Development of Gastric Carcinoma from Intestinal Metaplasia in Cdx2-transgenic Mice


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ABSTRACT

In the progression of chronic gastritis, gastric mucosal cells deviate from the normal pathway of gastric differentiation to an intestinal phenotype. Many epidemiologic studies have found an association between the formation of intestinal metaplasia and the development of gastric carcinoma. However, there is no direct evidence that shows intestinal metaplasia is a precursor lesion of gastric carcinoma, to date. We periodically examined the intestinal metaplastic mucosa of Cdx2-transgenic mice we have previously generated. Gastric polyps developed from intestinal metaplastic mucosa in all stomachs of Cdx2-transgenic mice examined. These gastric polyps consisted of intestinal-type adenocarcinoma that invaded the submucosa and muscularis propria and occasionally spread into the subserosa. p53 and APC gene mutations were recognized in the adenocarcinomas. The participation of APC and p53 gene mutations in gastric carcinogenesis from the intestinal metaplasia was verified by the Cdx2-transgenic mice, carrying Apc

INTRODUCTION

Gastric carcinoma remains the second most common cause of cancer deaths on a worldwide basis (1). Histologically, human gastric carcinoma is usually divided into intestinal type and diffuse type. Intestinal-type carcinoma is more common than diffuse-type carcinoma and is particularly associated with gastric atrophy and intestinal metaplasia. It arises in older patients in a part of the stomach where inflammation has been present for a long period. It is strongly associated with Helicobacter pylori infection. Correa (2) presented a hypothesis with respect to the mechanism of gastric carcinogenesis because of H. pylori infection. H. pylori infection is involved in the process of progression from normal gastric mucosa to superficial gastritis, chronic active gastritis, atrophic gastritis, and finally to intestinal metaplasia (2). The terminal stage of this process is gastric carcinoma. It is possible that the intestinal-type carcinoma may develop from the gastric epithelium that has undergone intestinal metaplasia, although there is no direct evidence to support this hypothesis. We and others have reported that the intestine-specific transcription factor Cdx2 is expressed in the human gastric intestinal metaplastic mucosa (3–7). We established Cdx2-transgenic mice expressing transcription factor Cdx2 in the gastric mucosa (8). The gastric fundic mucosa of the Cdx2-transgenic mice was completely changed into intestinal metaplastic mucosa. The Cdx2-transgenic mouse is therefore a good model for investigating the relationship between intestinal metaplasia and gastric carcinogenesis.

Thus far, experiments on gastric carcinogenesis in animal models have been conducted under H. pylori infection and/or carcinogens such as N-methyl-N’-nitro-N-nitrosoguanidine (9–11). Multiple factors are involved in H. pylori-related gastric carcinogenesis, and these experiments have been unable to prove that intestinal metaplasia itself actually leads to gastric carcinoma. To clarify whether intestinal metaplasia itself is a cause of intestinal-type gastric adenocarcinoma, we periodically examined the intestinal metaplastic mucosa of Cdx2-transgenic mice. All of the Cdx2-transgenic mouse stomachs examined at 100 weeks of age had developed gastric polyps that histopathologically showed invasive gastric carcinoma. The implication of intestinal metaplasia in gastric carcinogenesis was clarified with our animal model.

MATERIALS AND METHODS

Mice. We used Cdx2-transgenic mice with stomach-specific expression of Cdx2 using the β-subunit gene promoter of rat H+/K+-ATPase (8). The Cdx2-transgenic mice used were originally in the background of C57BL/6J (8). The Cdx2-transgenic mice that carry Apc

Immunohistochemistry. Three-micron thick sections were cut, deparaffinized, rehydrated in PBS, placed in 10 mmol/L citrate buffer (pH 6.0), and heated in an 850-W microwave for 15 minutes. Endogenous peroxidase activity was blocked by incubation for 30 minutes in 0.3% H2O2. After washing twice with PBS, including 0.1% Triton X-100, the sections were preincubated with blocking buffer (DAKO, Carpinteria, CA) or anti-proliferating cell nuclear antigen antibody (1:100; BD Biosciences Pharmingen, San Diego, CA), or anti-MUC5AC antibody (1:30; Novocastra, Newcastle upon Tyne, United Kingdom), anti-MUC6 antibody (1:50; Kanto Chemical, Tokyo, Japan), anti-β-catenin antibody (1:100; BD Biosciences Pharmingen, San Diego, CA), or anti-p53 antibody (1:500; Novocastra) overnight at 4°C. Slides were then washed in PBS and incubated with Envision (DAKO, Carpinteria, CA). After development with 3, 3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan), the slides were counterstained with hematoxylin and viewed under a light microscope.

