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Effect of Daikenchuto On Spontaneous Intestinal Tumors in $Apc^{Min/+}$ Mice

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Daikenchuto (TU-100) is herbal medicine which predominantly contains ginger, Japanese pepper, and ginseng. We investigated whether TU-100 can affect the composition of gut flora and intestinal tumor development using $Apc^{Min/+}$ mice, a murine model of intestinal tumor. Bacterial 16S rRNA sequencing and short-chain fatty acid analysis were performed on faecal samples. Tumor number and size were analysed. Any change in gene expression of the tumor tissues was assessed by real-time PCR. Principal coordinate analysis (PCoA) showed that the faecal microbiota cluster of TU-100-fed mice was different from the microbiota of control mice. However, no significant difference was observed in the concentration of short-chain fatty acids, tumor number, and gene expression levels between the two groups. Our data showed that TU-100 can affect the intestinal environment; however, it does not contribute in tumor progression or inhibition in our setting.

INTRODUCTION

Colon cancer is the third most prevalent cancer following lung cancer and breast cancer, and is the second leading cause of mortality worldwide [1]. The data implies that about 10% men and 8% women will be diagnosed with colon cancer during their lifetime in Japan. Lately, survival rates have improved due to development in treatment strategies such as surgical procedures, anticancer medicines, and radiation therapy [2,3]. However, these therapies affect the physical, mental, and financial quality of life of the patients and often cause severe adverse effects [4]. Thus, novel therapies that are safer and more effective are intensively being investigated. Ultimately, if a prophylactic treatment is discovered, it would be highly beneficial.

Furthermore, it is well known that gut microbiota can have a systemic effect on human health [5]. Studies have shown that ingested food can alter the components of gut microbiota and many reports further suggest that dietary factor (such as western diet) may be influencing the development and progression of diseases such as obesity, diabetes, inflammatory bowel disease, and colon cancer [6-8]. Most of the colon cancers develop from the tumorous growth of colonic benign polyps called adenomas via adenoma-carcinoma sequence [9]. This suggests that alteration in the gut flora can either inhibit intestinal adenoma growth or promote tumor progression and the risk of cancer.

Daikenchuto (TU-100), also called as Kampo, is a Japanese traditional herbal medicine which has prokinetic effects in the gut and is widely prescribed in Japan for the treatment of constipation [10,11]. TU-100 consists of processed ginger root (Zingiber officinale), ginseng radix root (Panax ginseng), and Japanese green pepper (Zanthoxylum piperitum) which is mixed in the ratio of 5:3:2 by weight [12-14]. Previous studies have shown that TU-100 and its ingredients can alter the components of gut flora [15,16]. Furthermore, this suggests that TU-100 might influence the development of intestinal disorders such as inflammatory diseases and intestinal cancer. If TU-100 shows therapeutic influence on intestinal tumor then it would be extremely beneficial. Additionally, it could be considered as a prophylactic medicine to demote colon cancer development besides alleviating constipation.

In this study, we used $Apc^{Min/+}$ mice, an animal model of intestinal adenomas with mutations in the adenomatous polyposis coli (Apc) gene which are similar to human adenomas and investigated the role of TU-100 in tumor progression.

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MATERIALS AND METHODS

Animals

$Apc^{Min/+}$ male mice in the C57BL/6 genetic background were obtained from Jackson Laboratories (Jackson Laboratories, ME, USA) and bred under specific pathogen-free conditions in the Animal Facility at Kobe University Graduate School of Medicine. All mice were allowed free access to food and water. Mice body weight was recorded weekly until 15 weeks old. All animal studies were approved by Institutional Animal Care and Use Committee of Kobe University (P170808).

TU-100 treatment

TU-100 was generously gifted by Tsumura & Co. (Tokyo, Japan). Six-week old mice ($Apc^{Min/+}$ and $Apc^{+/+}$ mice) were administered with 2% wt/wt TU100 mix (2g TU-100 added in 100g total diet) in a standard powdered CE-2 diet (CLEA Japan Inc., Japan) or standard powder alone for 9 weeks. The dosage of TU-100 for experiment was decided based on previous reports [14,15]. Due to the nature of powdered food, small piece of sterile wood was placed in every cage as an environmental enrichment equipment for mice to chew on.

