Development of Gastric Tumors in $Apc^{Min/+}$ Mice by the Activation of the β-Catenin/Tcf Signaling Pathway

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Abstract

Although several lines of evidence suggest the involvement of the Wnt pathway in the development of gastric cancers, the functional significance of the pathway in gastric carcinogenesis is still poorly defined. To examine the role of the Apc/β-catenin signaling pathway in the development of gastric cancers, we investigated the gastric mucosa of the $Apc^{Min/+}$ mouse, which is a murine model for familial adenomatous polyposis, carrying a germ-line mutation at codon 850 of Apc. We found that aged $Apc^{Min/+}$ mice spontaneously develop multiple tumors in the stomach, which are accompanied by loss of heterozygosity of Apc. Such tumors consisted of adenomatous glands with strong nuclear accumulation of β-catenin. Even a single adenomatous gland already showed nuclear accumulation of β-catenin, suggesting that Apc/β-catenin pathway is an initiating event in gastric tumorigenesis in $Apc^{Min/+}$ mice. Myc and cyclin D1 expressions, which are transcriptional targets of β-catenin/Tcf, increased in the adenomatous lesions. Furthermore, β-catenin/Tcf reporter transgenic mice with Apc^{Min} allele showed higher levels of the transcriptional activity of β-catenin/Tcf in the gastric tumors. We also treated $Apc^{Min/+}$ and wild-type mice with N-methyl-N-nitrosourea (MNU), an alkylating agent that induces adenomas and adenocarcinomas in the stomach. Consequently, MNU-treated $Apc^{Min/+}$ mice significantly enhanced the tumor development in comparison with $Apc^{Min/+}$ mice or MNU-treated wild-type mice. Several gastric tumors in MNU-treated $Apc^{Min/+}$ mice showed invasion into the submucosal layer. These results indicate that the Apc/β-catenin pathway may play an important role in at least subset of gastric carcinomas. In addition, $Apc^{Min/+}$ mice combined with MNU could be a useful short-term model to investigate multistage carcinogenesis in the stomach. [Cancer Res 2007;67(9):4079–87]

Introduction

Familial adenomatous polyposis (FAP) is regarded as an autosomal dominant disease in which many adenomatous polyps develop in the colon, thereafter progressing to colorectal carcinoma. Genetic linkage studies have shown that the inactivation of the *adenomatous polyposis coli* (APC) gene, located on chromosome 5q21, is responsible for such phenotypes in FAP (1, 2). Therefore, inactivating APC is considered to initiate the multistep progression of colorectal cancer (3). The knowledge about how *APC* acts as a tumor suppressor gene was considerably advanced by the demonstration that APC contributes to the degradation of cytosolic β-catenin, thereby linking APC to the Wnt/β-catenin pathway as a negative regulator (4–7). The critical event in the Wnt pathway activation is the elevation of the cytoplasmic pool of β-catenin and its resultant transport to the nucleus (5, 8). Consequently, the inactivation of APC leads to a constitutive accumulation of β-catenin, which triggers an aberrant transcriptional activation of the β-catenin/Tcf target genes, such as *Myc* and *Cyclin D1* (9, 10).

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death in the world (11). Although several lines of evidence suggest the involvement of the Wnt pathway in the development of sporadic gastric cancers, the functional significance of the Apc/β-catenin pathway in gastric carcinogenesis is still poorly defined in comparison with that in colon carcinogenesis. The nuclear accumulation of β-catenin, a hallmark of Wnt activation, has been found in 17% to 54% of gastric adenocarcinomas (12–14), and mutations in *APC* and *β-catenin* are found in a subset of gastric adenocarcinomas. However, the incidence of such mutations varies according to evidence from different studies (0–60% for *APC* mutations and 0–27% for *β-catenin* mutations; refs. 13–18). In addition, FAP patients have been reported to have an increased risk for gastric cancer in Japan but not in Western countries (19–22). Taken together, the functional significance of the Wnt pathway in gastric carcinogenesis therefore remains controversial.

$Apc^{Min/+}$ mouse is a mouse model for FAP and harbors a dominant mutation at the Apc, the mouse homologue of the human APC gene, resulting in the truncation of the gene product at amino acid 850 (23). Originally, this lineage was established from an ethylnitrosourea-treated C57BL/6J male mouse, and its phenotype is an autosomal dominant trait. Although homozygous $Apc^{Min/Min}$ mice die as embryos, $Apc^{Min/+}$ mice develop multiple neoplasias in their intestinal tracts within several weeks after birth (23). It is also known that most of the intestinal tumors in the mice that are heterozygous for a mutant allele of Apc have lost the Apc function due to a loss of heterozygosity (LOH; refs. 24, 25). Although mice generated by gene targeting with a mutation at codon 1638 of the Apc have been reported to occasionally develop gastric tumors after 30 weeks of age (26, 27), the relationship between gastric tumorigenesis and the activation of the Apc/β-catenin pathway has not yet been well characterized. $Apc^{Min/+}$ mice combined with $Foxl1^{-/-}$ mice (28) or $Cdx2$ transgenic mice (29) have been shown to develop gastric tumors. However, previous studies have indicated no evidence of a spontaneous development of gastric tumors in $Apc^{Min/+}$ mice.

In the present study, we examined the gastric lesions of $Apc^{Min/+}$ mice and found, for the first time, that aged $Apc^{Min/+}$ mice develop multiple adenomas in the stomach, which are accompanied by Apc LOH and the activation of the β-catenin/Tcf signaling pathway. We also treated $Apc^{Min/+}$ mice with *N*-methyl-*N*-nitrosourea (MNU), an alkylating agent that induces adenomas and adenocarcinomas in the stomach, to elucidate the mode of gastric carcinogenesis in $Apc^{Min/+}$ mice. Consequently, MNU-treated $Apc^{Min/+}$ mice were found to strongly promote tumor development, and several gastric tumors showed invasive adenocarcinomas. These results indicate that the Apc/β-catenin pathway plays a significant role in a subset of gastric carcinogenesis, thus suggesting that this model could be a short-term model to investigate multistage carcinogenesis in the stomach.

Materials and Methods

Animals. This study was approved by the Institutional Ethics Review Committee for Animal Experiments at Gifu University. $Apc^{Min/+}$ mice in the C57BL/6J background were obtained from The Jackson Laboratory and maintained by breeding $Apc^{Min/+}$ males to C57BL/6J females. All mice were maintained under specific pathogen-free conditions with isolated ventilation cages in an air-conditioned room with a 12-h light/12-h dark cycle. They were bred and maintained on a basal diet, CE-2 (CLEA Japan, Inc.), until the termination of the study. Heterozygous progeny were identified by a PCR analysis of tail DNA using allele-specific primers (24). To examine gastric lesions, $Apc^{Min/+}$ and age-matched wild-type littermates, 15 to 35 weeks of age, were used in the present study. It is known that the life span of $Apc^{Min/+}$ mice on C57BL/6J background is ∼20 weeks (23), whereas the mice in our facility have a better survival as was also observed in several previous studies (30–33). Elderly $Apc^{Min/+}$ mice (i.e., >25 weeks of age) were carefully monitored and sacrificed before become moribund.

Transgenic mice. To generate β-catenin/Tcf reporter mice, Tcf/Lef binding sites plus a minimal thymidine kinase promoter were PCR amplified from the TOPFLASH plasmid (Upstate), ligated to an EGFP-pA cassette (Clontech), and then injected into fertilized eggs from C57BL/6J mice.[5] Next, the β-catenin/Tcf reporter mice were crossed with $Apc^{Min/+}$ mice to generate the reporter mice with the Apc^{Min} allele.