RNA Isolation and cDNA Synthesis. Tissue specimens were stored at −80°C and homogenized with an ultrasound homogenizer in the presence of IsoGen (Nippon Gene, Tokyo, Japan). Total RNA was extracted from frozen tissues by the acid/guanidinium and phenol/chloroform extraction method. Total RNA (1 μg) was reverse transcribed at 37°C for 1 hour in a final volume of 20 μL of reverse transcription buffer [50 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 10 mmol/L MgCl2, 0.5 mmol/L spermidine, and 10 mmol/L DTT] containing reverse transcriptase (ReverTraAce; TOYOBO, Osaka, Japan), 16 units of RNase inhibitors, 200 pmol of random primer, 1.0 mmol/L deoxynucleoside triphosphates (Sigma). The reaction was terminated by incubating the mixture at 95°C for 10 minutes.

Mutation Analyses of the β-Catenin, APC, and p53 Genes. Detection of mutations in +1 to +249 of the β-catenin gene was carried out by sequencing.
reverse transcription-PCR products. Using the primer pair, cDNA from each sample was amplified by PCR (Table 1). These primers amplified a 298-bp fragment encoding the glycogen synthase kinase-3 \(\beta\)-subunit gene.

| β-catenin-sense | 5'-AGAGCTTCTGGGACCCG-3' | −29 to −10 |
| β-catenin-antisense | 5'-CTAAGCTTCTGCTGCTGCTG-3' | +269 to +250 |

**Table 1 Primers for sequencing**

**APC**

| APC-1-sense | 5'-CACGTTTACGTTGAGAAG-3' | 3772 to 3791 |
| APC-1-antisense | 5'-CTCCTGAGAAAGAACTCACA-3' | 4069 to 4050 |
| APC-2-sense | 5'-AGGTCTGCTGCTTACCTC-3' | 4007 to 4026 |
| APC-2-antisense | 5'-TCTGGCTGCTGCTGCTGCTG-3' | 4302 to 4283 |
| APC-3-sense | 5'-GGCATCTAAGAGGCTGAG-3' | 4243 to 4262 |
| APC-3-antisense | 5'-AAATGCTGCTACCCGCCAGG-3' | 4542 to 4523 |
| APC-4-sense | 5'-ACTCCAGACGGGTTTTCTTG-3' | 4483 to 4502 |
| APC-4-antisense | 5'-GGCTAGTCTTGGCTTGGCTG-3' | 4779 to 4760 |

| p53 | 5'-CCACAGAGGGTGTACGGTTT-3' | −32 to −13 |
| p53-1-sense | 5'-AGCAGTATGCCGAGAGGAAGG-3' | 387 to 367 |
| p53-2-sense | 5'-CTGGGAGACCCAGACCTG-3' | 334 to 354 |
| p53-2-antisense | 5'-GCGGACCGCTGCTGCTGCTG-3' | 666 to 646 |
| p53-3-sense | 5'-GAGGAGAGGGCCAGACG-3' | 613 to 633 |
| p53-3-antisense | 5'-CTTTTGGGGGGGAGGCGC-3' | 945 to 925 |
| p53-4-sense | 5'-GGGAGGCGACAGGACCCG-3' | 892 to 912 |
| p53-4-antisense | 5'-GCTGGTGATGAGGAGGCGAT-3' | 1200 to 1181 |

**RESULTS**

We previously generated a mouse strain expressing the transcription factor Cdx2 in the gastric mucosa with the \(\beta\)-subunit gene promoter of rat H\(^{\dagger}\)/K\(^{\dagger}\)-ATPase (8). The Cdx2-transgenic mice developed normally into superficially healthy adults and showed intestinal metaplasia in the stomach up to 12 weeks of age. The gastric mucosa was completely replaced by intestinal metaplastic mucosa consisting of terminally differentiated intestinal epithelial cells (absorptive enterocytes, goblet cells, and enteroendocrine cells). These mice could serve as a model for human gastric intestinal metaplasia.

In this study, we used the Cdx2-transgenic mice to examine the relationship between the intestinal metaplasia and gastric carcinoma. When 15 Cdx2-transgenic mice were extensively examined at 50 weeks of age, no gastric polyps were found in any of the mice. To determine the long-term consequences of intestinal metaplastic mucosa, transgenic mice were killed for pathological examination at the ages of 80 and 100 weeks. Eight of 8 mice killed at 80 weeks of age had developed very small polyps in the gastric fundic region with complete intestinal metaplastic change. Upon examination of the 100-week-old Cdx2-transgenic mice, gastric polyps were found in the intestinal metaplastic mucosa (Fig. 1A). The incidence of gastric polyps in the transgenic mice was 100% (10 of 10; 5 males and 5 females). In contrast, none of the nontransgenic littermate mice of the same age developed any gastric polyps (Fig. 1B), which is consistent with the lack of incidence of gastric polyps in the strain C57BL/6 (17, 18).
The polyp multiplicity ranged from one to three per stomach (Table 2). As shown in Fig. 1A, gastric polyps were found in the glandular part of the stomach harboring intestinal metaplasia and showed a sessile or pedunculated morphology with sizes ranging from 5 to 15 mm in diameter.

