HHS Public Access

Author manuscript

Cancer Prev Res (Phila). Author manuscript; available in PMC 2016 February 01.

Published in final edited form as:

Cancer Prev Res (Phila). 2015 February ; 8(2): 139–148. doi:10.1158/1940-6207.CAPR-14-0128.

Combined use of vitamin D3and metformin exhibits synergistic chemopreventive effects on colorectal neoplasia in rats and mice

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Abstract

Vitamin D3 and metformin are widely used in humans for regulating mineral metabolism and as an anti-diabetic drug respectively; and both of them have been shown to have chemopreventive effects against various tumors. This study was designed to investigate the potential synergistic chemopreventive effects of vitamin D3 and metformin against the development of early colon neoplasia in two models. The first model was a 1, 2-dimethylhydrazine dihydrochloride (DMH) induced colon cancer rat model and the second, a DMH-dextran sodium sulfate (DSS) induced colitis-associated colon neoplasia mouse model. Compared to either vitamin D3 or metformin alone, combined use of vitamin D3 and metformin showed more pronounced effect in reducing the numbers of aberrant crypt foci (ACF) and tumor in the colon. The most prominent inhibitory effects were observed in the vitamin D3 medium dose (100 IU/kg/day) and metformin medium dose (120 mg/kg/day) combination group. Furthermore, our results showed that enhancement of metformin's chemopreventive effects by vitamin D3 was associated with down-regulation of S6P expression, via the AMPK (IGF-1)/mTOR pathway. In addition, and enhancement of vitamin D3's chemopreventive effects by metformin was associated with inhibition of the protein expressions of c-Myc and Cyclin D1, via the vitamin D receptor/β-catenin pathway. These findings show that combined use of vitamin D3 and metformin exhibits synergistic effects against the development of early colon neoplasia. They suggest that the combined use of vitamin D3 and metformin may represent a novel strategy for chemoprevention of colorectal cancer.

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Conflict of Interest: The authors disclose no potential conflicts of interest.

Keywords

Vitamin D3; Metformin; Colorectal neoplasia; Rat; Mouse

Introduction

Colorectal cancer (CRC) is one of the most common forms of cancer and the fourth leading cause of cancer worldwide (1). It is well documented that patients with inflammatory bowel disease (IBD), such as ulcerative colitis (UC) and Crohn's disease, are at increased risk for developing colorectal cancer (CRC) (2, 3). The incidence of CRC in colitis patients is nearly 40% within thirty years after the onset of UC (4). It has been well established that the development of most sporadic colorectal cancer follows the fundamental "aberrant crypt foci-adenoma-carcinoma sequence", which is a stepwise progression from normal to dysplastic epithelium to carcinoma (5). Aberrant crypt foci (ACF) are now recognized as the earliest and smallest observable precancerous lesions, whose quantity are positively related to the incidence of colorectal cancer (6, 7).

Metformin, an anti-hyperglycemic drug, widely used for the treatment of type 2 diabetes, is emerging as a potential anti-tumor agent. Several epidemiological studies reported decreased cancer incidence, including colorectal cancer, in patients with type 2 diabetes receiving metformin treatment compared with those patients who did not (8, 9). Corroborating evidence has been reported in both a genetic engineered and a chemical induced rodent colon cancer model (10, 11). *In vitro* study has also demonstrated growth inhibitory effects of metformin in colon cancer cells via activating AMP-activated protein kinase (AMPK) pathway (12). Furthermore, in clinical trials, metformin suppressed colonic epithelial proliferation and rectal ACF formation in humans (13, 14). It is hypothesized that metformin has both direct and indirect anti-neoplastic actions. The direct effects of metformin are mainly mediated through activation of AMPK, which further leads to the inhibition of mammalian target of rapamycin (mTOR) signaling and protein synthesis in cancer cells (15, 16). Metformin also acts through an indirect, insulin-dependent mechanism, resulting in increased insulin sensitivity, reduced hepatic gluconeogenesis, and decreased circulating insulin level. Reduced circulating levels of insulin decrease the activation of insulin/insulin like growth factor-1 hybrid receptors (IR/IGF-1R), a receptor tyrosine kinase, thereby reducing the activation of PI3K/AKT/mTOR signaling in cancer cells (17, 18).

Vitamin D3 is synthesized from its precursor 7-dehydrocholesterol in the skin upon exposure to ultraviolet irradiation (UV) or obtained via diet. The active form of vitamin D, 1, 25(OH)2D3, contributes to calcium and phosphate homeostasis, skeletal mineralization, and regulates cell proliferation, differentiation and apoptosis (19, 20). Following Garland's hypothesis that the intensity of local sunlight was inversely correlated with the risk of CRC (21), a large number of experimental and epidemiological studies investigating the potential chemopreventive effects of vitamin D have been carried out, most of which are consistent with an inverse relationship (22–25). 1, 25(OH)2D3 exerts its biological effects mainly through the vitamin D receptor (VDR), which belongs to the nuclear receptor super-family, and regulates gene expression in a ligand-dependent manner. The Wnt/β- catenin signaling

pathway, one of the key pathways aberrantly activated in colon cancer (26), is often considered among the initial events in colon carcinogenesis. Recent studies have demonstrated that 1, 25(OH)2D3 inhibits the Wnt/β-catenin pathway and the activation of its target genes such as c-myc and cyclin D1, which play an important role in the proliferation and apoptosis of cancer cells (27).

Although an increasing number of studies demonstrate the anti-tumour effects of metformin or vitamin D3 (15, 16, 27), relatively little is known about their effects in combination. Therefore, the goal of the present study was to examine the combined effects of metformin and vitamin D3 both in an 1, 2-dimethyl-hydrazine (DMH) induced rat colon cancer and in a DMH-dextran sodium sulfate (DSS) induced colitis-associated colon neoplasia mouse models. The underlying mechanisms were also investigated in the mouse model.