Tumor counts

Small intestine and colon were cut open longitudinally, flattened with tweezers, dyed with indigocarmine (Daiichi-Sankyo, Japan). Subsequently, tumor number and size were noted by a single recorded blindfolded researcher (LL.K) throughout the study.

RNA extraction and real-time PCR

At 15 weeks, tumor and control healthy tissues were incised from small intestine and colon for tissue collection and placed in RNAlater (Qiagen, CA, USA) and stored at -80 ℃ until further use. Real-time PCR was performed according to previously described method [17,18]. Briefly, total RNA was extracted from tissues using TRIzol reagent (Thermo Fisher Scientific, MA, USA) and reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Real-time PCR was analysed using SYBR Green (Applied Biosystems, CA, USA) on ABI 7500 real-time PCR system (Applied Biosystems, CA, USA). Relative expression levels of the target genes were standardized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) expression. The primers used for Real-time in this study are listed as follows: Tumor necrosis factor α (TNF-α): 5′-AAAATTCGAGTGACAAGCCTGTAG-3′ (forward), 5′-CCCTTGAAGAGAACCTGGGAGTAG-3′(reverse),Interleukin6(IL-6):5'-GACAAAGCCAGAGTCCTTCAGAGAGATACAG-3′ (forward), 5′-TTGGATGGTCTTGGTCCTTAGCCAC-3′(reverse), Keratinocyte carcinomas (KC): 5′-GCTGGGATTCACCTCAAGAA-3′(forward), 5′-TCTCCGTTACTTGGGGACAC-3′(reverse), Cyclooxygenase-2 (COX-2): 5′-ACCCGGACTGGATTCTAT-3′(forward), 5′-GCTTCCCAGCTTTTGTAA-3′(reverse), IL-1β: 5′-TCCAGGATGAGGACATGAGCAC-3′(forward), 5′-GAACGTCACACACCAGCAGGTTA-3′(reverse), L-type amino acid transporter (LAT-1): 5′-CTGGATCGAGCTGCTCATC-3′(forward), 5′-GTTCACAGCTGTGAGGAGC-3′(reverse), HPRT: 5′-GTTGGATACAGGCCAGACTTTGTTG-3′(forward), 5′-CCAGTTTCACTAATGACACAAACG-3′(reverse).

Short-chain fatty acid measurement

To evaluate the effect on short-chain fatty acids, 0.1 gm of faeces was added to 2.0 mL-tube with zirconia beads and suspended in MilliQ water. Samples were heated at 85℃ for 15 min, vortexed at 5 m/s for 45 s using FastPrep-24 5G (MP Biomedicals, CA, USA), and centrifuged at 15,350 ×g for 10 min. Supernatant was filtered using 0.2 μm filter. Concentration of short-chain fatty acids such as acetic acid, propionic acid, and butyric acid in faeces was evaluated using organic acid analysis system (Shimadzu Corp., Japan) consisting of: a high performance liquid chromatography, Prominence; a detector, CDD-10A; tandemly-arranged two columns, Shim-pack SCR-102(H) (300 mm × 8 mm ID); a guard column, Shim-pack SCR-102(H) (50 mm × 6 mm ID); a mobile phase, 5 mM p-toluenesulfonic acid; a reaction solution, 5 mM p-toluenesulfonic acid, 100 μM EDTA, and 20 mM Bis-Tris. Flow rate and oven temperature were maintained at 0.8 mL/min and 45 ℃, respectively.

Microbiome analysis

Fresh faecal pellets were collected from 15 weeks old mice, immediately frozen on dry ice, and stored at -80 ℃ until further use. Bacterial DNA content was extracted using QIAamp PowerFecal DNA kit (MO BIO Laboratories, CA, USA). For 2-step PCR amplification, bacterial V3-V4 variable region of the 16S rRNA gene was amplified with primers containing Illumina overhang nucleotide adapter sequences (Illumina, CA, USA) followed by amplification using barcoded primers. DNA concentration of each amplicons was measured using

