Biomedicine & Pharmacotherapy 164 (2023) 114968

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Azithromycin induces read-through of the nonsense *Apc* allele and prevents intestinal tumorigenesis in C3B6F1 $Apc^{Min/+}$ mice

Ryoko Semba [a,b], Takamitsu Morioka [a], Hiromi Yanagihara [a], Kenshi Suzuki [a], Hirotaka Tachibana [a], Takahiro Hamoya [a], Yoshiya Horimoto [b], Tatsuhiko Imaoka [a], Mitsue Saito [b], Shizuko Kakinuma [a,*], Masami Arai [c]

[a] Department of Radiation Effects Research, National Institute of Radiological Sciences, National Institutes for Quantum Science and Technology (NIRS/QST), Japan
[b] Department of Breast Oncology, Juntendo University School of Medicine, Japan
[c] Department of Clinical Genetics, Juntendo University School of Medicine, Japan

ARTICLE INFO

Keywords:
Read-through
Nonsense mutation
Apc
Chemoprevention
Azithromycin

ABSTRACT

Therapeutic strategies that promote read-through of a mutant gene have proved effective for certain non-neoplastic diseases. However, the efficacy of this approach is unproven regarding neoplastic diseases with germline nonsense mutations, including familial adenomatous polyposis. Here we examined the cancer-preventive efficacy of the macrolide antibiotic azithromycin, with a reported read-through effect, on intestinal tumorigenesis in C3B6F1 $Apc^{Min/+}$ mice harboring a nonsense *Apc* mutation resulting in a truncated Apc protein. Mice were given drinking water lacking azithromycin or containing 0.0125–0.2 mg/mL azithromycin from 3 weeks of age. The small intestine and cecum were analyzed for pathological changes and alterations of intestinal flora. Azithromycin suppressed the number of tumors and the proportion of adenocarcinomas, with the most effective drinking-water concentration being 0.0125 mg/mL. Furthermore, azithromycin recovered the cellular level of full-length Apc, resulting in downregulation of β-catenin and cyclin D1. Conversely, the effect of azithromycin on the diversity of the intestinal microbiota depended on the drinking-water concentration. These results suggest that the balance between azithromycin-mediate read-through of mutant *Apc* mRNA and antibacterial effects influences intestinal tumorigenesis. Thus, azithromycin is a potential anticancer agent for familial adenomatous polyposis patients harboring nonsense mutations.

1. Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death in Japan [1]. The majority of patients with CRC have sporadic cancer with no evidence of a relationship with inheritance [2]. On the other hand, an estimated 5–10 % of all CRC cases have a hereditary basis, so they are called inherited CRC syndromes [3] that have a much greater likelihood of leading to cancer development.

The three major types of inherited CRC syndromes are familial adenomatous polyposis (FAP) [4], hereditary nonpolyposis CRC [5], and *MUTYH*-associated polyposis [6]. FAP is an autosomal dominant disease characterized by the early onset of hundreds to thousands of adenomatous polyps throughout the large bowel [4]. A monoallelic mutation in the tumor-suppressor gene *APC* (adenomatous polyposis coli) is causal for FAP. Deletions of the other allele result in loss of heterozygosity and tumor development (i.e., the two-hit theory) [7]. If left untreated, almost all FAP patients develop CRC by age 50 [3]. The standard treatment regimen for FAP patients is based on “preventive total colectomy” at approximately age 20 [3]. However, this approach inevitably impairs the patient’s quality of life, particularly for young patients; therefore, young patients often receive preventative endoscopic polypectomy [8], although it is not an established therapy. Therefore, establishing new preventative measures other than surgical resection would provide substantial benefits to FAP patients, particularly young patients.

Nonsense point mutations result in a premature stop codon in mRNA sequences, which, upon translation, produce truncated proteins [9]. Nonsense mutations account for ~42% of all variants of the *APC* gene in

Abbreviations: AZM, azithromycin; CRC, colorectal cancer; FAP, Familial adenomatous polyposis.

* Corresponding author.
E-mail address: kakinuma.shizuko@qst.go.jp (S. Kakinuma).

https://doi.org/10.1016/j.biopha.2023.114968
Received 11 November 2022; Received in revised form 23 May 2023; Accepted 29 May 2023

FAP patients [10]. In FAP patients harboring an *APC* nonsense mutation, the translation of the mutant *APC* mRNA is prematurely terminated, with the consequent loss of APC function [7]. APC is a multifunctional tumor suppressor that negatively regulates WNT signaling via the degradation of β-catenin. Therefore, adenomatous polyps in FAP patients harboring a nonsense mutation of *APC* accumulate β-catenin, a target of APC, in the cytoplasm and nucleus, leading to the upregulation of WNT signaling and the consequent overexpression of cyclin D1 and c-Myc, which physically interact with β-catenin [11].

Research has demonstrated that certain aminoglycoside antibiotics can promote the read-through of premature stop codons and facilitate the synthesis of the full-length protein [12]. Therefore, these antibiotics have been proposed as possible therapeutic interventions for patients who carry such mutations [13]. In fact, the aminoglycoside antibiotic gentamycin improved the symptoms in both mouse models and human patients with Duchenne muscular dystrophy with nonsense mutations [14]. This antibiotic-mediated read-through has also been reported for treatments of other genetic diseases with nonsense mutations such as cystic fibrosis [15]. However, the therapeutic use of aminoglycosides is limited by the potential for nephrotoxicity or ototoxicity [15]. The synthetic compound PTC124 (ataluren) was developed for long-term use without marked side effects and has been conditionally approved for read-through therapies [16]. Clinical trials utilizing PTC124 have consistently yielded encouraging results for patients with cystic fibrosis or Duchenne muscular dystrophy [16]. However, data are lacking for the treatment of tumors having a hereditary basis, and thus the development of read-through therapies for such tumors would have substantial clinical impact.

Macrolide antibiotics act via the same mechanism as aminoglycosides and are efficacious mainly against Gram-positive bacteria. Therefore, these antibiotics are generally used to treat infectious diseases such as mycoplasma pneumonia, nontuberculous mycobacterial infection, and chlamydia trachomatis urethritis/cervicitis. Recent studies have demonstrated that certain macrolide antibiotics, such as tylosin, erythromycin, and azithromycin (AZM), induce read-through of premature stop codons in mammalian cells and mouse models with nonsense mutations [17]. Among the macrolide antibiotics, AZM is effective at lower doses and has fewer side effects, raising the possibility of long-term administration [18]. Therefore, AZM is considered a promising candidate for the prevention of hereditary diseases associated with nonsense mutations, including hereditary tumor syndromes, although its effectiveness has not been tested.

C57BL/6J $Apc^{Min/+}$ mice, which harbor a c.2549T>A (p.L850*) nonsense mutation, are commonly used as a mouse model of FAP. Hence numerous investigators have used this model mouse to study intestinal carcinogenesis and cancer chemoprevention. However, almost all C57BL/6J $Apc^{Min/+}$ mice die by age 120 days owing to bleeding caused by the intestinal tumors [19]. Therefore, this mouse model precludes long-term studies. On the other hand, C3B6F1 $Apc^{Min/+}$ mice (F1Min mice) have sixfold fewer tumors and thus live longer than C57BL/6J $Apc^{Min/+}$ mice [20], enabling pathological observation of the full multi-stage carcinogenesis process that ends in invasive cancer, and therefore F1Min mice are suitable for tests of chemoprevention of malignant progression [21]. We utilized these mice to investigate the cancer-preventive effects of AZM as well as its read-through effect. Because AZM also affects the makeup of the intestinal bacterial flora [22], we also monitored its effect on the bacterial community structure of the intestinal microbiome.

2. Materials and methods

2.1. Mice

Male C57BL/6J $Apc^{Min/+}$ mice carrying a nonsense mutation at codon 850 of *Apc* were originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in our laboratory. Female C3H/HeJ mice were obtained from the Jackson Laboratory Japan Inc. (Kanagawa, Japan). F1Min mice were generated and genotyped as reported previously [21].

2.2. Chemicals

AZM (purity >98.0%) (CAS number 117772–70–0, Tokyo Chemical Industry Co., Tokyo, Japan) was dissolved in ethanol (maximal final concentration, 0.4%), then diluted with sterile distilled water to concentrations of 0.0125, 0.05 and 0.2 mg/mL, and used as drinking water. The maximum concentration of AZM was calculated based on the typical dose used in clinical treatment without side effects as well as the average weight and expected water consumption of mice.

