*Clonorchis sinensis*). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* 61: 121–175, 1994.
39. Inoue S, Ito K, Yamamoto K, and Kawanishi S. Caffeic acid causes metal-dependent damage to cellular and isolated DNA through $H_2O_2$ formation. *Carcinogenesis* 13: 1497–1502, 1992.
40. Inoue S and Kawanishi S. Hydroxyl radical production and human DNA damage induced by ferric nitrilotriacetate and hydrogen peroxide. *Cancer Res* 47: 6522–6527, 1987.
41. Inoue S and Kawanishi S. Oxidative DNA damage induced by simultaneous generation of nitric oxide and superoxide. *FEBS Lett* 371: 86–88, 1995.
42. Iwasa M, Iwata K, Kaito M, Ikoma J, Yamamoto M, Takeo M, Kuroda M, Fujita N, Kobayashi Y, and Adachi Y. Efficacy of long-term dietary restriction of total calories, fat, iron, and protein in patients with chronic hepatitis C virus. *Nutrition* 20: 368–371, 2004.
43. Juedes MJ and Wogan GN. Peroxynitrite-induced mutation spectra of pSP189 following replication in bacteria and in human cells. *Mutat Res* 349: 51–61, 1996.
44. Kato J, Kobune M, Nakamura T, Kuroiwa G, Takada K, Takimoto R, Sato Y, Fujikawa K, Takahashi M, Takayama T, Ikeda T, and Niitsu Y. Normalization of elevated hepatic 8-hydroxy-2′-deoxyguanosine levels in chronic hepatitis C patients by phlebotomy and low iron diet. *Cancer Res* 61: 8697–8702, 2001.
45. Kawanishi S, Hiraku Y, Murata M, and Oikawa S. The role of metals in site-specific DNA damage with reference to carcinogenesis. *Free Radic Biol Med* 32: 822–832, 2002.
46. Kawanishi S, Hiraku Y, and Oikawa S. Mechanism of guanine-specific DNA damage by oxidative stress and its role in carcinogenesis and aging. *Mutat Res* 488: 65–76, 2001.
47. Kawanishi S, Inoue S, and Kawanishi M. Human DNA damage induced by 1,2,4-benzenetriol, a benzene metabolite. *Cancer Res* 49: 164–168, 1989.
48. Kawanishi S, Inoue S, Oikawa S, Yamashita N, Toyokuni S, Kawanishi M, and Nishino K. Oxidative DNA damage in cultured cells and rat lungs by carcinogenic nickel compounds. *Free Radic Biol Med* 31: 108–116, 2001.
49. Kawanishi S, Inoue S, and Sano S. Mechanism of DNA cleavage induced by sodium chromate(VI) in the presence of hydrogen peroxide. *J Biol Chem* 261: 5952–5958, 1986.
50. Kawanishi S, Inoue S, and Yamamoto K. Site-specific DNA damage induced by nickel(II) ion in the presence of hydrogen peroxide. *Carcinogenesis* 10: 2231–2235, 1989.
51. Kawanishi S and Oikawa S. Mechanism of telomere shortening by oxidative stress. *Ann NY Acad Sci* 1019: 278–284, 2004.
52. Kawanishi S, Oikawa S, Murata M, Tsukitome H, and Saito I. Site-specific oxidation at GG and GGG sequences in double-stranded DNA by benzoyl peroxide as a tumor promoter. *Biochemistry* 38: 16733–16739, 1999.
53. Kim MY, Dong M, Dedon PC, and Wogan GN. Effects of peroxynitrite dose and dose rate on DNA damage and mutation in the *supF* shuttle vector. *Chem Res Toxicol* 18: 76–86, 2005.
54. Kino K, Saito I, and Sugiyama H. Product analysis of GG-specific photooxidation of DNA via electron transfer: 2-aminoimidazolone as a major guanine oxidation product. *J Am Chem Soc* 120: 7373–7374, 1998.