MNU treatment. MNU (Sigma Chemical) was dissolved in distilled water at a concentration of 240 ppm and freshly prepared thrice weekly for the administration in drinking water in light-shielded bottles *ad libitum*. $Apc^{Min/+}$ and wild-type littermates, from 4 to 6 weeks of age, were given drinking water containing 240 ppm MNU on alternate weeks for a total of 10 weeks of exposure according to the protocol described in previous reports (34, 35). The MNU-treated $Apc^{Min/+}$ and wild-type littermates were sacrificed at 15, 20, 25, 30, and 35 weeks of age.

Preparation of tissue samples for tumor counting and histologic analysis. All mice underwent a thorough postmortem examination at the time of sacrifice. The stomach was removed and opened along the greater curvature. The number, as well as the long diameter, of the tumors in the stomach was measured using a dissected microscope at ×7 magnification. Tumors >0.5 mm in diameter were mapped and counted. To eliminate interobserver error, all counts were done by a single observer blinded to the genotype of the mice. All cases were also counted by a second observer to confirm the results of the first observer. After tumor counting, all of the excised stomachs, including the neoplastic nodules, were fixed for 24 h in neutral-buffered 10% formalin and then cut into eight strips, which were processed by standard methods, embedded in paraffin, sectioned at 5 μm, and stained with H&E. The defining characteristics for adenoma and adenocarcinoma were adapted from both the consensus guidelines on murine models of intestinal cancer (36) and previous reports in the literature (35, 37).

Human tissue samples. A total of 81 formalin-fixed, paraffin-embedded sporadic gastric adenomas (*n* = 20) and early adenocarcinomas (intramucosal adenocarcinomas; *n* = 61), dissected in 2003 to 2006 at Gifu

University Hospital, was examined for β-catenin expression. The experiments were carried out according to the protocol approved by the Ethics Committee of Gifu University.

Immunohistochemistry. The avidin-biotin peroxidase complex (ABC) technique was used for immunohistochemical studies. Sections (5 μm thick) were cut, deparaffinized, rehydrated in PBS, placed in 10 mmol/L citrate buffer (pH 6.0), and heated in a 750-W microwave four times for 6 min. The endogenous peroxidase activity was blocked by incubation for 30 min in 0.3% H_2O_2. After washing thrice with PBS, the sections were preincubated with normal blocking serum for 20 min at room temperature and then incubated with β-catenin (1:1,000; BD Biosciences PharMingen), c-myc (1:200; Santa Cruz Biotechnology), cyclin D1 (1:200; Santa Cruz Biotechnology), Ki-67 (1:200; DAKO Corp.), AE1/AE3 (1:100; DAKO), and green fluorescent protein (GFP) antibody (1:1,500; Molecular Probes) overnight at 4°C. Subsequently, the sections were incubated with biotinylated secondary antibodies (Vectastain ABC kit, Vector Laboratories) for 30 min followed by incubation with avidin-coupled peroxidase (Vector Laboratories) for 30 min. The sections were developed with 3,3′-diaminobenzidine (DAB) using DAKO Liquid DAB Substrate-Chromogen System (DAKO) and then counterstained with hematoxylin. For immunofluorescence, FITC- or TRITC-conjugated secondary antibodies (Jackson ImmunoResearch) were used and then counterstained with 4′,6-diamidino-2-phenylindole. No specific staining was observed in the negative control slides prepared without primary antibody.

LOH analysis and laser capture microdissection. In the current study, the DNA for the analysis of the Apc allelic loss was extracted from the cells isolated with laser microdissected tissue sections as described previously (38, 39). For laser capturing, the slides were put into xylene for 30 min to dissolve the paraffin that otherwise interfered with laser capture microdissection (LCM). Next, the slides were washed for 10 min in 100% ethanol. After staining with H&E, the sections were dehydrated in 100% ethanol, incubated for 2 min in xylene, and then dried at room temperature. Microdissection was done using a laser microdissection system (PALM Microlaser Technologies). The microdissected tissues of normal-appearing epithelium and tumors from $Apc^{Min/+}$ and MNU-treated $Apc^{Min/+}$ mice were used for the analysis. They were digested overnight at 50°C in 20 μL of lysis buffer containing 500 μg/mL proteinase K, 10 mmol/L Tris-HCl (pH 8.0), 50 mmol/L KCl, 0.45% NP40, and 0.45% Tween 20. The proteinase K was heat inactivated (10 min at 95°C). LOH of the Apc gene was checked using PCR with mismatched primers as described previously (24). Briefly, the amplification of the Apc^{Min} allele resulted in a 155-bp PCR product with one *Hin*dIII site, whereas the 155-bp product from Apc^+ allele contained two *Hin*dIII sites. *Hin*dIII digestion of PCR-amplified DNA from $Apc^{Min/+}$ heterozygous tissues resulted in a 123-bp product from Apc^+ allele and a 144-bp product from the Apc^{Min} allele. Therefore, the PCR products from tissue with LOH displayed only one band (144 bp) from the Apc^{Min} allele. The samples were assayed at least twice independently.

Mutation analysis of the Apc gene. The mutation cluster region of the mouse Apc gene (nucleotides 1,991–5,333; Genbank M88127) was screened for sequence alterations by PCR of four overlapping fragments (segment A, nucleotides 1,991–2,954; segment B, nucleotides 2,919–3,942; segment C, nucleotides 3,862–4,852; segment D, nucleotides 4,807–5,333). PCR products were gel purified using the DNA Gel Extraction kit (Millipore). These samples were also subcloned into pCR 2.1 (Invitrogen) and sequenced using T7 and M13 primers according to the manufacturer's protocol. The standard cycle sequencing was done in the presence of fluorescently labeled dideoxynucleotides by an ABI automated sequencer. Sequencing analyses of all samples were repeated twice to exclude PCR errors.

Quantitative real-time reverse transcription-PCR. Gastric tumors from $Apc^{Min/+}$ mice and MNU-treated $Apc^{Min/+}$ mice were examined for mRNA expression by quantitative real-time reverse transcription-PCR (RT-PCR). RNA was extracted using the RNAqueous-4PCR kit (Ambion) according to the manufacturer's protocol. cDNA was synthesized from 0.2 μg of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen). Real-time PCR was done in a LightCycler (Roche) with SYBR Premix Ex Taq (TaKaRa). The expression level of each gene was normalized to the β-actin expression level using the standard curve method. Each experiment was done

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[5] T. Oyama, et al., manuscript in preparation.

in either duplicate or triplicate, and then, the average was calculated. The primers for amplifications are listed in Supplementary Table S1.

Results

Spontaneous development of gastric tumors in the elderly *Apc*^Min/+ mice. We examined the gastric lesions in the *Apc*^Min/+ mice of 15, 20, 25, 30, and 35 weeks of age (n = 12, 13, 15, 16, and 13, respectively) and then compared the findings with those in age-matched wild-type littermates (Fig. 1). We found multiple gastric tumors in the *Apc*^Min/+ mice >20 weeks of age, whereas no gastric tumors were found in the wild-type littermates at any age. Macroscopically, the gastric tumors in the *Apc*^Min/+ mice showed a sessile and/or polypoid morphology (Fig. 1*A*). Although the *Apc*^Min/+ mice tended to develop multiple tumors in the small intestine as early as a few weeks after birth, no gastric tumors were found in the stomach of *Apc*^Min/+ mice before 20 weeks of age. The incidence of gastric tumors in the *Apc*^Min/+ mice at each age is given in Fig. 1*B*. The incidence of gastric tumors in *Apc*^Min/+ mice at 35 weeks of age was 100% (n = 13). The number and size of the tumors have increased as it appears from 20 weeks of age by aging (Fig. 1*C* and *D*). When the glandular stomach was divided into the antrum and the corpus, most gastric tumors developed in the pyloric glands of the antrum (Fig. 1*C*).