2.3. Experimental procedures

Female F1Min mice were divided into four experimental groups and orally given 0 (as a control), 0.0125, 0.05, or 0.2 mg/mL AZM in drinking water continuously from 3 weeks of age. The mice in the control group were given drinking water containing the vehicle (i.e., 0.4% ethanol in water) (Fig. 1A). Five mice each were dissected at 8, 12, and 16 weeks of age, and 10 mice were dissected at age 20 weeks; euthanasia was carried out via isoflurane anesthesia. Liver, kidney, spleen, bone marrow, intestinal tumors, and feces were collected for histological, immunohistochemical, and intestinal bacterial examinations. For all groups, mice were housed at five per cage and weighed weekly during the experimental period. Water consumption in each cage was measured three times a week as water bottles were replaced. All mice were handled according to the principles and procedures outlined in our institutional protocols after authorization by the Institutional Animal Care and Use Committee (authorization number 20–1001).

2.4. Macroscopic examination

The small intestine was collected for macroscopic examination, and tumor number and size were measured as reported previously [21]. The 'tumor load' was defined as the sum of the longitudinal diameter (in mm) of all tumors per mouse, as described elsewhere [21,23].

2.5. Histopathology

All collected organs and tumors were embedded in paraffin, sectioned at 4 μm thickness, and stained with hematoxylin and eosin for histopathologic analysis. The intestinal tumor sections were also stained with alcian blue solution (pH 2.5; Muto Pure Chemicals Corp., Tokyo, Japan) to detect acidic mucosal proteins for evaluating goblet-cell differentiation [24]. Images of all stained specimens were transformed into digital virtual slides using a NanoZoomer-XR slide scanner (Hamamatsu Photonics Corp., Ltd., Hamamatsu, Japan) and stored in a J-SHARE archive (Japan Storehouse of Animal Radiobiology Experiments) [25]. Histopathologic diagnosis and measurement of tumor diameter were performed with NDP.view2 viewing software (Hamamatsu Photonics Corp. Ltd.), which provides digital histopathologic images and measurements of area and length. The adverse effects of AZM were evaluated by analyzing histological changes in the liver, kidney, spleen, and bone marrow. Tumors were histologically classified into two diagnostic categories, namely adenoma or adenocarcinoma according to established criteria [26]. The correlation between tumor size and tumor progression and the ratio of adenomas to adenocarcinomas were analyzed statistically as described below. The alcian blue–positive index was calculated as the fraction of alcian blue–positive area in each tumor using Patholoscope image analysis software (Mitani Corp., Tokyo, Japan).

2.6. Immunohistochemistry

The cellular levels of Apc, β-catenin, and cyclin D1 were quantified

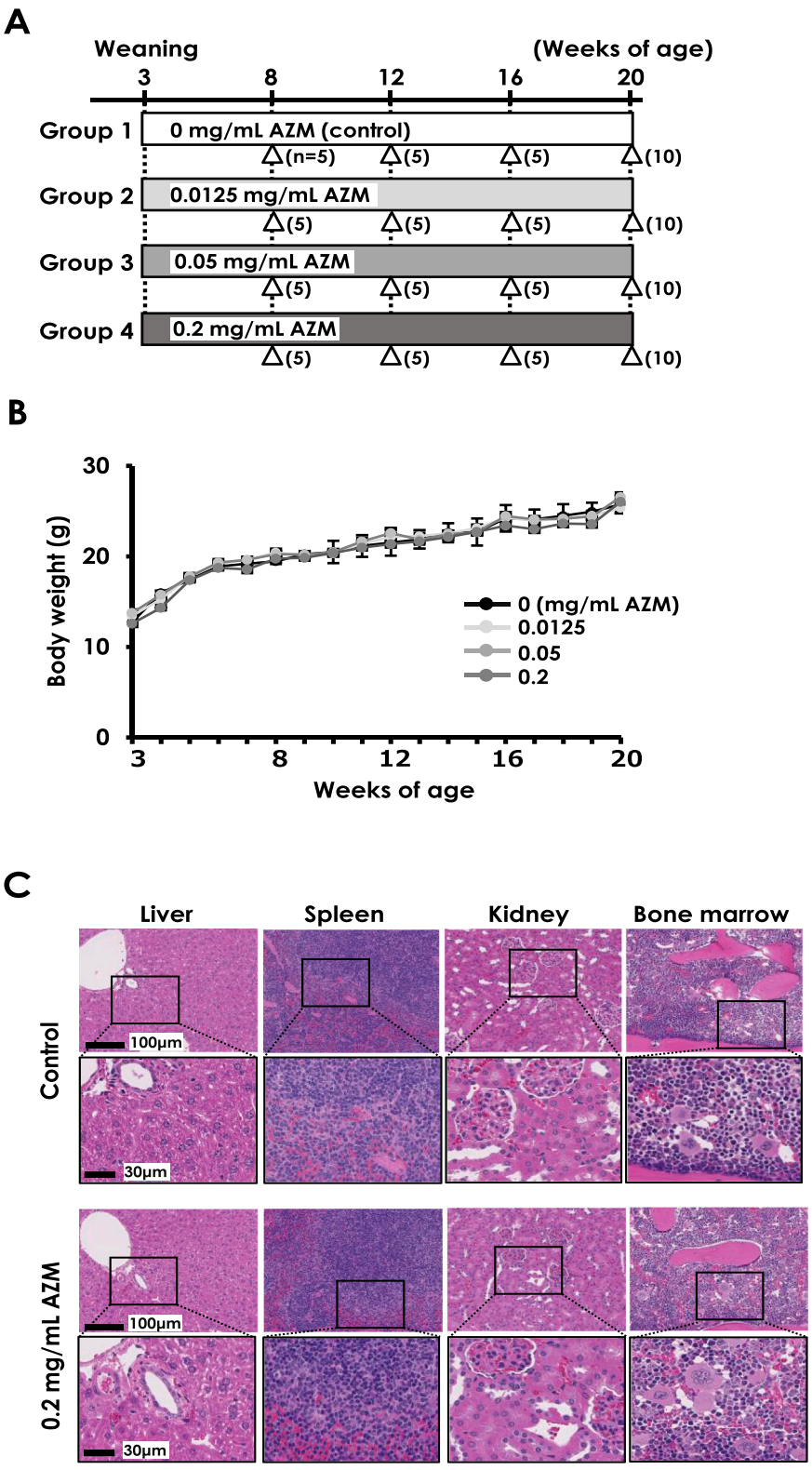

Fig. 1. Experimental design and assessment of AZM toxicity. A. Protocol for the mouse experiment. A total of 100 female F1Min mice were divided into four groups, and drinking water with or without AZM was provided ad libitum from 3 weeks of age. White arrowheads indicate the date of sacrifice and dissection. Numbers in parentheses indicate the number of mice. B. Change in average body weight for each group over the course of the experiment. C. Hematoxylin and eosin staining of thin sections of selected organs of control mice (20 weeks) and mice that were given AZM in drinking water at the highest dose for the longest period (0.2 mg/mL, 20 weeks). Images in the panels are magnified views of the black frame in the upper figures. Scale bars in the leftmost panels apply to all images.

by immunohistochemical staining of serial 4-μm-thick sections of both normal epithelium and tumors. The antibodies used were directed against the following proteins: Apc (amino-acid residues 2770–2850, C-terminal, LSBio Inc., catalog no. LS-C382049; 1:100 dilution), β-catenin (BD Biosciences, catalog no. 610154; 1:400 dilution), and cyclin D1 (Cell Signaling Technologies, clone 92G2; 1:400 dilution). Standard protocols for immunohistochemistry were followed [27]. The Apc-, β-catenin-, and cyclin D1–positive indexes were calculated using the same method as the alcian blue index.

2.7. cDNA synthesis, real-time PCR

Total RNA was isolated from fresh-frozen intestinal mucosa using the AllPrep DNA/RNA/Protein Mini kit (Qiagen, Hilden, Germany). The

resulting RNA solution was treated with DNase to degrade any residual DNA. First-strand complementary DNA was synthesized from RNA using SuperScript III reverse transcriptase (Thermo Fisher Scientific, Waltham, MA) and primers *Apc* C-terminal portion, *Apc*-wild, *Apc*-Min, β-catenin, and cyclin D1, which are listed in Supplementary Table 1. Expression level data were normalized to values for the housekeeping gene *Gapdh*. Analysis was carried out for samples of the distant small intestinal mucosa of one wild-type C3B6F1 (F1wild) mouse and three F1Min mice in each dosage group at 12 weeks of age.