55. Kitada T, Seki S, Iwai S, Yamada T, Sakaguchi H, and Wakasa K. *In situ* detection of oxidative DNA damage, 8-hydroxydeoxyguanosine, in chronic human liver disease. *J Hepatol* 35: 613–618, 2001.
56. Langholz E, Munkholm P, Davidsen M, and Binder V. Colorectal cancer risk and mortality in patients with ulcerative colitis. *Gastroenterology* 103: 1444–1451, 1992.
57. Li CQ, Pignatelli B, and Ohshima H. Increased oxidative and nitrative stress in human stomach associated with cagA+ *Helicobacter pylori* infection and inflammation. *Dig Dis Sci* 46: 836–844, 2001.
58. Li Y, Togashi Y, Sato S, Emoto T, Kang JH, Takeichi N, Kobayashi H, Kojima Y, Une Y, and Uchino J. Abnormal copper accumulation in non-cancerous and cancerous liver tissues of LEC rats developing hereditary hepatitis and spontaneous hepatoma. *Jpn J Cancer Res* 82: 490–492, 1991.
59. Loeb LA and Preston BD. Mutagenesis by apurinic/apyrimidinic sites. *Annu Rev Genet* 20: 201–230, 1986.
60. Ma N, Adachi Y, Hiraku Y, Horiki N, Horiike S, Imoto I, Pinlaor S, Murata M, Semba R, and Kawanishi S. Accumulation of 8-nitroguanine in human gastric epithelium induced by *Helicobacter pylori* infection. *Biochem Biophys Res Commun* 319: 506–510, 2004.
61. Maeda S, Akanuma M, Mitsuno Y, Hirata Y, Ogura K, Yoshida H, Shiratori Y, and Omata M. Distinct mechanism of *Helicobacter pylori*-mediated NF-κB activation between gastric cancer cells and monocytic cells. *J Biol Chem* 276: 44856–44864, 2001.
62. McNaughton L, Puttagunta L, Martinez-Cuesta MA, Kneteman N, Mayers I, Moqbel R, Hamid Q, and Radomski MW. Distribution of nitric oxide synthase in normal and cirrhotic human liver. *Proc Natl Acad Sci USA* 99: 17161–17166, 2002.
63. Midorikawa K, Murata M, and Kawanishi S. Histone peptide AKRHRK enhances $H_2O_2$-induced DNA damage and alters its site specificity. *Biochem Biophys Res Commun* 333: 1073–1077, 2005.
64. Mignogna MD, Fedele S, Lo Russo L, Lo Muzio L, and Bucci E. Immune activation and chronic inflammation as the cause of malignancy in oral lichen planus: is there any evidence ? *Oral Oncol* 40: 120–130, 2004.
65. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Matsuura Y, Kimura S, Miyamura T, and Koike K. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 4: 1065–1067, 1998.
66. Murata M and Kawanishi S. Oxidation of 5′-site guanine at GG and GGG sequences induced by a metabolite of carcinogenic heterocyclic amine PhIP in the presence of Cu(II) and NADH. *Carcinogenesis* 23: 855–860, 2002.
67. Murata M, Kobayashi M, and Kawanishi S. Mechanism of oxidative DNA damage induced by a heterocyclic amine, 2-amino-3,8-dimethylimidazo[4,5*f*]quinoxaline. *Jpn J Cancer Res* 90: 268–275, 1999.
68. Murata M, Kobayashi M, and Kawanishi S. Nonenzymatic reduction of nitro derivative of a heterocyclic amine IQ by NADH and Cu(II) leads to oxidative DNA damage. *Biochemistry* 38: 7624–7629, 1999.
69. Murata M, Tamura A, Tada M, and Kawanishi S. Mechanism of oxidative DNA damage induced by carcinogenic