Nuclear accumulation of β-catenin in the gastric tumors of the *Apc*^Min/+ mice. The gastric tumors in *Apc*^Min/+ mice were evaluated to determine the histopathologic features (Fig. 2). In H&E staining, such tumors revealed a disturbed glandular architecture, an increased nuclear to cytoplasmic ratio, and nuclear atypia with surrounding hyperplastic glands (Fig. 2*A*). Gastric tumors in the *Apc*^Min/+ mice were classified as adenomas with mild to severe cellular atypia.

To clarify the possible involvement of the Apc/β-catenin pathway in the gastric tumors, we determined the subcellular localization of β-catenin by immunohistochemical staining. We found nuclear/cytoplasmic staining of β-catenin in adenomatous lesions (Fig. 2*A*), suggesting that the β-catenin signaling pathway is activated in such tumors. No nuclear/cytoplasmic β-catenin staining was observed in the elongated glands adjacent to these tumors, thus suggesting that these glands are reactive hyperplasia.

In addition to macroscopically detectable tumors, we found small adenomatous lesions in H&E staining (Fig. 2*A*). Such small lesions consisted of a few adenomatous glands or even a single adenomatous gland located near the proliferative compartment of the pyloric glands. β-Catenin immunostaining revealed marked nuclear/cytoplasmic staining even in such a single atypical gland (100%; n = 16), thus suggesting that the accumulation of β-catenin protein is an initiating event in the development of gastric tumors.

To examine the cell proliferative activity in such gastric tumors, we did double immunofluorescence for β-catenin and Ki-67 (Fig. 2*B*). Adenomatous lesions accompanied by β-catenin accumulation showed a high frequency of Ki-67–positive epithelial cells, whereas the adjacent hyperplastic glands showed such activity only in the proliferative compartment.

Increased β-catenin/Tcf transcriptional activity in the gastric lesions. To evaluate the possible activation of the downstream targets of Apc/β-catenin signaling, we investigated the β-catenin/Tcf transcriptional activity. We analyzed the expression of Myc and cyclin D1, both of which are known targets of β-catenin/Tcf transcription. Immunostainings revealed that adenomatous glands with the accumulation of β-catenin showed an increased expression of both Myc and cyclin D1 (Fig. 3*A*). The cyclin D1 mRNA expression in gastric tumors also increased in comparison with the normal epithelium of the stomach by

Figure 1. The spontaneous development of gastric tumors in aged *Apc*^Min/+ mice. *A,* macroscopic photograph of the grandular stomach of an *Apc*^Min/+ mouse at 30 weeks of age. Bar, 2 mm. *B,* incidence of *Apc*^Min/+ mice with gastric tumors increased by aging. *C,* number of gastric tumors increased by aging and gastric tumors preferentially developed in the antrum. *Columns,* mean; *bars,* SD. *, P < 0.05, Mann-Whitney *U* test; **, P < 0.01, Mann-Whitney *U* test. *D,* the size (the maximum long diameter) of gastric tumors in *Apc*^Min/+ mice increased over time. *Columns,* mean; *bars,* SD.
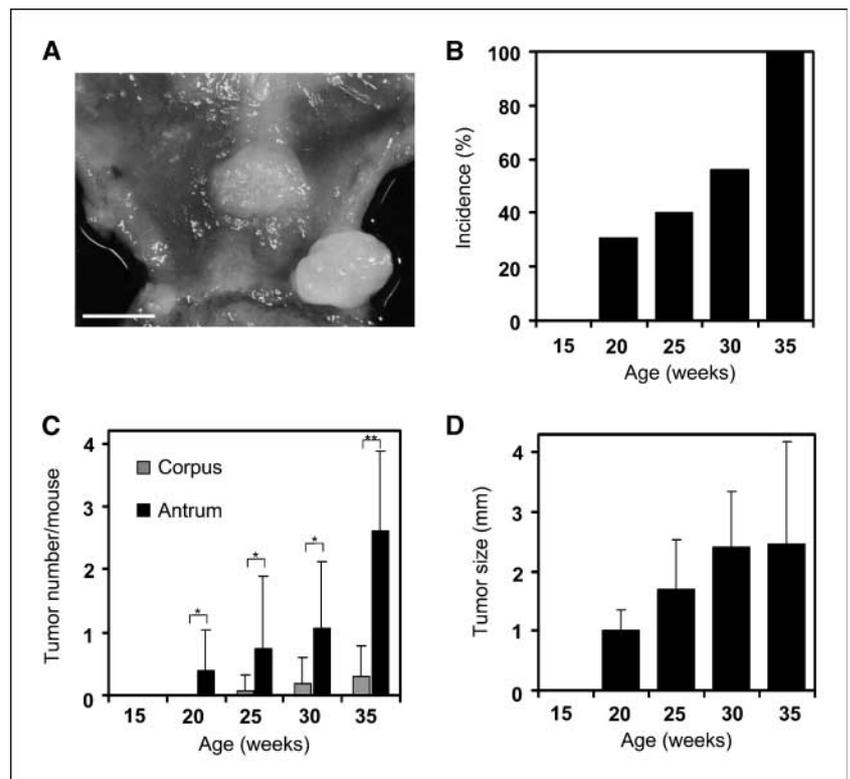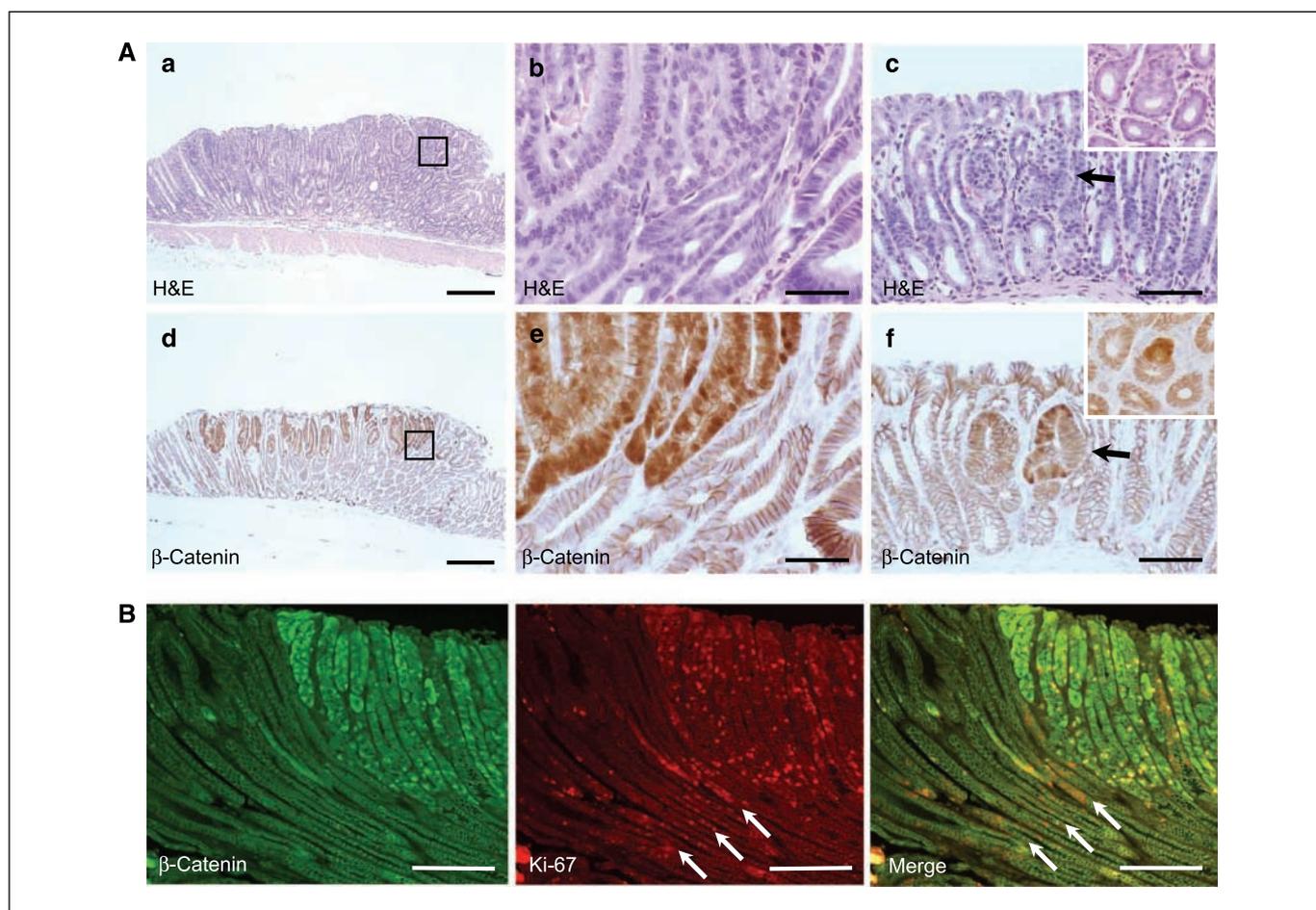
Figure 2. Gastric tumors in $Apc^{Min/+}$ mice show adenomatous lesions with nuclear/cytoplasmic accumulation of β-catenin. *A,* H&E-stained sections (*a–c*) and immunostaining for β-catenin on serial sections (*d–f*). Strong nuclear immunostaining for β-catenin was recognized in the adenomatous glands, whereas the adjacent hyperplastic glands only revealed membranous staining. Note that the small lesion (*c*) already shows β-catenin accumulation (*f*). *c* and *f, arrow,* small adenomatous lesion. Bars, 200 μm (*a* and *d*), 50 μm (*b* and *e*), and 100 μm (*c* and *f*). *B,* proliferating Ki-67–positive cells increase in the adenomatous glands with nuclear/cytoplasmic β-catenin accumulation, whereas surrounding hyperplastic glands include positive cells only at proliferative compartment (*arrows*). Bar, 100 μm.