2.8. Analysis of the intestinal microbiota

Analysis of the intestinal microbiota using fecal specimens was outsourced to Techno Suruga Laboratory (Shizuoka, Japan). The microbiota was analyzed by targeting bacterial 16S rDNA amplicon sequences. Generation of an amplicon-sequence variant table, including quality filtering and chimeric variant filtering, was carried out using the DADA2 plugin of Quantitative Insights Into Microbial Ecology 2 (QIIME2) version 2020.6 (https://docs.qiime2.org). The 16S rDNA sequence data for each fecal sample were subjected to a homology search using a publicly available analytical database provided by the ribosomal database project (http://rdp.cme.msu.edu) to identify bacterial families. In addition, α-diversity and β-diversity were determined by the UniFrac metric to evaluate distances between the samples and visualized by a principal coordinate analysis score plot [28].

2.9. Statistical analysis

The data were analyzed using SPSS ver. 28 (IBM Corp., Tokyo, Japan) and Prism 8.0 (GraphPad Inc., San Diego, CA, USA) software. Differences between two independent data were determined using the Student's or Welch's *t*-test based on the results of the *F*-test. Two-way analysis of variance (ANOVA) was used to analyze the relationship between concentration and duration for the administration of AZM for both macroscopic and histopathologic examinations. The significance of a difference in β-diversity of intestinal flora between the two groups was analyzed with the non-parametric analysis of similarities (commonly known as ANOSIM) based on unweighted UniFrac distances using QIIME2. The significance level was set at $p < 0.05$ for all analyses.

3. Results

3.1. General observations of AZM-treated F1Min mice

For all experimental mouse groups, body weight increased gradually with age toward the endpoint of the experimental period, with no significant differences between groups (i.e., the three AZM-treatment groups and control group) (Fig. 1B). In addition, there were no histological changes such as hepatic inflammation or hepatocellular necrosis, extramedullary hematopoiesis in the spleen, renal inflammation, acute tubular necrosis, or hypoplastic marrow (Fig. 1C). These results showed that the range of drinking-water concentration of AZM (hereafter "dose") used did not lead to serious side effects.

3.2. AZM suppresses spontaneous intestinal tumorigenesis in F1Min mice

Next, we examined the inhibitory effects of AZM on tumor number and size. Macroscopic views of small intestinal tumors revealed round or oval tumors of varying size (Fig. 2A). The average number of tumors in all AZM-treated groups tended to be fewer than that of the control group at each of 12, 16, and 20 weeks of age (Fig. 2B). The average number of tumors changed significantly over the experimental course of AZM treatment ($p = 0.0053$, two-way ANOVA). In the control group, the number of tumors increased gradually over time, whereas in the 0.0125 and 0.05 mg/mL AZM groups the increase in tumor number was suppressed until 16 weeks of age. In particular, in the 0.0125 mg/mL AZM group at age 16 weeks, the tumor number was significantly lower than that of the control group ($p = 0.047$). In all groups, tumor size increased gradually with age (Fig. 2C-F). Meanwhile, AZM administration tended to decrease the number of tumors with a diameter of 1–2 mm compared with the control group at 12, 16, and 20 weeks of age. The lowest and middle doses of AZM (0.0125, 0.05 mg/mL) were particularly effective and suppressed tumor size at 16 weeks of age ($p = 0.064$, $p = 0.050$). Tumor load also increased with age in all groups, whereas AZM administration significantly suppressed it ($p = 0.000002$, two-way ANOVA; Fig. 2G). Similar to what we observed for tumor number, the lowest AZM dose significantly suppressed tumor load compared with 12-week-old controls ($p = 0.041$).

Next, we evaluated the possible suppressive effect of AZM on tumor progression by grading tumors based on histopathologic diagnoses. The size of adenocarcinomas increased with age over the period 12–16 weeks ($n = 5$ per group) and 20 weeks ($n = 10$). The number of adenocarcinomas tended to decrease in the AZM-treated groups compared with the control group (Fig. 3A). In the control group, the tumors progressed to adenocarcinoma despite their small size (i.e., relative to tumors measured for the AZM-treated groups). The proportion of adenocarcinomas in the control group increased over time. On the other hand, in all AZM-treated groups, the proportion of adenocarcinomas decreased compared with the control group. ($p = 0.022$, two-way ANOVA; Fig. 3B) The proportion of adenocarcinomas in the three AZM-treated groups (0.0125, 0.05, and 0.2 mg/mL) was significantly lower than that of the control group over the entire course of the experiment ($p = 0.00063$, 0.00044, and 0.00091, respectively). The proportion of adenocarcinomas in the 0.05 or 0.2 mg/mL groups at 12 weeks of age was significantly lower than that of the control group (both $p = 0.026$), and the proportion in the 0.0125, 0.05, and 0.2 mg/mL groups at 20 weeks of age was also significantly lower than that of the control group ($p = 0.000024$, 0.00065, and 0.00033 compared with the control group, respectively). These results indicated that AZM reduced the number of tumors and suppressed malignant progression from adenoma to adenocarcinoma. Moreover, the lowest dose of AZM was the most effective with respect to slowing tumorigenesis.

3.3. AZM induces read-through of the Apc nonsense mutation in normal intestinal epithelia and tumors of F1Min mice

We next investigated the read-through effect of AZM in the normal intestinal villus by immunohistochemical staining with an antibody against the C-terminal portion of Apc. The normal villus cells of the F1wild mice expressed the C-terminal part of Apc, whereas the crypt cells did not (Fig. 4A). Compared with the F1wild mice, however, the villus cells of F1Min mice expressed a lower level of the C-terminal part of Apc (Fig. 4A). Of note, the immunopositivity of the C-terminal part was apparently more intense after AZM administration compared with that observed in control mice; however, there was no obvious dependence of immunopositivity on AZM dose (Fig. 4A).

Next, we examined the levels of mRNAs encoding Apc, cyclin D1, and β-catenin in normal intestinal mucosa to confirm that the increase in Apc protein upon AZM administration depended on alterations in the expression of genes related to WNT signaling (Fig. 4B-G). F1wild mice have two copies of the normal *Apc* allele, whereas F1Min mice have one copy of each of the normal and mutant alleles. The level of *Apc* C-terminal mRNA was in the same range in all experimental groups (Fig. 4B). Reflecting the genome copy number, the level of *Apc* wild mRNA was approximately half that measured in the F1Min mouse groups (Fig. 4C). The level of *Apc* mutant mRNA was in the same range in the F1Min mouse groups regardless of AZM dose (Fig. 4D). The ratio of the levels of wild and mutant mRNAs was approximately 1.0 regardless of AZM dose (Fig. 4E). These data demonstrated that AZM (at any dose) did not affect the level of mutant mRNA in F1Min mice. There was no significant difference in the level of β-catenin mRNA between groups (Fig. 4F). However, the level of cyclin D1 mRNA was significantly greater in

