obesity from those of prolonged insulin resistance. In fact, these conditions may promote liver fibrosis by distinct mechanisms since obesity and type 2 diabetes are independent predictors for cirrhosis. Obesity alters the bio-availability of several adipose tissue products that are known to modulate fibrosis:

Leptin. Leptin is produced by adipocytes and activated hepatic stellate cells (HSC; the major source of collagen in cirrhotic liver) express leptin receptors, and respond to leptin by proliferating and increasing collagen gene expression. Moreover, obese ob/ob mice that are genetically incapable of producing leptin are relatively resistant to cirrhosis (36). Unlike *ob/ob* mice, most obese humans do not have mutated leptin genes, and thus, their adiposity increases leptin production. Because leptin is a pro-fibrogenic factor, obesity may promote cirrhosis because it causes hyperleptinemia. It is not known, however, whether chronic hyperleptinemia necessarily increases leptin activity. At least two lines of evidence suggest that it does not: 1) obese humans are clearly resistant to the satiety-inducing actions of leptin and 2) although most obese humans are hyperleptinemic, relatively few become cirrhotic. Thus, it seems very likely that HSC (like certain neurons) become refractory to leptin's effects, or that the development of cirrhosis requires additional factors. The latter possibility, in particular, is supported by recent evidence that leptin, itself, regulates other factors that have direct fibrogenic actions on hepatic stellate cells.

Neurotransmitters. Leptin-deficient *ob/ob* mice have reduced levels of norepinephrine (NE), and liver fibrosis can be induced in these mice merely by replacing NE. Like angiotensin, NE functions as a growth factor for HSC. Indeed, adrenoceptor activity appears necessary for optimal HSC responses to leptin, because adrenergic antagonists inhibit the actions of leptin on cultured HSC. Rodent strains with defective leptin receptors, but excessive NE production, are unusually vulnerable to cirrhosis, providing further support for the concept that this neurotransmitter may dictate whether or not hyperleptinemia promotes hepatic fibrosis. Although NE can interact directly with its receptors on HSC to induce fibrogenesis, more complicated mechanisms may mediate NE's actions in intact animals.

Cytokines. Various types of immune cells, including natural killer T (NKT) cells, also express adrenoceptors. NKT cell populations produce cytokines, including IL-4 and IL-10, that promote hepatic fibrosis. NE is a potent viability factor for hepatic $CD4^+$ NKT cells. During NE deficiency, these cells disappear and hepatic levels of IL-4 and IL-10 decline. The low hepatic levels of IL-4 and IL-10 may help to explain why NE-deficient mice are relatively resistant to liver fibrosis. Treating NE-deficient mice with NE restores their hepatic NKT cell populations, increases hepatic IL-4 and IL-10, and permits a fibrotic response to liver injury.

Adiponectin. Support for the importance of adiponectin in the pathogenesis of cirrhosis is also growing. Obesity is generally associated with low levels of adiponectin. Serum levels of adiponectin are inversely related to the severity of the metabolic syndrome. Conversely, treatments that improve the metabolic syndrome increase adiponectin. Because the severity of insulin resistance tends to increase as liver disease progresses to cirrhosis, it is not surprising that adiponectin is decreased in experimental animals with cirrhosis. In fact, low adiponectin may promote cirrhosis because adiponectin knock-out mice are exquisitely sensitive to CCl_4-induced cirrhosis and cirrhosis is prevented by replenishing adiponectin. Additional work is needed to delineate the cellular and molecular mechanisms involved.

8.5. Obesity-related liver disease can expand hepatic progenitor cell populations

Obesity has been associated with HCC. Obesity also increases the risk of dying from HCC. Compared to men with a normal BMI, obese men have ~ 5 fold increased risk of HCC-related mortality. A positive, albeit weaker, correlation between HCC mortality and obesity also occurs in women. In humans, HCC is rarely detected in non-cirrhotic livers. Therefore, obesity may increase HCC simply by increasing the incidence of cirrhosis. However, HCC is also extremely prevalent in murine models of NASH that rarely develop cirrhosis. Therefore, carcinogenic programs may be activated by factors that are altered either during chronic liver disease per se or by obesity or insulin resistance.

Hepatic accumulation of liver progenitor cells is an early event in chemically induced carcinogenesis, and is also typical of humans with chronic viral hepatitis, who are acknowledged to have an increased risk for HCC. An analysis of liver biopsy samples from patients and mice with fatty liver disease of diverse etiologies suggests significant expansion of hepatic progenitor cell populations. Moreover, hepatic accumulation of progenitors becomes progressively greater as liver damage progressed from steatosis to cirrhosis. Therefore, HCC may be related to the expansion of hepatic progenitor cell populations that occurs during the progression of obesity-related liver disease. More research is needed to clarify the mechanisms that regulate the growth and differentiation of these cells.

Conclusion

In summary, obesity is associated with the risk of death from all cancers and from cancers at individual sites including liver cancer, and increases the incidence of HCC due to accelerating the progression from steatohepatitis to cirrhosis. Currently, patients with obesity-related fatty liver are less likely to

be enrolled in HCC surveillance programs resulting in delay in diagnosis and poorer prognosis. More frequent surveillance for HCC may be warranted in obese patients with fatty liver and attempts should be made to interrupt the progression from simple hepatic steatosis to steatohepatitis, cirrhosis and HCC. Weight reduction may reasonably decrease the incidence of liver diseases or interrupt their progression, ultimately reducing the incidence of HCC and related death. Calle et al. [13] have estimated that 90 000 deaths due to cancer could be prevented each year in the United States if men and women could maintain normal weight. It is unlikely that this goal can be achieved without concerted effort and substantial investment of policymakers, educators, clinicians, employers, and schools to promote physical activity and healthy dietary practices as a cultural norm. Effective prevention and treatment of obesity, fatty liver and related liver cancer must have a long way to go. In this context, a recent report by Muto et al. [60] is quite interesting. They conducted a multicenter, randomized, controlled trial to investigate the effect of long-term oral supplementation with branched-chain amino acids (BCAA) on the event-free survival in 622 patients with decompensated cirrhosis, and found that the risk for HCC is significantly higher for males, patients with concurrent diabetes mellitus, patients with an alpha-fetoprotein (AFP) level of 20 ng/mL or higher, patients with higher BMI, and patients with lower serum albumin levels. Also, they observed that oral supplemental treatment with BCAA reduces the risk of HCC in cirrhotic patients with these specific factors.

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Induction of Prostaglandin E_2 Pathway Promotes Gastric Hamartoma Development with Suppression of Bone Morphogenetic Protein Signaling

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Abstract

Mutations in bone morphogenetic protein (BMP) receptor IA (*BMPR1A*) are responsible for a subset of cases of juvenile polyposis (JP) syndrome that develops hamartomatous tumors in the gastrointestinal tract. Mouse genetic studies have shown that suppression of BMP signaling in the intestines causes JP-type hamartoma development. Here, we generated *K19-Nog* transgenic mice expressing noggin, a BMP antagonist, in gastric epithelium. However, inhibition of BMP signaling did not cause gastric phenotypes. We thus crossed *K19-Nog* with *K19-C2mE* mice that expressed *Ptgs2* and *Ptges* in the stomach to generate compound transgenic mice. Expression of *Ptgs2* and *Ptges* results in prostaglandin E_2 (PGE_2) biosynthesis, and both enzymes are induced in most human gastrointestinal tumors. Importantly, *K19-Nog/C2mE* compound mice developed gastric hamartomas that were morphologically similar to those found in JP with mucin-containing dilated cysts and inflammatory infiltration. Notably, treatment of *K19-Nog/C2mE* mice with a cyclooxygenase-2 inhibitor, celecoxib, significantly reduced tumor size with suppression of angiogenesis, suggesting that induction of the PGE_2 pathway together with inhibition of BMP signaling is required for gastric hamartoma development. Moreover, microarray analyses revealed that canonical Wnt signaling target genes were not induced in *K19-Nog/C2mE* hamartomas, indicating that BMP inhibition and PGE_2 induction lead to gastric hamartoma development independent of the Wnt/β-catenin pathway. These results, taken together, suggest that the PGE_2 pathway is an effective preventive target against BMP-suppressed gastric hamartomas, as well as for Wnt/β-catenin–activated adenocarcinomas. [Cancer Res 2009;69(7):2729–33]

Introduction

Juvenile polyposis (JP) is a hereditary gastrointestinal hamartomatous polyposis syndrome (1). Germline mutations in bone morphogenetic protein (BMP) receptor type IA gene (*BMPR1A*) have been found in a subpopulation of JP patients (2). BMP ligands bind to a complex of the BMP receptor type II and type I, which leads to phosphorylation of Smad1,5,8, allowing them to form a complex with Smad4 (3, 4). These Smad complexes translocate to nuclei and function as transcription enhancers. BMP signaling inhibits epithelial cell proliferation and promotes differentiation (5, 6), and suppression of BMP signaling in mice results in intestinal hamartomatous polyp development through activation of the PI3K-Akt pathway (6, 7). Moreover, intestinal epithelial cell–specific deletion of *Bmpr1a* results in elongated villi and crypt fission (8). These results indicate that BMP signaling promotes intestinal epithelial differentiation, and, thus, suppression of the BMP pathway causes tumorigenesis. Although the main affected site of tumors in JP patients is the colon, gastric polyps have been found in 14% of JP patients, and cancer risk in JP patients increases both in the colon and stomach (9, 10). Recently, it was reported that disruption of *Bmpr1a* in mouse stomach results in development of tumorous lesions in squamocolumnar and gastrointestinal transition zones, suggesting that suppression of BMP signaling triggers tumor development also in the stomach (11).

On the other hand, we found that expression of cyclooxigenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) is induced simultaneously in gastrointestinal tumor tissues (12). COX-2 and mPGES-1 are functionally coupled for biosynthesis of prostaglandin E_2 (PGE_2; ref. 13) that plays a critical role in tumorigenesis in the gastrointestinal tract (14–17). However, the role of the PGE_2 pathway in hamartomatous tumors is not understood. We constructed transgenic mice expressing *Nog* encoding noggin in the gastric mucosa and crossed them with another transgenic mice expressing both *Ptgs2* and *Ptges* encoding COX-2 and mPGES-1, respectively, (16). We show that inhibition of BMP signaling is not sufficient for gastric tumorigenesis, but that BMP suppression together with PGE_2 induction causes development of JP-type gastric hamartoma.