quantitative real-time RT-PCR (Fig. 3*B*). We further examined the β-catenin/Tcf transcriptional activity using the β-catenin/Tcf reporter mice carrying the Apc^{Min} mutation. Immunostaining in serial sections revealed a colocalization of the β-catenin and the reporter (GFP) in such adenomatous lesions (Fig. 3*C*). These results suggest that the transcriptional activation of the β-catenin/Tcf targets thus plays an important role in the pathogenesis of gastric tumors in $Apc^{Min/+}$ mice.

MNU accelerates gastric tumorigenesis in $Apc^{Min/+}$ mice. We treated $Apc^{Min/+}$ mice with MNU, an alkylating agent that induces adenomas and adenocarcinomas in the stomach. The MNU-treated $Apc^{Min/+}$ mice of 15, 20, 25, and 30 weeks of age (*n* = 7, 6, 7, and 8, respectively) were examined for gastric lesions and then compared with either the age-matched $Apc^{Min/+}$ mice (as described above) or the wild-type littermates with MNU treatment (*n* = 8, 6, 11, and 12, respectively). Two MNU-treated $Apc^{Min/+}$ mice (22 and 28 weeks of age) and one MNU-treated wild-type mouse (32 weeks of age) became morbid and therefore were sacrificed during the experiment. The macroscopic appearance of typical gastric lesions in $Apc^{Min/+}$, MNU-treated $Apc^{Min/+}$, and MNU-treated wild-type mice at 25 weeks of age is shown in Fig. 4*A*. MNU-treated $Apc^{Min/+}$ mice developed gastric tumors as early as at 15 weeks of age, whereas $Apc^{Min/+}$ and MNU-treated wild-type mice did not develop any

tumors before 20 weeks of age. The incidence of gastric tumors in the MNU-treated $Apc^{Min/+}$ mice was significantly higher than that of $Apc^{Min/+}$ mice or MNU-treated wild-type mice (Fig. 4*B*). MNU-treated $Apc^{Min/+}$ mice also had a greater tumor multiplicity in the stomach when compared with that in $Apc^{Min/+}$ or MNU-treated wild-type mice (Fig. 4*C*). The average number of the gastric tumors in $Apc^{Min/+}$, MNU-treated $Apc^{Min/+}$, and MNU-treated wild-type mice at 25 weeks of age was 0.8 ± 1.26, 4.3 ± 1.38, and 0.2 ± 0.40 (±SD) per mouse, respectively. These results indicate that MNU accelerates gastric tumorigenesis in $Apc^{Min/+}$ mice.

Invasive adenocarcinomas in the stomach of $Apc^{Min/+}$ mice treated with MNU. Gastric tumors in $Apc^{Min/+}$, MNU-treated $Apc^{Min/+}$, and MNU-treated wild-type mice were evaluated for their histopathologic features (Fig. 5). MNU-treated $Apc^{Min/+}$ mice often developed intramucosal adenocarcinomas as well as benign adenomas, although all gastric tumors in $Apc^{Min/+}$ mice were adenomas as described above. The majority of the tumors in MNU-treated wild-type mice were also classified as adenomas at each age. Surprisingly, 3 of 12 (25%) MNU-treated $Apc^{Min/+}$ mice developed invasive adenocarcinomas after 30 weeks of age (Fig. 5*B*), and this was never observed in either the $Apc^{Min/+}$ mice or the MNU-treated wild-type mice during these experiments. Undifferentiated cells in the submucosal layer were confirmed to be invasive

adenocarcinomas using AE1/AE3 staining, which recognizes the epithelial cells (Fig. 5A). The histologic grade of gastric tumors in MNU-treated $Apc^{Min/+}$ mice progressed over time (Fig. 5B), possibly representing the multistage process of the gastric carcinogenesis.

β-Catenin accumulation in gastric tumors of MNU-treated $Apc^{Min/+}$ mice. Most of the adenomatous lesions in MNU-treated $Apc^{Min/+}$ mice revealed nuclear/cytoplasmic β-catenin staining, similar to the staining in $Apc^{Min/+}$ mice (Fig. 6A), indicating the involvement of β-catenin activation. However, the majority of small tumors (<1.0 mm) in the stomach of MNU-treated wild-type mice revealed no evidence of β-catenin accumulation, thereby showing only membranous staining and thus suggesting that MNU-induced tumorigenesis is initiated by pathways other than β-catenin activation. The incidences of β-catenin accumulation in gastric tumors (<2.0 mm) of $Apc^{Min/+}$, MNU-treated $Apc^{Min/+}$, and MNU-treated wild-type mice were 100% (12 of 12), 84.6% (11 of 13), and 12.5% (1 of 8), respectively (Fig. 6B). Gastric tumors in MNU-treated $Apc^{Min/+}$ mice had a significantly higher frequency of β-catenin accumulation in comparison with the MNU-treated wild-type mice.