4-aminobiphenyl. *Free Radic Biol Med* 30: 765–773, 2001.
70. Naito S, Ono Y, Somiya I, Inoue S, Ito K, Yamamoto K, and Kawanishi S. Role of active oxygen species in DNA damage by pentachlorophenol metabolites. *Mutat Res* 310: 79–88, 1994.
71. Neeley WL, Delaney JC, Henderson PT, and Essigmann JM. *In vivo* bypass efficiencies and mutational signatures of the guanine oxidation products 2-aminoimidazolone and 5-guanidino-4-nitroimidazole. *J Biol Chem* 279: 43568–43573, 2004.
72. Oda T, Tsuda H, Scarpa A, Sakamoto M, and Hirohashi S. p53 gene mutation spectrum in hepatocellular carcinoma. *Cancer Res* 52: 6358–6364, 1992.
73. Ohkuma Y, Hiraku Y, Oikawa S, Yamashita N, Murata M, and Kawanishi S. Distinct mechanisms of oxidative DNA damage by two metabolites of carcinogenic *o*-toluidine. *Arch Biochem Biophys* 372: 97–106, 1999.
74. Ohkuma Y and Kawanishi S. Oxidative DNA damage by a metabolite of carcinogenic and reproductive toxic nitrobenzene in the presence of NADH and Cu(II). *Biochem Biophys Res Commun* 257: 555–560, 1999.
75. Ohkuma Y and Kawanishi S. Oxidative DNA damage induced by a metabolite of carcinogenic *o*-anisidine: enhancement of DNA damage and alteration in its sequence specificity by superoxide dismutase. *Arch Biochem Biophys* 389: 49–56, 2001.
76. Ohnishi S and Kawanishi S. Double base lesions of DNA by a metabolite of carcinogenic benzo[*a*]pyrene. *Biochem Biophys Res Commun* 290: 778–782, 2002.
77. Ohshima H, Tatemichi M, and Sawa T. Chemical basis of inflammation-induced carcinogenesis. *Arch Biochem Biophys* 417: 3–11, 2003.
78. Oikawa S, Hirosawa I, Hirakawa K, and Kawanishi S. Site specificity and mechanism of oxidative DNA damage induced by carcinogenic catechol. *Carcinogenesis* 22: 1239–1245, 2001.
79. Oikawa S and Kawanishi S. Copper-mediated DNA damage by metabolites of *p*-dichlorobenzene. *Carcinogenesis* 17: 2733–2739, 1996.
80. Oikawa S, Tada-Oikawa S, and Kawanishi S. Site-specific DNA damage at the GGG sequence by UVA involves acceleration of telomere shortening. *Biochemistry* 40: 4763–4768, 2001.
81. Okuda M, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, and Weinman SA. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 122: 366–375, 2002.
82. Overvad K, Wang DY, Olsen J, Allen DS, Thorling EB, Bulbrook RD, and Hayward JL. Copper in human mammary carcinogenesis: a case-cohort study. *Am J Epidemiol* 137: 409–414, 1993.
83. Peek RM Jr and Blaser MJ. *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. *Nat Rev Cancer* 2: 28–37, 2002.
84. Pignatelli B, Bancel B, Plummer M, Toyokuni S, Patricot LM, and Ohshima H. *Helicobacter pylori* eradication attenuates oxidative stress in human gastric mucosa. *Am J Gastroenterol* 96: 1758–1766, 2001.
85. Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, and Ben-Neriah Y. NF-κB functions as a tumour promoter in inflammation-associated cancer. *Nature* 431: 461–466, 2004.
86. Pinlaor S, Hiraku Y, Ma N, Yongvanit P, Semba R, Oikawa S, Murata M, Sripa B, Sithithaworn P, and Kawanishi S. Mechanism of NO-mediated oxidative and nitrative DNA damage in hamsters infected with *Opisthorchis viverrini*: a model of inflammation-mediated carcinogenesis. *Nitric Oxide* 11: 175–183, 2004.
87. Pinlaor S, Ma N, Hiraku Y, Yongvanit P, Semba R, Oikawa S, Murata M, Sripa B, Sithithaworn P, and Kawanishi S. Repeated infection with *Opisthorchis viverrini* induces accumulation of 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanine in the bile duct of hamsters via inducible nitric oxide synthase. *Carcinogenesis* 25: 1535–1542, 2004.
88. Pinlaor S, Sripa B, Ma N, Hiraku Y, Yongvanit P, Wongkham S, Pairojkul C, Bhudhisawasdi V, Oikawa S, Murata M, Semba R, and Kawanishi S. Nitrative and oxidative DNA damage in intrahepatic cholangiocarcinoma patients in relation to tumor invasion. *World J Gastroenterol* 11: 4644–4649, 2005.
89. Pinlaor S, Tada-Oikawa S, Hiraku Y, Pinlaor P, Ma N, Sithithaworn P, and Kawanishi S. *Opisthorchis viverrini* antigen induces the expression of Toll-like receptor 2 in macrophage RAW cell line. *Int J Parasitol* 35: 591–596, 2005.
90. Pinlaor S, Yongvanit P, Hiraku Y, Ma N, Semba R, Oikawa S, Murata M, Sripa B, Sithithaworn P, and Kawanishi S. 8-nitroguanine formation in the liver of hamsters infected with *Opisthorchis viverrini*. *Biochem Biophys Res Commun* 309: 567–571, 2003.
91. Podolsky DK. Inflammatory bowel disease. *N Engl J Med* 347: 417–429, 2002.
92. Powrie F, Leach MW, Mauze S, Caddle LB, and Coffman RL. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *Int Immunol* 5: 1461–1471, 1993.
93. Poynard T, Yuen MF, Ratziu V, and Lai CL. Viral hepatitis C. *Lancet* 362: 2095–2100, 2003.
94. Rahman MA, Dhar DK, Yamaguchi E, Maruyama S, Sato T, Hayashi H, Ono T, Yamanoi A, Kohno H, and Nagasue N. Coexpression of inducible nitric oxide synthase and COX-2 in hepatocellular carcinoma and surrounding liver: possible involvement of COX-2 in the angiogenesis of hepatitis C virus-positive cases. *Clin Cancer Res* 7: 1325–1332, 2001.
95. Rajentheran R, McLean NR, Kelly CG, Reed MF, and Nolan A. Malignant transformation of oral lichen planus. *Eur J Surg Oncol* 25: 520–523, 1999.
96. Sakano K, Oikawa S, Murata M, Hiraku Y, Kojima N, and Kawanishi S. Mechanism of metal-mediated DNA damage induced by metabolites of carcinogenic 2-nitropropane. *Mutat Res* 479: 101–111, 2001.
97. Saucier MA, Wang X, Re RN, Brown J, and Bryan SE. Effects of ionic strength on endogenous nuclease activity in chelated and nonchelated chromatin. *J Inorg Biochem* 41: 117–124, 1991.
98. Sawa T, Akaike T, Ichimori K, Akuta T, Kaneko K, Nakayama H, Stuehr DJ, and Maeda H. Superoxide generation mediated by 8-nitroguanosine, a highly redox-active