Materials and Methods

Animals

Male Wistar rats (Animal Experiment Center of Southern Medical University, Guangzhou, China) weighing 80–120 g and male ICR (CD-1) mice aged 5 weeks (Beijing Vital River Laboratory Animal Technological Company, Beijing, China) were used in this study. All animals were housed in plastic cages (temperature 22±2 °C, relative humidity 50±10%, 12 hour light/dark cycle) with free access to drinking water and a pelleted basal diet (Chengdu Dashuo Biotechnology Co. Ltd., Chengdu, China). All animal experiments were conducted according to the principles of National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (28) and were approved by the ethics committee for laboratory animal care and use of Southern Medical University (Wistar rats) or Lanzhou University (ICR mice). All efforts were made to minimize the suffering of animals used in this study.

Drugs and agents preparation

DMH was dissolved at 20 mg/ml in 0.9% saline solution and the pH value was adjusted to 6.5. The DMH solution was filtered with 0.22 μm membrane and used immediately. Vitamin D3 was dissolved from 10 to 600 IU/ml into soybean oil. Metformin was dissolved in triple-distilled water with a concentration of 10–720 mg/ml.

Experimental procedure

DMH induced colon neoplasia in rats—One hundred and ten rats were randomly divided into 2 control groups and 9 experimental groups (Supplemental Table 1). All the animals except those in the normal group received 30 mg/kg body weight DMH (Tokyo Kasei Kogyo, Tokyo, Japan) by intraperitoneal (i.p.) injections once a week for 18 weeks. Vitamin D3 (Shanghai General Pharmaceutical Co. Ltd., Shanghai, China) and/or metformin hydrochloride (Zhengzhou Lion Biological Technology Co. Ltd., Zhengzhou, China) were orally administered once daily starting on the first day of intraperitoneal administration of DMH and continued for 18 weeks. At week 18 after DMH and drug treatment, all the rats were euthanized by exsanguination under deep ether anesthesia for collection of colorectal samples. The design for drug treatment is shown in Supplemental Table 1.

DMH and dextran sodium sulfate (DSS) induced colitis-associated colon neoplasia in mice—One hundred and twenty-five male ICR mice were quarantined for the first 7 days, and then randomized by body weight into 2 control groups and 3 experimental groups (Supplemental Table 2). Groups 2–5 were given a single intraperitoneal injection of DMH at a dose of 20 mg/kg body weight. Starting 1 week after the injection, animals in groups 2–5 received 2% (W/V) DSS (molecular weight 36,000–50,000; MP Biomedicals, Aurora, OH, USA) in drinking water for 7 days. Group 1 received no treatment. Vitamin D3 (Zhejiang Garden Biochemical High-tech Co. Ltd., Dongyang, China) or/and metformin hydrochloride (Qufu Maidesen Fine Chemical Co. Ltd., Qufu, China) were orally administered once daily form the fourth day after intraperitoneal administration of DMH, and continued for 137 days. All mice were sacrificed at the end of 20th week, with large intestine and serum samples collected. The design for drug treatment was shown in Supplemental Table 2.

The experiment protocols were shown in Supplemental Figure 1. All the animals were carefully observed for clinical welfare and weighed weekly throughout the experimental period.

Counting of Tumor and aberrant crypt foci (ACF) in colon and rectum of rats and mice

ACF were determined by the method of Bird (6). The entire colon was removed from distal cecum to rectum, opened longitudinally and flushed thoroughly with ice-cold phosphate-buffered saline (PBS) solution to remove the fecal contents. Tumors of large bowel were observed and counted by naked eye, and then the colon was fixed flatly in a 10% buffered formalin solution between two pieces of filter paper. After fixation for a minimum of 24 hours in formalin solution, the colon was stained with 0.2% methylene blue dissolved in PBS for 5–10min at room temperature. Excess dye was rinsed off with PBS and the colon was placed with mucosal side up on a microscope slide. The total number of ACF/colon and the number of aberrant crypts (AC) per focus, categorized as containing 1, 2, 3, 4 or more aberrant crypts, were counted under an observation microscope.

Tumor volumes in DMH and DSS induced mouse colitis-associated colon neoplasia model were calculated. The tumor size was measured with calipers, and tumor volume was calculated using the following formula:

$$V = \frac{(a * b^2)}{2}$$

Where a is the length (mm), b is the width (mm), and V is the volume (mm^3) of the tumor (29, 30). The volumes of all tumors from each mouse were added to give the overall tumor burden per animal.

Pathological evaluation in mice

After ACF evaluation, the colons with tumors were embedded in paraffin and histological evaluation was carried out on hematoxylin-eosin (HE)-stained colon sections. Colon tumors were classified as adenomas, non-invasive (i.e, carcinoma in situ) or invasive

adenocarcinomas, as previously described (31). Photomicrographs were taken with equal exposure on Motic microscope (Motic DMB5-2232PL-5; 4×, 10× or 40× magnification) coupled to a computer running Motic Images Advanced 3.2 software for windows.

Fasting blood glucose in mice

Mouse tail veins were transversely sectioned by scissors, and the tail blood was used to determine fasting blood glucose level using a glucometer (AccuCheck Performa, Roche, Mannheim, Germany).

ELISA determination of Insulin, IGF-1, IGFBP-1 and IGFBP-3 levels in mouse serum

Before mice were sacrificed, blood was collected from retro-orbital sinus, centrifuged at 1200×g for 10 min at 4°C, and serum was stored at −80°C until assay. ELISA kits were used for measurement of serum concentrations of insulin (Mercodia, Uppsala, Sweden), IGF-1 (RayBiotech, Inc., Norcross, GA), IGFBP-1 (Genway Biotech Inc, San Diego, CA, USA), and IGFBP-3 (RayBiotech, Inc., Norcross, GA) according to the manufacturers' instructions.

Western blotting analyses in mice

For the untreated controls, normal colonic tissues were sampled whereas for the other groups, samples of colonic tumor tissues were used. In brief, colon with tumors was lysed in RIPA lysis buffer (Beyotime, China) at 4°C for 30 min. The lysates were clarified by centrifugation at 12,000×g for 15 min at 4°C and assayed for total protein concentration by using BCA Kit (Beyotime, China). Cleared lysates (50 μg for p-Akt, 75 μg for p-AMPK and 100μg for all other proteins) were resolved by 8% for p-mTOR or 10% for all other proteins SDS-PAGE, and separated proteins were transferred to a PVDF membrane (Millipore, USA). Membranes were blocked in 0.5% BSA in TBS containing 0.1% Tween 20 and then the protein levels were detected by using the primary antibodies with appropriate dilutions. The primary antibodies included those to p-Akt (4058), p-AMPKα (2535), p-mTOR (2971), p-P70S6K (9205), p-S6P (4857), Cyclin D1 (2978), β-catenin (9563), c-Myc (9402), β-actin (4967, Cell Signaling, USA), CYP27B1 (sc-67261) and VDR (sc-1008, Santa Cruz, CA, USA). The primary antibodies were washed with 0.1%Tween-20/TBS and then incubated with horseradish peroxidase-conjugated secondary antibody. The bound antibodies were visualized using an enhanced chemiluminescence kit (Beyotime, China) and quantified by integral optical density (IOD) using Image-Pro Plus Software. The data was expressed as the relative IOD of the protein normalized to β-actin. All Western blot analyses were carried out at least three times.