Qubit® and dsDNA HS assay kit (Invitrogen, CA, USA). DNA libraries adjusted to 6 pM were pooled with 20% 6 pM PhiX spike-in control (Illumina, CA, USA) and sequenced on MiSeq® platform (Illumina, CA, USA). The raw data was converted to FASTQ file and demultiplexed using MiSeq® reporter. Sequence data was quality filtered and analysed using QIIME 8.1.0 open-source software. 30,000 paired-end reads were randomly selected per sample and analysed. 3′-end low quality bases were trimmed by PRINSEQ (version 0.20.4.) [19] and paired-end reads were merged using fastq-join program. Chimeric sequences were removed using USEARCH 6.1.544 and operational taxonomic unit clustering was performed using UCLUST at a similarity threshold of 97%. Further, the sequence reads were examined against Greengages reference database (version 13.8). Principal coordinate analysis (PCoA) was performed using QIIME™ based on weighted UniFrac distances. Differences in microbial composition which were described in weighted UniFrac distances, were assessed using multivariate analysis of variance (MANOVA). $P < 0.05$ was considered statistically significant. To study the differences in intestinal microbiota between two groups, Linear discriminant analysis effect size (LDA Effect Size: LEfSe) [20] was performed. Significant differences were identified by LEfSe analysis as a p-value less than 0.05 using the Kruskal–Wallis test, together with the threshold set at 2.0 on the logarithmic LDA score.

Statistical analysis

Excluding microbiome analysis, data are presented as mean ± standard error of the mean (SEM) and were analysed using Student's t-test using Prism 7 (GraphPad Software, Inc., CA, USA). $p < 0.05$ was considered as statistically significant.

RESULTS

TU-100 alters faecal microbiota composition

It is well known that gut microbiota composition can be different in different facilities and that is often used to explain when the similar experiments showed different results. An earlier study has reported that TU-100 can alter the component of gut flora and faecal short-chain fatty acids [15]. Subsequently, we investigated whether TU-100 can cause similar alteration in the intestinal microenvironment in our facility. After feeding mice with TU-100 for 9 weeks, faecal microbiota was analysed using 16S rRNA sequencing. As anticipated, the data revealed that the microbiota from faecal samples of TU-100-fed mice (Group 2, Figure 1A) was clustered together and distinct from the microbiota of control mice (Group 1, Figure 1A). Furthermore, the microbiota of control group appeared to be more randomly distributed than the microbiota of TU-100-fed mice. Linear discriminant analysis effect size (LDA Effect Size: LEfSe) was employed to search for features most likely to explain differences between two group (Figure 1B). Seven taxa were found different between two groups based on $LDA > 2$. The significant increase was observed in the relative abundance of *Prevotella* (family of *paraprevotellaceae*), *Bacteroides*, unclassified genera of *Helicobacteraceae*, and genus AF12 in the Rikenellaceae family, and the significant reduction was observed in *Coprococcus* and *Rikenella* in the TU-100 group compared to the control group.