nucleic acid derivative. *Biochem Biophys Res Commun* 311: 300–306, 2003.
99. Schipper DL, Wagenmans MJ, Peters WH, and Wagener DJ. Significance of cell proliferation measurement in gastric cancer. *Eur J Cancer* 34: 781–790, 1998.
100. Scully C, Beyli M, Ferreiro MC, Ficarra G, Gill Y, Griffiths M, Holmstrup P, Mutlu S, Porter S, and Wray D. Update on oral lichen planus: etiopathogenesis and management. *Crit Rev Oral Biol Med* 9: 86–122, 1998.
101. Seike K, Murata M, Hirakawa K, Deyashiki Y, and Kawanishi S. Oxidative DNA damage induced by benz[*a*]anthracene dihydrodiols in the presence of dihydrodiol dehydrogenase. *Chem Res Toxicol* 17: 1445–1451, 2004.
102. Seike K, Murata M, Oikawa S, Hiraku Y, Hirakawa K, and Kawanishi S. Oxidative DNA damage induced by benz[*a*]anthracene metabolites via redox cycles of quinone and unique non-quinone. *Chem Res Toxicol* 16: 1470–1476, 2003.
103. Senesse P, Meance S, Cottet V, Faivre J, and Boutron-Ruault MC. High dietary iron and copper and risk of colorectal cancer: a case-control study in Burgundy, France. *Nutr Cancer* 49: 66–71, 2004.
104. Shibutani S, Takeshita M, and Grollman AP. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature* 349: 431–434, 1991.
105. Shimoda R, Nagashima M, Sakamoto M, Yamaguchi N, Hirohashi S, Yokota J, and Kasai H. Increased formation of oxidative DNA damage, 8-hydroxydeoxyguanosine, in human livers with chronic hepatitis. *Cancer Res* 54: 3171–3172, 1994.
106. Singer II, Kawka DW, Scott S, Weidner JR, Mumford RA, Riehl TE, and Stenson WF. Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. *Gastroenterology* 111: 871–885, 1996.
107. Smith MF, Jr., Mitchell A, Li G, Ding S, Fitzmaurice AM, Ryan K, Crowe S, and Goldberg JB. Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for *Helicobacter pylori*-induced NF-κB activation and chemokine expression by epithelial cells. *J Biol Chem* 278: 32552–32560, 2003.
108. Steenken S and Jovanovic S. How easily oxidizable is DNA? One-electron reduction potentials of adenosine and guanosine radicals in aqueous solution. *J Am Chem Soc* 119: 617–618, 1997.
109. Stevens RG, Jones DY, Micozzi MS, and Taylor PR. Body iron stores and the risk of cancer. *N Engl J Med* 319: 1047–1052, 1988.
110. Sugiyama H and Saito I. Theoretical studies of GG-specific photocleavage of DNA via electron transfer: significant lowering of ionization potential and 5′-localization of HOMO of stacked GG bases in B-form DNA. *J Am Chem Soc* 118: 7063–7068, 1996.
111. Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, and Lee SS. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation. *Mutat Res* 480–481: 243–268, 2001.
112. Suzuki N, Yasui M, Geacintov NE, Shafirovich V, and Shibutani S. Miscoding events during DNA synthesis past the nitration-damaged base 8-nitroguanine. *Biochemistry* 44: 9238–9245, 2005.
113. Takahashi T, Nau MM, Chiba I, Birrer MJ, Rosenberg RK, Vinocour M, Levitt M, Pass H, Gazdar AF, and Minna JD. p53: a frequent target for genetic abnormalities in lung cancer. *Science* 246: 491–494, 1989.
114. Tanaka T, Iwasa Y, Kondo S, Hiai H, and Toyokuni S. High incidence of allelic loss on chromosome 5 and inactivation of p15INK4B and p16INK4A tumor suppressor genes in oxystress-induced renal cell carcinoma of rats. *Oncogene* 18: 3793–3797, 1999.
115. Tatemichi M, Ogura T, Nagata H, and Esumi H. Enhanced expression of inducible nitric oxide synthase in chronic gastritis with intestinal metaplasia. *J Clin Gastroenterol* 27: 240–245, 1998.
116. Thoren F, Romero A, Lindh M, Dahlgren C, and Hellstrand K. A hepatitis C virus-encoded, nonstructural protein (NS3) triggers dysfunction and apoptosis in lymphocytes: role of NADPH oxidase-derived oxygen radicals. *J Leukoc Biol* 76: 1180–1186, 2004.
117. Tyldesley WR and Appleton J. Observations on the ultrastructure of the epithelium in oral lichen planus. *J Oral Pathol* 2: 46–57, 1973.
118. Uttaravichien T, Buddhiswasdi V, and Pairojkul C. Bile duct cancer and the liver fluke: pathology, presentation and surgical management. *Asian J Surg* 19: 267–270, 1996.
119. Wiseman H and Halliwell B. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem J* 313 (Pt 1): 17–29, 1996.
120. Yamamoto K, Inoue S, Yamazaki A, Yoshinaga T, and Kawanishi S. Site-specific DNA damage induced by cobalt(II) ion and hydrogen peroxide: role of singlet oxygen. *Chem Res Toxicol* 2: 234–239, 1989.
121. Yamamoto K and Kawanishi S. Hydroxyl free radical is not the main active species in site-specific DNA damage induced by copper(II) ion and hydrogen peroxide. *J Biol Chem* 264: 15435–15440, 1989.
122. Yermilov V, Rubio J, Becchi M, Friesen MD, Pignatelli B, and Ohshima H. Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite *in vitro*. *Carcinogenesis* 16: 2045–2050, 1995.
123. Zaki MH, Akuta T, and Akaike T. Nitric oxide-induced nitrative stress involved in microbial pathogenesis. *J Pharmacol Sci* 98: 117–129, 2005.