Altered gene expressions in gastric tumors of $Apc^{Min/+}$ and MNU-treated $Apc^{Min/+}$ mice. To examine gene expression patterns on the gastric tumors of $Apc^{Min/+}$ mice, we analyzed 18 genes (*Ptgs2, Erbb2, Kras, Ccne1, Cdh1, Cdkn1a, Cdkn1b, Tgfa, Tgfb1, Egf, Egfr, Vegfa, Vegfr2, Fgfr2, Met, Stat3, Runx3,* and *Cdx2*), which have previously been shown to be differentially expressed in human gastric adenomas and carcinomas (15, 40, 41), by quantitative real-time RT-PCR (Fig. 6C; Supplementary Fig. S1). The expression of three genes (*Cyclin E1, TGFβ1,* and *STAT3*), in addition to *Cyclin D1* (Fig. 3B), significantly increased in the gastric tumors of $Apc^{Min/+}$

mice, and five genes (*COX-2, Cyclin E1, TGFβ1, EGFR,* and *K-sam*) were significantly up-regulated in tumors of MNU-treated $Apc^{Min/+}$ mice in comparison with the adjacent normal mucosa. Furthermore, the expression of four genes (*COX-2, E-cadherin, TGFα,* and *EGFR*) was significantly higher in tumors of MNU-treated $Apc^{Min/+}$ mice in comparison with those in untreated $Apc^{Min/+}$ mice. Among these up-regulated genes, the expression of cyclooxygenase-2 (COX-2) mRNA was markedly increased in gastric tumors of both MNU-treated wild-type and $Apc^{Min/+}$ mice (Fig. 6C).

Frequent LOH of the Apc in gastric tumors of $Apc^{Min/+}$ mice. To determine whether Apc LOH is involved in the development of gastric tumors, we did Apc LOH analysis on the adenomatous lesions isolated using the LCM system (Fig. 6D). Twenty-seven of 32 (84.3%) dissected tumors from $Apc^{Min/+}$ mice showed a predominant signal of the Apc^{Min} allele, thus indicating a loss of the wild-type allele. These results suggest that Apc LOH is involved in the development of gastric tumors in $Apc^{Min/+}$ mice. Gastric tumors in MNU-treated $Apc^{Min/+}$ mice showed a reduced frequency of allelic loss at Apc (8 of 21, 38.1%; $P < 0.001$ versus $Apc^{Min/+}$ mice, by Fisher's exact test). To determine whether mutations of Apc are involved in the tumor initiation by MNU, three gastric tumors without allelic loss at Apc in MNU-treated $Apc^{Min/+}$ mice were examined for somatic mutations at Apc. However, no mutation of the Apc was detected in these gastric tumors except for a nonsense mutation of Apc^{Min} allele (T/A → A/T transversion at nucleotide 2,549 of the Apc; ref. 42).

Nuclear accumulation of β-catenin in human gastric adenomas and early adenocarcinomas. To clarify roles of Wnt/β-catenin pathway in the early stage of human gastric carcinogenesis, we investigated the β-catenin expression in sporadic adenomas and
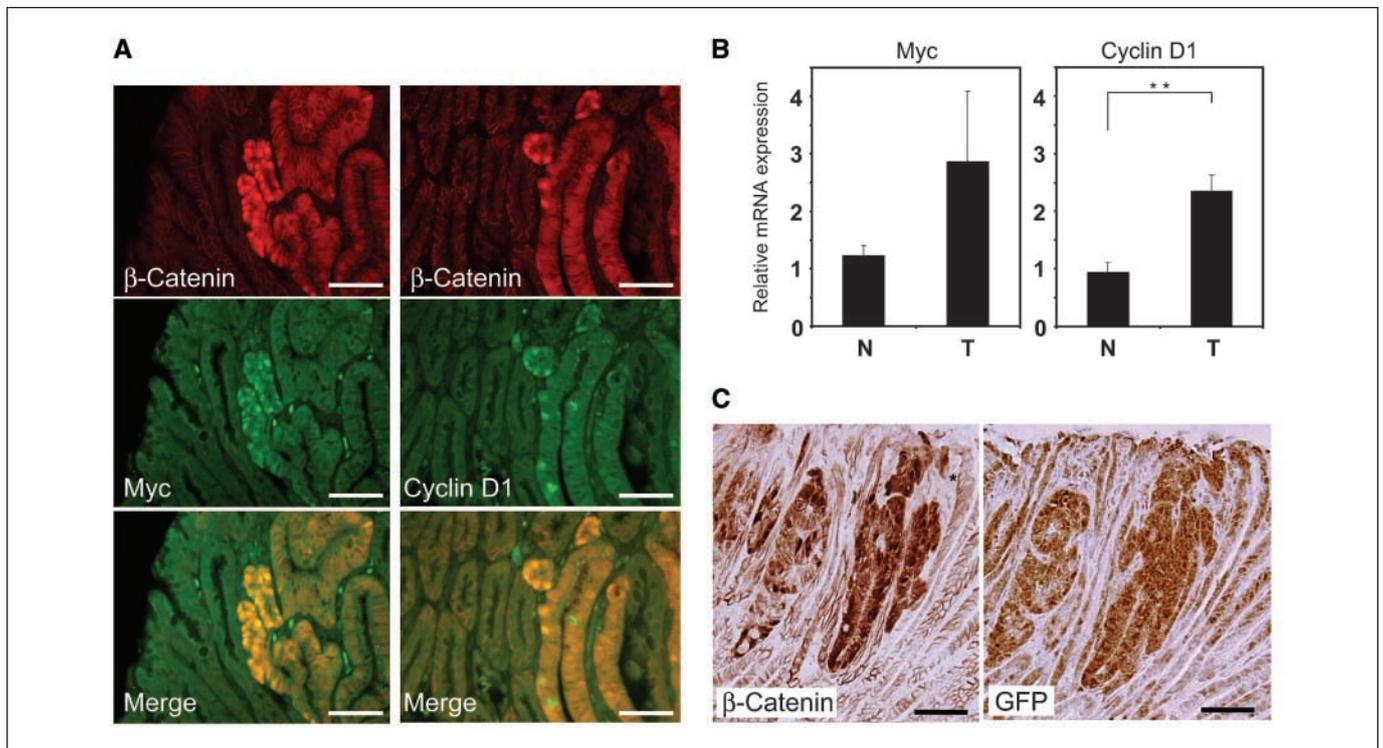