All of the polyps of the Cdx2-transgenic mice showed similar histology. Proliferation of atypical neoplastic glands was seen in the area of the intestinal metaplastic mucosae (Fig. 2A). The whole of the polyp in Fig. 2A consisted of neoplastic cells. Histopathologically, the tumors were quite similar to human intestinal-type adenocarcinomas of the stomach. Invasive adenocarcinomas were also observed in some tumors were quite similar to human intestinal-type adenocarcinomas.

To clarify the possible activation of the Wnt signaling pathway in the neoplasm, we determined the subcellular localization of β-catenin and p53 staining, and APC and p53 mutations of gastric polyps from 10 Cdx2-transgenic mouse stomachs (A to J).

We examined the relationship between intestinal metaplastic cells and tumor cells by Alcian blue staining and the immunohistochemical staining for Cdx2, MUC5AC, and MUC6. Alcian blue staining and Cdx2 were observed in both intestinal metaplastic mucosa and tumor (Fig. 3) but not in the normal gastric mucosa (data not shown). On the other hand, MUC5AC and MUC6 were recognized in the normal gastric mucosa (data not shown) but not in intestinal metaplastic mucosa and tumor (Fig. 4). These results indicate that the tumors showed the intestinal phenotype.

As shown in Fig. 1, gastric polyps were found in the glandular part of the stomach harboring intestinal metaplasia and showed a sessile or pedunculated morphology with sizes ranging from 5 to 15 mm in diameter.
detected nine mutations in the APC gene (Table 2). Three of these were missense mutations resulting in amino acid substitutions, two were 1-bp insertions resulting in frameshift, and one was a 1-bp insertion leading to a stop codon. The other three were silent third-position alterations resulting in no amino acid changes. The mutations, except for the silent ones, are summarized in Table 2. We detected 13 mutations in the p53 gene (Table 2). Ten of these were missense mutations resulting in amino acid substitutions, and one was a 1-bp insertion leading to a stop codon. The other two were silent third-position alterations resulting in no amino acid changes. The missense mutations are summarized in Table 2. In contrast, no β-catenin gene mutations were detected in this study.

To examine whether a lack of APC or p53 activity affects the generation of the gastric polyps, we generated the Cdx2-transgenic mice that carry ApcMin mutation or p53 deficiency. Upon examination of the 27 to 30-week-old Cdx2-transgenic mice that carry ApcMin mutation, gastric polyps were found in the intestinal metaplastic mucosa (Fig. 6C). The incidence of gastric polyps in the transgenic mice (Cdx2Tg ApcMin) was 90% (9 of 10; Table 2). None of the 10 Cdx2-transgenic mice (Fig. 6A) and the 10 ApcMin mice (Fig. 6B) had...
developed gastric polyps at 30 weeks of age. We examined whether the Apc gene shows LOH in the gastric polyps of Cdx2Tg Apc<sup>+/H11001</sup> mice because all intestinal polyps develop in Apc mutant mice due to loss of the wild-type Apc gene. One hundred percent of the gastric polyps (n=9) of Cdx2Tg Apc<sup>+/Min</sup> mice also showed LOH. Upon examination of the 50-week-old Cdx2-transgenic mice that carry p53 deficiency, gastric polyps were found in the intestinal metaplastic mucosa (Fig. 7C). The incidence of gastric polyps in the transgenic mice (Cdx2Tg p53<sup>+/−</sup>) was 50% (5 of 10; Table 2). None of the 10 Cdx2-transgenic mice (Fig. 7A) and the 10 p53-deficient mice (Fig. 7B) had developed gastric polyps at 50 weeks of age. Histomorphological features of the gastric polyps (Fig. 8, B and E) and the surrounding intestinal metaplasia (Fig. 8, A and D; H&E staining) in both Cdx2Tg Apc<sup>+/Min</sup> (Fig. 8, A and B) and Cdx2Tg p53<sup>+/−</sup> (Fig. 8, D and E) were same as those in Cdx2 transgenic mice (Fig. 2). Immunohistochemical staining for Cdx2 in the gastric polyps (Fig. 8, A) and the surrounding intestinal metaplasia (Fig. 8, B and E) shows positive staining for Cdx2.