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# Accumulation of 8-nitroguanine in human gastric epithelium induced by *Helicobacter pylori* infection

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**Abstract**

*Helicobacter pylori* infection causes chronic inflammation, which can lead to gastric carcinoma. A double immunofluorescence labeling study demonstrated that the level of 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) apparent in gastric gland epithelium was significantly higher in gastritis patients with *H. pylori* infection than in those without infection. A significant accumulation of proliferating cell nuclear antigen, a prognostic factor for gastric cancer, was observed in gastric gland epithelial cells in patients with *H. pylori* infection as compared to those without infection, and its accumulation was closely correlated with the formation of 8-nitroguanine and 8-oxodG. These results suggest that nitrosative and oxidative DNA damage in gastric epithelial cells and their proliferation by *H. pylori* infection may lead to gastric carcinoma. 8-Nitroguanine could be not only a promising biomarker for inflammation but also a useful indicator of the risk of gastric cancer development in response to chronic *H. pylori* infection.


*Keywords:* *Helicobacter pylori*; Gastric cancer; DNA damage; 8-Nitroguanine; 8-Oxo-7,8-dihydro-2′-deoxyguanosine; Proliferating cell nuclear antigen; Gastric gland epithelial cell; Infection; Inflammation; Double immunofluorescence labeling

*Helicobacter pylori* (*H. pylori*) infection, which is the major cause of atrophic gastritis, is a high risk factor for gastric carcinoma [1]. Recent studies have provided evidence that inflammation plays a role in the pathogenesis of cancer [2]. Reactive oxygen species (ROS) and reactive nitrogen species generated by inflammatory cells may contribute to carcinogenesis through the formation of DNA base lesions, such as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), which can lead to a G:C-to-T:A transversion [3]. 8-oxodG, a marker of oxidative DNA damage, is found at a significantly increased level in the gastric epithelium of *H. pylori*-infected patients [4,5]. Recently, we have reported that 8-nitroguanine is formed in hamster livers in response to an inflammatory reaction caused by a parasitic infection in association with cholangiocarcinoma development [6]. Therefore, in addition to 8-oxodG formation, the accumulation of 8-nitroguanine may play a key role in the initiation and/or promotion of inflammation-mediated carcinogenesis. However, whether 8-nitroguanine is formed in the gastric epithelium of patients with gastric carcinoma that are infected with *H. pylori* has yet to be determined.

To evaluate whether nitrosative DNA damage plays a role early in the carcinogenic process triggered by *H. pylori*, we used a double-immunofluorescent staining procedure to compare the formation of both 8-nitroguanine and 8-oxodG in the gastric epithelium of gastritis patients with and without *H. pylori* infection. Proliferating cell nuclear antigen (PCNA), which

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functions as a cofactor for DNA polymerase δ, is associated with DNA replication [7]. As Schipper et al. [7] reviewed, several studies have demonstrated that PCNA is an independent prognostic factor for gastric cancer in patients with *H. pylori* infection. To evaluate the proliferating activity of gastric epithelial cells in patients infected with *H. pylori*, we examined accumulation of PCNA by immunohistochemical technique.

## Materials and methods

*Patients.* We tested eight gastritis patients with *H. pylori* infection that was confirmed by histology, bacterial culture, and a rapid urease test (five men and three women; mean age, 58.1 ± 15.1 years), and eight gastritis patients without *H. pylori* infection (five men and three women; mean age, 44.9 ± 15.7 years). There was no significant difference in the age or sex ratio of the patients with or without *H. pylori* infection. The permission No. 300 was obtained from the Ethics Committee of the Mie University School of Medicine (9 May, 2002). Informed consent was obtained from each patient. Four biopsy samples, consisting of two antrum and two corpus samples, were taken from each patient.

*Production of anti-8-nitroguanine antibody.* A polyclonal antibody raised against 8-nitroguanine was produced using a modified method [8]. 8-Nitroguanosine was incubated with sodium metaperiodate for 20 min at room temperature and was then allowed to conjugate to rabbit serum albumin (RSA) for 1 h, followed by a 1-h incubation with sodium borohydride. The conjugate was dialyzed overnight against 150 mM NaCl. The resulting product, 8-nitroguanine-aldehyde-RSA, along with Freund's complete adjuvant was injected in rabbit by intracutaneous administration. Four weeks after the initial immunization, the same antigen was again administered, followed by serum collection after subsequent ten days. The antibody was purified using an 8-nitroguanine conjugated column. Specificity of the purified antibody was evaluated using a dot immunobinding assay and an absorption test [9,10].

*Immunohistochemical study.* 8-Nitroguanine and 8-oxodG immunoreactivity in the gastric epithelium was assessed by a double immunofluorescence labeling study as previously described [11]. Briefly, paraffin sections (6-μm thickness) were incubated with a rabbit polyclonal anti-8-nitroguanine antibody (2 μg/ml) and a mouse monoclonal anti-8-oxodG antibody (5 μg/ml, Japan Institute for the Control of Aging, Fukuroi, Japan) overnight at room temperature. Next, the sections were incubated for 3 h with an Alexa 594-labeled goat antibody against rabbit IgG and an Alexa 488-labeled goat antibody against mouse IgG (1:400) (Molecular Probes, Eugene, Oregon, USA). The stained sections were examined under an inverted Laser Scan Microscope (LSM 410, Zeiss, Gottingen, Germany).

The accumulation of PCNA was also assessed by immunohistochemistry. Briefly, paraffin sections (6-μm thickness) were incubated overnight at room temperature with a mouse monoclonal anti-PCNA antibody (1:100; Novocastra Laboratories, Newcastle, United Kingdom). Then, the sections were incubated with a goat anti-mouse IgG conjugate to HRP (1:200). Sections were visualized with 3,3-diaminobenzidine tetrahydrochloride as the chromogen.

Sections were blindly evaluated by two anatomists (M.D., Ph.D.) who were unaware of the clinical status of each patient.

*Statistical analysis.* Comparisons between the data sets were made using the $\chi^2$ test. Spearman's rank correlation coefficient was used to analyze the relative correlation of the qualitative data. Quantitative data from the two groups were compared using the Student's $t$ test. $P < 0.05$ was considered to be statistically significant.