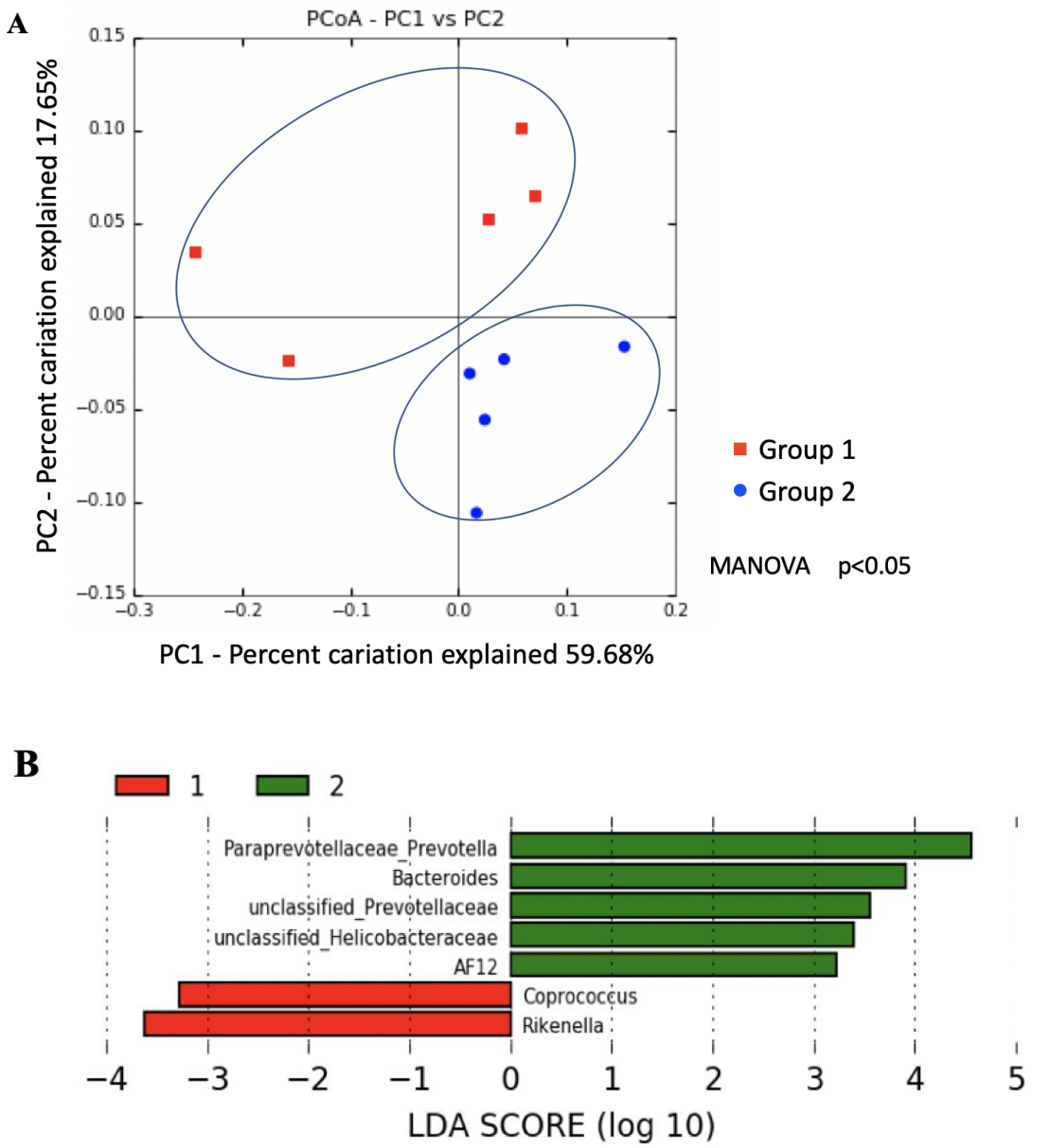

Figure 1. Comparison of faecal microbiota of Apc$^{Min/+}$ mice with and without TU-100 administration
DNA was extracted from faecal pellets and analysed for bacterial 16S rRNA sequence in gut. (A)Principal coordinate analysis (PCoA) plot was generated using QIIME, the first two principal coordinates, PC1 and PC2. Group 1: TU-100(-), n=5; Group 2: TU-100(+), n=5. (B) The most differentially abundant taxa between two groups were generated using LEfSe analysis. The threshold of LDA score was set to 2.0. The p-value less than 0.05 analysed by Kruskal-Wallis test was considered significantly different.

Dietary TU-100 does not increase short-chain fatty acid production

To further evaluate the alterations in intestinal environment, we investigated whether TU-100 can alter the concentration of short-chain fatty acids. TU-100 is a mixture of different herbs, and indigestible dietary fibres of the herbs can be fermented in the cecum and colon by the gut microbes to form short-chain fatty acids such as acetate, propionic acid, and butyric acid. Remarkably, none of investigated fatty acids showed significant variation on consumption of dietary TU-100 (Figure 2A-C).