Materials and Methods

Mouse models. *K19-C2mE* mice expressing *Ptgs2* and *Ptges*; *K19-Wnt1* mice expressing *Wnt1*; and *K19-Wnt1/C2mE* mice expressing *Wnt1*, *Ptgs2*, and *Ptges*, were described previously (16, 17). pK19-Nog was constructed using keratin 19 gene promoter, mouse *Nog* cDNA, and SV40 p(A) cassette (Fig. 1*A*). The expression vector was microinjected into the fertilized eggs of F1 (C3H and C57BL/6) mice (CLEA) to generate *K19-Nog* mice. Primer sequences used for genotyping were as follows: F-5′-GTACGCGTGGAATGACCTAGG-3′, F-5′-GCAAAGGGTCGCTACAGACGT-3′. Transgenic vector constructs are shown in Fig. 1*A*. *K19-Nog* and *K19-C2mE* mice were crossed to generate *K19-Nog/C2mE* mice. Gastric phenotypes of these mice were examined at age 30 wk. For inhibition of COX-2, mice were given p.o. with celecoxib (Pfizer) at 100 mg/kg/d for 3 wk. All animal experiments were carried out according to the protocol approved by Ethics Committees on Animal Experimentation of Kanazawa University.

Real-time reverse transcription-PCR. Total RNA was reverse-transcribed and PCR-amplified. Primer sets used in real-time reverse

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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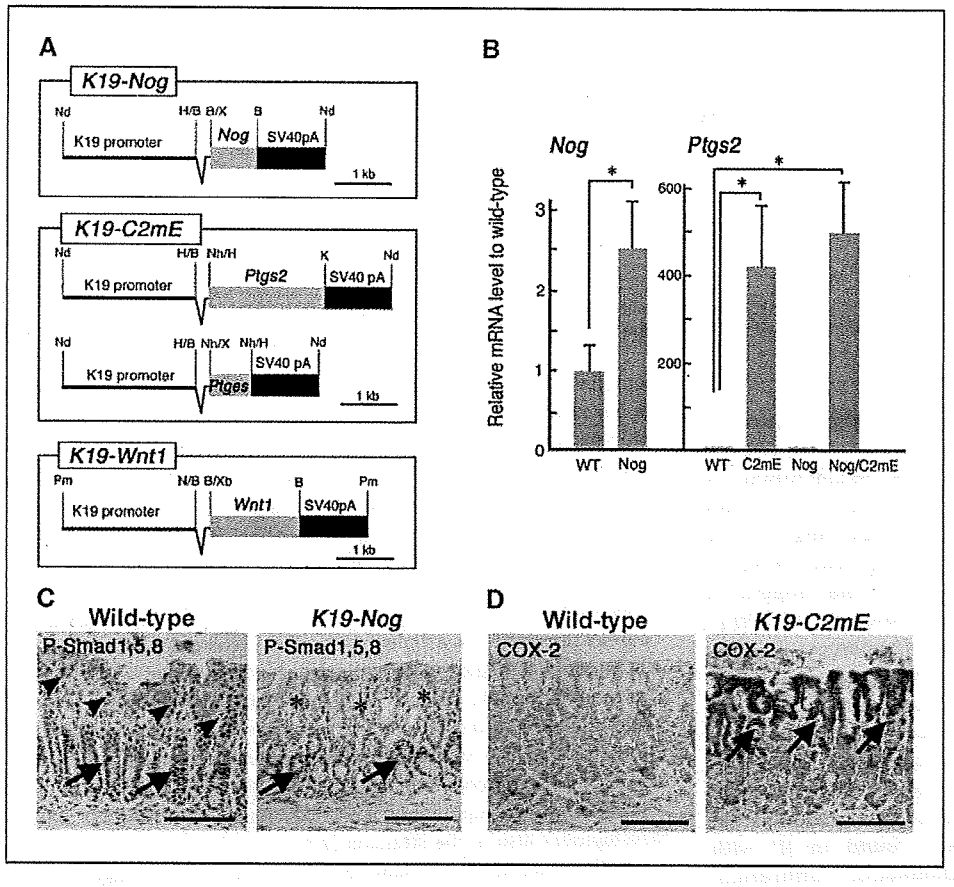

Figure 1. Generation of transgenic mice. *A*, transgenic vectors. *B*, relative mRNA levels (mean ± SD) of *Nog* and *Ptgs2* in the gastric mucosa of *K19-Nog* (*Nog*), *K19-C2mE* (*C2mE*), and *K19-Nog/C2mE* (*Nog/C2mE*) mice to the wild-type (*WT*). *, $P < 0.05$. *C*, immunohistochemistry of phosphorylated Smad1,5,8 in gastric mucosa of wild-type and *K19-Nog* mice. *Arrowheads* and *arrows*, positive nuclear staining in surface and gland bottom, respectively. *Asterisks*, decreased immunostaining signal in *K19-Nog* mouse. *D*, immunohistochemistry of COX-2 in wild-type and *K19-C2mE* mouse stomach. *Arrows*, transgenic expression of COX-2 in *K19-C2mE* gastric mucosa where *K19* promoter is transcriptionally active. *Bars*, 100 μm.

transcription-PCR for detection of *Nog, Ptgs2, Ptger1, Ptger2, Ptger3*, and *Ptger4* were purchased (TakaraBio).

Histology. Tissues were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at 4-μm thickness. The following antibodies were used for immunostaining: anti-COX-2 (Cayman Chemical), anti-F4/80 (Serotec), anti-α-smooth muscle actin (Sigma), anti-Ki-67, anti-von Willebrand factor (DakoCytomation), and anti-phosphorylated Smad1,5,8 (Chemicon). Staining signal was visualized using the Vectorstain Elite kit (Vector Laboratories).

X-ray computed tomography. *K19-Nog/C2mE* mice were subjected to X-ray computed tomography using LaTheta LCT-100 (Aloka). Computed tomography analyses were performed 1 wk before celecoxib treatment and at 0, 1, 2, and 3 wk after treatment. Tumor size on computed tomography images was measured using NIH Image software (NIH).

Immunoblotting. Tissue samples were homogenized in lysis buffer. Protein samples were separated in a SDS-polyacrylamide gel. Antibody for the active β-catenin (Upstate) was used. The enhanced chemiluminescence detection system (Amersham) was used to detect specific signals.

Microarray analyses. Total RNA were prepared from mouse stomach at age 30 wk. Expression profiles of the Wnt target genes,[4] cytokines, and chemokines were examined with the Affymetrix GeneChip system and Mouse Genome 430 2.0 Arrays (Affymetrix).

Statistical analyses. Statistical analyses were carried out using Student's *t* test.

Results and Discussion

Generation of *K19-Nog* transgenic mice. To suppress BMP signaling in the stomach, we constructed *K19-Nog* mice that expressed *Nog* encoding noggin in gastric epithelial cells (Fig. 1*A*). Noggin is a polypeptide that inhibits BMP signaling by binding BMP ligands (4). We confirmed increased levels of *Nog* mRNA in *K19-Nog* mouse stomach compared with that in the wild-type by real-time RT-PCR (Fig. 1*B*). BMP type I receptor phosphorylates Smad1,5,8 upon complex formation with BMP ligand and type II receptor (3). We found phosphorylated Smad1,5,8 by immunohistochemistry in the nuclei of differentiated epithelial cells both at the surface and bottom of the gastric gland (Fig. 1*C*), which is consistent with a previous report (11). Notably, in the *K19-Nog* mice, the immunostaining signals of phosphorylated Smad1,5,8 decreased significantly in the upper gastric gland where the K19 promoter is transcriptionally active (Fig. 1*C* and *D*). These results indicate that exogenous *Nog* expression inhibits BMP signaling in the stomach.

Construction of compound mutant mice. We previously constructed *K19-C2mE* transgenic mice expressing both *Ptgs2* and *Ptges* encoding COX-2 and mPGES-1, respectively, in gastric mucosa (Fig. 1*A* and *D*). Expression of COX-2 and mPGES-1 leads to increase of PGE_2 level in *K19-C2mE* mouse stomach (16). To investigate the effect of the PGE_2 pathway in BMP-suppressed gastric mucosa, we crossed *K19-Nog* and *K19-C2mE* mice to generate *K19-Nog/C2mE* compound mice. We confirmed expression of *Ptgs2* by real-time RT-PCR in the stomach of *K19-C2mE* and *K19-Nog/C2mE* mice but not in wild-type and *K19-Nog* mice (Fig. 1*B*). We also used *K19-Wnt1/C2mE* mice for this

[4] http://www.stanford.edu/~rnusse/wntwindow.html

study that develop gastric adenocarcinoma caused by simultaneous activation of the Wnt and PGE_2 pathways (17). Genotypes of respective transgenic strains were confirmed by genomic PCR (Supplementary Fig. S1).

Gastric tumor development in *K19-Nog/C2mE* mice. *K19-Nog* mice did not develop tumorous lesions in the stomach, and the histology of gastric glands was normal (Supplementary Fig. S2; Fig. 2*A*). In contrast, *K19-C2mE* mice developed inflammation-associated hyperplasia, and *K19-Wnt1* mice developed dysplastic preneoplastic lesions, which were consistent with previous reports (16, 17). Importantly, *K19-Nog/C2mE* mice develop large tumors in the glandular stomach (Supplementary Fig. S2; Fig. 2*A*). These results suggest that suppression of BMP signaling is insufficient for gastric tumorigenesis, but that cooperation of BMP inhibition and PGE_2 induction cause gastric tumor development. Such an effect of PGE_2 on tumorigenesis was similar to that found in *K19-Wnt1/C2mE* mice. Activation of Wnt/β-catenin alone leads to development of small preneoplastic lesions, whereas simultaneous activation of the Wnt/β-catenin and PGE_2 pathways cause gastric adenocarcinoma (Fig. 2*A*; ref. 17). Therefore, PGE_2 plays an important role in tumorigenesis regardless of the types of genetic alterations.