## Results

### *Formation of 8-nitroguanine and 8-oxodG in gastric epithelium*

The formation of 8-nitroguanine and 8-oxodG in gastric epithelium in gastritis patients with *H. pylori* infection is shown in Fig. 1. Notably, intense immunoreactivity of both compounds was observed to colocalize in gastric gland epithelial cells in patients with *H. pylori* infection (Fig. 1, *HP*(+)). On the other hand, in gastritis patients without *H. pylori* infection, little or no immunoreactivity was observed in gastric gland epithelial cells (Fig. 1, *HP*(−)). 8-Nitroguanine formation was observed in both the nuclei and the cytoplasm of the labeled epithelial cells, suggesting that it can form in both DNA and RNA. The 8-oxodG immunoreactivity was coincident with that of 8-nitroguanine within the nuclei of gastric gland cells and surface epithelial cells in *H. pylori*-infected patients (Fig. 1, merged labeling in yellow). Regardless of the *H. pylori* infection status, immunoreactivity of 8-oxodG and 8-nitroguanine was observed in inflammatory cells.

### *Accumulation of PCNA in gastric epithelium*

PCNA accumulation in gastric gland epithelial cells is shown in Fig. 2. PCNA immunoreactivity was not observed in the gastric epithelium of patients without *H. pylori* infection. In the *H. pylori*-infected patients, intense PCNA immunoreactivity was observed in the nucleus of gastric gland epithelial cells.

### *Relationship between the accumulation of 8-nitroguanine, 8-oxodG, and PCNA in patients with H. pylori infection*

We tabulated the number of patients with and without *H. pylori* infection that exhibited immunoreactivity to 8-nitroguanine, 8-oxodG, and PCNA (Table 1). The level of immunoreactivity of 8-nitroguanine, 8-oxodG, and PCNA in patients with *H. pylori* infection was significantly ($P < 0.01$) higher than that observed in patients without infection. The formation of 8-nitroguanine ($R = 0.848$, $P < 0.001$ in corpus and $R = 0.893$, $P < 0.001$ in antrum) and 8-oxodG ($R = 0.882$, $P < 0.001$ in corpus and $R = 0.907$, $P < 0.001$ in antrum) was significantly correlated with PCNA accumulation in the epithelium. 8-Nitroguanine formation was significantly correlated with 8-oxodG formation ($R = 0.976$, $P < 0.001$ in corpus and $R = 0.958$, $P < 0.001$ in antrum).

## Discussion

In this study, we have first demonstrated that *H. pylori* infection can promote 8-nitroguanine formation

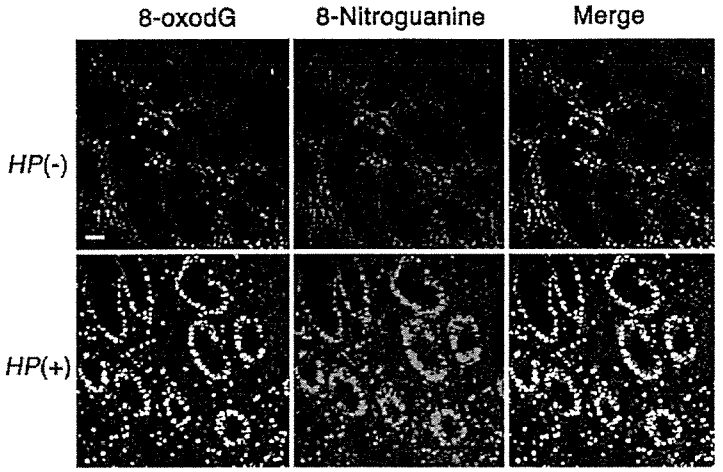


Fig. 1. 8-oxodG and 8-nitroguanine formation in gastritis patients with and without *H. pylori* infection. Double immunofluorescence staining of paraffin sections shows the localization of 8-oxodG and 8-nitroguanine in the gastric epithelium. In *H. pylori*-infected patients (*HP*(+)), the immunoreactivity of 8-oxodG and 8-nitroguanine colocalizes primarily in the nuclei of gastric gland epithelial cells and in some inflammatory cells in the corpus (Merge). In chronic gastritis patients without *H. pylori*-infection (*HP*(−)), the immunoreactivity of 8-oxodG and 8-nitroguanine is observed mainly in the inflammatory cells, while the gastric gland epithelial cells displayed little or no immunoreactivity. Scale bar represents 50 μm.

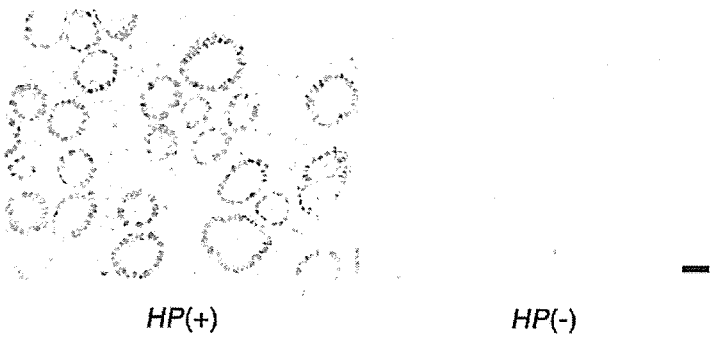


Fig. 2. PCNA accumulation in gastric gland epithelial cells of patients with and without *H. pylori* infection. PCNA accumulation was analyzed by immunohistochemistry with an HRP-conjugated antibody. Intense immunoreactivity of PCNA is observed in the gastric epithelium in the corpus of patients with *H. pylori* infection. Scale bar represents 50 μm.