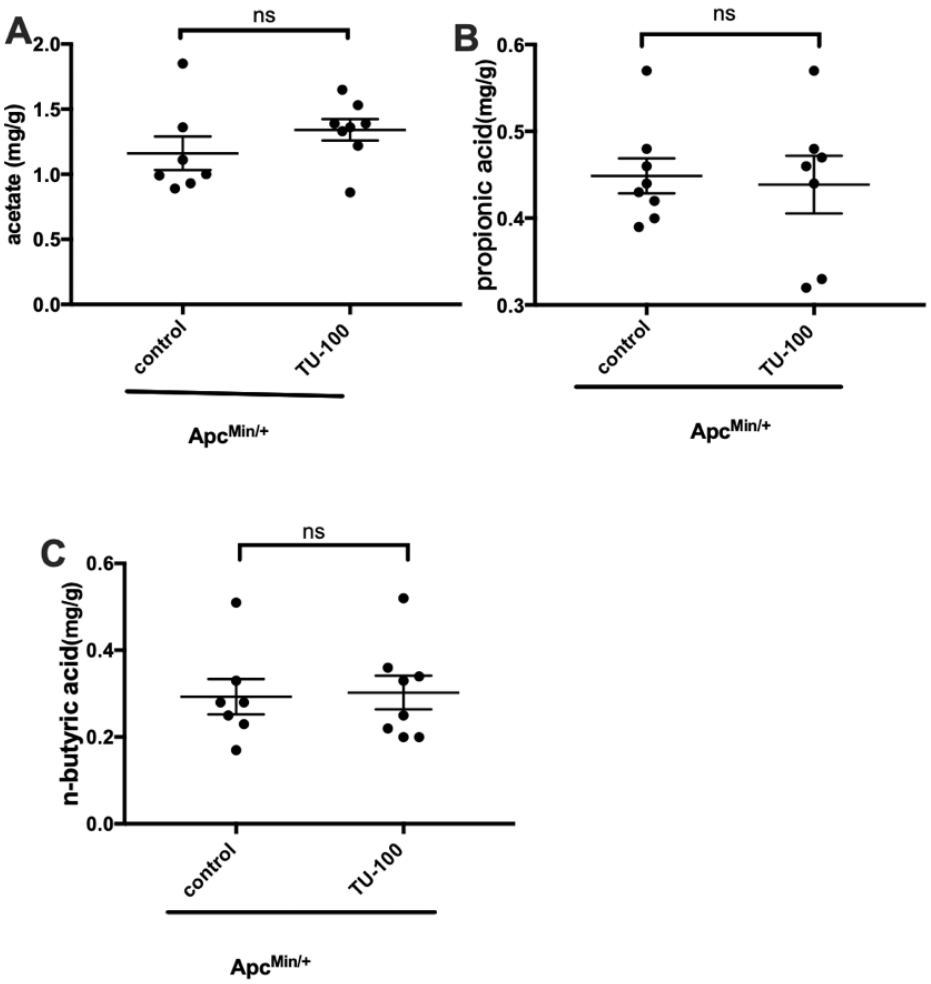

Figure 2. No change in faecal short-chain fatty acids on feeding mice with TU-100

Faecal samples were collected after feeding mice with or without TU-100 for 9 weeks and analysed for the concentration of short-chain fatty acids using organic acid analysis system. Apc$^{Min/+}$ TU-100(+), n=8; Apc$^{Min/+}$ TU-100(-), n=7. Statistical analysis was performed using Student's t-test. Data is presented as mean ± standard error of the mean (SEM), ns: no significance, $p > 0.05$.

TU-100 does not affect body weight and tumor growth

To investigate whether TU-100-based change in microbiota composition influences tumor development, difference in body weight and tumor of the small intestine and colon were analysed. However, there was no significant difference in body weight on feeding mice with TU-100 (Fig.3A). Accordingly, we did not observe any reduction in number of tumors or tumors size in the small intestine and colon of both the groups (Figure 3B-E). Thus, the results suggested that TU-100 diet neither suppresses nor promotes tumor growth.