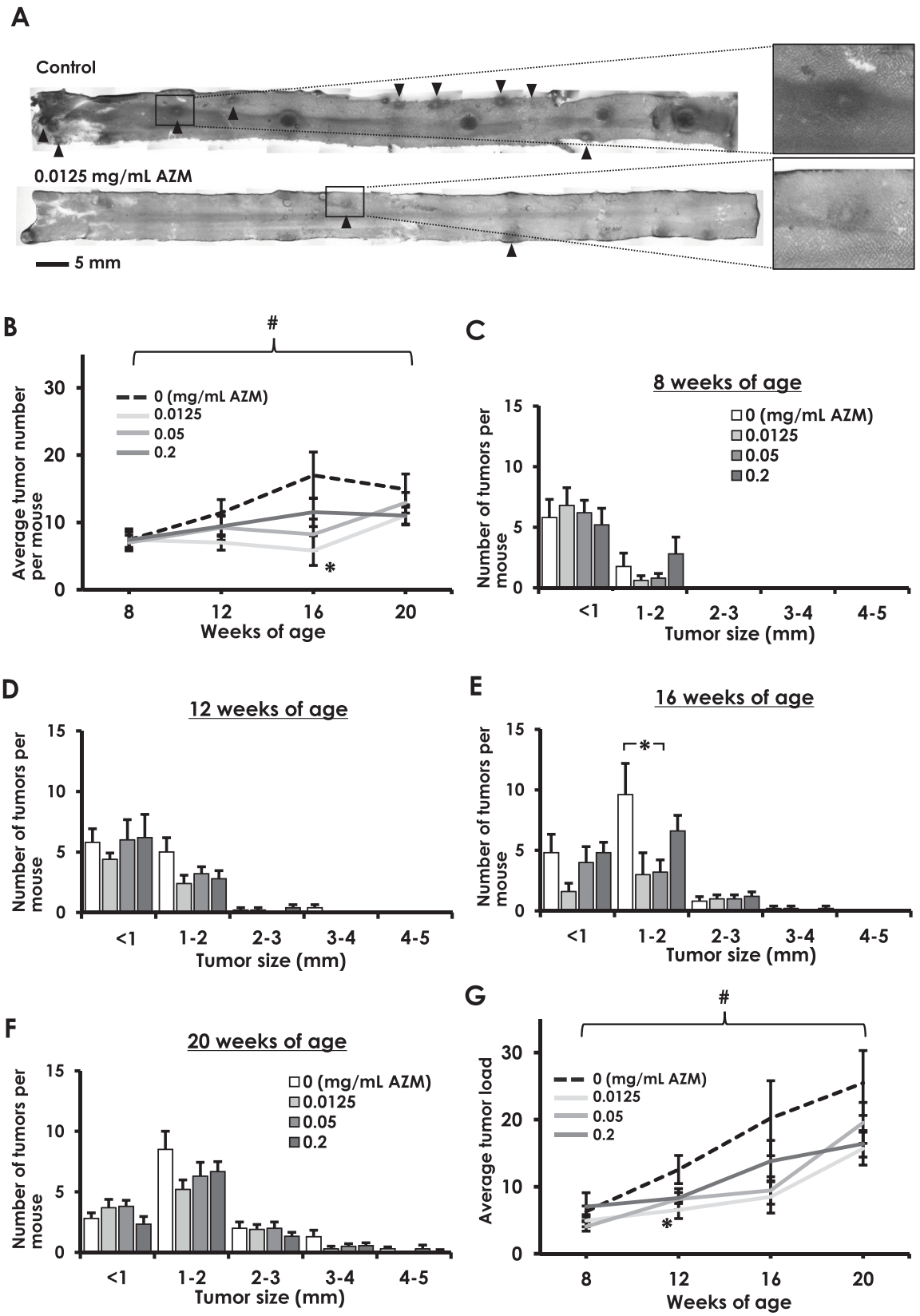

Fig. 2. Effect of AZM on tumor number, size distribution, and tumor load. A. Various sizes of tumors in the small intestine as shown in macroscopic views (arrowheads) of control mice (16 weeks) and mice treated with 0.0125 mg/mL AZM for 16 weeks. B. AZM treatment reduced the average number of tumors per mouse throughout the experiment. C–F. AZM treatment reduced the average number of tumors per mouse based on size distribution at 8 (C), 12 (D), 16 (E), and 20 (F) weeks of age. G. AZM treatment decreased the tumor load per mouse. $^{*}p < 0.05$ vs. control group by *t*-test. $^{\#}p < 0.05$ among groups by two-way ANOVA.

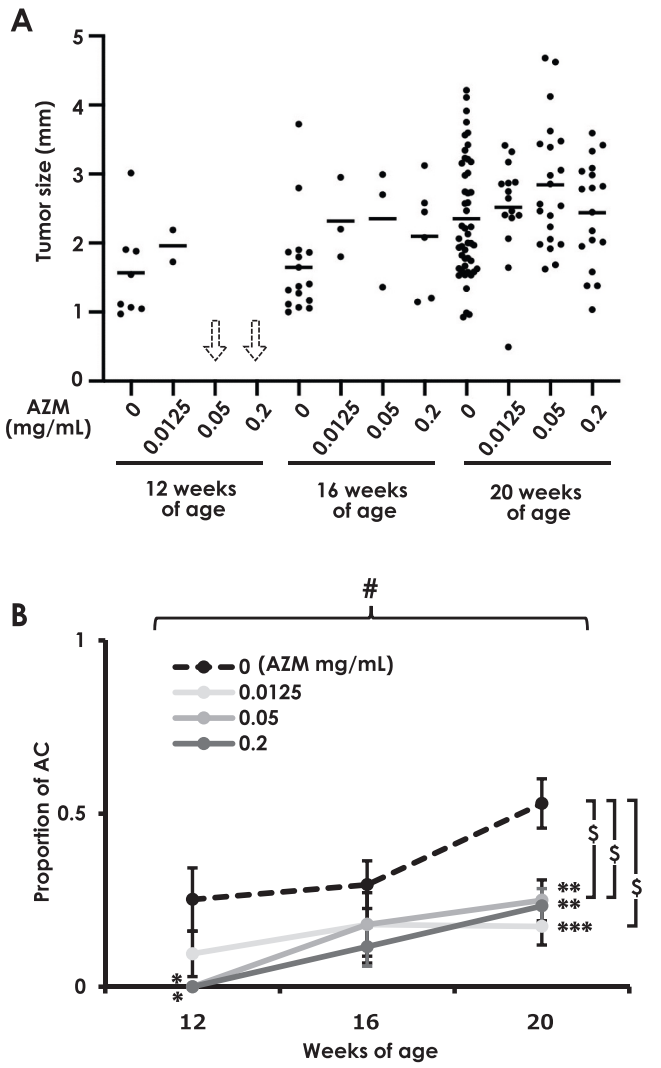

Fig. 3. Effect of AZM on tumor progression in F1Min mice at 12, 16, and 20 weeks of age. A. AZM treatment reduced the number of adenocarcinomas. The horizon bars indicate the median tumor size in each group. B. AZM treatment reduced the proportion of adenocarcinomas. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control group by *t*-test. $^{\#}p < 0.05$ among groups by two-way ANOVA. $^{\$}p < 0.001$ for each AZM dose group vs. control group by two-way ANOVA. AC, adenocarcinoma.

F1Min mice than in F1wild mice, and AZM administration decreased the level of cyclin D1 mRNA in F1Min mice to the level measured in F1wild mice without any dependence on AZM dose (Fig. 4G).

We subsequently quantified the area of Apc expression in tumor samples to evaluate the read-through effect of AZM. We also measured WNT signaling using antibodies specific for β-catenin and cyclin D1. AZM administration increased the cellular level of the C-terminal part of Apc in intestinal tumors of 12-week-old F1Min mice based on the greater fraction of Apc-immunopositive area in the AZM-treated groups than in the control group, which was statistically significant for the 0.0125 and 0.05 mg/mL AZM groups; there was also a significant difference between the 0.0125 mg/mL and 0.2 mg/mL groups (Fig. 5A and B). Conversely, β-catenin and cyclin D1 levels were apparently decreased by AZM administration; the percentage of immunopositive cells for these proteins was significantly reduced in the AZM-treated groups compared with the control group (Fig. 5C and D). In the AZM-treated groups, the alcian blue–positive index, which reflects the differentiation status in tumor areas, tended to be greater than that measured for the control group ($p = 0.076$) (Fig. 5E). These results indicated that AZM, via its read-through activity, could suppress WNT signaling by inducing the production of full-length Apc, thereby promoting the differentiation of tumor cells into goblet cells.

3.4. AZM affects the intestinal bacterial profile

We hypothesized that the antibacterial action of AZM might be related to the observed inverse relationship between AZM dose and its cancer-preventive effect. Therefore, we examined the influence of AZM on the fecal microbiota based on 16S rDNA metagenome sequencing. The Simpson diversity (1D) index was used to represent the diversity of microbial communities. The DNA sequence was determined for each sample, and diversity was compared among the AZM groups and control group. The average Simpson (1D) index was 0.98 (standard deviation [SD], 0.003) for the control group, 0.94 (SD 0.003) for the 0.0125 mg/mL group, 0.95 (SD 0.001) for the 0.05 mg/mL group, and 0.94 (SD 0.01) for the 0.2 mg/mL group. The range of these values was 0.92–0.99. UniFrac β-diversity principal coordinate analysis plots were used to reflect bacterial community diversity in each sample (Fig. 6A). A similarity between any two principal coordinate analysis plot patterns would suggest that bacterial community diversity among the same AZM-treated samples was smaller than that among different dose groups. In fact, there were three aggregates of groups, the first comprising the control, the second comprising the samples treated with 0.0125 mg/mL AZM, and the third comprising samples treated with 0.05 or 0.2 mg/mL AZM (Fig. 6A).