Fig. 4. Immunohistochemical staining for MUC5AC and MUC6. MUC 5AC is not recognized in intestinal metaplastic mucosa (A) and tumor (B). MUC6 is not recognized in intestinal metaplastic mucosa (C) and tumor (D). Scale bars: 100 μm.

Fig. 5. Immunohistochemical staining for β-catenin and p53 in the neoplastic polyp and intestinal metaplasia. Strong nuclear staining for β-catenin is observed in the polyp epithelium (A). The nuclear staining for β-catenin is not recognized in the intestinal metaplastic mucosa (B). Intranuclear overexpression of p53 protein is observed immunohistochemically in the tumor (C). Intranuclear overexpression of p53 protein is not observed immunohistochemically in the intestinal metaplastic mucosa (D). Scale bars: 50 μm (A and B), 100 μm (C and D).
GASTRIC CARCINOMA FROM INTESTINAL METAPLASIA

DISCUSSION

Intestinal-type carcinoma arises in older patients in a part of the stomach where inflammation has been present for a long period. We successfully showed that long-term intestinal metaplasia induces gastric adenocarcinoma in the Cdx2-transgenic mouse stomach. No significant changes were noted in wild-type littermate in this study. This indicates that the Cdx2-induced intestinal metaplasia itself causes the gastric carcinoma. The tumor incidence was 100% (10 of 10 mice) at 100 weeks after birth. Although intestinal metaplasia has been categorized as a risk factor for gastric carcinoma based on the results of various epidemiologic studies (19), no direct evidence of this relationship has been presented to date. To the best of our knowledge, this study is the first showing a direct relationship between intestinal metaplasia itself and gastric carcinogenesis.

All of the tumors induced in the Cdx2-transgenic mice showed similar histologic features. They were composed of intestinal-type adenocarcinoma and located in the fundic region that was completely replaced by intestinal metaplasia. The tumors were positive for Alcian blue staining and Cdx2 immunostaining, whereas the expressions of MUC5AC and MUC6 were not recognized. It can be concluded that intestinal metaplasia itself is a precancerous lesion leading to gastric carcinoma in this model.

To generate intestinal metaplasia by expressing Cdx2 in the gastric mucosa, it is ideal to express Cdx2 in the gastric stem cells. However, the promoters that express Cdx2 exclusively in the gastric stem cells do not exist, to date. We used H⁺/K⁺-ATPase β-subunit gene promoter to express Cdx2 specifically in the gastric mucosa because H⁺/K⁺-ATPase β-subunit gene promoter affects not only parietal cell lineage but also chief and mucus neck cell lineage (20, 21). First, administration of the antiherpetic drug ganciclovir to transgenic mice, in which H⁺/K⁺-ATPase β-subunit gene promoter was used to target expression of herpes simplex virus 1 thymidine kinase to parietal cells, not only caused a rapid and specific ablation of parietal cells but also led to the loss of other gastric epithelial cells (chief and mucus-producing cells) that were not expression sites of the herpes simplex virus 1 thymidine kinase suicide gene (20). Second, administration of the toxin to transgenic mice, in which H⁺/K⁺-ATPase β-subunit gene promoter was used to express Cdx2 in the gastric stem cells by Cdx2. By the above reasons, we used the intestinal metaplastic mucosa of Cdx2-transgenic mouse generated with H⁺/K⁺-ATPase β-subunit gene promoter to analyze the development of gastric carcinoma from intestinal metaplasia.

Fig. 6. Gastric polyps observed in the intestinal metaplastic mucosa of the transgenic mice (C, Cdx2Tg Apc⁻/⁺Min). Macroscopic dissection views of the stomachs from 50-week-old mice (A, Cdx2Tg; B, Apc⁺/+Min; C, Cdx2Tg Apc⁻/⁻Min) opened along the greater curvature. Gastric polyps are indicated by arrows (C). Scale bars: 5 mm.