Figure 3. Increased β-catenin/Tcf transcriptional activity in the adenomatous lesions of the stomach in $Apc^{Min/+}$ mice. *A,* double immunofluorescent staining for β-catenin, Myc, or cyclin D1. β-Catenin accumulation (*red*) is colocalized (*yellow*) with Myc (*left; green*) and cyclin D1 (*right; green*) overexpressions in the adenomatous glands within the tumor. *B,* increased mRNA expression of *Myc* and *cyclin D1* in the gastric tumors of $Apc^{Min/+}$ mice. The expression of each gene was examined by quantitative real-time RT-PCR and normalized to β-actin expression. *N,* normal epithelium (*n* = 10); *T,* tumor (*n* = 10). *Columns,* mean of three independent experiments; *bars,* SE. **, $P < 0.01$, Mann-Whitney *U* test. *C,* β-catenin/Tcf reporter mice show GFP (*right*) expression in adenomatous glands with β-catenin accumulation (*left*). Bar, 100 μm (*A* and *C*).
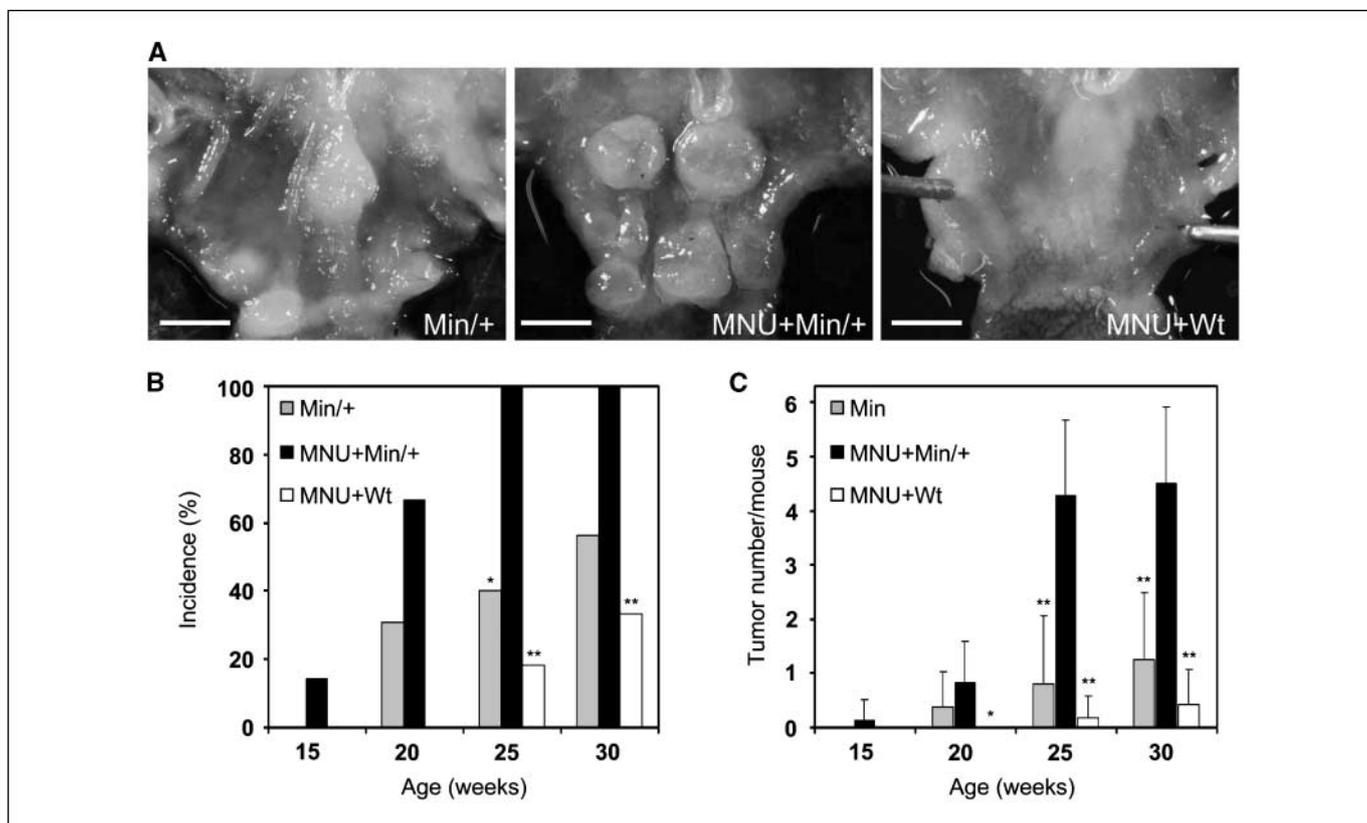
Figure 4. MNU treatment accelerates gastric tumorigenesis in $Apc^{Min/+}$ mice. *A,* representative macroscopic photographs of the stomach in $Apc^{Min/+}$, MNU-treated $Apc^{Min/+}$, and MNU-treated wild-type mice at 25 weeks of age. Bar, 2 mm. *Min/+,* $Apc^{Min/+}$; *Wt,* wild-type. *B,* incidence of mice with gastric tumors in $Apc^{Min/+}$, MNU-treated $Apc^{Min/+}$, and MNU-treated wild-type mice at each age. The incidence of MNU-treated $Apc^{Min/+}$ mice with gastric tumors was significantly increased when compared with that of $Apc^{Min/+}$ or MNU-treated wild-type mice at 25 to 30 weeks of age. *Bars,* SD. *, $P < 0.05$ versus $Apc^{Min/+}$ mice, by Fisher's exact test; **, $P < 0.01$ versus MNU-treated wild-type mice, by Fisher's exact test. *C,* number of gastric tumors per mouse in $Apc^{Min/+}$, MNU-treated $Apc^{Min/+}$, and MNU-treated wild-type mice at each age. MNU-treated $Apc^{Min/+}$ mice developed significantly higher numbers of gastric tumors than $Apc^{Min/+}$ or MNU-treated wild-type mice at 20 to 35 weeks of age. *Columns,* mean; *bars,* SD. *, $P < 0.05$ versus MNU-treated $Apc^{Min/+}$ mice, by Mann-Whitney U test; **, $P < 0.01$ versus MNU-treated $Apc^{Min/+}$ mice, by Mann-Whitney U test.

intramucosal adenocarcinomas of the stomach by immunohistochemistry (Supplementary Fig. S2). The nuclear accumulation of β-catenin was detected in 8 (40%) and 24 (39%) cases of 20 adenomas and 61 intramucosal adenocarcinomas, respectively. These results suggest that Wnt/β-catenin signaling is involved in the early stage of gastric carcinogenesis in humans.

Discussion

The $Apc^{Min/+}$ mouse, a murine model for FAP, carrying a germline mutation at codon 850 of *Apc* (23), has been used extensively to clarify the pathogenesis of intestinal tumorigenesis. In this study, we carefully monitored $Apc^{Min/+}$ mice over 25 weeks of age and found that aged $Apc^{Min/+}$ mice develop multiple gastric tumors. Gastric tumors in $Apc^{Min/+}$ mice showed adenomatous lesions with a strong nuclear/cytoplasmic accumulation of β-catenin, thus suggesting that an altered β-catenin expression is involved in tumorigenesis. Furthermore, the accumulation of β-catenin is already detectable at a single adenomatous gland in the stomach. These results suggest that β-catenin accumulations could therefore be an initiating event in gastric carcinogenesis. We consistently found the nuclear accumulation of β-catenin in ∼40% of adenomas and early carcinomas in human gastric tissues.

β-Catenin accumulation is caused by a loss of the *Apc* function through LOH in intestinal tumors in mice that are heterozygous for

a mutant allele of *Apc* (24). In the present study, adenomatous lesions in the stomach were also found to have lost the remaining allele of *Apc*, indicating that a loss of *Apc* leads to the formation of such adenomatous glands. Similar to the intestinal tumorigenesis, *Apc* LOH may lead to the accumulation of β-catenin, which could activate its downstream pathway in the gastric tumors. Indeed, we showed that *Myc* and *Cyclin D1* expressions, which are targets of β-catenin/Tcf transcription (9, 10), also increased in adenomatous lesions, and they were colocalized with the nuclear accumulation of β-catenin. In addition, the transcriptional activity of β-catenin/Tcf, which is assessed by the use of β-catenin/Tcf reporter mice, was also found to increase in the adenomatous glands with β-catenin accumulation. These results suggest that a loss of the *Apc* function followed by the activation of β-catenin/Tcf transcription plays a causal role in the development of gastric cancers.