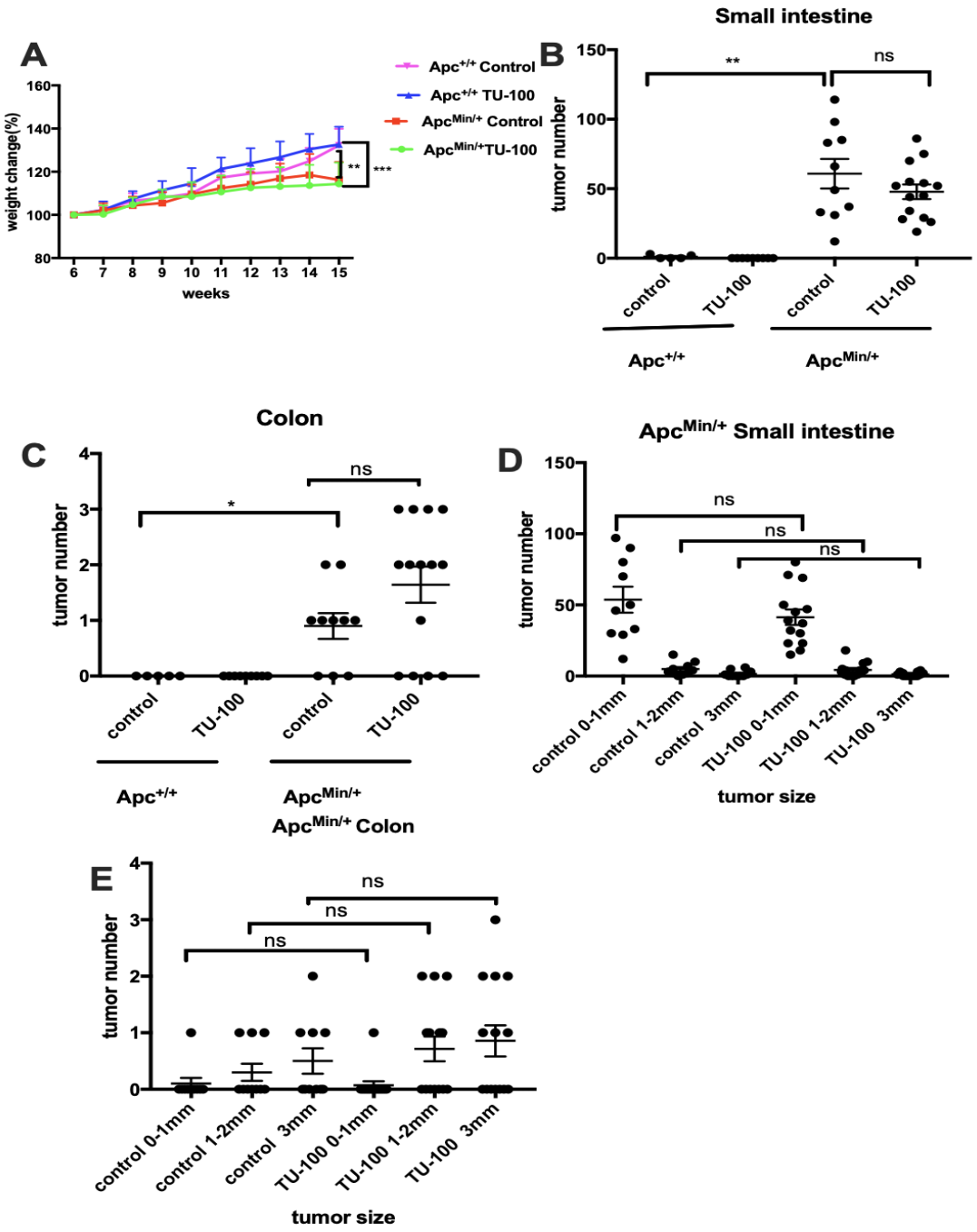

Figure 3. Change in body weight and tumor development in TU-100 treated mice.
(A) Change in body weight. Total tumor burden in small intestine (B) and colon (C) till 15 weeks. Small intestine (D) and colon (E) were assessed for tumor size and number in 15 weeks old mice. $Apc^{Min/+}$ TU-100(+), n=14; $Apc^{Min/+}$ control, n=10; $Apc^{+/+}$ TU-100(+), n=9; $Apc^{+/+}$ control, n=5. ns: no significance, $p > 0.05$. Statistical analysis was performed using Student's t-test. $^{**}p < 0.01$, $^{***}p < 0.001$.

TU-100 does not alter the expression of inflammatory genes in $Apc^{Min/+}$mice

Several studies have proved that intestinal gut microbiota affects immune status of the intestine [21]. Furthermore, inflammation is significantly associated with cancer progression. For instance, it has been reported that IL-6 over-expression stimulates cachexia and increases number of intestinal polyps in $Apc^{Min/+}$ mouse [22,23]. Until now, our data suggests that TU-100 has no effect on tumor development. However, TU-100 has been shown to have anti-inflammatory effects [10,12,15]. Thus, we further investigated whether TU-100 can modify gene expression levels of inflammatory cytokines. However, no change was observed. Subsequently, we assessed the expression of COX-2, a positive regulator of intestinal tumor [24,25], and LAT-1, a tumor-specific amino acid transporter [26,27] for reference. Overall, our experiments revealed that TU-100 did not alter the inflammatory cytokine expressions (Figure 4).