When the proportion of bacterial families in each dosage group was analyzed and compared with the control group, the familial diversity in the AZM treatment group was found to decrease with dose (Fig. 6B). The proportions of the major three bacterial families, i.e., *Lachnospiraceae*, *Porphyromonadaceae*, and *Lactobacillaceae*, were relatively well maintained in the 0.0125 mg/mL AZM group compared with the two higher-dose AZM groups. Next, we examined bacterial family alterations closely related to colorectal carcinogenesis and intestinal inflammation. The proportion of family *Lactobacillaceae*, which is decreased in CRC patients [29], was reduced in the 0.05 and 0.2 mg/mL AZM groups but not in the 0.0125 mg/mL group (Fig. 6C). On the other hand, the proportion of *Rikenellaceae*, which helps control inflammation in the intestine [30], was significantly increased in the 0.0125 mg/mL AZM group ($p = 0.0015$, Fig. 6C). At age 16 and 20 weeks, *Lactobacillaceae* was maintained at a relatively large proportion in the control and 0.0125 mg/mL AZM groups, in contrast to the 0.05 and 0.2 mg/mL groups (especially, *Lactobacillaceae* was not detected at 16 weeks in the highest-dose group). *Rikenellaceae* was maintained at a constant proportion in the control group, as expected. In the 0.0125 mg/mL group, its proportion was highest at 12 weeks and gradually decreased thereafter, reaching the same level as the control group at 20 weeks; it was not detected in the middle and highest dose groups at most time points (Fig. 6C). Finally, we analyzed the ratio of species of phylum *Bacteroidetes* compared with those of phylum *Firmicutes*, i.e., the B/F ratio, which is inversely correlated with obesity and inflammation [31,32]. The B/F ratio decreased with age in all groups including the control group (Fig. 6D). Over the time course of the experiment, the B/F ratio of the 0.0125 mg/mL AZM group did not differ significantly from that of the control group. In contrast, the ratio for each of the remaining two AZM groups (0.05 and 0.2 mg/mL) decreased significantly (Fig. 6D). These results suggested that the makeup of the intestinal microbiota in the two higher AZM dose groups was substantially altered compared with that of the control and 0.0125 mg/mL groups.

4. Discussion

This study demonstrates, for the first time, the potential for AZM to prevent intestinal tumorigenesis in a mouse model of FAP harboring a nonsense mutation in *Apc*, with the possible involvement of the read-through activity of AZM. In addition, the antibacterial effect of long-

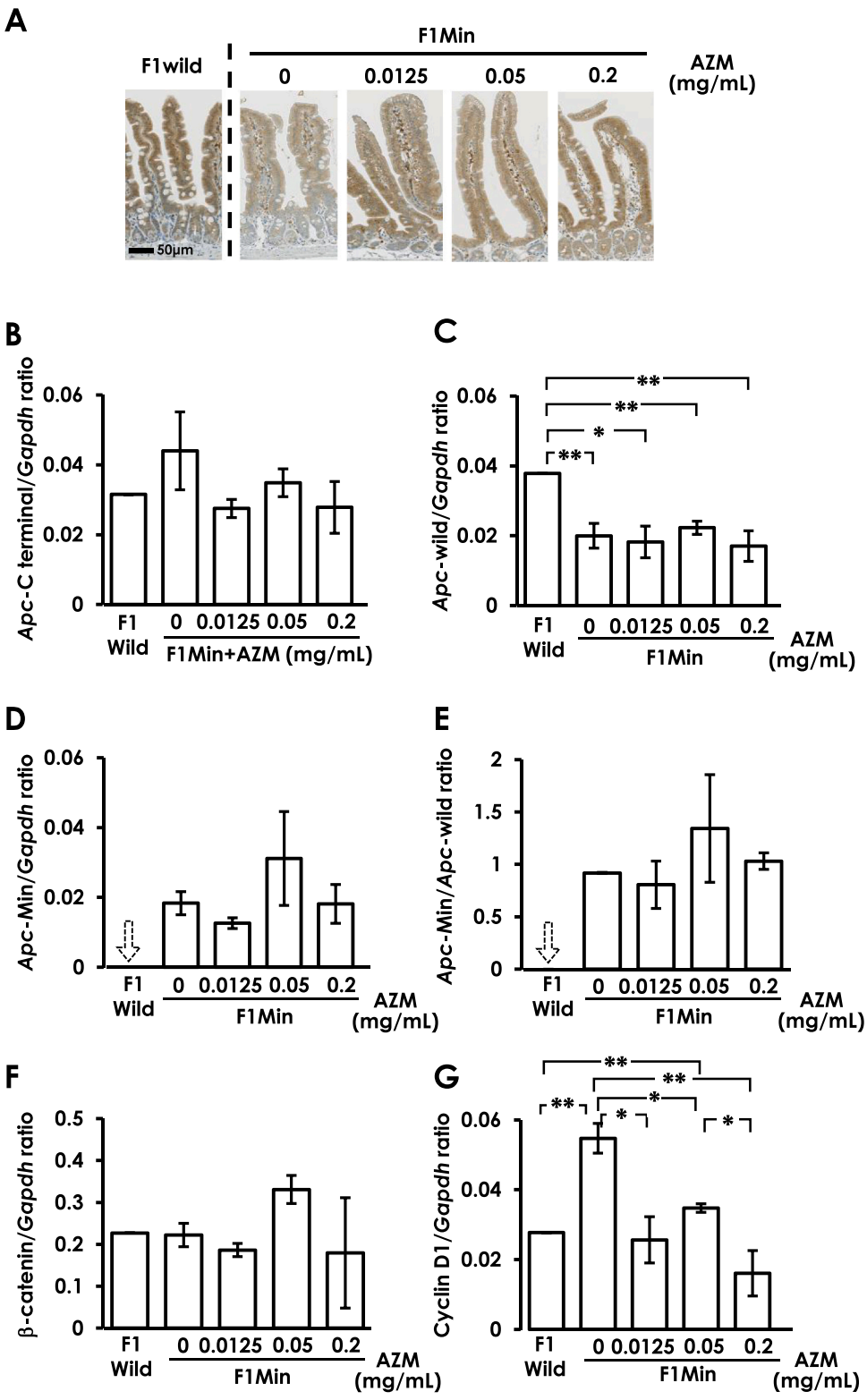

Fig. 4. Expression of proteins and mRNAs related to WNT signaling in normal epithelium. A. AZM increased the cellular abundance of the C-terminal part of Apc in the villus cells of F1Min mice. B-F. AZM treatment did not affect the levels of the mRNAs encoding the *Apc* C-terminal portion (B), the *Apc*-wild allele (C), the *Apc-Min* allele (D), or β-catenin (F); likewise, AZM did not affect the mRNA ratio *Apc*-Min: *Apc*-wild (E) in F1Min mice. G. AZM treatment suppressed the level of cyclin D1 mRNA in F1Min mice, which was increased compared with F1wild mice. F1wild: C3B6F1 wild mice; F1Min: C3B6F1 $Apc^{Min/+}$ mice. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ by *t*-test.

term AZM administration—especially at high doses—alters the intestinal microbiota, and this aspect seems to correlate with the weakened tumor suppressive effect of AZM at high doses.

The chemopreventive effect of macrolide antibiotics on tumors with *APC* nonsense mutations has been reported in both animal experiments and clinical trials. In a mouse model, treatment with tylosin for 10 weeks significantly reduced the number of adenomas larger than 2 mm at age 12 weeks, with concomitant extension of lifespan [12]; treatment with erythromycin for 8 weeks significantly reduced the number of proximal adenomas at age 13 weeks [31]. In a clinical trial with FAP patients, the chemopreventive effect of erythromycin on the number and size of colonic adenomas was evaluated endoscopically before treatment, after completing erythromycin treatment for 4 months as well as at 8 months after completing treatment (i.e., 12 months after treatment onset) [33]. After 4 months of treatment, 7 of 10 patients responded to the read-through therapy. This result suggests that the variability in the