Statistical analysis

Differences (body weight changes, numbers of AC and ACF, tumor numbers and volumes, fasting blood glucose, ELISA and Western blot) between groups were examined for statistical significance using one-way analysis of variance (ANOVA). The incidence of colorectal non-invasive adenocarcinomas was compared by Fisher's exact test. Statistical significance was set at $\alpha = 0.05$ (2-sided).

Results

DMH Induced Colon Neoplasia in Rats

General observation—Compared with untreated control group, the body weight of rats in the DMH control group was significantly decreased at 18 weeks. Compared with the DMH control group, the body weight of rats was significantly increased in the vitamin D3 (30, 100, 300 IU/kg/day) group while it was significantly decreased in the metformin high dose (360 mg/kg/day) group, likely due to the well know weight losing effect of metformin taken at high dose (32, 33). In contrast, no significant differences in weight were observed in the three combination groups when compared with DMH control group (Supplemental Table 3). No other notable clinical symptoms were observed in the rat models.

Numbers of colorectal tumors—At 18 weeks, nearly all rats receiving DMH (Group 2–11) developed colorectal tumors, and the tumor incidence showed no significant difference among groups (Supplemental Table 3). All rats treated with vitamin D3 and/or metformin had decreased numbers of tumor compared with the DMH control group, with the medium dose combination group (vitamin D3 100 IU/kg/d plus metformin 120 mg/kg/d) showing the greatest tumor inhibiting effect. Notably, compared with vitamin D3 medium dose or the metformin medium dose group, the tumor numbers per colon were statistically significantly reduced in the medium dose combination group (Table 1).

ACF analysis—Multiple ACF (Figure 1A) were observed in the colon and rectum of DMH treated rats, most of which were located in the middle and distal region. Compared with the DMH control group, all treatment groups showed significantly reduced total counts of AC and ACF per colon. Similar to the tumor multiplicity analysis, the medium dose vitamin D3 and metformin combination group showed the greatest preventive effect. Again, compared with either the vitamin D3 medium dose group or the metformin medium dose group, the AC and ACF numbers per colon were statistically significantly reduced in the medium dose combination group (Table 1).

DMH+DSS Induced Colon Neoplasia in Mice

General observations—Bloody stool and slightly decreased body weight were found in a few mice 3 days after receiving 2% DSS in drinking water. Rectal prolapse was observed in a few mice 8 weeks after receiving DMH and DSS. These lasted to the end of the experiment (20 weeks). However, there were no statistically significantly differences in the mean body weights (Supplemental Table 4) of mice across all groups at the end of the study.

Numbers and volumes of colorectal tumors—At 20 weeks, nearly all mice in the DMH+DSS treated control groups (groups 2–5) developed colorectal tumors, and the tumor incidence showed no significant difference among groups (Supplemental Table 4). As shown in Figure 1 panel B and panel C, treatment with vitamin D3, but not with metformin, resulted in decreased tumor number and volume compared with the DMN+DSS treated control mice. As shown, the volumes of colorectal tumors in DMH and DSS treated mice were further significantly reduced by combining metformin with vitamin D3 (RAW DATA were shown in Supplemental Table 5).

ACF analysis—ACF were only observed in the colon and rectum of mice receiving both DMH and DSS, most of which were located in the middle and distal regions at 20 weeks. Compared with DMH+DSS control group, the total numbers of AC and ACF were significantly lower in both vitamin D3 groups and the metformin groups. Combined use of metformin and vitamin D3 also significantly reduced the total numbers of AC and ACF (Figure 1D, raw data were shown in Supplemental Table 6). However, when compared with either vitamin D3 or metformin treatment alone, the combination treated showed no difference (Figure 1D).

Histopathological analyses—Histopathological analyses at the end of 20 weeks revealed that all tumors in mice were adenomas or non-invasive adenocarcinomas with the lesion limited to the mucosa. No invasive adenocarcinomas beyond the mucosa were observed (Figure 1E). Compared with DMH+DSS control group, the incidence of non-invasive adenocarcinoma was decreased in both the vitamin D3 group and the metformin group (Table 2), and it was further reduced in the combination treatment group (Figure 1E and Table 2). No differences in the number of adenoma were observed across group (data not shown).

Combined use of vitamin D3 and metformin increased the serum level of insulin like growth factor binding protein 3 (IGFBP-3)—To evaluate the effects of vitamin D3 and metformin on insulin signaling pathway, we measured the serum levels of glucose, insulin, IGF-1, IGFBP-1, IGFBP-3 (Figure 2A, B, C, D, E, raw data were shown in Supplemental Table 7–11), and colonic tissue phosphorylation of Akt in mice (Figure 2F, raw data were shown in Supplemental Table 12). DMH+DSS treated mice showed significant decreases in serum levels of insulin, IGF-1 and IGFBP3 as compared to the untreated mice (Figures 2A, B, C, E). Compared with the DMH+DSS control group, vitamin D3 or metformin treated mice showed no differences in serum levels of glucose level, serum concentrations of insulin, IGF-1 or IGFBP-1 (Figures 2A, B, C, D). There was, however, a significant elevation of serum IGFBP-3 in the combination group (Figure 2E). Phosphorylation of Akt was significantly reduced in the metformin group, but it was not further decreased in the combination group (Figure 2F,).

