in tissues exposed to chronic inflammation (29), suppres-
sion of aberrant methylation might become an effective
preventive approach for these types of cancers. This study
also showed that induction of aberrant methylation is an
important mechanism for gastric carcinogenesis by H. pylori
infection.

As mechanisms of suppression of gastric cancers by 5-
aza-dC, at least 2 modes of action were present. The first
one was the DNA demethylating effect. 5-Aza-dC treat-
ment decreased global methylation level in GECs and
suppressed hypermethylation of CGIs. These results sug-
gested that 5-aza-dC was capable of removing aberrant
DNA methylation induced by H. pylori infection and
thereby prevented cancer development. The second one
was the effect on inflammation. It was previously shown
that inflammation triggered by H. pylori infection is pivotal
for aberrant methylation induction, and expression of
inflammation-related genes (Il1b, Nos2, and Tnf) in the
stomach is associated with the induction (14, 41). The
present study showed that 5-aza-dC treatment dysregu-
lated expression of these genes. Therefore, there is a pos-
sibility that altered balances among the related cytokines
might have led to the reduced methylation induction.

Recently, in the stomach of hypergastrinemic INS-
GAS mice, inhibition of H. pylori (H. felis)-induced
global demethylation by folic acid supplementation
was reported to suppress gastric dysplasia (44). The
data are seemingly discordant with our data showing a
cancer preventive effect by DNA demethylation. How-
ever, in the INS-GAS mice, global hypomethylation by H. felis
was evident, suggesting that the demethylation plays
important roles in the carcinogenesis. Hypermethylation
by folic acid might exert the cancer preventive effect via
suppression of global demethylation. In contrast, in our gerbil study, hypermethylation of CGIs by H. pylori
infection, rather than global demethylation, was evident, suggesting that hypermethylation of CGIs rather than global demethylation was the major mechanism for the carcinogenesis. Thus,
demethylation by 5-aza-dC was considered to have
exerted the preventive effect via suppression of hyper-
methylation of CGIs.

No obvious adverse effect of 5-aza-dC treatment was
observed besides hypopermetogenesis in the testes. Hy-
permethogenesis due to 5-aza-dC treatment was
reported in mice (24). As global hypomethylation was
not detected in the testes of the 5-aza-dC-treated gerbils,
the effect was speculated to be independent of its DNA
demethylating activity. However, we cannot exclude the
possibility that decrease of methylation was not detected
due to immediate elimination of spermatozoa/sperma-
tids with decreased DNA methylation. Regardless of the
mechanism, the presence of this adverse effect precludes
5-aza-dC as a chemoprevention agent for the general
population. However, chemoprevention by a DNA de-
methylating agent itself still might become a promising
strategy if a DNA demethylating agent without such
toxicity is developed.

Demethylating effects by 5-aza-dC were observed in the
stomach and the small intestine, but not in the liver and
the kidneys. Specific global demethylation in the stomach
and intestine was also observed in the female Apcfl/fl
mice administrated a demethylating agent, zebularine
(23). Turnover of epithelial cells in these tissues is
known to be very rapid, being 3 to 4 days in mice (45,
46). As demethylating effects of 5-aza-dC and zebularine
are exerted after their incorporation into gDNA and DNA
replication, rapid cell turnover in the stomach and
intestine could explain the organ-specific global
demethylation.

Individuals with a severe epigenetic field defect, in
contrast with the general population, can be considered
as a target population for cancer prevention using 5-aza-
dC after careful balancing of its preventive and adverse
effects. In the case of gastric cancers, eradication of H.
pylori is the primary strategy for prevention (47), but the
incidence of gastric cancers remains high, even after H.
pylori eradication, especially in persons with intestinal
metaplasia and gastric atrophy (48). Notably, aberrant
methylation in gastric mucosa decreases by H. pylori
eradication, but it does not disappear completely
(14, 49). The level of the remaining methylation reflects
the risk of gastric cancers (8, 9). As DNA demethylating
agents are likely to remove such accumulated methyla-
tion and suppress gastric cancers development, these
individuals with a severe epigenetic field defect may
benefit from epigenetic chemoprevention.

In summary, treatment with 5-aza-dC effectively pre-
vented gastric cancers induced by H. pylori infection in
gerbils, suppressed DNA methylation in GECs, and induced
dysregulation of inflammation. Chemoprevention with a
DNA demethylating agent is expected to become an ef-
fective strategy for prevention of chronic inflammation–asso-
ciated cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Authors' Contributions
Conception and design: T. Niwa, M. Tatematsu, T. Ushijima
Development of methodology: T. Niwa, T. Tsukamoto
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Niwa, T. Toyoda
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Niwa, T. Toyoda
Writing, preparation, or revision of the manuscript: T. Niwa, T. Ushijima
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Mori
Study supervision: M. Tatematsu

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References

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