JP-type hamartoma in *K19-Nog/C2mE* mouse stomach. Histologically, gastric tumors in *K19-Nog/C2mE* mice displayed irregular branching of epithelial cell layers, combined with dilated cysts that were filled with mucin (Fig. 2*B* and *C*). Such histological characteristics were different from dysplastic tumors of the *K19-Wnt1/C2mE* mice (Fig. 2*B*; ref. 17). We also found abundant α-smooth muscle actin–positive myofibroblasts in stroma. Moreover, we detected infiltration of F4/80-positive macrophages and the accumulation of lymphocytes in the *K19-Nog/C2mE* tumors (Fig. 2*C*). These histological characteristics are typical of the hamartoma of JP patients (9, 10, 18), indicating that suppression of BMP signaling associated with PGE_2 induction causes development of JP-type gastric hamartoma. However, tumor incidence in *K19-Nog/C2mE* mice was 23%, whereas that in *K19-Wnt1/C2mE* mice was 100% (Supplementary Table). Notably, expression of inflammatory cytokine tumor necrosis factor-α (TNF-α) increased in *K19-Nog/C2mE* hamartomas as well as in *K19-C2mE* hyperplasia (Supplementary Fig. S3). However, TNF-α was not induced in nontumor stomach of *K19-Nog/C2mE* mice, whereas transgenic expression of *Ptgs2* stayed at the same level as that in tumor tissues. These results suggest that inflammatory response is also important for hamartoma development together with BMP suppression and PGE_2 induction.

Suppression of gastric hamartoma by COX-2 inhibition. To investigate whether the PGE_2 pathway is required for gastric hamartoma development, we treated *K19-Nog/C2mE* mice with a COX-2 selective inhibitor, celecoxib, at 100 mg/kg/day for 3 weeks. We examined gastric tumor size by X-ray computed tomography scanning, and found that the tumor volume of *K19-Nog/C2mE* mice decreased significantly by celecoxib treatment (Fig. 3*A*). The mean relative tumor size on computed tomography images reduced to 58% after celecoxib treatment (Fig. 3*B*). Histologically, cystic structures were no longer found, and necrotic area was detected in the celecoxib-treated *K19-Nog/C2mE* tumors (Fig. 3*C*). The PGE_2 pathway is important for angiogenesis of gastrointestinal tumorigenesis (15, 19). Consistently, the number of capillary vessels decreased significantly in celecoxib-treated *K19-Nog/C2mE* tumors

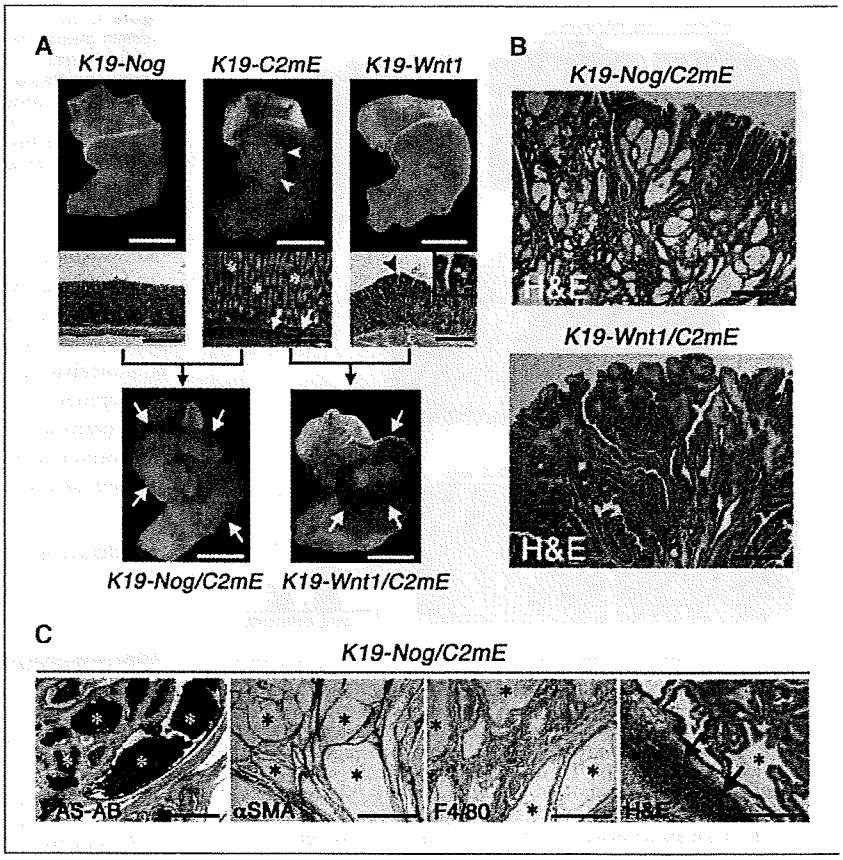

Figure 2. Gastric tumors developed in *K19-Nog/C2mE* mice. *A*, macroscopic photographs and H&E of *K19-Nog*, *K19-C2mE*, and *K19-Wnt1* mouse stomach (*top*). Arrowheads and asterisks in *K19-C2mE* indicate hyperplasia; *arrows*, inflammatory infiltration. Arrowhead and inset in *K19-Wnt1* indicate preneoplastic lesion. Gastric tumors in *K19-Nog/C2mE* and *K19-Wnt1/C2mE* mice are shown (*arrows*, *bottom*). *Bars*, 10 mm. *B*, histology of gastric tumors of *K19-Nog/C2mE* and *K19-Wnt1/C2mE* (H&E). *Bars*, 200 μm. *C*, PAS-Alcian blue, immunostaining for αSMA, F4/80, and H&E of *K19-Nog/C2mE* hamartomas (from left to right). *, dilated cysts. *Arrows*, lymphocyte accumulation. *Bars*, 100 μm.

(Fig. 3*D*). Accordingly, it is possible that angiogenesis is one of the important functions of PGE_2 for hamartoma development.

Wnt-independent development of gastric hamartoma. In the intestinal crypt, inhibition of BMP signaling results in activation of the Wnt/β-catenin pathway (7). We thus examined activation of Wnt signaling in *K19-Nog/C2mE* gastric tumors. The level of the active β-catenin did not increase in *K19-Nog/C2mE* hamartomas, whereas it elevated markedly in *K19-Wnt1/C2mE* tumors (Fig. 4*A*). Consistently, expression of Wnt target genes in *K19-Nog/C2mE* tumors stayed at the same level as that in wild-type mouse stomach, whereas these genes were up-regulated in *K19-Wnt1/C2mE* tumors (Fig. 4*B*). We confirmed that inflammatory cytokines and chemokines were induced in both *K19-Nog/C2mE* and *K19-Wnt1/C2mE* tumors. Accordingly, activation of Wnt signaling is not involved in hamartoma development in BMP-suppressed gastric mucosa, although PGE_2 signaling or PGE_2-dependent inflammation may be required for both adenocarcinoma and hamartoma.

We next examined expression of PGE_2 receptors, EP1 to EP4, in tumor tissues. Notably, the expression of *Ptger1, Ptger2*, and *Ptger3* encoding EP1, EP2, and EP3, respectively, decreased significantly in tumors of *K19-Nog/C2mE* and *K19-Wnt1/C2mE* mice (Fig. 4*C*). In contrast, expression of *Ptger4* encoding EP4 increased dramatically in both *K19-Nog/C2mE* hamartomas and *K19-Wnt1/C2mE* adenocarcinomas. These results suggest that PGE_2 signaling through EP4 is important for development of both gastric hamartoma and adenocarcinoma. Namely, it is possible that the type of genetic alterations determines the histological phenotype of tumors, hamartoma or adenocarcinoma, and that the induced PGE_2 pathway promotes tumor growth through EP4 receptor regardless of histological types (Supplementary Fig. S4).

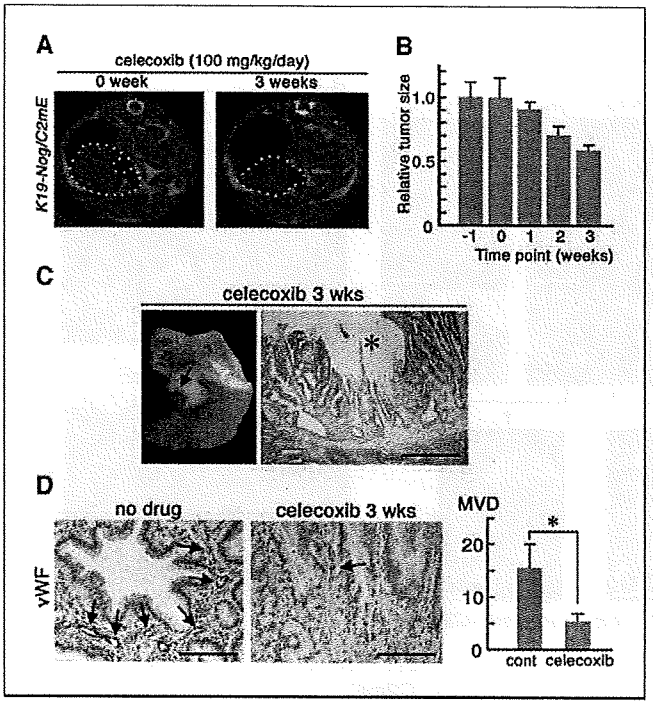

Figure 3. Suppression of hamartoma development by COX-2 inhibition. *A*, X-ray computed tomography images of gastric tumors of the same *K19-Nog/C2mE* mouse (*yellow dashed lines*) at 0 and 3 wk of celecoxib treatment. *B*, relative tumor size at each time point of celecoxib treatment to the level at 0 wk (mean ± SD). *C*, representative photograph and H&E of celecoxib-treated *K19-Nog/C2mE* tumors. *Arrow* and *, necrotic area. *D*, immunostaining for vWF. *Arrows*, vWF-positive capillary vessels. Microvessel densities (*MVD*) are shown (mean ± SD). *, $P < 0.05$. *Cont*, control.