Combined use of vitamin D3 and metformin inhibited mTOR/S6P pathway via enhanced activation of AMPK—We further analyzed the effects of vitamin D3 and metformin alone or in combination on the key proteins of the AMPK/mTOR pathway (Figure 3A, raw data were shown in Supplemental Table 12). Compared with the normal untreated group, the phosphorylation of AMPK in DMH+DSS treated control group was decreased, while the phosphorylation of its downstream targets, including mTOR, P70S6K and S6P were significantly increased, indicating the activation of this pathway in the mouse colitis-associated neoplasia model. Compared with the DMH+DSS treated control group, the phosphorylation of AMPK was significantly increased in the metformin group, and it was further enhanced in the combination metformin and vitamin D3 group (Figure 3B). No significant differences in the phosphorylation of mTOR and P70S6K were observed in the vitamin D3 or metformin groups. However, the phosphorylations of mTOR and P70S6K were both decreased significantly in the combination group (Figure 3C, D). Furthermore, the

phosphorylation of S6P was significantly decreased in both the vitamin D3 group and the metformin group, and this effect was further enhanced in the combination group (Figure 3E).

Metformin enhanced vitamin D3's chemopreventive effects targeting VDR/β-catenin pathway—To study the effects of vitamin D3 and metformin, the expression of key proteins in VDR/ β-catenin pathway was further investigated by SDS PAGE and Western blot (Figure 4A, raw data were shown in Supplemental Table 13). Compared with the untreated control group, the expression of VDR in the DMH+DSS treated group decreased, while the protein expressions of β-catenin, c-Myc and Cylin D1 increased significantly, demonstrating reduced VDR/β-catenin signaling in the DMH+DSS induced colon neoplasia mouse model.

Compared with the DMH+DSS treated group, the expression of CYP27B1 increased significantly in the vitamin D3 treated groups (Figure 4B), and the expression of VDR increased significantly in both the vitamin D3 group and the metformin group, which was further enhanced in the combination group (Figure 4C). Both vitamin D3 and metformin significantly decreased the protein expression of β- catenin and c-Myc and they were even further decreased by the combined use of vitamin D3 and metformin (Figure 4D, E). Surprisingly, although numerous studies have shown that metformin decreases the expression of Cyclin D1 in various cancer cells (34, 35), our results showed that metformin alone significantly increased the protein expression of Cyclin D1 in DMH+DSS induced colitis-associated colorectal tumors. Nevertheless, the protein expression of Cyclin D1 was significantly decreased in the combination group in the colon tumor tissues, which needs to be further investigated in future studies (Figure 4F).

Discussion

Vitamin D3 and metformin both have been shown to have chempreventive effects against colorectal neoplasia in previous studies (15, 16, 27, 28). Employing either a DMH induced colon carcinogenesis rat model or a DMH+DSS induced colitis-associated colon neoplasia mouse model, our present study demonstrates that combined use of vitamin D3 and metformin was more effective than each agent alone in reducing colorectal tumor formation either in a DMH induced colon carcinogenesis rat model or in a DMH+DSS induced colitis-associated colon neoplasia mouse model.

Metformin is a widely used anti-hyperglycemic drug characterized as an insulin sensitizer in reducing hepatic insulin resistance. We measured the effects of metformin alone or in combination with vitamin D3 on circulating glucose and insulin to determine its impact on insulin signaling. We founded that metformin and vitamin D3 used alone or in combination have no effect on the circulating levels of glucose or insulin in the colitis-associated colon neoplasia mouse model. Our results are consistent with previously reported studies that metformin's anti-tumor effect is not always correlated with decreased insulin resistance in azoxymethane-induced colorectal tumor mouse model and in the $APC^{min/+}$ mouse model (10, 11). Insulin-like growth factor I (IGF-I) shares a common receptor IR/IGF-1R with insulin, and has been associated with increased risks of colorectal and other cancers (36).

IGF binding proteins (IGFBPs) attenuate the carcinogenic effects of IGF- I by binding to IGF- I, thus limiting the binding of free fraction IGF- I to IR/IGF-1R (37). Some IGFBPs, especially IGFPB-3, have also been shown to inhibit proliferation and induce apoptosis of several cancer cells independent of IGF-I (38, 39). In the present study, we found that metformin alone or combined use with vitamin D3 did not affect serum levels of IGF-1 and IGFBP-1 in the experimental mice. However, their combined use significantly increased the serum concentration of IGFBP-3, suggesting that the combined chemopreventive effects of metformin and vitamin D3 may in part be mediated by IGFBP-3, but independent of IGF- I. We also observed that metformin alone inhibited the phosphorylation of Akt, but this inhibitory effect was not enhanced by concurrently treating with vitamin D3 treatment, indicating that vitamin D3 and metformin have no synergistic effect on Akt phosphorylation.

To further examine the indirect chemopreventive effects of metformin that might be enhanced by vitamin D3, we analyzed the activities of the key proteins of the AMPK/mTOR signaling pathway in mouse colorectal tumor tissue. Our data revealed that the combined use of vitamin D3 and metformin enhanced the phosphorylation of AMPK, resulting in the inhibition of mTOR, P70S6K and S6P protein activation. These results suggest that potentiating AMPK-dependent inhibition of mTOR signaling is one possible mechanism underlying the enhancing effect of vitamin D3 on metformin's chemoprevention action.

1, 25-dihydroxyvitamin D3 (1, 25(OH)2D3), the active metabolite of vitamin D3, is synthesized by the enzyme, 25-hydroxyvitamin D3 1-α-hydroxylase (CYP27B1) (40), and is degraded by the enzyme 25-hydroxyvitamin D(3) 24-hydroxylase (CYP24A1) (41). 1, 25(OH)2D3 signals through the VDR, and then interferes with several other signaling pathways, which may partially mediate its anti-tumor effects. In the present study, we found that the expressions of CYP27B1 and VDR in colorectal tumors were decreased in DMH-DSS treated mice, and treatment with vitamin D3 (200 IU/kg) increased the expression of both. Combined use of vitamin D3 and metformin further increased the expressions of VDR and CYP 27B1. These results suggest that metformin enhances the chemopreventive effects of vitamin D3 by inhibiting its degradation and promoting its synthesis and binding to VDR. Wnt/β-catenin signaling pathway is aberrantly activated in nearly all colon neoplasia, leading to the disassociation of β-catenin from cell membrane, and its migration into nucleus (26, 42–44). Acting as a transcriptional co-activator of T-cell factor/lymphoid enhancer factor (TCF/LEF), β-catenin in the nucleus can lead to uncontrolled cell proliferation by increasing the expression of certain genes such as c-myc and cyclin D1. These genes have been previously shown to be highly expressed in colon cancer cells and the adenomas of $APC^{Min/+}$ mice (45–47). In this study, protein expression of β-catenin and c-Myc in colorectal tumors were reduced by vitamin D3 alone and were further decreased by combined use of vitamin D3 and metformin. Although Cyclin D1 was increased in DMH-DSS induced tumors treated with either vitamin D3 or metformin alone its protein expression was significantly reduced by combined use of vitamin D3 and metformin were combined. These results suggest that metformin may enhance the chemopreventive effects of vitamin D3 by reducing the protein expression of Cyclin D1, the downstream target of the VDR/β-catenin pathway.