in human gastric epithelium in addition to 8-oxodG formation. A high level of 8-oxodG and 8-nitroguanine formation was observed, particularly in the gastric gland epithelial cells of patients with *H. pylori* infection; this phenomenon was associated with the persistence of inflammatory cell infiltration. The accumulation of DNA damage in human gastric epithelium induced by *H. pylori* infection can be reasonably explained by the following mechanisms. Neutrophils, the first line of defense against *H. pylori* infection, are not able to kill the bacteria living in the gastric mucus, and persistent *H. pylori* infection will then promote additional infiltration by other inflammatory cells, including macrophages. In patients with *H. pylori*-induced gastritis or gastric ulcers, inducible nitric oxide synthase (iNOS) is expressed in the infiltrating inflammatory cells [12]. Sustained generation of ROS and NO derived from these inflammatory cells due to iNOS expression may contribute to 8-oxodG and 8-nitroguanine formation in the surrounding gastric gland epithelial cells [13]. However, inflammatory cells can also infiltrate into mucosa even in *H. pylori*-negative gastritis. Therefore, *H. pylori* infection must have some additional effects on the host to induce the observed higher levels of 8-oxodG and 8-nitroguanine formation. Fu et al. [14] demonstrated that the expression of iNOS mRNA was significantly increased in *H. pylori*-positive compared to *H. pylori*-negative gastritis, and that the iNOS protein could be detected at a higher level in the epithelial cells of *H. pylori*-positive gastritis patients. We have previously demonstrated that repeated parasitic infections can induce stronger iNOS expression in the epithelium of a target organ in an animal model, probably through cytokine production [9]. There are some reports suggesting that specific cytokines, such as TNF-α [15] and IL-8 [16], induced iNOS. The host immune response to *H. pylori* mediated by cytokines, and the

Table 1
Relationship of 8-nitroguanine, 8-oxodG, and PCNA in gastric epithelium of patients with and without *H. pylori* infection

| Area | Immunoreactivity | 8-Nitroguanine | | 8-oxodG | | PCNA | |
|---|---|---|---|---|---|---|---|
| | | *H. pylori* | | *H. pylori* | | *H. pylori* | |
| | | (−) | (+) | (−) | (+) | (−) | (+) |
| Corpus | − | 7 | 0 | 8 | 0 | 6 | 0 |
| | + | 1 | 0 | 0 | 0 | 2 | 0 |
| | ++ | 0 | 5 | 0 | 5 | 0 | 6 |
| | +++ | 0 | 3 | 0 | 3 | 0 | 2 |
| | | $P = 0.00113$ | | $P = 0.00034$ | | $P = 0.00113$ | |
| Antrum | − | 8 | 0 | 8 | 0 | 4 | 0 |
| | + | 0 | 0 | 0 | 0 | 4 | 0 |
| | ++ | 0 | 2 | 0 | 4 | 0 | 2 |
| | +++ | 0 | 6 | 0 | 4 | 0 | 6 |
| | | $P = 0.00034$ | | $P = 0.00034$ | | $P = 0.00113$ | |
| *Correlation with PCNA* | | | | | | | |
| Corpus | | $R = 0.848$ | | $R = 0.882$ | | | |
| | | $P = 0.00003$ | | $P = 0.00001$ | | | |
| Antrum | | $R = 0.893$ | | $R = 0.907$ | | | |
| | | $P = 0.00000$ | | $P = 0.00000$ | | | |

subsequent iNOS expression, may lead to an increase in the accumulation of 8-nitroguanine and 8-oxodG in epithelium.

The formation of 8-oxodG is known to cause a G → T transversion, which may promote carcinogenesis [17,18]. 8-Nitroguanine undergoes spontaneous depurination, which leads to apurinic sites in DNA [19]. The resulting apurinic sites in DNA can also lead to G → T transversions [20]. The mutation frequency in the gastric gland was 4-fold higher in mice infected with *H. pylori* than in uninfected mice, and it was associated with a high frequency of G → T transversions [21]. Because 8-nitroguanine can also cause G → T transversions, it may contribute to *H. pylori*-induced carcinogenesis in addition to 8-oxodG. This study also showed that PCNA accumulates in gastric epithelial cells in patients with *H. pylori* infection, suggesting an increased proliferation rate of these cells [7]. This observation accords with a previous study which highlighted the importance of an increased proliferation rate in gastric carcinogenesis in response to *H. pylori* infection [22]. Ongoing tissue repair in response to tissue damage, as well as the secretion of cytokines such as growth factors, both stimulate epithelial cell proliferation. Since rapid DNA replication allows the accumulation of potential oncogenic mutations [23], *H. pylori* infection-mediated DNA damage and epithelial cell proliferation may promote gastric carcinogenesis.

3-Nitrotyrosine is a well-known marker of inflammation [24]. 8-oxodG is a representative marker of oxidative DNA damage caused by oxidative stress, including inflammation, mitochondrial respiratory chain reactions, and several $O_2^{\cdot-}$-generating enzymes. We have shown that 8-nitroguanine formation is significantly correlated with PCNA expression. In conclusion, 8-nitroguanine could be not only a promising biomarker for inflammation but also a useful indicator of the risk of gastric cancer development induced by chronic *H. pylori* infection.