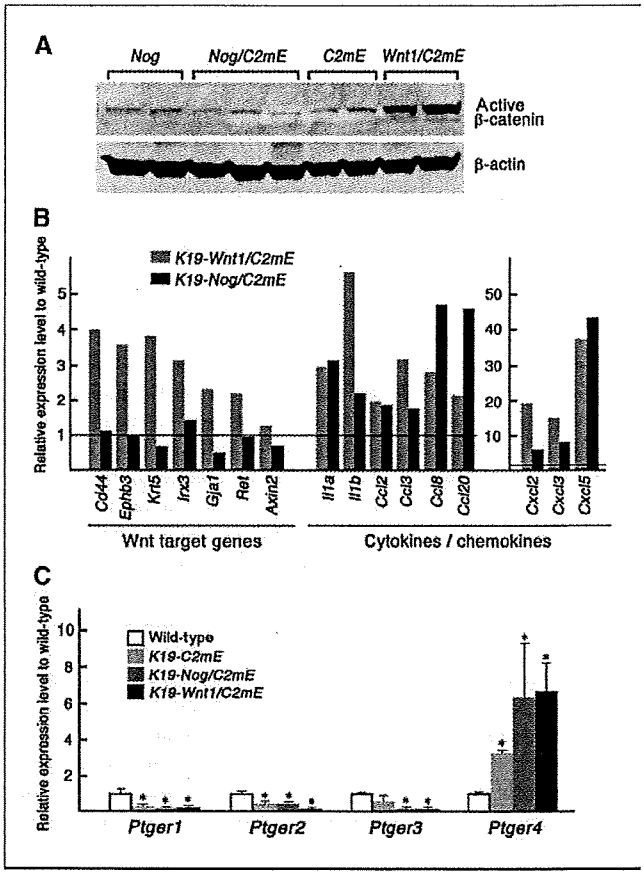

Figure 4. Wnt activity and EP expression in gastric hamartomas. *A*, Western blotting for active β-catenin in the stomach of each genotype. *B*, relative expression levels determined by microarray analyses of Wnt target genes and cytokines/chemokines in *K19-Wnt1/C2mE* and *K19-Nog/C2mE* tumors to the wild-type level. *C*, relative expression levels of PGE_2 receptors, *Ptger1, Ptger2, Ptger3*, and *Ptger4* in *K19-C2mE* hyperplasia, *K19-Nog/C2mE* hamartoma, and *K19-Wnt1/C2mE* adenocarcinoma to the wild-type level (mean ± SD). *, $P < 0.05$.

In human stomach, expression of COX-2 is induced in *Helicobacter pylori*–associated gastric lesions (20). Accordingly, it is conceivable that *H. pylori* infection contributes to the development of gastric hamartoma through induction of the PGE_2 pathway. Therefore, inhibition of the PGE_2 pathway as well as eradication of *H. pylori* may be an effective preventive strategy not only for gastric cancer but also for gastric hamartoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Roles of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 expression and β-catenin activation in gastric carcinogenesis in *N*-methyl-*N*-nitrosourea-treated K19-C2mE transgenic mice

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K19-C2mE transgenic (Tg) mice, simultaneously expressing cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) in the gastric mucosa under the cytokeratin 19 gene promoter, were here treated with *N*-methyl-*N*-nitrosourea (MNU) and inoculated with *Helicobacter pylori* (*H. pylori*) to investigate gastric carcinogenesis. Wild-type (WT) and Tg mice undergoing MNU treatment frequently developed tumors in the pyloric region (100% and 94.7%, respectively); multiplicity in Tg was higher than that in WT ($P < 0.05$) with *H. pylori* infection. Larger pyloric tumors were more frequently observed in Tg than in WT ($P < 0.05$). In addition, Tg developed fundic tumors, where WT did not. No gastric tumors were observed without MNU treatment. Transcripts of TNF-α, iNOS, IL-1β, and CXCL14 were up-regulated with *H. pylori* infection in both genotypes and were also increased more in Tg than in WT within *H. pylori*-inoculated animals. Immunohistochemical analysis demonstrated significantly greater β-catenin accumulation in pyloric tumors, compared with those in the fundus ($P < 0.01$) with mutations of exon 3; 18.2% and 31.6% in MNU-alone and MNU + *H. pylori*-treated WT, whereas 21.4% and 62.5% was observed in the Tg, respectively; the latter significantly higher ($P < 0.05$), suggesting the role of *H. pylori* in Wnt activation. In conclusion, K19-C2mE mice promoted gastric cancer in both fundic and pyloric regions. Furthermore β-catenin activation may play the important role of pyloric carcinogenesis especially in *H. pylori*-infected Tg. Induction of various inflammatory cytokines in addition to overexpression of COX-2/mPGES-1 could be risk factors of gastric carcinogenesis and may serve as a better gastric carcinogenesis model. (*Cancer Sci* 2008; 99: 2356–2364)

There is a large body of evidence that *Helicobacter pylori* (*H. pylori*) infection is involved in development of chronic gastritis, peptic ulceration, and gastric cancer.[1,2] Recent reports have revealed that *H. pylori* infection induces cyclooxygenase-2 (COX-2) expression and microsomal prostaglandin E synthase-1 (mPGES-1), enzymes responsible for synthesizing prostaglandin E_2 (PGE_2) in gastric mucosa.[3–5] In the stomach, prostaglandins are major molecules for maintaining the gastric mucosa.[6] PGE_2 plays distinct roles in tumor growth and metastasis in several cancers.[7]

Oshima *et al.* recently constructed transgenic mice (K19-C2mE) that simultaneously overexpress COX-2 and mPGES-1 in the gastric mucosa under the influence of the cytokeratin 19 gene promoter.[4] The transgenic (Tg) mice develop inflammation-associated hyperplastic lesions in the proximal glandular stomach, similar to those found in the *Helicobacter*-infected stomach.[4] Furthermore, K19-Wnt1/C2mE Tg mice, simultaneously expressing Wnt1 as well as COX-2/mPGES-1, develop dysplastic gastric tumors,[8] indicating that COX-2/mPGES-1 and Wnt pathway activation might be involved in mouse gastric neoplasia.

The Wnt/β-catenin signaling pathway plays important roles in cell–cell adhesion and cell cycle regulation and its alternation is implicated in genesis of many cancers. Abnormal nuclear accumulation of β-catenin due to mutation of the β-catenin gene stimulates the expression of β-catenin/Tcf target genes, such as *c-myc*, *c-jun*, and *cyclin D1*.[9,10] Mutations of β-catenin gene exon 3, where serine and threonine residues are physiologically phosphorylated by glycogen synthase kinase (GSK)-3β, prevent degeneration by APC/GSK-3β/Axin complex. With human gastric cancers, nuclear accumulation of β-catenin has been estimated to occur in 12–37% of cases,[11–15] with mutations in exon 3 reported in a few to over 20%.[12,16,17] However, the degree of involvement of Wnt pathway alteration in the development of mouse gastric cancers remains unclear.

In the present study, Tg mice were treated with a stomach carcinogen, *N*-methyl-*N*-nitrosourea (MNU), and inoculated with *H. pylori* to investigate the influence of COX-2/mPGES-1 expression and *H. pylori* infection on mouse gastric carcinogenesis. Furthermore, we analyzed the frequency of β-catenin activation and gene mutations to assess involvement of the Wnt pathway.

Materials and Methods

Experimental design. The experimental design is shown in Fig. 1. K19-C2mE Tg mice and littermate wild-type (WT) mice were randomly divided into four groups (groups A–D). The mice of groups B and D were inoculated intragastrically with 0.8 mL of broth culture containing *H. pylori*. After 1 week, the mice of groups C and D were given MNU (Sigma Chemical Co., St Louis, MO, USA) in drinking water at the concentration of 120 p.p.m. on alternate weeks (total exposure was 5 weeks) and then normal tap water until the end of experiment. MNU was dissolved in distilled water and freshly prepared three times per week. At the end of the experiment, all surviving mice were sacrificed under deep anesthesia 60 min after an intraperitoneal

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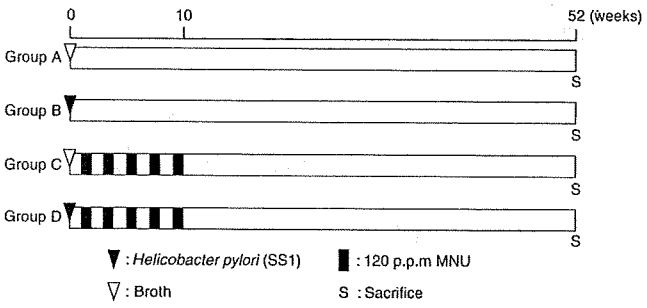

Fig. 1. Experimental design. Five- to 6-week-old K19-C2mE transgenic mice and littermate wild-type mice were inoculated with *Helicobacter pylori* SS1 (groups B and D) or broth (groups A and C). After 1 week, animals of groups C and D were administered 120 p.p.m. *N*-methyl-*N*-nitrosourea on alternate weeks (total exposure, 5 weeks).

injection of 5′-bromo-2′-deoxyuridine (BrdU) at a dose of 100 mg/kg. The excised stomachs were fixed in 10% neutral-buffered formalin or 95% ethanol plus 1% acetic acid for histology and immunohistochemistry.

Animals. K19-C2mE transgenic (Tg) mice produced by Oshima *et al.*[4] were maintained by breeding male K19-C2mE Tg with female C57BL/6 N at the Animal Facility of Aichi Cancer Center Research Institute. WT mice were used as controls. All were housed in plastic cages with hardwood chips in an air-conditioned room with 12 h light–12 h dark cycle and given a basal diet (CA-1; CLEA Japan Inc., Tokyo, Japan) and water *ad libitum*. For genotyping of each mouse, DNA samples were extracted from the tails using a DNeasy tissue kit (Qiagen, Tokyo, Japan) and subjected to polymerase chain reaction (PCR) as reported elsewhere.[4]

Bacterial culture. *H. pylori* strain SS1 was inoculated on Brucella agar plates (Merck, Darmstadt, Germany) containing 7% v/v heat-inactivated fetal calf serum and incubated at 37°C under microaerobic conditions using an Anaero Pack Campylo (Mitsubishi Gas Chemical Co., Tokyo, Japan) at high humidity for 2 days. Then, bacteria grown on the plates were introduced into Brucella broth (Becton Dickson, Cockeysville, MD, USA) supplemented with 7% v/v fetal calf serum, and cultures of *H. pylori* were checked under a phase contrast microscope for bacterial shape and mobility.