In conclusion, our studies demonstrate that the combined use of vitamin D3 and metformin significantly reduces the development of colorectal neoplasia in two distinct colorectal carcinogenesis models. In the colitis –associated colorectal neoplasia mouse model, vitamin D3 enhanced the chemopreventive effects of metformin by the phosphorylation of AMPK, resulting in inhibition of the mTOR/S6P signaling pathway. Metformin in turn can also enhance the chemopreventive effects of vitamin D3 by targeting the VDR/β-catenin pathway and subsequently down-regulating Cyclin D1 synthesis. Vitamin D3 and metformin are already widely used in humans as a nutritional supplement and anti-diabetic drug respectively; therefore their combined use might be a safe and promising strategy for the chemoprevention of CRC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work is partially supported by the National Cancer Institute grants (R01CA136726, U01CA181770 to L. Li; and P50CA50964 to L. Li and N. A. Berger)

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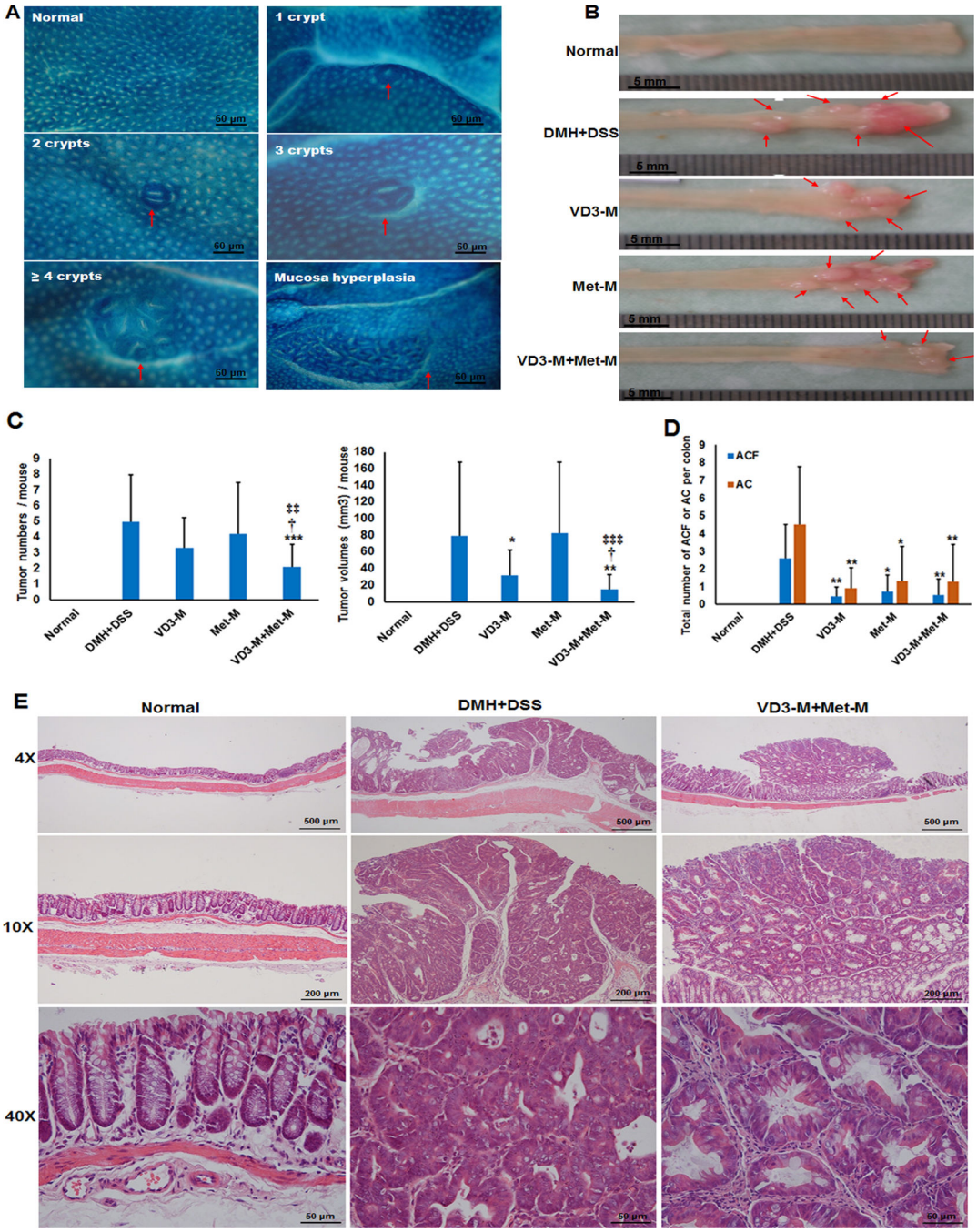