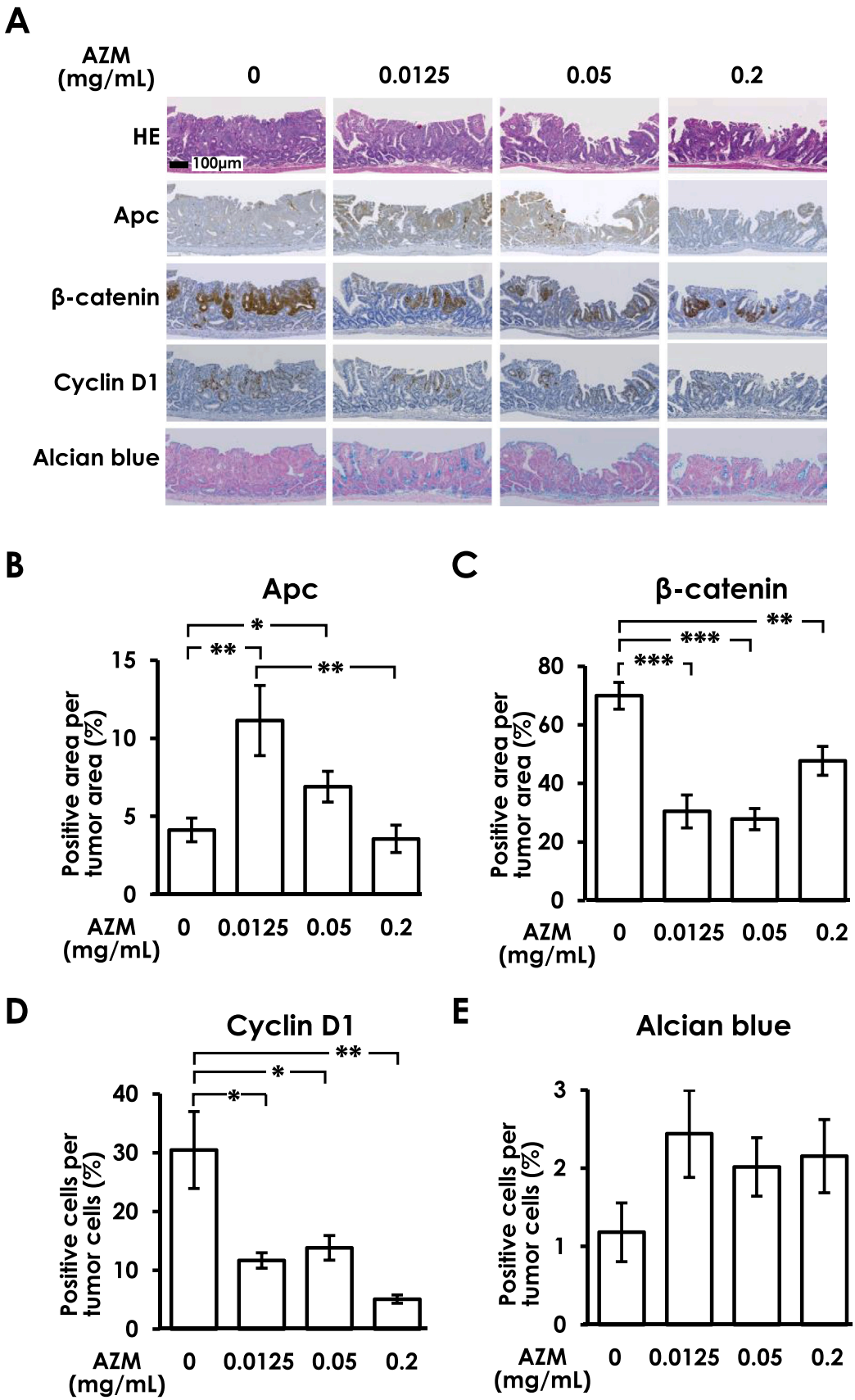

Fig. 5. Expression of proteins related to WNT signaling in tumor areas. A. AZM treatment altered the abundance of Apc, β-catenin, and cyclin D1 as well as the proportion of alcian blue–positive cells in intestinal tumors of 12-week-old F1Min mice. The tumor sections were stained with hematoxylin/eosin and alcian blue and immunostained with an antibody against Apc, β-catenin, or cyclin D1. B–E. AZM treatment increased the percentage of cells positive for Apc (C) and alcian-blue staining (F) and decreased the percentage of cells positive for β-catenin (D) and cyclin D1 (E). $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ by *t*-test.

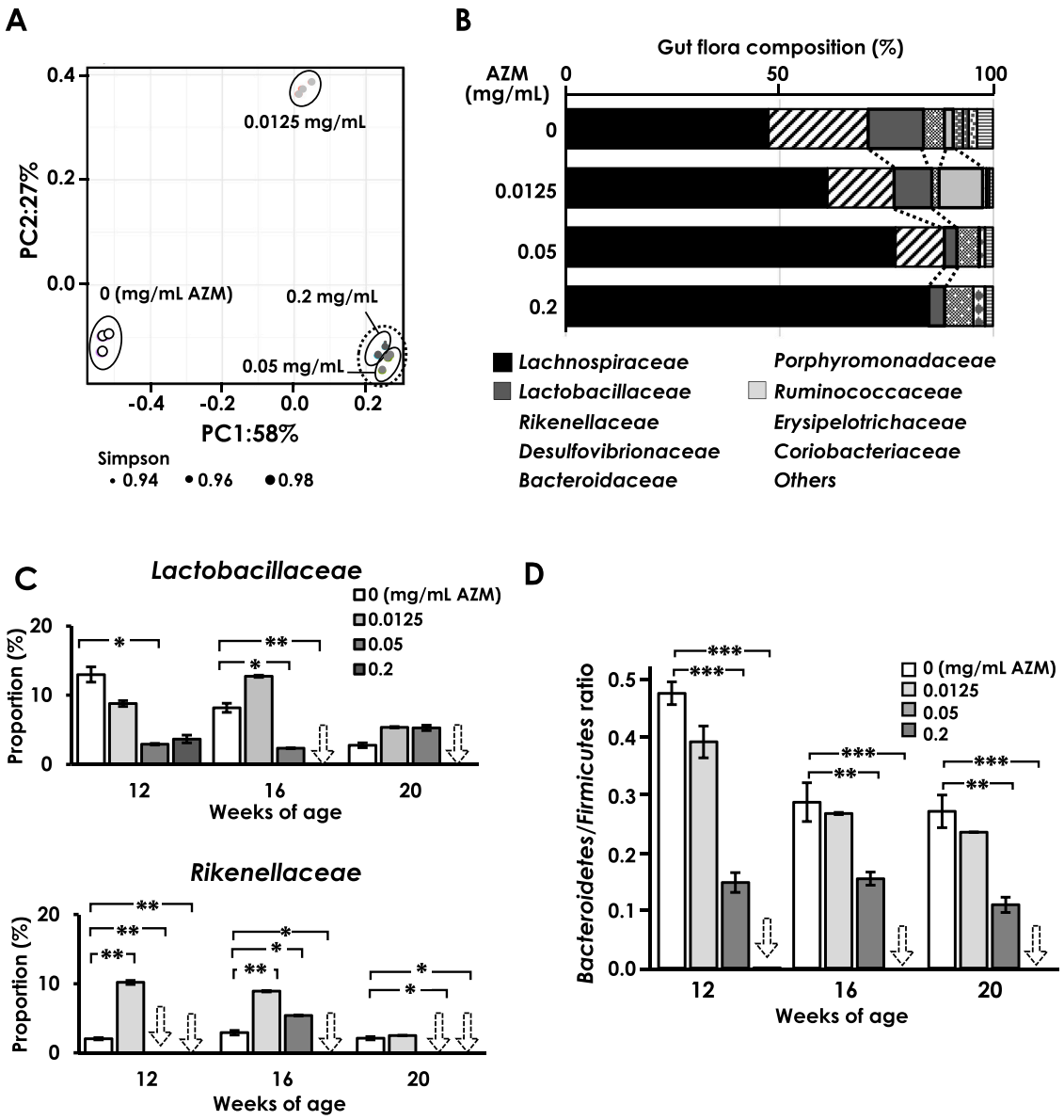

Fig. 6. Analysis of the intestinal microbiota in AZM-treated F1Min mice at 12 weeks of age (three mice per group). A. AZM treatment altered the β-diversity of the microbiota depending on dose. The Bray-Curtis matrix was used to construct the principal component analysis plot. The Simpson index is represented by dot size for α-diversity. B. AZM treatment reduced familial diversity in a dose-dependent manner. C. Treatment with 0.0125 mg/mL AZM maintained the relative proportions of *Lactobacillaceae* (upper) and *Rikenellaceae* (lower) compared with the two higher-dose AZM levels. D. Treatment with 0.0125 mg/mL AZM maintained the ratio of *Bacteroidetes*-to-*Firmicutes*, but the two higher-dose AZM levels reduced it. The *Bacteroidetes*-to-*Firmicutes* ratio in the four AZM dose groups at the designated ages. The ratio for the 0.0125 mg/mL AZM group did not differ significantly from that of the control group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control group by *t*-test.

chemopreventive effect of erythromycin might be a consequence of inter-individual differences in physiology, e.g., owing to gender, age, age at disease onset, adenoma burden, genetic background, and the potential effects of erythromycin on the microbiome. In this study, we used only female mice, although future studies with male mice also would produce informative data with regard to the efficacy of AZM for CRC.