Histopathological analysis. Tissue sections were stained with hematoxylin–eosin and Alcian blue (pH 2.5)–periodic acid-Schiff (AB-PAS) for histological analysis. Tumor location was categorized into 'fundic' and 'pyloric', the former included tumors developing in the gastric fundic mucosa and border areas of fundic and pyloric glands. The glandular mucosa was examined histologically for any inflammatory and epithelial changes. Active chronic gastritis was estimated according to criteria modified from the updated Sydney System, characterized by infiltration of neutrophils and lymphocytes. The degree of change was graded in a scale from 0 to 3, (0 [normal], 1 [mild], 2 [moderate], and 3 [marked]). Mucosa thickness was measured using AxioVision 4.6 (Carl Zeiss, Jena, Germany). Tumor size was evaluated by the largest tumor area using NIH image version 1.62 (National Institutes of Health, USA) on hematoxylin–eosin sections. Serial sections were also stained immunohistochemically with antibodies against COX-2 (Cayman Chemical, Ann Arbor, MI, USA), β-catenin (clone 14; BD Transduction Laboratories, KY, USA), and BrdU (Dako, Glostrup, Denmark). BrdU labeling index was calculated as the percentages of BrdU-positive epithelial cells within glands at five different arbitrarily selected points in gastric mucosa.

Analysis of inflammatory cytokine mRNA by quantitative real-time reverse transcription (RT)–PCR. Total RNA was extracted from the border areas of fundic and pyloric regions in the glandular stomach mucosa using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany). First strand cDNAs were synthesized using a Super Script III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Relative quantitative PCR for COX-2, tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), IL-6, and CXCL14 was performed using the mouse glyceraldhyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control with the StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using a QuantiTect SYBR Green PCR kit (Qiagen). The primer sequences are listed in Table 1. Quantification was performed as earlier established.[18] The expression levels were expressed relative to 1.00 in WT mice in the control group A.

Table 1. Primer sequences used for quantitative reverse transcription–polymerase chain reaction

Target	Primer sequence	Product size (bp)
COX-2	5′-AAGCCCTCTACAGTGACATC-3′	115
	5′-GAGAATGGTGCTCCAAGCTCTA-3′	
TNF-α	5′-GCCGATGGGTTGTACCTTGTCTACT-3′	134
	5′-ACGGCAGAGAGGAGGTTGACTT-3′	
iNOS	5′-CCGGCAAACCCAAGGTCTACGTT-3′	128
	5′-CACATCCCGAGCCATGCGCACATCT-3′	
IL-1β	5′-TTGACTTCACCATGGAATCCGTGTC-3′	126
	5′-GAGTCCCCTGGAGATTGAGC-3′	
IL-6	5′-CCTACCCCAATTTCCAATGCTCT-3′	143
	5′-CACTAGGTTTGCCGAGTAGATCTCA-3′	
CXCL14	5′-TGGTTGAGACCGTTCACAGCACTAC-3′	122
	5′-GAAACTCTGACCAGTCATAAGCC-3′	
GAPDH	5′-CAACTCCCACTCTTCCACCTTCGAT-3′	106
	5′-CCTGTTGCTGTAGCCGTATTC-3′	

COX-2, cyclooxygenase-2; GAPDH, glyceraldhyde-3-phosphate dehydrogenase; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-α.

DNA extraction and direct sequencing. Immunoreactivity of β-catenin was classified into 'nuclear/cytoplasmic' or 'membranous' according to the intracellular localization of β-catenin protein. Tumors with 5% or more section area of nuclear/cytoplasmic β-catenin were judged as β-catenin accumulating. Tumor areas with nuclear/cytoplasmic or membranous β-catenin localization and surrounding gastric mucosa in serial paraffin sections (5-μm thick) were microdissected using a laser microdissection system (AS LMD; Leica Microsystems, Wetzlar, Germany). Microdissection, PCR, and sequencing were performed as previously reported.[19,20] The PCR primer sequences to amplify exon 3 of mouse β-catenin gene were 5′-AGCCACTGGCAG-CAGCAGTCTTAC-3′ and 5′-ATAAAGGACTTGGGAGGTGTC-AACA-3′. Sequencing was performed using a BigDye Terminator Cycle Sequencing Kit (v 3.1; Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Statistical analysis. The incidences of gastric tumors and frequencies of β-catenin accumulation and gene mutation were analyzed using Fisher's exact probability test. Differences of inflammation scores were assessed with the Mann–Whitney *U*-test.

Results

Incidence, multiplicity, and size of gastric tumors. The observed incidences and multiplicities of gastric tumors are summarized in Table 2. In the fundic region, Tg mice of groups C and D developed dysplastic gastric tumors (Fig. 2Aa,b), whereas WT mice did not. In contrast, WT (Fig. 2Ba,b) and Tg (Fig. 2Ca,b)

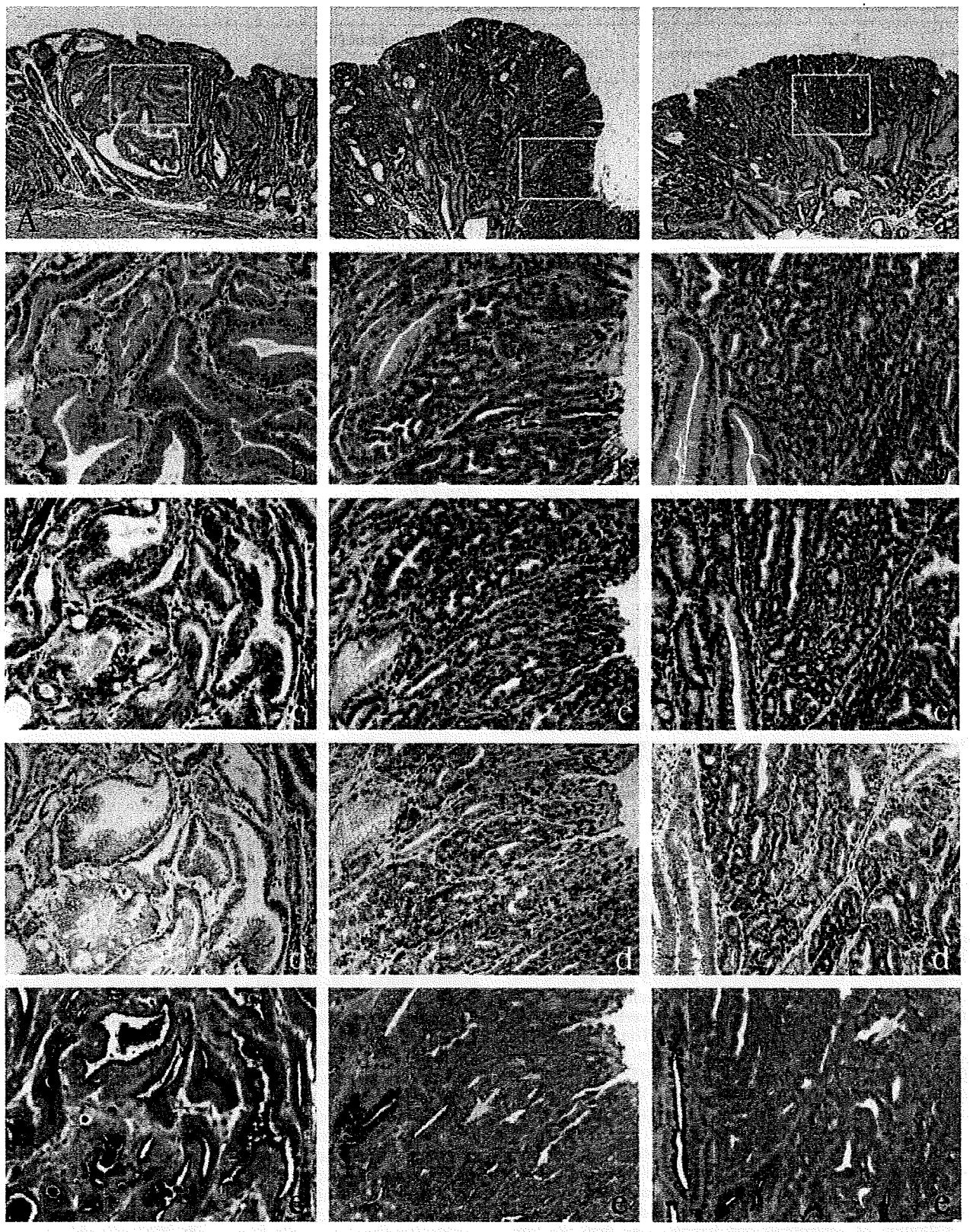

Fig. 2. Histopathological and immunohistochemical analysis of COX-2 and β-catenin in *N*-methyl-*N*-nitrosourea (MNU)–induced tumors in K19-C2mE Tg mice. (A) Gastric tumors in the fundic region of MNU-treated Tg mice. (B,C) Gastric tumors with nuclear β-catenin accumulation in the pyloric region of MNU-induced WT (B) and Tg mice (C). (a,b) Hematoxylin–eosin staining. (c,d) Immunohistochemistry for COX-2 (c) and β-catenin (d). (e) Alcian blue (pH 2.5)–periodic acid-Schiff (AB-PAS). Yellow arrow shows PAS-positive mucin. Yellow boxes in (a) are magnified in (b–e), respectively. Original magnification, 50× (a), 200× (b–e).