Figure 1. Effects of vitamin D3 and/or metformin on colon tumor formation

(A) Representative aberrant crypt foci (ACF) of rat colon (scale bar = 60 μm). **(B)** Macroscopic view of large bowels of mice. **(C)** Tumor numbers and tumor volumes of mice. Data were means ± SD, n= 20–25 per group. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. DMH +DSS group; †*P*<0.05 vs. VD3-M group; ⌗ *P*<0.01, ⌗⌗*P*<0.001 vs. Met-M group. **(D)** Total numbers of ACF and ACF of the mice. Data were means ± SD, n=10–12 per group. **P*<0.05, ***P*<0.05 *vs*. DMH+DSS group. **(E)** Representative H&E images of colorectal neoplasms developed in mice (4×, scale bar = 500 μm; 10×, scale bar = 200 μm; 40×, scale

bar = 50 μm). Normal colon tissues in normal group (left panel). The epithelial surface was smooth without villi. The mucosa was consisted of simple columnar epithelium made up of absorptive cells and columnar cells. In laminae propria, there were a number of crypts of bowel glands in a single tube containing numerous absorptive cells and goblet cells. Tubular adenoma with non-invasive adenocarcinoma in the DMH+DSS group (middle panel). The higher cell density and rich cytoplasmic protein resulted in a lower transmittance. The normal tubular structure was lost. The glands were arranged irregularly and branching with common wall glands, bridge-like growth, and sieve-like structure formation in glandular epithelium without mucus secretion. The cells were enlarged, multilayer and polarity disappeared. Their nuclei were irregular with obvious nucleolus and more frequent karyokinetic phenomena. VD3-M+Met-M group with low-grade adenoma (right panel) showing tumor with remaining tubular structure. The number of glands increased with marked variation in size and shape. The nuclei were rod-shaped and moving away from the cell base area and display like pseudostratified epithelium. VD3-M, vitamin D3 200 IU/kg; Met-M, metformin 240 mg/kg; VD3-M+Met-M, vitamin D3 200 IU/kg plus metformin 240 mg/kg; DMH, 1, 2-dimethyl-hydrazine; DSS, dextran sodium sulfate.

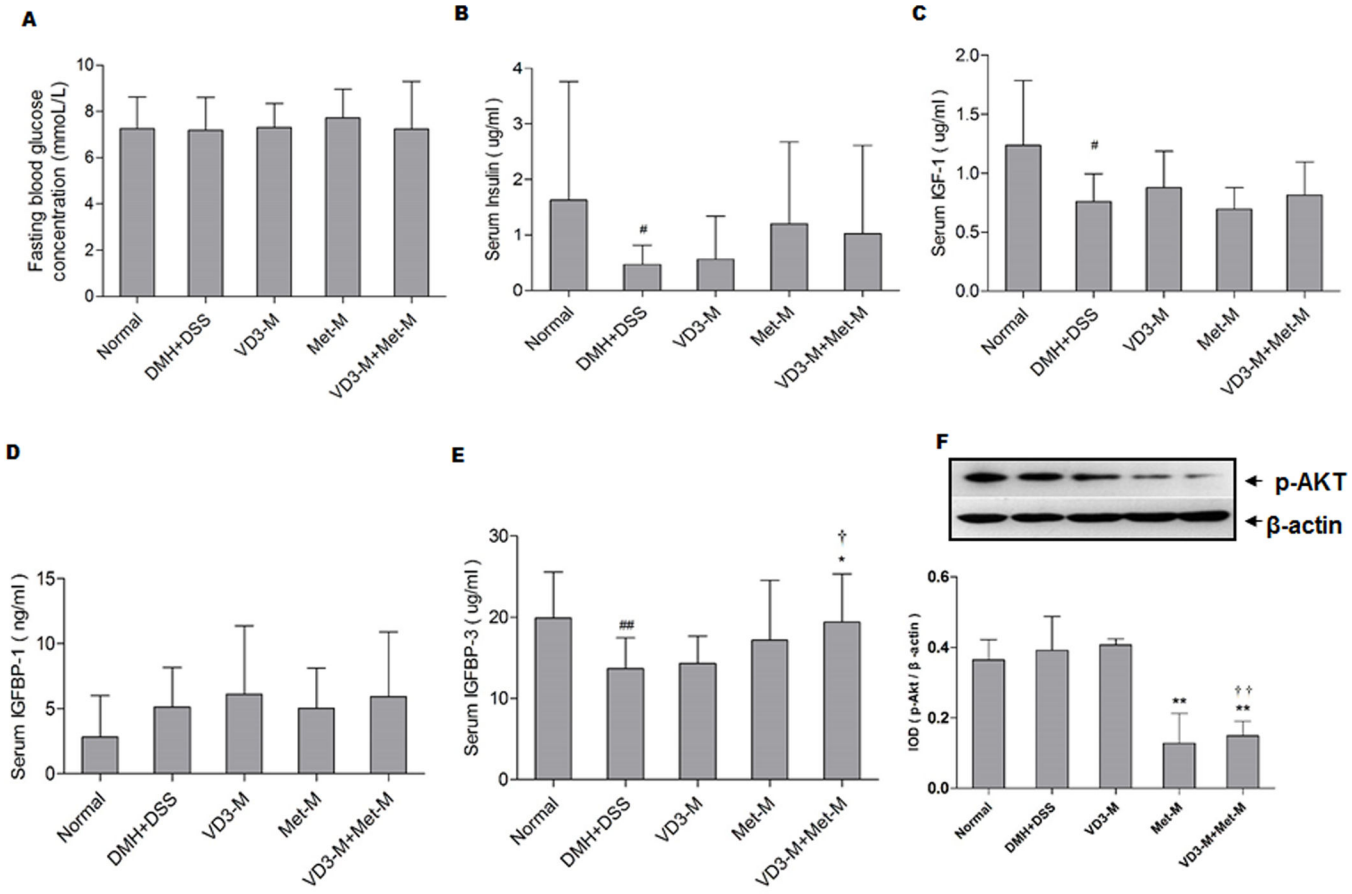

Figure 2. Effects of vitamin D3 and/or metformin on IGF/AKT pathway in mouse model of colorectal neolpasia

(A–E) Serum concentration of blood glucose, serum insulin, IGF-1, IGFBP-1and IGFBP-3 were revealed by ELISA. **(F)** Western blot analysis for phosphorylated AMPK and β-actin. Data were mean ± SD. A and C-E, n=10–12 per group; B, n=12–15 per group; f, n=3 per group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs*. normal group; $^{*}P<0.05$, $^{**}P<0.01$ *vs*. DMH+DSS group; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ *vs*. VD3-M group. VD3-M, vitamin D3 200 IU/kg; Met-M, metformin 240 mg/kg; VD3-M+Met-M, vitamin D3 200 IU/kg plus metformin 240 mg/kg; DMH, 1, 2-dimethyl-hydrazine; DSS, dextran sodium sulfate.