The glandular stomach consists of two different types of glands: the pyloric glands of the antrum and the fundic glands of the corpus. The highest frequency of gastric tumors in $Apc^{Min/+}$ mice was observed in the pyloric glands of the antrum. Our results suggest that the activation of the Apc/β-catenin pathway is closely associated with gastric tumorigenesis in the pyloric glands. The biological characteristics of the pyloric glands in the antrum have recently been shown to be developmentally similar to those of the small intestinal epithelium (40, 43). In addition to the frequent development of tumors in both the small intestine and the antrum

of the stomach in $Apc^{Min/+}$ mice, our results suggest that the mechanisms of gastric tumorigenesis in the pyloric glands may be similar to those of intestinal tumorigenesis. In contrast, we did not detect any fundic gland polyps that are often associated with FAP patients. The importance of the Apc/β-catenin pathway in the development of fundic gland polyps in mice remains to be elucidated.

The MNU-induced rodent models for gastric carcinogenesis have been widely used to study carcinogenesis of the stomach. These animal models have been used not only for investigating the pathogenesis of gastric carcinogenesis but also for identifying possible tumor promoters and chemopreventive agents (34, 35, 44, 45). However, mice have been known to be relatively resistant to MNU, and therefore, previous studies showed that 48 to 52 weeks were required to induce gastric carcinoma in C57BL/6J mice (35, 37, 46, 47). In the present study, we treated $Apc^{Min/+}$ mice with MNU and found MNU to strongly promote tumor development in the stomach of $Apc^{Min/+}$ mice in comparison with MNU-treated wild-type mice. Furthermore, MNU-treated $Apc^{Min/+}$ mice developed invasive adenocarcinomas, which were not detectable in either $Apc^{Min/+}$ mice or MNU-treated wild-type mice at the same age. These results indicate that this model could be a short-term model for gastric carcinogenesis with β-catenin accumulation in mice.

It is interesting to note that the majority of small gastric tumors (<1.0 mm) induced by MNU in wild-type mice showed no evidence of β-catenin accumulations, whereas all tumors in $Apc^{Min/+}$ mice showed an accumulation of the protein even in a single adenomatous gland. These findings suggest that an initiating event in the stomach of an MNU-induced model is independent of Wnt activation. This notion is consistent with previous findings in rodents, which suggest that β-catenin accumulation may be a later event of gastric carcinogenesis in MNU-induced models (48–50). The fact that $Apc^{Min/+}$ mice treated with MNU tend to develop more aggressive tumors in the stomach may be attributable to the combined activation of different oncogenic pathways in both models. It is noteworthy that the expression of COX-2 mRNA specifically increased in gastric tumors of MNU-treated mice. It has recently been reported that the simultaneous activation of Wnt signaling and the COX-2 pathway leads to the development of gastric cancers in mice (12). The cross-talk between Wnt and the COX-2 pathway may contribute to the rapid development of gastric tumors in MNU-treated $Apc^{Min/+}$ mice. In this context, the MNU-treated $Apc^{Min/+}$ mice could be a useful model to investigate multistage carcinogenesis of the stomach.