Several research groups have documented the read-through effect of macrolide antibiotics [12,17,31,33]. The effect of tylosin on the read-through of a nonsense mutation in *APC* was demonstrated by a dual luciferase read-through reporter construct containing the *APC* wild-type or nonsense mutation at codon 1450, and the production of the full-length protein product was verified by the hemagglutinin tag method. Similar results were obtained via western blotting of tylosin-treated SW1417 cells (colon-cancer line), which also carry a nonsense *APC* mutation at codon 1450 [12]. In the present study, we clarified the read-through effect of AZM in the normal intestinal epithelium and tumors with a nonsense mutation at codon 850 by immunohistochemical staining using an antibody against the C-terminal part of Apc. In normal intestinal mucosa, AZM administration increased the cellular abundance of full-length Apc. In contrast, the levels of mRNAs encoding Apc wild and mutant alleles were essentially unaffected by AZM dose. These results strongly suggest that the read-through effect of AZM led to the production of full-length Apc via constitutive expression of the mutant allele mRNA. Interestingly, the expression of cyclin D1 mRNA was increased in F1Min mice compared with F1wild mice and could be restored by treatment with AZM (Fig. 4G). However, the abundance of cyclin D1 protein in normal intestinal mucosa was very low compared with tumor regions (data not shown), although the abundance of the corresponding mRNA was unaffected by AZM

treatment. Samson et al. reported that homozygous deletion of *Apc* in *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice resulted in increased cellular abundance of cyclin D1 and consequent progression to adenoma [34]. This suggests that the heterozygous status of *Apc* in F1Min mice may not be sufficient for overproduction of cyclin D1. We recently reported that the loss of wild-type alleles, which is a key tumorigenic event in F1Min mice, was predicated on cell proliferation–dependent chromosomal recombination [35]. These data suggest that regulation of cell proliferation is critical for tumorigenesis in F1Min mice. In tumors, the cellular levels of β-catenin and cyclin D1 decreased in conjunction with increased expression of full-length Apc. These results suggest that the full-length Apc produced by the AZM-induced read-through effect may facilitate the formation of the protein complex required for β-catenin degradation. Thence the read-through effect of AZM in normal epithelium and tumor cells may lead to suppression of both tumor development and progression. On the other hand, recovery of full-length Apc level and suppression of tumor number was inversely related to AZM dose, and the underlying reason for this discrepancy must be studied in the future.

Our analysis of the mouse intestinal microbiota indicated that maintenance of families *Lactobacillaceae* and *Rikenellaceae* was related to the suppression of tumorigenesis. In contrast, the proportion of family *Lachnospiraceae*, which has been reported to counter the onset of CRC in humans by producing butyric acid [36], decreased with AZM dose but was not related to suppression of tumorigenesis, indicating an inconsistency. Other research has also suggested that the longer the treatment period and the higher the dose of AZM, the greater the effect on the intestinal flora. The B/F ratio is related to obesity and inflammation [32]. In our study, this ratio was significantly smaller in the two higher AZM dose groups (Fig. 6D), suggesting that the higher doses may have induced inflammation and thus limited the ability of AZM to suppress tumorigenesis. This effect was smaller at the lowest AZM dose (0.0125 mg/mL), at least through age 20 weeks. Overall, these results suggest that although macrolide antibiotics can suppress inflammation [31], they may alter the species composition of the intestinal microbiota and thereby induce inflammation.

The efficacy of AZM to prevent tumorigenesis might be maximized at a much lower dose than used in our present study. In other words, the cancer-preventive effect of AZM (i.e., resulting from its ability to read-through the *Apc* nonsense mutation) decreases above a certain threshold of AZM dose.

5. Conclusion

Taken together, the cancer chemopreventive efficacy of AZM may depend on the balance between its read-through and antibacterial effects. In addition, lifelong administration of an antibiotic(s) for cancer prevention is not a realistic strategy owing to the substantial risk of developing antibiotic-resistant bacteria as well as issues with medication compliance. Towards future clinical applications, it will be necessary to verify the relationship among duration of antibiotic treatment, effects on the intestinal microbiota, and cancer-preventive effects of AZM. Finally, treatment with AZM may also be applicable to the prevention of other hereditary tumors and diseases caused by germline nonsense mutations.

CRediT authorship contribution statement

Conceptualization: **Takamitsu Morioka, Shizuko Kakinuma, Masami Arai**. Methodology: **Ryoko Semba, Morioka Takamitsu, Shizuko Kakinuma, Masami Arai**. Formal Analysis: **Ryoko Semba, Takamitsu Morioka, Shizuko Kakinuma**. Investigation: **Ryoko Semba, Takamitsu Morioka, Hiromi Yanagihara, Kenshi Suzuki, Hirotaka Tachibana, Takahiro Hamoya**. Resources: **Takamitsu Morioka, Shizuko Kakinuma**. Writing – Original Draft: **Ryoko Semba, Takamitsu Morioka, Shizuko Kakinuma**. Writing – Review & Editing: **Hiromi Yanagihara, Kenshi Suzuki, Hirotaka Tachibana, Takahiro Hamoya, Yoshiya Horimoto, Tatsuhiko Imaoka, Mitsue Saito, Masami Arai**. Visualization: **Ryoko Semba**. Supervision: **Mitsue Saito**. Project Administration: **Takamitsu Morioka, Shizuko Kakinuma**. Funding Acquisition: **Masami Arai, Shizuko Kakinuma**.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The Authors thank Ms. M. Ogawa, Y. Nishimura, M. Okabe, M. Kaminishi, H. Seo, M. Fujita, U. Enzaka, Y. Kin, and Y. Amasaki for assistance with animal experimentation. We also thank Professor H. Eguchi at Juntendo University School of Medicine, Japan, for useful discussions. This work was supported by the Japan Society for the Promotion of Science via KAKENHI Grant Numbers JP20K07684 and JP21H04932.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114968.

References

[1] A. Ouchi, D. Shida, T. Hamaguchi, A. Takashima, Y. Ito, H. Ueno, M. Ishiguro, Y. Takii, S. Ikeda, M. Ohue, S. Fujita, M. Shiozawa, K. Kataoka, M. Ito, Y. Tsukada, T. Akagi, M. Inomata, Y. Shimada, Y. Kanemitsu, Challenges of improving treatment outcomes for colorectal and anal cancers in Japan: the colorectal cancer study group (CCSG) of the Japan clinical oncology group (JCOG), Jpn J. Clin. Oncol. 50 (2020) 368–378.
[2] R.M. Byrne, V.L. Tsikitis, Colorectal polyposis and inherited colorectal cancer syndromes, Ann. Gastroenterol. 31 (2018) 24–34.
[3] K. Wells, P.E. Wise, Hereditary colorectal cancer syndromes, Surg. Clin. North Am. 97 (2017) 605–625.
[4] K.W. Kinzler, B. Vogelstein, Lessons from hereditary colorectal cancer, Cell 87 (1996) 159–170.
[5] S. Senba, F. Konishi, T. Okamoto, H. Kashiwagi, K. Kanazawa, M. Miyaki, M. Konishi, T. Tsukamoto, Clinicopathologic and genetic features of nonfamilial colorectal carcinomas with DNA replication errors, Cancer 82 (1998) 279–285.
[6] K.S. Boparai, E. Dekker, S. Van Eeden, M.M. Polak, J.F. Bartelsman, E.M. Mathus-Vliegen, J.J. Keller, C.J. van Noesel, Hyperplastic polyps and sessile serrated adenomas as a phenotypic expression of MYH-associated polyposis, Gastroenterology 135 (2008) 2014–2018.
[7] F. Ghadamyari, M.M. Heidari, S. Zeinali, M. Khatami, S. Merat, H. Bagherian, L. Rejali, F. Ghasemi, Mutational screening through comprehensive bioinformatics analysis to detect novel germline mutations in the APC gene in patients with familial adenomatous polyposis (FAP), J. Clin. Lab Anal. 35 (2021), e23768.
[8] H.F. Vasen, G. Moslein, A. Alonso, S. Aretz, I. Bernstein, L. Bertario, I. Blanco, S. Bulow, J. Burn, G. Capella, C. Colas, C. Engel, I. Frayling, W. Friedl, F.J. Hes, S. Hodgson, H. Jarvinen, J.P. Mecklin, P. Moller, T. Myrhoi, F.M. Nagengast, Y. Parc, R. Phillips, S.K. Clark, M.P. de Leon, L. Renkonen-Sinisalo, J.R. Sampson, A. Stormorken, S. Tejpar, H.J. Thomas, J. Wijnen, Guidelines for the clinical management of familial adenomatous polyposis (FAP), Gut 57 (2008) 704–713.
[9] B. Roy, J.D. Leszyk, D.A. Mangus, A. Jacobson, Nonsense suppression by near-cognate tRNAs employs alternative base pairing at codon positions 1 and 3, Proc. Natl. Acad. Sci. USA 112 (2015) 3038–3043.
[10] S.E. Kerr, C.B. Thomas, S.N. Thibodeau, M.J. Ferber, K.C. Halling, APC germline mutations in individuals being evaluated for familial adenomatous polyposis: a review of the Mayo Clinic experience with 1591 consecutive tests, J. Mol. Diagn. 15 (2013) 31–43.
[11] K. Tamura, J. Utsunomiya, T. Iwama, J. Furuyama, T. Takagawa, N. Takeda, Y. Fukuda, T. Matsumoto, T. Nishigami, K. Kusuhara, K. Sagayama, K. Nakagawa, T. Yamamura, Mechanism of carcinogenesis in familial tumors, Int. J. Clin. Oncol. 9 (2004) 232–245.
[12] A. Zilberberg, L. Lahav, R. Rosin-Arbesfeld, Restoration of APC gene function in colorectal cancer cells by aminoglycoside- and macrolide-induced read-through of premature termination codons, Gut 59 (2010) 496–507.
[13] M. Gomez-Grau, E. Garrido, M. Cozar, V. Rodriguez-Sureda, C. Dominguez, C. Arenas, R.A. Gatti, B. Cormand, D. Grinberg, L. Vilageliu, Evaluation of aminoglycoside and non-aminoglycoside compounds for stop-codon readthrough therapy in four lysosomal storage diseases, PLOS One 10 (2015), e0135873.
[14] V. Malik, L.R. Rodino-Klapac, L. Viollet, C. Wall, W. King, R. Al-Dahhak, S. Lewis, C.J. Shilling, J. Kota, C. Serrano-Munuera, J. Hayes, J.D. Mahan, K.J. Campbell, B. Banwell, M. Dasouki, V. Watts, K. Sivakumar, R. Bien-Willner, K.M. Flanigan, Z. Sahenk, R.J. Barohn, C.M. Walker, J.R. Mendell, Gentamicin-induced