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Table 2. Incidence and multiplicity of MNU-treated K19-C2mE mice with gastric tumors

Groups	Treatments	Genotypes	Effective nos.	Incidence (%)		Tumor multiplicity in pyloric mucosa (no. of tumors/mouse)†
				Fundic mucosa	Pyloric mucosa	
A	Broth	WT	10	0 (0%)	0 (0%)	0
		Tg	10	0 (0%)	0 (0%)	0
B	*H. pylori*	WT	10	0 (0%)	0 (0%)	0
		Tg	10	0 (0%)	0 (0%)	0
C	Broth + MNU	WT	12	0 (0%)	12 (100%)	1.40 ± 0.70
		Tg	15	4 (26.7%)	15 (100%)	2.00 ± 0.88
D	*H. pylori* + MNU	WT	24	0 (0%)	24 (100%)	1.75 ± 0.74
		Tg	19	4 (21.1%)*	18 (94.7%)	2.28 ± 0.96*

H. pylori, *Helicobacter pylori*; MNU, *N*-methyl-*N*-nitrosourea; Tg, Transgenic; WT, wild type.
*$P < 0.05$ *versus* WT within group D.
†Values are expressed as average ± SD.

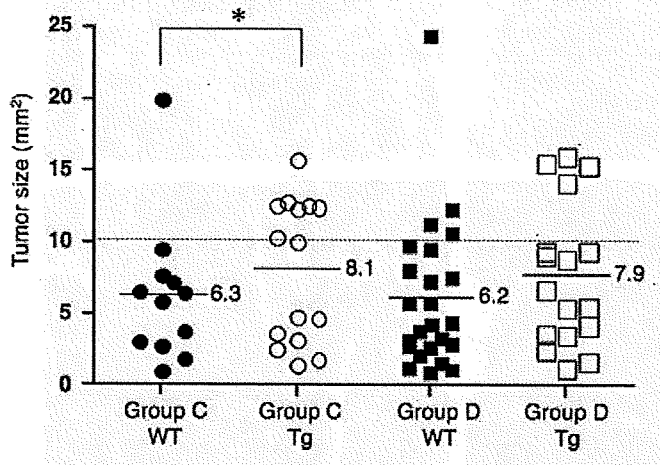

Fig. 3. Maximum tumor size in individual *N*-methyl-*N*-nitrosourea (MNU)–induced K19-C2mE mice in the pyloric region. Ratio of larger tumors (> 10.0 mm²): Tg *versus* WT in group C, 8/15 = 53.3% *versus* 1/12 = 8.3% (*$P < 0.05$); 4/24 = 16.7% *versus* 4/17 = 23.5% (not significant) in group D. Dotted line, cut-off value (10 mm²). Horizontal lines and values, mean.

mice of groups C and D developed dysplastic gastric tumors in the pyloric region; incidences were 100% and 94.7%, respectively, the difference not being significant among the groups and genotypes. Tumor multiplicity in Tg mice in group D (2.28 ± 0.96 tumors/mouse) was higher than those in WT mice in group C (1.40 ± 0.70) and in group D (1.75 ± 0.74) ($P < 0.01$ and $P < 0.05$, respectively). The maximum tumor size in each animal is plotted in Fig. 3. Averages ± SD were 6.3 ± 5.0 and 8.1 ± 5.0 in WT and Tg mice in group C. The corresponding figures were 6.2 ± 5.2 and 7.9 ± 5.1 in group D. The ratio of larger tumors (> 10.0 mm²) in Tg mice (8/15 = 53.3%) was significantly more frequent than that in the WT (1/12 = 8.3%) mice within group C ($P < 0.05$), but not within group D (4/24 = 16.7% and 4/17 = 23.5% in WT and Tg, respectively, $P = 0.70$). No gastric tumors were observed in groups A and B.

Status of gastritis. Data for the gastritis status in each group are summarized in Table 3. The gastric mucosa of *H. pylori* infected groups (B and D) showed significantly higher scores for infiltration of neutrophils and lymphocytes than the non-*H. pylori*-infected groups (A and C). There were no significant differences in scores for infiltration of neutrophils and lymphocytes between genotypes. Gastric mucosa of Tg mice was significantly thickened compared with that of WT. Correspondingly, gastric mucosa of mice with *H. pylori* infection was also significantly thickened compared with mice without *H. pylori* infection. BrdU labeling indices were increased with *H. pylori* infection irrespective of genotypes (groups B and D), associated with hyperplastic change in fundic mucosa. In pyloric mucosa, the BrdU labeling index was higher in Tg mice in group D.

Alteration of expression of inflammatory cytokines. Data for the expression levels of inflammatory factors in each group are summarized in Table 4. The mRNA expressions of TNF-α, iNOS, IL-1β, and CXCL14 in *H. pylori*-infected groups were significantly increased compared with non-*H. pylori*-infected groups in both genotypes. COX-2 was not up-regulated with *H. pylori* infection in WT animals. Furthermore, the mRNA expression levels of COX-2, TNF-α, iNOS, IL-1β, and CXCL14 in Tg mice were significantly higher than those in WT mice in group D. In the groups A–C, there were no significant differences in expression of inflammatory factors between both genotypes except for COX-2.

Immunolocalization of COX-2 and β-catenin, and mucin staining. COX-2 expression was retained in the fundic tumor cells (Fig. 2Ac) as well as surrounding normal foveolar epithelium (Fig. 2Cc, left image). But it was attenuated in pyloric tumor cells in WT (Fig. 2Bc) and Tg (Fig. 2Cc, right image) mice. Besides the epithelial tumor cells, infiltrating and/or stromal cells also expressed COX-2 in tumor stroma (right surface area in Fig. 2Bb). β-Catenin was localized on the membrane in fundic tumors (Fig. 2Ad) as well as in normal glands. In contrast, most pyloric tumors harbored β-catenin in cytoplasms or nuclei both in WT and Tg mice (Fig. 2Bd and Cd, respectively) (Table 5). Gastric cancer cells in the pyloric region contained little PAS-positive mucin (Fig. 2Be and Ce), whereas those in fundic area retained AB- and PAS-positive mucin (Fig. 2Ae).

β-Catenin accumulation and gene mutations in gastric tumors. Data for number and frequency of fundic and pyloric tumors demonstrating β-catenin accumulation are summarized in Table 5. β-Catenin accumulation was significantly more common in tumors in pyloric mucosa compared with those in the fundic region in both WT and Tg genotypes ($P < 0.01$).

To analyze β-catenin gene mutation in gastric tumors with β-catenin accumulation, microdissection was performed for corresponding regions from serial paraffin sections. All mutations of β-catenin gene exon 3 were identified in β-catenin accumulating (nuclear/cytoplasmic) regions except for one membranous staining case in group D (Table 6). Mutations of β-catenin gene exon 3 were more frequently observed in the Tg mice of group D than in the Tg mice of group C ($P < 0.05$). Mutation frequency of β-catenin accumulating regions in WT and Tg mice in group C were 18.2% and 21.4%. The corresponding

Table 3. Histopathological response in gastric musoca of K19-C2mE Tg mice

Groups	Treatments	Genotypes	Effective nos.	Neutrophilis	Lymphocytes	Fundic mucosa thickness (μm)		BrdU labeling index (%)
						Fundic mucosa	Pyloric mucosa	
A	Broth	WT	10	0.33 ± 0.82	0.17 ± 0.41	342.8 ± 38.7	7.46 ± 1.94	12.16 ± 2.22
		Tg	10	0.18 ± 0.40	0.27 ± 0.47	520.8 ± 283.0****	7.56 ± 2.00	12.62 ± 3.01
B	*H. pylori*	WT	10	2.70 ± 0.48*	2.80 ± 0.42*	593.2 ± 111.2*	12.38 ± 1.64*	14.97 ± 3.18
		Tg	10	2.60 ± 0.52*	2.70 ± 0.48*	772.1 ± 214.1*****	13.96 ± 1.76*	14.33 ± 2.69
C	Broth + MNU	WT	12	0 ± 0	0.18 ± 0.40	293.3 ± 88.2	7.32 ± 2.37	12.88 ± 3.65
		Tg	15	0.43 ± 0.76	0.07 ± 0.27	438.2 ± 162.1******	7.70 ± 1.45	12.86 ± 3.11
D	*H. pylori* + MNU	WT	24	1.58 ± 1.02**	2.00 ± 0.83**	441.9 ± 151.8**	13.43 ± 7.18**	15.66 ± 3.78
		Tg	19	2.11 ± 0.68**	2.67 ± 0.69**,***	517.5 ± 129.2	16.95 ± 4.23**,***	16.04 ± 5.46*******

BrdU, 5′-bromo-2′-deoxyuridine; *H. pylori*, *Helicobacter pylori*; MNU, *N*-methyl-*N*-nitrosourea; Tg, Transgenic; WT, wild type.
*$P < 0.01$ *versus* corresponding genotypes in group A; **$P < 0.01$ *versus* corresponding genotypes in group C; ***$P < 0.01$ *versus* WT within group D; ****$P < 0.05$ *versus* WT within group A; *****$P < 0.01$ *versus* WT within group B; ******$P < 0.01$ *versus* WT within group C; *******$P < 0.05$ *versus* Tg in group D. Values for results are expressed as averages ± SD.