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## References

[1] R.M. Peek Jr., M.J. Blaser, *Helicobacter pylori* and gastrointestinal tract adenocarcinomas, Nat. Rev. Cancer 2 (2002) 28–37.
[2] L.M. Coussens, Z. Werb, Inflammation and cancer, Nature 420 (2002) 860–867.
[3] S. Kawanishi, Y. Hiraku, S. Oikawa, Mechanism of guanine-specific DNA damage by oxidative stress and its role in carcinogenesis and aging, Mutat. Res. 488 (2001) 65–76.
[4] S.C. Baik, H.S. Youn, M.H. Chung, W.K. Lee, M.J. Cho, G.H. Ko, C.K. Park, H. Kasai, K.H. Rhee, Increased oxidative DNA damage in *Helicobacter pylori*-infected human gastric mucosa, Cancer Res. 56 (1996) 1279–1282.
[5] B. Pignatelli, B. Bancel, M. Plummer, S. Toyokuni, L.M. Patricot, H. Ohshima, *Helicobacter pylori* eradication attenuates oxidative stress in human gastric mucosa, Am. J. Gastroenterol. 96 (2001) 1758–1766.
[6] S. Pinlaor, P. Yongvanit, Y. Hiraku, N. Ma, R. Semba, S. Oikawa, M. Murata, B. Sripa, P. Sithithaworn, S. Kawanishi, 8-Nitroguanine formation in the liver of hamsters infected with *Opisthorchis viverrini*, Biochem. Biophys. Res. Commun. 309 (2003) 567–571.
[7] D.L. Schipper, M.J. Wagenmans, W.H. Peters, D.J. Wagener, Significance of cell proliferation measurement in gastric cancer, Eur. J. Cancer 34 (1998) 781–790.
[8] B.F. Erlanger, S.M. Beiser, Antibodies specific for ribonucleosides and ribonucleotides and their reaction with DNA, Proc. Natl. Acad. Sci. USA 52 (1964) 68–74.

[9] S. Pinlaor, N. Ma, Y. Hiraku, P. Yongvanit, R. Semba, S. Oikawa, M. Murata, B. Sripa, P. Sithithaworn, S. Kawanishi, Repeated infection with *Opisthorchis viverrini* induces accumulation of 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanine in the bile duct of hamsters via inducible nitric oxide synthase. Carcinogenesis (in press).

[10] R. Hawkes, E. Niday, J. Gordon, A dot-immunobinding assay for monoclonal and other antibodies, Anal. Biochem. 119 (1982) 142–147.

[11] N. Ma, X. Ding, T. Miwa, R. Semba, Immunohistochemical localization of taurine in the rat stomach, Adv. Exp. Med. Biol. 526 (2003) 229–236.

[12] E.E. Mannick, L.E. Bravo, G. Zarama, J.L. Realpe, X.J. Zhang, B. Ruiz, E.T. Fontham, R. Mera, M.J. Miller, P. Correa, Inducible nitric oxide synthase, nitrotyrosine, and apoptosis in *Helicobacter pylori* gastritis: effect of antibiotics and antioxidants, Cancer Res. 56 (1996) 3238–3243.

[13] S.P. Hussain, L.J. Hofseth, C.C. Harris, Radical causes of cancer, Nat. Rev. Cancer 3 (2003) 276–285.

[14] S. Fu, K.S. Ramanujam, A. Wong, G.T. Fantry, C.B. Drachenberg, S.P. James, S.J. Meltzer, K.T. Wilson, Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in *Helicobacter pylori* gastritis, Gastroenterology 116 (1999) 1319–1329.

[15] M. Tatemichi, T. Ogura, H. Nagata, H. Esumi, Enhanced expression of inducible nitric oxide synthase in chronic gastritis with intestinal metaplasia, J. Clin. Gastroenterol. 27 (1998) 240–245.

[16] C.Q. Li, B. Pignatelli, H. Ohshima, Increased oxidative and nitrative stress in human stomach associated with cagA+ *Helicobacter pylori* infection and inflammation, Dig. Dis. Sci. 46 (2001) 836–844.

[17] S.D. Bruner, D.P. Norman, G.L. Verdine, Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA, Nature 403 (2000) 859–866.

[18] S. Shibutani, M. Takeshita, A.P. Grollman, Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG, Nature 349 (1991) 431–434.

[19] V. Yermilov, J. Rubio, H. Ohshima, Formation of 8-nitroguanine in DNA treated with peroxynitrite in vitro and its rapid removal from DNA by depurination, FEBS Lett. 376 (1995) 207–210.

[20] L.A. Loeb, B.D. Preston, Mutagenesis by apurinic/apyrimidinic sites, Annu. Rev. Genet. 20 (1986) 201–230.

[21] E. Touati, V. Michel, J.M. Thiberge, N. Wuscher, M. Huerre, A. Labigne, Chronic *Helicobacter pylori* infections induce gastric mutations in mice, Gastroenterology 124 (2003) 1408–1419.

[22] T. Rokkas, C. Liatsos, A. Karameris, E. Petridou, A. Lazaris, D. Antoniades, E. Kalafatis, Proliferating cell nuclear antigen (PCNA) immunostaining in *Helicobacter pylori* infection: impact of eradication, Pathol. Oncol. Res. 5 (1999) 304–308.

[23] S. Normark, C. Nilsson, B.H. Normark, M.W. Hornef, Persistent infection with *Helicobacter pylori* and the development of gastric cancer, Adv. Cancer Res. 90 (2003) 63–89.

[24] A. van der Vliet, J.P. Eiserich, H. Kaur, C.E. Cross, B. Halliwell, Nitrotyrosine as biomarker for reactive nitrogen species, Methods Enzymol. 269 (1996) 175–184.