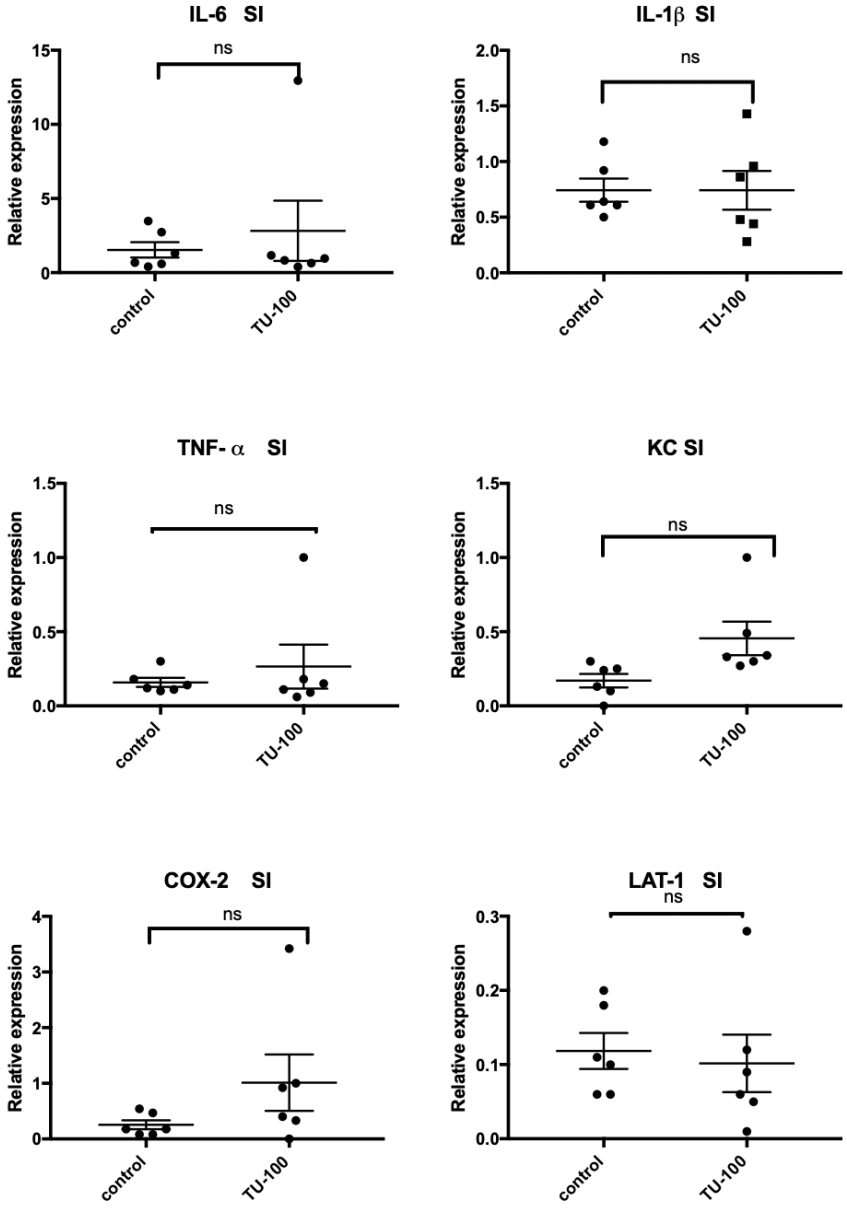

Figure 4. Effect of TU-100 on real-time PCR analysis of tumor tissue of small intestine.

Tumor tissue samples were harvested from Apc$^{Min/+}$ mice. Gene expression levels were analysed. n=6, (A-F). Data is presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.005$, ns: no significance, $p > 0.05$. Statistical analysis was performed using Student's t-test.

DISCUSSION

Our results showed that TU-100 influences the components of gut flora. However, TU-100 induced alteration was not significant enough to influence tumor progression in the mouse model of intestinal adenomas.