Table 4. Relative mRNA expression levels of inflammatory cytokines in gastric mucosa of K19-C2mE mice

Groups	Treatments	Genotypes	Relative mRNA expression levels (range)					
			COX-2	TNF-α	iNOS	IL-1β	IL-6	CXCL14
A	Broth	WT	1.00 (0.55–1.81)	1.00 (0.49–2.02)	1.00 (0.58–1.73)	1.00 (0.31–3.20)	1.00 (0.38–2.63)	1.00 (0.52–1.93)
		Tg	1824.28 (1448.04–2298.28)******	0.68 (0.37–1.24)	0.68 (0.18–2.52)	0.97 (0.37–2.49)	1.22 (0.64–2.29)	0.76 (0.41–1.43)
B	*H. pylori*	WT	2.07 (0.76–5.64)	27.74 (16.21–47.46)*	18.85 (8.56–41.49)*	31.45 (20.52–48.20)*	2.74 (1.83–4.10)**	3.96 (2.94–5.34)*
		Tg	1272.96 (694.86–2332.02)******	42.98 (15.66–117.96)*	38.59 (9.57–155.70)*	32.41 (14.04–74.81)*	2.55 (2.08–3.14)*	5.12 (3.25–8.06)*
C	Broth + MNU	WT	2.00 (1.18–3.39)	5.61 (2.55–12.32)*	4.23 (2.92–6.12)*	8.42 (4.35–16.30)*	1.23 (0.35–4.27)	4.94 (2.21–11.07)*
		Tg	2333.23 (1588.37–3427.38)******	4.81 (2.75–8.43)*	5.98 (3.11–11.51)*	9.61 (4.73–19.55)*	1.59 (0.13–19.12)	6.35 (3.35–12.03)*
D	*H. pylori* + MNU	WT	2.18 (1.21–3.91)	26.63 (8.78–80.76)****	19.08 (5.13–71.02)****	25.18 (10.67–59.45)****	3.22 (1.61–6.44)	4.53 (2.42–8.47)***
		Tg	901.64 (492.31–1651.30)******	61.19 (20.55–182.24)****,******	42.31 (12.94–138.40)****,******	54.03 (19.62–148.80)****,******	3.74 (1.29–10.85)	8.04 (5.09–12.71)***,******

COX-2, cyclooxygenase-2; *H. pylori*, *Helicobacter pylori*; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; MNU, *N*-methl-*N*-nitrosourea; Tg, Transgenic; TNF-α, tumor necrosis factor-α; WT, wild type.
*$P < 0.01$ or **$P < 0.05$ *versus* corresponding genotypes in group A; ***$P < 0.01$ *versus* corresponding genotypes in group B; ****$P < 0.01$ or *****$P < 0.05$ *versus* corresponding genotypes in group C; ******$P < 0.01$ *versus* WT in same groups. Values are expressed as mean. Ranges in parenthesis are expressed as mean − SD to mean + SD.

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Table 5. Number and frequency of β-catenin accumulation in fundic and pyloric tumors

Groups	Treatments	Genotypes	Number (frequency) of β-catenin accumulation	
			Fundic mucosa	Pyloric mucosa
C	Broth + MNU	WT	NA	12/12 (100%)
		Tg	0/4 (0%)	15/15 (100%)*
D	*H. pylori* + MNU	WT	NA	22/24 (91.6%)
		Tg	1/4 (25%)	18/18 (100%)**

H. pylori, *Helicobacter pylori*; MNU, *N*-methyl-*N*-nitrosourea; NA, not applicable due to no tumors; Tg, Transgenic; WT, wild type.
*$P < 0.01$ *versus* that of fundic mucosa in Tg within group C; **$P < 0.01$ *versus* that of fundic mucosa in Tg within group D.

figures in group D were 31.6% and 62.5%, respectively. All mutations observed in pyloric tumors were transitions: C→T (9/22 = 41%), G→A (9/22 = 41%), T→C (4/22 = 18%) (Table 7). No mutations were detected in surrounding normal mucosa.

Discussion

In the present study, K19-C2mE Tg mice developed gastric tumors not only in the pyloric mucosa but also the fundic region, whereas WT mice developed tumors only in pyloric areas with the carcinogen treatment but irrelevant to *H. pylori* infection. Furthermore, Tg mice possessed larger tumors even without *H. pylori* infection. On the other hand, Tg mice showed increased tumor multiplicity compared with the corresponding WT mice only with *H. pylori* infection. These findings indicated that transgenes in combination with *H. pylori* infection and subsequent inflammatory response should play important roles in promotion of gastric carcinogenesis in various ways in this mouse model.

Immunohistochemical analysis here demonstrated that tumor cells in the fundic region more markedly express COX-2 than those in the pyloric region. COX-2 expression was predominantly observed in foveolar epithelial cells in K19-C2mE Tg mice. Thus fundic tumors might be derived from foveolar epithelial cells and be more significantly affected by COX-2 expression compared with pyloric tumors. In human gastric neoplasia, proximal gastric tumors are suggested to be specific subtypes of gastric carcinoma based on histological and genetic research.[21,22] K19-C2mE Tg mice may serve as a new animal model for proximal gastric carcinogenesis.

In the present study, *H. pylori* infection did not promote gastric carcinogenesis in the fundic region, and influence could not be evaluated in the pyloric region in terms of cancer incidence. However, there is abundant evidence from rodent gastric cancer models that *H. pylori* infection promotes gastric cancers induced by stomach carcinogens, MNU and *N*-methyl-*N'*-nitroso-*N*-nitrosoguanidine (MNNG),[23–27] although not without exceptions.[28] *H. pylori* infection induced gastritis and caused hyperplasia of the gastric mucosa in the current mouse system, but heterotopic proliferating glands arising with long-term infection of *H. pylori* and considered as high-grade inflammation in the Mongolian gerbil,[29,30] were not observed here. Such lesions are reversible and are considered as regenerative lesions due to excessive cell proliferation. The observations indicate that the influence of *H. pylori* infection may depend on the animal species with clear differences between the mouse and Mongolian gerbil. In addition, host immune responses or *H. pylori* virulence factors may affect gastritis and gastric carcinogenesis.[31,32] By the fact of increased multiplicity of pyloric tumors in *H. pylori*-infected Tg mice, overexpression of COX-2 and mPGES-1 may serve as a better mouse model for mimicking human cases.

Table 6. Localization of β-catenin in gastric tumors and mutation frequency

Groups	Treatments	Genotypes	Animal nos.	Frequency of mice with mutations	β-Catenin mutation frequency in fundic tumors		β-Catenin mutation frequency in pyloric tumors		Surrounding normal mucosa
					Nuclear + Cytoplasmic	Membranous	Nuclear + Cytoplasmic	Membranous	
C	Broth + MNU	WT	10	2/10 (20%)	NA	NA	2/11 (18.2%)	0/7 (0%)	0/7 (0%)
		Tg	10	2/10 (20%)	NA	0/4 (0%)	3/14 (21.4%)	0/10 (0%)	0/6 (0%)
D	*H. pylori* + MNU	WT	10	4/10 (40%)	NA	NA	6/19 (31.6%)	0/10 (0%)	0/7 (0%)
		Tg	10	8/10 (80%)*	0/1 (0%)	0/4 (0%)	10/16 (62.5%)**,***	1/14 (7.1%)	0/7 (0%)

H. pylori, *Helicobacter pylori*; MNU, *N*-methyl-*N*-nitrosourea; NA, not applicable due to no tumors; Tg, Transgenic; WT, wild type.
*$P < 0.05$ *versus* Tg in group C; **$P < 0.05$ *versus* Tg in group C; ***$P < 0.05$ *versus* Tg in 'membranous' in within group D.

Table 7. Mutation of β-catenin exon 3 in gastric tumors

Groups	Treatments	Genotypes	Mice nos.	Sample nos.	Tumor location	β-Catenin localization	Mutations	Amino acid changes	Events
C	Broth + MNU	WT	W-6	T4	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
			W-11	T9	Pylorus	Nuclear/Cytoplasmic	codon 34: GGA→GAA	Gly→Glu	Transition
		Tg	T-1	T12	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
				T13	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
			T-12	T22	Pylorus	Nuclear/Cytoplasmic	codon 34: GGA→GAA	Gly→Glu	Transition
D	*H. pylori* + MNU	WT	W-8	T27	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
			W-11	T75	Pylorus	Nuclear/Cytoplasmic	codon 35: ATC→ATT	Silent	Transition
				T75	Pylorus	Nuclear/Cytoplasmic	codon 45: TCC→TTC	Ser→Phe	Transition
			W-23	T85	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
			W-24	T34	Pylorus	Nuclear/Cytoplasmic	codon 33: TCT→CCT	Ser→Pro	Transition
				T35	Pylorus	Nuclear/Cytoplasmic	codon 33: TCT→CCT	Ser→Pro	Transition
		Tg	T-2	T37	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
				T38	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
				T39	Pylorus	Membranous	codon 41: ACC→ATC	Thr→Ile	Transition
			T-4	T40	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
				T41	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
			T-5	T88	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
			T-6	T90	Pylorus	Nuclear/Cytoplasmic	codon 33: TCT→CCT	Ser→Pro	Transition
			T-16	T158	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
			T-17	T147	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
			T-18	T92	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
			T-19	T149	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition

H. pylori, *Helicobacter pylori*; MNU, *N*-methyl-*N*-nitrosourea; Tg, Transgenic; WT, wild type.

For further analysis of factors promoting gastric carcinogenesis, we investigated the expression of inflammatory cytokines in gastric mucosa. In the present study, expression of those such as TNF-α, iNOS, IL-1β, and CXCL14 were significantly increased in Tg mice with *H. pylori* infection and MNU treatment. Among them, CXCL14 has been known to selectively attract monocytes, where PGE_2 up-regulates their responsiveness.[33] The combination of these factors may contribute to the participation of macrophages in increased tumorigenesis in Tg mice with *H. pylori* infection plus MNU treatment. We previously demonstrated that the severity of chronic gastritis, characterized by high-level expression of IL-1β, TNF-α, COX-2, and iNOS was concerned with glandular gastric carcinogenesis in *H. pylori*-infected Mongolian gerbils.[34] Thus, in Tg mice with *H. pylori* infection a higher level of inflammatory cytokines may be induced that eventually promotes gastric carcinogenesis.