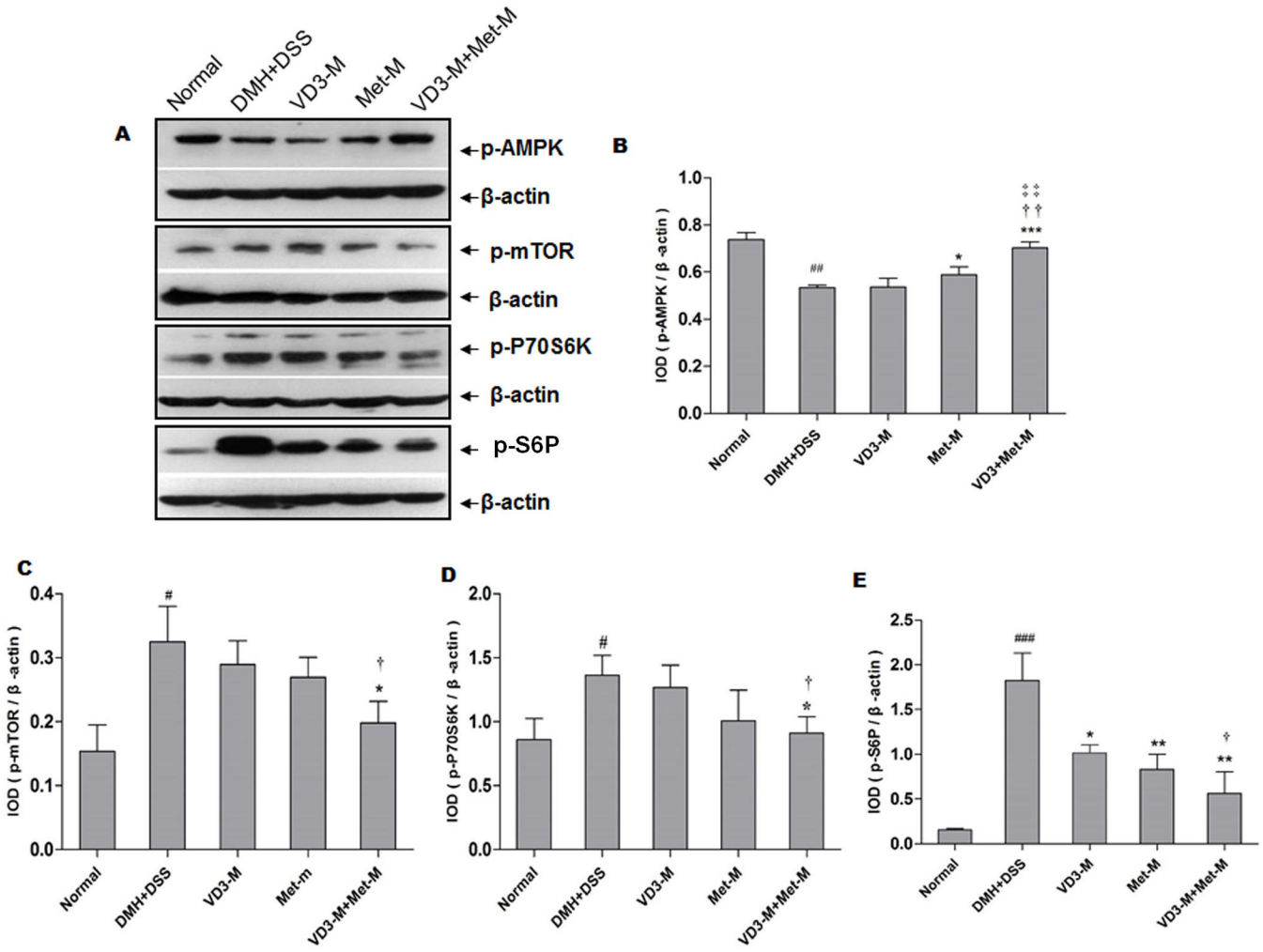

Figure 3. Vitamin D3 enhanced chemopreventive effects of metformin on mouse colorectal tumors via AMPK/mTOR/S6P pathway

(A) Western blot analysis for phosphorylated AMPK, mTOR, P70S6K, S6P and β-actin in colorectal tumor tissue from mice in each group. **(B–E)** Graph showing the ratios of phosphorylated protein to β-actin. IOD represents intergrated optical density. Data were means ± SD, n=3 per group. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs*. Normal group; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs*. DMH+DSS group; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ *vs*. VD3-M group; $^{\ddagger\ddagger}P<0.01$ *vs*. Met-M group. VD3-M, vitamin D3 200 IU/kg; Met-M, metformin 240 mg/kg; VD3-M+Met-M, vitamin D3 200 IU/kg plus metformin 240 mg/kg; DMH, 1, 2-dimethyl-hydrazine; DSS, dextran sodium sulfate.