Fig. 7. Gastric polyps observed in the intestinal metaplastic mucosa of the transgenic mice (C, Cdx2Tg p53⁻/⁻). Macroscopic dissection views of the stomachs from 30-week-old mice (A, Cdx2Tg; B, p53⁻/+; C, Cdx2Tg p53⁻/⁻) opened along the greater curvature. Gastric polyps are indicated by arrows (C). Scale bars: 5 mm.
In our transgenic mice, nuclear staining for β-catenin was recognized in the neoplastic lesions. Nuclear-translocated β-catenin serves as a transcriptional factor through binding with the Tcf-Lef family, which has been proposed as an important oncogenic step in various tumors, including gastric carcinoma (22–27). Intracellular levels of β-catenin are mainly regulated by degradation, which is initiated by interaction with the APC protein and glycogen synthase kinase-3β. The APC protein binds to β-catenin directly and promotes targeted phosphorylation of highly conserved serine and threonine residues in the NH2 terminus by way of glycogen synthase kinase-3β, thereby targeting β-catenin for degradation by the proteosome system (28, 29). Therefore, the NH2 terminus of β-catenin is an important area in the regulatory mechanism of β-catenin turnover. However, mutations in the NH2 terminus of β-catenin were not recognized in our gastric tumors. On the other hand, mutations in the APC gene have been frequently detected in gastric carcinomas, particularly in intestinal-type gastric carcinoma (15, 30). Although mutations in β-catenin were not present, we detected mutations in the APC gene in our transgenic mouse adenocarcinoma, indicating that the nuclear translocation of β-catenin might be due to the mutations in the APC gene. Furthermore, alterations in the p53 gene have been showed in gastric adenocarcinomas. The frequencies of p53 mutations in early and advanced intestinal-type gastric carcinomas are consistent at ~40% (31–33). However, p53 mutations are rare in early undifferentiated carcinomas (34, 35). Thus, mutations in the p53 gene are considered to be critical early events in the development of intestinal-type carcinomas. Many studies have used immunohistochemical analysis of tumors in an effort to detect excessive nuclear expression of p53 as an indirect means of identifying mutations in this gene. We also detected overexpression of p53 protein in the present model and mutations in the p53 gene. These mutations in the APC and p53 genes detected in the neoplastic lesions are quite similar to those of intestinal-type gastric carcinoma in humans. The importance of the mutations in the APC and p53 genes was verified by Cdx2-transgenic mice that carry ApcMin mutation (Cdx2Tg Apc+/Min) and p53 deficient Cdx2-transgenic mice (Cdx2Tg p53+/-).

In the present study, we showed that Cdx2-transgenic mice develop intestinal-type adenocarcinoma when aged older than 80 weeks. In general, development of gastric adenocarcinoma from complete intestinal metaplastic mucosa also occurs in elderly people (sixties to eighties). However, it is difficult to combine Cdx2 expression directly with Wnt-dependent intestinal-type tumorigenesis because it takes a long time for intestinal-type adenocarcinoma to develop from intestinal metaplastic mucosa. The present experimental and the previous clinical data indicate that p53 and APC mutations occur in the intestinal metaplastic mucosa of long duration (15, 30–33) and induce the tumorigenesis of intestinal-type adenocarcinoma, although the role of Cdx2 expression and intestinal metaplasia in Wnt-dependent intestinal-type tumorigenesis has not been clarified, to date.

Taken together, the histopathological and molecular features of the gastric carcinomas induced in this model closely resemble those of human intestinal-type gastric carcinomas. The protruded form of human gastric well-differentiated adenocarcinoma is often developed in the intestinal metaplastic mucosa of the elderly people and is macroscopically and microscopically quite similar to the protruded form of gastric adenocarcinoma in our transgenic mice. Although careful interpretation is required in extrapolating the data from such an animal model to humans, our present findings are highly suggestive of the involvement of intestinal metaplasia in gastric carcinogenesis in humans. Correa (2) hypothesized that severe atrophic gastritis accompanying intestinal metaplasia caused by H. pylori infection is closely related to the development of intestinal-type gastric carcinoma. Uemura et al. (36) have also shown that patients with H. pylori infection and severe atrophic gastritis along with intestinal metaplasia are at high risk for intestinal-type gastric carcinoma development. Our present results clearly indicate that intestinal metaplasia itself might promote the steps of the intestinal metaplasia-dysplasia-carcinoma sequence. It is controversial whether H. pylori eradication could stop the progression or even lead to a regression of intestinal metaplasia (37, 38). H. pylori plays an important role as a trigger of the process that leads to intestinal metaplasia; however, in the case that regression of intestinal metaplasia does not occur after H. pylori eradication, our results suggest that there is the possibility that H. pylori eradication cannot interrupt the progression from intestinal metaplasia toward carcinoma once intestinal metaplasia has developed. In patients with severe atrophy accompanying intestinal metaplasia, it may be therefore necessary to perform careful follow-up examinations to...
detect intestinal-type gastric carcinoma even after the eradication of *H. pylori*.

In conclusion, we have presented that intestinal metaplasia itself plays a key role in causing malignant and invasive gastric adenocarcinoma using Cdx2-transgenic mice.

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