Contrary, it has been shown that TU-100 hinders the development of intestinal tumors in azoxymethane (AOM) colon tumor model as well as Apc$^{Min/+}$ mice model via inhibiting epidermal growth factor receptor (EGFR)-mediated downstream pathways [12]. As Balb/c mice were used in the study, the discrepancy between our results and the data from AOM tumor model maybe due to use of different mouse models and diverse genetic background. However, the variation in the data should be justified to be environmental difference as Apc$^{Min/+}$ mice was purchased from the same company. Apc$^{Min/+}$ mice express a truncating mutation in the Apc and is reported to develop about 60 to 80 intestinal adenomas as seen in our study [28,29]. Interestingly, number of small intestinal tumors were reasonably fewer (around 40 in 24 weeks old mice) compared to number of tumors in mice used in this study which was almost 60 in 15 weeks old mice [12]. One important difference between the two study was that mice of the earlier study were nourished with AIN76A diet while mice in our study were fed CE-2 diet. Variety in diet can have a huge impact on the composition of gut flora together with the metabolic content of the intestine. In fact, genetic study of the same research group has shown that TU-100

significantly upregulates butyrate and acetate producing bacteria belonging to *Clostridiaceae* family. They have even confirmed the alteration in the concentrations of acetate and butyrate [15]. Many studies have reported direct relation between increase in short-chain fatty acids and reduction in tumor growth [30-32]. We found that the genus *Bacteroides*, which can produce propionate[33], was increased in our study. It is shown that short-chain fatty acids produced by the gut flora can increase those levels in the plasma, subsequently, it modifies the phenotypes in the remote organs such as adipose tissue [34]. We expected the change of microbiota could increase the levels of short-chain fatty acid and modify the tumor growth in both small intestine and the colon. However, it did not affect even faecal propionate level. After all, our 16S rRNA data does not exactly match to the previous paper [15]. Those discrepancy may be attributed to the different diet used to feed the mice. Furthermore, mucous production could be another candidate which have been contributed to the discrepancies. Intestinal surface is covered by mucous layers [35,36] that is influenced by the ingested food [37,38]. Mucous layers can affect the host response towards intestinal environment which is evidently shown by the fact that mucin 2 (Muc2)-deficient mice can develop spontaneous colitis and intestinal tumor [39-41]. Further investigations are required to elucidate the significance of mice chow type on mucous production or its quality, and its role in causing discrepancies in our data. It would be interesting to investigate if different chow diet can influence the tumor occurrence and/or growth in the same set-up. Comprehensive investigation of the ingredients of mice chow may further determine their role in modifying intestinal tumor growth in $Apc^{Min/+}$ mice model.

Chronic inflammation is considered as a risk factor for cancer development. Targeting inflammatory pathways has been demonstrated to be effective in suppressing cancer development [42]. Ingredients of TU-100 such as ginger and ginseng are known to have anti-inflammatory effects [10,43]. In addition, reports have revealed TU-100 to have anti-inflammatory activity by reducing T-cell-induced TNF-α production. Subsequently, downregulated TNF-α expression inhibits NF-κB activation in epithelial cells and causes amelioration of murine enteritis. The mechanism has been observed to be independent of gut commensal flora [42]. Furthermore, tumor growth in $Apc^{Min/+}$ mice is known to be significantly modified by the function of T cells [44,45]. Thus in the present study, we hypothesized TU-100 to reveal anti-inflammatory and/or anti-tumorigenesis activity through previously reported mechanisms. However, as shown by our real-time PCR data, TU-100 did not affect the expression levels of the investigated inflammatory cytokines. Accordingly, no change was observed in the tumor phenotype nor in the expression levels of COX-2 and LAT-1.

Conventionally, similar experiments often show different or even contradictory results [46,47]. However, systematic investigations of these observations could be beneficial in understanding the unknown phenotypic mechanisms. Notable information from this study and previously published report is that TU-100 does not promote tumor progression which proves the safety in using TU-100 for treating constipation.

Our data revealed that TU-100 influences the composition of intestinal microbiota; however, does not alter the concentration of short-chain fatty acids nor supports intestinal tumor occurrence and progression. Overall, our current study suggests that TU-100 does not promote intestinal tumor growth.

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AUTHOR CONTRIBUTIONS

LL.K. and N.H. conceived and designed the experiments. LL.K., N.H., D.W, Y.Y, E.Y., S.A., J.I., YL.S. R.Y., E.T. and R.S. performed experiments and analysed the data. LL.K., H. M, H.T., M.O., Y.K., ZB.T., Y.K. and N.H. drafted the paper.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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