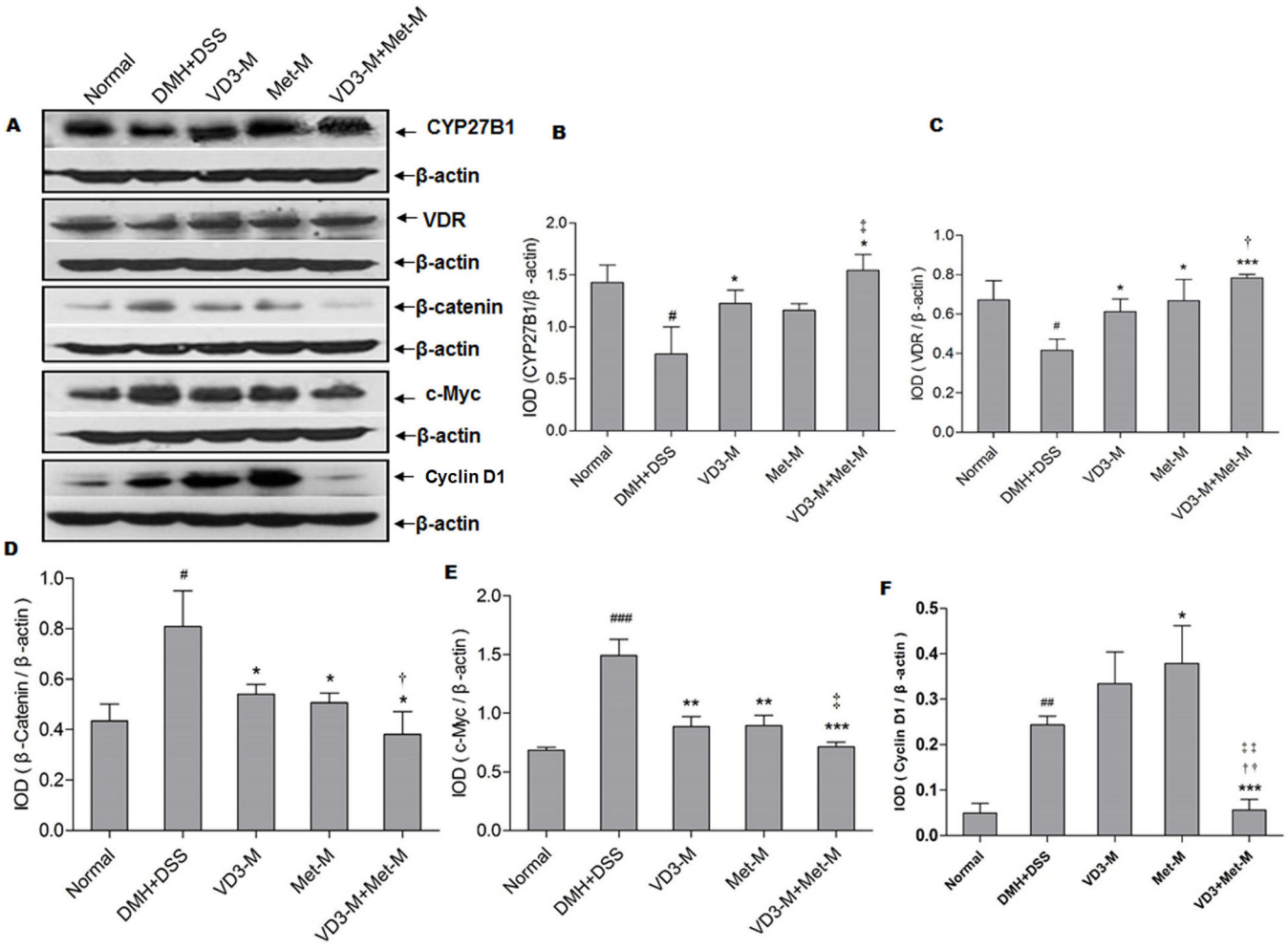

Figure 4. Metformin enhanced chemopreventive effects of vitamin D3 on mouse colorectal tumors via Wnt/β-catenin pathway

(A) Western blot analysis for the expression of CYP27B1, VDR, β-catenin, c-Myc and Cyclin D1 in colorectal tumor tissue from mice in each group. **(B–F)** Graph showing the ratios of the protein to β-actin. IOD represents intergrated optical density. Data were means ± SD, n=3 per group. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs*. normal group; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs*. DMH+DSS group; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ *vs*. VD3-M group; $^{\ddagger\ddagger}P<0.01$ *vs*. Met-M group. VD3-M, vitamin D3 200 IU/kg; Met-M, metformin 240 mg/kg; VD3-M+Met-M, vitamin D3 200 IU/kg plus metformin 240 mg/kg; DMH, 1, 2-dimethyl-hydrazine; DSS, dextran sodium sulfate.

Table 1

The number of tumor, AC and ACF per colon in different groups of rats

Groups	Total number of tumors/colon	Total number of AC/colon	Total number of ACF/colon
1. Normal	0.0±0.0	0.00±0.00	0.00±0.00
2. DMH control	2.8±0.8	242.90±31.64	141.3±15.2
3. VD3-L dose (30 IU/kg)	2.3±0.5	219.70±25.26***	125.6±12.18*
4. VD3-M dose (100 IU/kg)	1.7±0.7**	178.50±22.11***	104.7±14.3**
5. VD3-H dose (300 IU/kg)	1.4±0.8**	145.50±10.11***	91.5±7.2**
6. Met-L dose (40 mg/kg)	2.0±0.7*	189.90±21.35***	110.3±13.0**
7. Met-M dose (120 mg/kg)	1.8±0.4**	165.60±20.18***	98.3±11.7**
8. Met-H dose (360 mg/kg)	1.6±0.5**	139.90±22.43***	89.0±12.7**
9. VD3-M + Met-L dose (100 IU/kg+40 mg/kg)	1.5±0.5**	151.70±10.19***	93.4±6.9**
10. VD3-L + Met-M dose (30 IU/kg+120 mg/kg)	1.6±0.5**	148.60±20.98***	90.9±9.7**
11. VD3-M + Met-M dose (100 IU/kg+120 mg/kg)	0.9±0.7**†‡‡	118.10±8.58***†††‡‡‡	71.7±5.0***†††‡‡‡

[a]. DMH, 1,2-dimethyl-hydrazine; VD3, vitamin D3; Met, metformin; L, low; M, medium; H, high.

[b]. All data were expressed as mean± SD, n=10 per group.

[c]. *P<0.05, **P<0.01, ***P<0.001 vs. DMH control group; †P<0.05, †††P<0.001 vs. VD3-M group; ‡‡P<0.01, ‡‡‡P<0.001 *vs*. Met-M group.

[d]. AC = aberrant crypt; ACF = aberrant crypt foci.

Table 2

The incidence of non-invasive adenocarcinoma in mouse colorectal neoplasia model

Groups	N	n	Incidence
1. Normal	12	0	0%
2. DMH+DSS control	10	4	40%
3. VD3-M dose (200 IU/kg)	9	1	11%
4. Met-M dose (240 mg/kg)	9	1	11%
5. VD3-M+Met-M dose (200 IU/kg+240 mg/kg)	11	0	0%*

[a]. DMH, 1,2-dimethyl-hydrazine; DSS, dextran sodium sulfate; VD3, vitamin D3; Met, metformin; M, medium.

[b]. * $P<0.05$, vs. the control group.

[c]. N: total number of mice; n: number of mice having non-invasive adenocarcinoma. Incidence: (n/N)*100%.