The Tg mice feature increased PGE_2 synthesis due to overexpressed COX-2/mPGES-1 in gastric mucosa.[4] PGE_2 exerts its biological effects by binding to four isoform receptors, EP_1, EP_2, EP_3, and EP_4,[35,36] categorized in the family of seven transmembrane G protein coupled rhodopsin-type receptors. Accumulating evidence indicates that PGE_2 promotes tumor growth by stimulating EP receptor signaling with subsequent enhancement of cell proliferation, promotion of angiogenesis, and inhibition of apoptosis.[37] Previous reports based on mouse studies demonstrated that EP_1, EP_2, and EP_4 receptors play important roles in colon carcinogenesis.[38–41] Furthermore, expression of EP_1, EP_2, and EP_4 receptors has been found to be elevated in mouse mammary tumors as well as colon cancers.[42] EP_3 receptor activation was furthermore suggested to contribute to breast cancer progression.[43] These observations indicate that the expression pattern of EP receptors in cancer cells might determine the potential of PGE_2 to drive tumor progression. Although the expression level of EP receptors in gastric cancers was unclear in the present study, EP receptor signaling stimulated by PGE_2 might have influenced gastric carcinogenesis. It should be stressed that PGE_2 transactivates epidermal growth factor receptor (EGFR) and triggers PI3K/Akt signaling,[44–46] and Ras/MEK/ERKs,[47] pathways in gastric epithelial and colon cancer cells *in vitro* as well as rat gastric mucosa *in vivo*.[46]

To analyze the differences between present mouse gastric tumors developed in fundic and pyloric mucosas, we investigated β-catenin activation, which was suggested to play an important role in gastric carcinogenesis. Immunohistochemical analysis here showed that β-catenin activation characterized by its intracellular accumulation was frequently observed in tumors in the pyloric region, in contrast to those in the fundic region, indicating the unnecessariness of the Wnt pathway. Conversely, pyloric tumorigenesis might be promoted by Wnt activation. In a rat model, a type of adenocarcinoma resembling foveolar epithelium showed nuclear accumulation of cyclin D1 without β-catenin activation,[48] whereas β-catenin was accumulated in cytoplasms or nuclei in the majority of less-differentiated adenocarcinomas.[20] Since fundic tumors were histologically classified as being of the foveolar type and pyloric ones were classified as the less differentiated in this experiment, oncogene activation could depend on cell/tissue differentiation or vice versa.[49] Similarly, mucin expression characterized by AB-PAS staining was observed in fundic tumors as in the hyperplastic fundic tumors in the previous report;[4] pyloric tumors, however, lost most of those mucin production.

Several previous studies of gastric carcinogenesis models in rodents such as the mouse, rat, and Mongolian gerbil have indicated that β-catenin activation plays an important role in gastric carcinogenesis.[20,50–52] In the present study, this was more frequently observed than in previous reports (rat, 18.2%; mouse, 12.5%; and Mongolian gerbil, 2.2%).[20,50,51] Such variation in the frequency of β-catenin activation might be caused by differences in experimental design such as the experimental period, chemical carcinogen applied, or type of experimental animal.

In rat and Mongolian gerbil models, mutations of β-catenin gene in exon 3 have been identified in codons 34, 41, and 45 at GSK-3β phosphorylation sites, and are significantly associated with nuclear β-catenin accumulation.[20,51] In human gastric cancers,

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gene alternations have been found in the same sites including codons 29, 37, 41, and 47 as well as in adjacent sites at codons 28, 32, 34, 36, 38, 39, and 48.[12,13,19,53] In the present experiment, the mutation spectrum was codons 32, 33, 34, 35, 41, and 45, consistent with the previous reports. Furthermore, β-catenin mutations were particularly frequent in Tg mice with *H. pylori* infection. Tumor multiplicity was also increased in Tg mice with *H. pylori* infection, indicating the possibility that *H. pylori* infection in addition to COX-2/mPGES-1 expression might contribute to progression of gastric adenocarcinomas though β-catenin gene alternations at least in part. In humans, stomach cancers with intestinal differentiation markers feature more β-catenin mutations compared to those with gastric markers.[19] Furthermore, intestinal markers may be induced only in stomach tumors in *H. pylori*-infected gerbils.[54] Thus, alteration of β-catenin could be related to *H. pylori* infection, although further work is needed to reveal interactions between these two factors.

β-Catenin accumulation without β-catenin gene mutations was detected in some tumors in the present study, indicating involvement of other alterations of Wnt pathway regulatory genes. Indeed, there have been a large number of previous reports suggesting that *APC* gene mutation,[17,55–61] APC loss of heterozygosity,[59] over-expression of various Wnt ligands, and altered frizzled receptors[62–64] may be involved in β-catenin activation. Furthermore, degradation of E-cadherin and microsatellite instability might also be responsible for β-catenin accumulation. In one rodent model, the $APC^{Min/+}$ mouse which harbors a nonsense mutation at codon 850 of the *APC* gene, gastric tumors frequently develop with *APC* loss of heterozygosity.[50]

In conclusion, the present study indicated that over-expression of COX-2/mPGES-1 promotes gastric carcinogenesis, especially in the fundic region, further showing the K19-C2mE Tg mouse to be a new animal model for proximal gastric carcinogenesis. Furthermore Tg mice developed multiple tumors in the pyloric region with *H. pylori* infection partly with β-catenin gene mutation and activation. This indicates the risk of multiple or metachronous gastric cancers also in human cases with *H. pylori* infection and supports the idea to eradicate the bacterium or to suppress inflammatory response for the prevention of secondary malignancies.[65]

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CD44$^+$ slow-cycling tumor cell expansion is triggered by cooperative actions of Wnt and prostaglandin E_2 in gastric tumorigenesis

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Similar to normal tissue stem cells, cancer stem cells (CSCs) are thought to be quiescent or slow-cycling and, thereby, insensitive to chemo- and radiotherapies. CD44, a cell surface component that interacts with the extracellular matrix, has been found to be highly expressed in CSCs of several solid tumors. However, the relevancy between CD44$^+$ cells and slow-cycling cells and the underlying mechanisms for the emergence of CD44$^+$ CSCs during tumorigenesis have not been elucidated. Here we show that a gastric gland residing at the squamo-columnar junction (SCJ) in normal mouse stomach contains CD44$^+$ stem cell-like slow-cycling cells and that this characteristic CD44$^+$ gland was expanded by prostaglandin E2 (PGE_2) and Wnt signaling in *K19-Wnt1/C2mE* mouse, a genetic mouse model for gastric tumorigenesis. The analysis of three transgenic mouse lines, *K19-Wnt1*, *K19-C2mE* and *K19-Wnt1/C2mE*, revealed that the expansion of CD44$^+$ SCJ cells is triggered by PGE_2-mediated signaling and is prominently enhanced by the addition of Wnt activation. Furthermore, each expanded CD44$^+$ gland in gastric tumor of *K19-Wnt1/C2mE* mouse contains a few BrdU label-retaining quiescent or slow-cycling cells, suggesting that the CD44$^+$ SCJ cells in normal mouse are candidates for the cell-of-origin of gastric CSCs. These observations suggest that PGE_2-mediated inflammatory signaling and Wnt signaling cooperatively trigger the expansion of CD44$^+$ slow-cycling stem-like cells in SCJ, leading to development of lethal gastric tumors in mice. (*Cancer Sci* 2010; 101: 673–678)

CD44, a major cell adhesion molecule, has been implicated in a wide variety of physiological processes and pathological processes, including lymphocyte homing, wound healing, and cell migration, as well as cancer cell growth and metastasis.[1,2] CD44 has been recently detected as a cell surface marker of cancer stem cells (CSCs) in several types of cancer.[3] Moreover, a CD44$^+$ subpopulation of cells isolated from several gastric cancer cell lines has been reported to have stem cell-like properties, which are the capacities to self-renew and to differentiate.[4] However, the underlying mechanism by which CD44$^+$ CSC-like subpopulations emerge in the process of tumor development is largely unknown.

Cancer stem cells, also known as cancer-initiating cells, are responsible for tumor initiation and maintenance. CSCs possess the ability to drive tumor growth and are inherently resistant to chemo- and radiotherapies. Recent studies have revealed that melanoma-initiating cells[5] and leukemia-initiating cells are a slow-cycling or quiescent subpopulation.[6,7] These quiescent or slow-cycling properties are thought to be a major reason why CSCs are resistant to cancer therapies targeted at proliferating cells. It has been shown that cell adhesion molecules play an essential role in the quiescence of hematopoietic stem cells by regulating adhesion in the osteoblastic niche.[8] Therefore, we hypothesized that CD44, which is highly expressed in CSCs, is involved in the regulation of their quiescent or slow-cycling status.

In the present study, we discovered a gastric gland at the squamo-columnar junction (SCJ) in normal mice, which contains CD44-expressing stem cell-like slow-cycling cells. This unique CD44$^+$ SCJ gastric gland was rapidly expanded in response to the prostaglandin E2 (PGE_2)-mediated signaling in *K19-C2mE* mice, a mouse model of gastric hyperplasia. Furthermore, Wnt signal activation significantly enhanced the PGE_2-mediated expansion of the CD44$^+$ gland, resulting in increased numbers of the stem cell-like slow-cycling cells, which might contribute to development of lethal gastric tumors in mice.

Materials and Methods

Transgenic mice. *K19-Wnt1*, *K19-C2mE*, and *K19-Wnt1/C2mE* transgenic mice have been described previously.[9,10] All animal experiments were carried out according to protocols approved by the Ethics Committee of Keio University (Tokyo, Japan).

BrdU label-retention assays. Wild-type mice (8- to 10-weeks-old) were subjected to sublethal irradiation (8.0 Gy) then injected i.p. with BrdU solution (50 mg/kg body weight) twice a day for 2 days, and killed 12 days later. *K19-Wnt1/C2mE* mice were injected i.p. with BrdU solution twice a day for 7 days, and killed 35 days later without irradiation. Tissue samples were processed for immunohistochemical staining with anti-BrdU (clone Bu20a, 1:50; DakoCytomation, Carpinteria, CA, USA). The mean number of BrdU-positive cells per 300 tumor (epithelial) cells in four microscopic fields per mouse was calculated. We examined three *K19-Wnt1/C2mE* mice.

Histology and immunohistochemistry. Tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 4 μm. Sections were depleted of paraffin then rehydrated in a graded series of ethanol solutions. For histology, sections were stained with H&E. For immunohistochemistry, sections were washed with PBS, subjected to antigen retrieval by heating for 10 min at 100°C in 0.01 M sodium citrate (pH 6.0), and exposed to 3% hydrogen peroxide before incubation with primary antibodies. Immune complexes were detected using a Vectastain Elite Kit (Vector Laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine, and the sections were counterstained with hematoxylin.

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