In the current study, most of the adenomatous lesions in MNU-treated $Apc^{Min/+}$ mice revealed nuclear accumulations of β-catenin, similar to those in $Apc^{Min/+}$ mice (Fig. 6A and B), indicating the involvement of β-catenin activation. However, allelic loss at Apc in such tumors was less frequent than that in $Apc^{Min/+}$ mice (Fig. 6D). In addition, no mutation at the Apc was
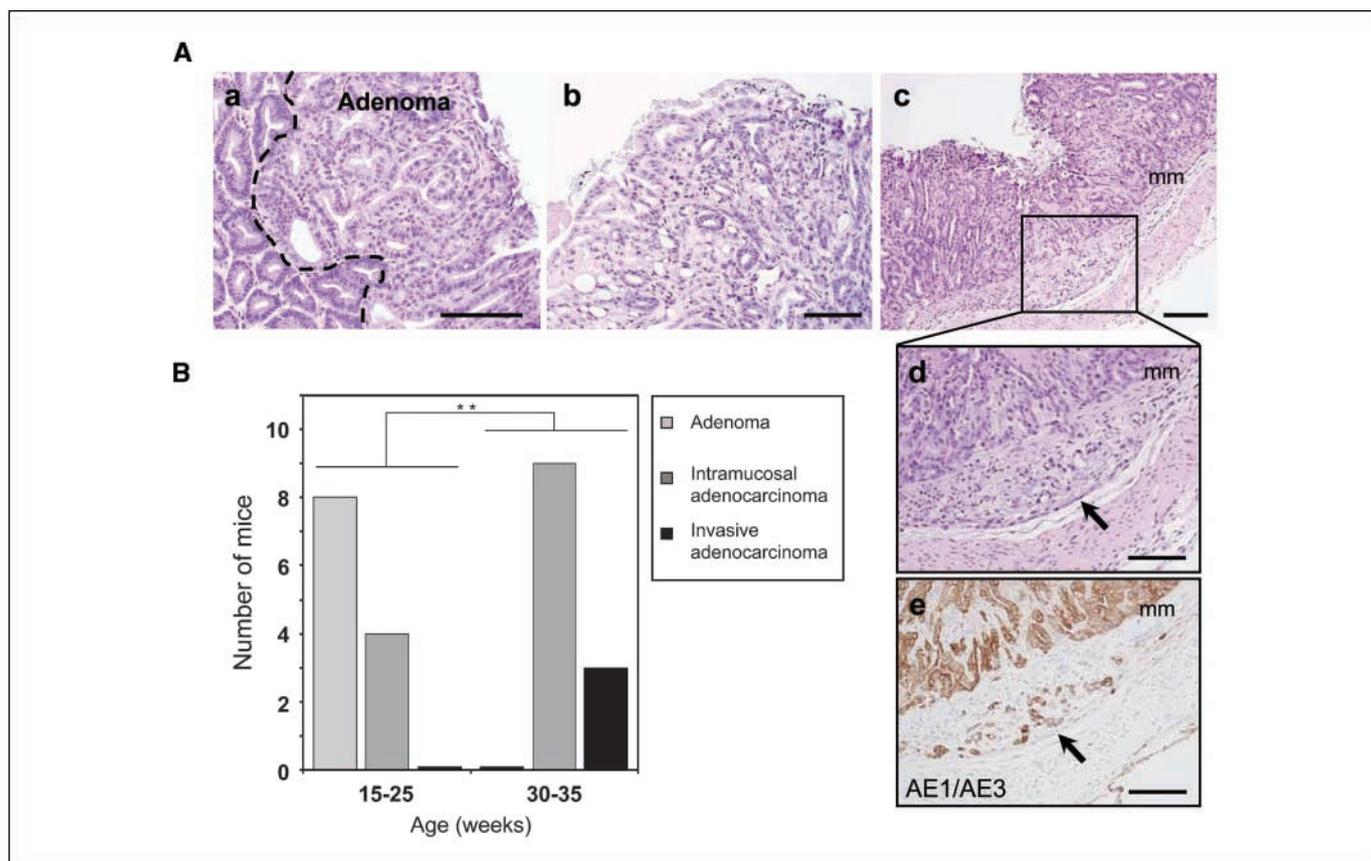
Figure 5. Gastric tumors in MNU-treated $Apc^{Min/+}$ mice show more aggressive lesions. *A,* histopathologic features of gastric tumors in MNU-treated $Apc^{Min/+}$ mice. *a,* adenomas in MNU-treated $Apc^{Min/+}$ mice were similar to that in $Apc^{Min/+}$ mice. *Dotted line,* border between hyperplastic and adenomatous (*Adenoma*) lesion. *b,* intramucosal adenocarcinomas with distinctive cellular and structural atypia. *c,* several gastric adenocarcinomas showed invasion into the submucosa. *d,* higher magnification of the boxed section in (*c*). *e,* invasive undifferentiated cells expressed an epithelial marker, AE1/AE3. *mm,* muscularis mucosae. Bar, 100 μm. *B,* histologic grade of the most advanced tumor in tumor-bearing MNU-treated $Apc^{Min/+}$ mice. The histologic grade of the tumors progressed over time. *Columns,* number of mice with each histologic type. **, $P < 0.01$, Spearman's rank correlation test.
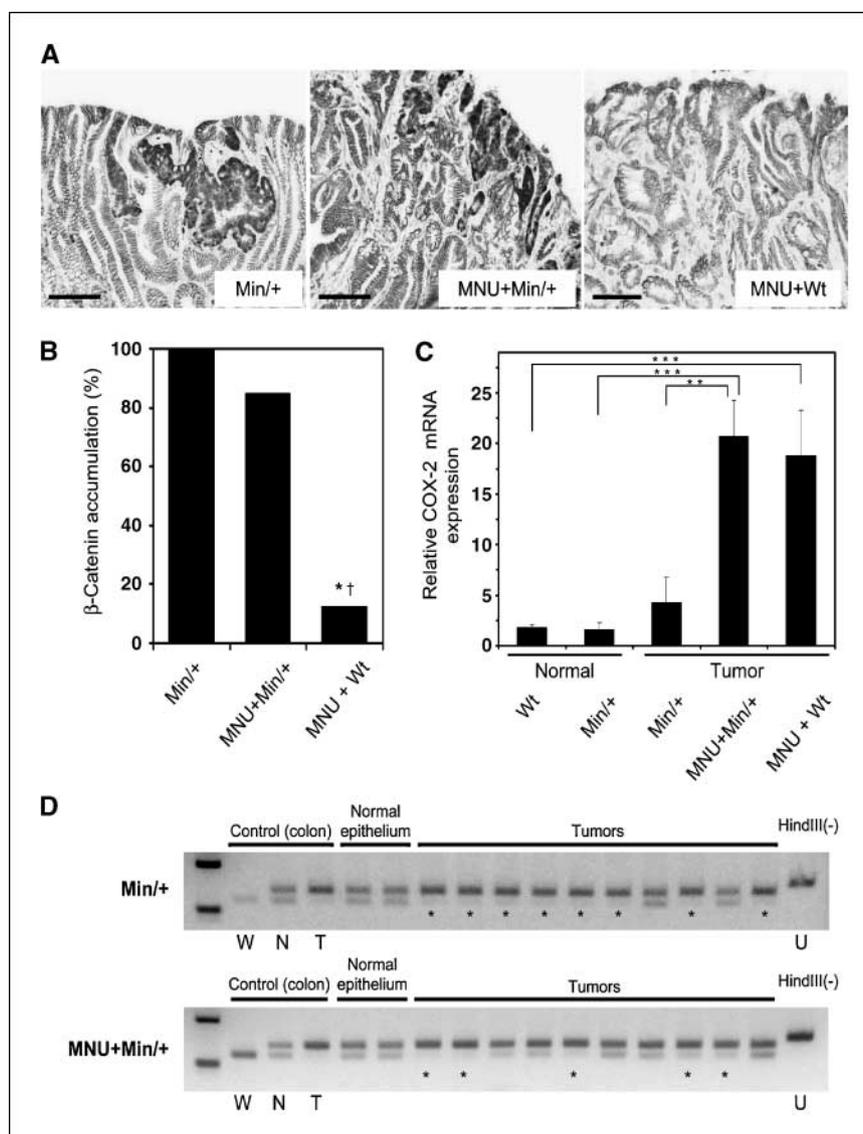
Figure 6. *A,* β-catenin accumulation of gastric tumors in *Apc*[Min/+] and MNU-treated *Apc*[Min/+] mice. The nuclear/cytoplasmic accumulation of β-catenin was prominent in *Apc*[Min/+] and MNU-treated *Apc*[Min/+] mice. Bar, 100 μm. *B,* incidence of β-catenin accumulation in gastric tumors (<2.0 mm). Gastric tumors in the MNU-treated wild-type mice had a significantly lower frequency of β-catenin accumulation than in either the *Apc*[Min/+] or the MNU-treated *Apc*[Min/+] mice. *, $P < 0.01$ versus *Apc*[Min/+] mice, by Fisher's exact test; †, $P < 0.01$ versus MNU-treated *Apc*[Min/+] mice, by Fisher's exact test. *C,* COX-2 mRNA expressions in gastric lesions of *Apc*[Min/+], MNU-treated *Apc*[Min/+], and MNU-treated wild-type mice were assessed by quantitative real-time RT-PCR. The expression was normalized to β-actin mRNA expression. Ten samples of each group were analyzed in triplicate. *Columns,* mean of three independent experiments; *bars,* SE. **, $P < 0.01$, Mann-Whitney *U* test; ***, $P < 0.001$, Mann-Whitney *U* test. *D, Apc* LOH analysis of gastric lesions. *Apc* LOH analysis of gastric tumors in *Apc*[Min/+] and MNU-treated *Apc*[Min/+] mice. *Top band,* HindIII-resistant *Apc*[Min] PCR product; *bottom band,* wild-type *Apc* allele cut by HindIII. *Lane W,* normal-appearing colonic crypts from a wild-type mice; *lane N,* normal-appearing colonic crypts from an *Apc*[Min/+] mouse; *lane T,* intestinal tumors from an *Apc*[Min/+] mouse showing LOH; *lane U,* undigested PCR product. The band ratio (*Apc*[+]/*Apc*[Min]) in each sample was compared with the control lane. *Asterisks,* gastric tumors showing a low band ratio (*Apc*[+]/*Apc*[Min]), thus suggesting LOH. *MNU+Min/+,* MNU-treated *Apc*[Min/+]; *MNU+Wt,* MNU-treated wild-type.

found in the tumors without *Apc* LOH. It is possible that haploinsufficiency of the *Apc* might contribute to the tumor development in MNU-treated *Apc*[Min/+] mice.

In conclusion, we herein reported a detailed analysis of gastric lesions in *Apc*[Min/+] mice, showing that *Apc*[Min/+] mice develop spontaneous tumors in the stomach. These tumors showed β-catenin accumulation accompanied by LOH of the *Apc* gene and the activation of the β-catenin/Tcf signaling pathway. Our results suggest that the Wnt pathway thus plays a causal role in the development of gastric cancer, and this animal model could provide a useful means to investigate the human gastric tumorigenesis in relation to Wnt activation. Furthermore, *Apc*[Min/+] mice

combined with MNU treatment could be a model to investigate multistage carcinogenesis in the stomach.

Acknowledgments

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