readthrough of stop codons in Duchenne muscular dystrophy, Ann. Neurol. 67 (2010) 771–780.
[15] K.M. Keeling, X. Xue, G. Gunn, D.M. Bedwell, Therapeutics based on stop codon readthrough, Annu Rev. Genom. Hum. Genet. 15 (2014) 371–394.
[16] S. Michorowska, Ataluren-promising therapeutic premature termination codon readthrough frontrunner, Pharmaceuticals 14 (2021).
[17] M. Caspi, A. Firsow, R. Rajkumar, N. Skalka, I. Moshkovitz, A. Munitz, M. Pasmanik-Chor, H. Greif, D. Megido, R. Kariv, D.W. Rosenberg, R. Rosin-Arbesfeld, A flow cytometry-based reporter assay identifies macrolide antibiotics as nonsense mutation read-through agents, J. Mol. Med. 94 (2016) 469–482.
[18] H. Lode, K. Borner, P. Koeppe, T. Schaberg, Azithromycin–review of key chemical, pharmacokinetic and microbiological features, J. Antimicrob. Chemother. 37 (Suppl C) (1996) 1–8.
[19] L.K. Su, K.W. Kinzler, B. Vogelstein, A.C. Preisinger, A.R. Moser, C. Luongo, K. A. Gould, W.F. Dove, Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene, Science 256 (1992) 668–670.
[20] R. Koratkar, K.A. Silverman, E. Pequignot, W.W. Hauck, A.M. Buchberg, L. D. Siracusa, Analysis of reciprocal congenic lines reveals the C3H/HeJ genome to be highly resistant to ApcMin intestinal tumorigenesis, Genomics 84 (2004) 844–852.
[21] T. Morioka, S. Yamazaki, H. Yanagihara, M. Sunaoshi, M. Kaminishi, S. Kakinuma, Calorie restriction suppresses the progression of radiation-induced intestinal tumours in C3B6F1 $Apc^{Min/+}$ mice, Anticancer Res. 41 (2021) 1365–1375.
[22] L. McDonnell, A. Gilkes, M. Ashworth, V. Rowland, T.H. Harries, D. Armstrong, P. White, Association between antibiotics and gut microbiome dysbiosis in children: systematic review and meta-analysis, Gut Microbes 13 (2021) 1–18.
[23] C. Neufert, C. Becker, M.F. Neurath, An inducible mouse model of colon carcinogenesis for the analysis of sporadic and inflammation-driven tumor progression, Nat. Protoc. 2 (2007) 1998–2004.
[24] E. Sakai, T. Morioka, E. Yamada, H. Ohkubo, T. Higurashi, K. Hosono, H. Endo, H. Takahashi, R. Takamatsu, C. Cui, M. Shiozawa, M. Akaike, H. Samura, T. Nishimaki, A. Nakajima, N. Yoshimi, Identification of preneoplastic lesions as mucin-depleted foci in patients with sporadic colorectal cancer, Cancer Sci. 103 (2012) 144–149.
[25] T. Morioka, B.J. Blyth, T. Imaoka, M. Nishimura, H. Takeshita, T. Shimomura, J. Ohtake, A. Ishida, P. Schofield, B. Grosche, U. Kulka, Y. Shimada, Y. Yamada, S. Kakinuma, Establishing the Japan-Store house of animal radiobiology experiments (J-SHARE), a large-scale necropsy and histopathology archive providing international access to important radiobiology data, Int. J. Radiat. Biol. 95 (2019) 1372–1377.
[26] G.P. Boivin, K. Washington, K. Yang, J.M. Ward, T.P. Pretlow, R. Russell, D. G. Besselsen, V.L. Godfrey, T. Doetschman, W.F. Dove, H.C. Pitot, R.B. Halberg, S. H. Itzkowitz, J. Groden, R.J. Coffey, Pathology of mouse models of intestinal cancer: consensus report and recommendations, Gastroenterology 124 (2003) 762–777.
[27] K. Taniguchi, S. Kakinuma, Y. Tokairin, M. Arai, H. Kohno, K. Wakabayashi, T. Imaoka, E. Ito, M. Koike, H. Uetake, M. Nishimura, K. Yamauchi, K. Sugihara, Y. Shimada, Mild inflammation accelerates colon carcinogenesis in Mlh1-deficient mice, Oncology 71 (2006) 124–130.
[28] T. Chi, Q. Zhao, P. Wang, Fecal 16S rRNA gene sequencing analysis of changes in the gut microbiota of rats with low-dose aspirin-related intestinal injury, Biomed. Res. Int. 2021 (2021), 8848686.
[29] M. Borges-Canha, J.P. Portela-Cidade, M. Dinis-Ribeiro, A.F. Leite-Moreira, P. Pimentel-Nunes, Role of colonic microbiota in colorectal carcinogenesis: a systematic review, Rev. Esp. Enferm. Dig. 107 (2015) 659–671.
[30] S.J. Kim, S.E. Kim, A.R. Kim, S. Kang, M.Y. Park, M.K. Sung, Dietary fat intake and age modulate the composition of the gut microbiota and colonic inflammation in C57BL/6J mice, BMC Microbiol. 19 (2019) 193.
[31] T. Hamoya, S. Miyamoto, S. Tomono, G. Fujii, R. Nakanishi, M. Komiya, S. Tamura, K. Fujimoto, J. Toshima, K. Wakabayashi, M. Mutoh, Chemopreventive effects of a low-side-effect antibiotic drug, erythromycin, on mouse intestinal tumors, J. Clin. Biochem. Nutr. 60 (2017) 199–207.
[32] F.J. Verdam, S. Fuentes, C. de Jonge, E.G. Zoetendal, R. Erbil, J.W. Greve, W. A. Buurman, W.M. de Vos, S.S. Rensen, Human intestinal microbiota composition is associated with local and systemic inflammation in obesity, Obesity 21 (2013) E607–E615.
[33] R. Kariv, M. Caspi, N. Fliss-Isakov, Y. Shorer, Y. Shor, G. Rosner, E. Brazowski, G. Beer, S. Cohen, R. Rosin-Arbesfeld, Resorting the function of the colorectal cancer gatekeeper adenomatous polyposis coli, Int. J. Cancer 146 (2020) 1064–1074.
[34] H.K. Knutsen, H.B. Olstorn, J.E. Paulsen, T. Husoy, I.L. Goverud, E.M. Loberg, K. Kristiansen, J. Alexander, Increased levels of PPARbeta/delta and cyclin D1 in flat dysplastic ACF and adenomas in Apc(Min/+) mice, Anticancer Res. 25 (2005) 3781–3789.
[35] H. Yanagihara, T. Morioka, S. Yamazaki, Y. Yamada, H. Tachibana, K. Daino, C. Tsuruoka, Y. Amasaki, M. Kaminishi, T. Imaoka, S. Kakinuma, Interstitial deletion of the Apc locus in beta-catenin-overexpressing cells is a signature of radiation-induced intestinal tumors in C3B6F1 $Apc^{Min/+}$ mice, J. Radiat. Res. 64 (2023) 622–631.
[36] C.J. Meehan, R.G. Beiko, A phylogenomic view of ecological specialization in the Lachnospiraceae, a family of digestive tract-associated bacteria, Genome Biol. Evol. 6 (2014) 703–713.