Combined Use of Vitamin D3 and 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 antidiabetic 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 with 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/d) and metformin medium dose (120 mg/kg/d) combination group. Furthermore, our results showed that enhancement of metformin’s chemopreventive effects by vitamin D3 was associated with downregulation of S6P expression, via the AMPK (IGF)/mTOR pathway. In addition, 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 the 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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Introduction

Colorectal cancer 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, such as ulcerative colitis, and Crohn’s disease, are at increased risk for developing colorectal cancer (2, 3). The incidence of colorectal cancer in colitis patients is nearly 40% within 30 years after the onset of ulcerative colitis (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 antihyperglycemic drug, widely used for the treatment of type II diabetes, is emerging as a potential anti-tumor agent. Several epidemiologic studies reported decreased cancer incidence, including colorectal cancer, in patients with type II 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). An in vitro study has also demonstrated growth-inhibitory effects of metformin in colon cancer cells via activating the 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 antineoplastic actions. The direct effects of metformin are mainly mediated through activation of AMPK, which further leads to the inhibition of 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-I hybrid receptors (IR/IGFIR), a receptor tyrosine kinase, thereby reducing the activation of PI3K/AKT/mTOR signaling in cancer cells (17, 18).

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Vitamin D3 is synthesized from its precursor 7-dehydrocholesterol in the skin upon exposure to UV irradiation or obtained via diet. The active form of vitamin D, 1,25-dihydroxyvitamin D3 (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 colorectal cancer (21), a large number of experimental and epidemiologic 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 biologic 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 antitumor 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-dimethylhydrazine (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 to 120 g and male ICR (CD-1) mice ages 5 weeks (Beijing Vital River Laboratory Animal Technological Company) were used in this study. All animals were housed in plastic cages (temperature 22°C, 10%, 12-hour light/dark cycle) with free access to drinking water and a pelleted basal diet (Chengdu Dashuo Biotechnology Co. Ltd.). All animal experiments were conducted according to the principles of 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 (Lanzhou, China; 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 PBS for 5 to 10 minutes at room temperature. Excess dye was flushed thoroughly with ice-cold 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 to 10 minutes 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 ACs, were counted under an observation microscope.

Experimental procedure

DMH induced colon neoplasia in rats. One hundred and ten rats were randomly divided into two control groups and nine experimental groups (Supplementary Table S1). All the animals except those in the normal group received 30 mg/kg body weight DMH (Tokyo Kasei Kogyo) by intraperitoneal injections once a week for 18 weeks. Vitamin D3 (Shanghai General Pharmaceutical Co. Ltd.) and/or metformin hydrochloride (Zhengzhou Lion Biotechnical Technology Co. Ltd.) 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 Supplementary Table S1.

DMH and 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 two control groups and three experimental groups (Supplementary Table S2). Groups 2 to 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 to 5 received 2% (W/V) DSS (molecular weight 36,000–50,000; MP Biomedicals) in drinking water for 7 days. Group 1 received no treatment. Vitamin D3 (Zhejiang Garden Biochemical High-tech Co. Ltd.) or/and metformin hydrochloride (Qufu Maidesen Fine Chemical Co. Ltd.) was orally administered once daily from 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 Supplementary Table S2.

The experiment protocols were shown in Supplementary Fig. S1. All the animals were carefully observed for clinical welfare and weighed weekly throughout the experimental period.

Counting of tumor and 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 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 to 10 minutes 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 ACs, 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:

$$V = \frac{a \times b^2}{2}$$

Where a is the length (mm), b is the width (mm), and V is the volume (mm³). The volume of all tumors from each mouse were added to give the overall tumor burden per animal.

Pathologic evaluation in mice

After ACF evaluation, the colons with tumors were embedded in paraffin and histologic evaluation was carried out on
hematoxylin & eosin (H&E)-stained colon sections. Colon tumors were classified as adenomas, noninvasive (i.e., carcinoma in situ) or invasive adenocarcinomas, as previously described (31). Photomicrographs were taken with equal exposure on Motic microscope (Motic DMBS-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).

ELISA determination of Insulin, IGF, IGFBP-1 and IGFBP-3 levels in mouse serum
Before mice were sacrificed, blood was collected from retro-orbital sinus, centrifuged at 1,200×g for 10 minutes at 4°C, and serum was stored at -80°C until assay. ELISA kits were used for measurement of serum concentrations of insulin (Merodia), IGF (RayBiotech, Inc.), IGF-binding protein (IGFBP)-1 (Genway Biotech Inc.) and IGFBP-3 (RayBiotech, Inc.) 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) at 4°C for 30 minutes. The lysates were cleared by centrifugation at 12,000×g for 15 minutes at 4°C and assayed for total protein concentration by using BCA Kit (Beyotime). Cleared lysates (50 μg for p-Akt, 75 μg for p-AMPK, and 100 μg 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 polyvinylidene difluoride membrane (Millipore). 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 (2971), p-AMPK (2535), p-mTOR (2971), p-70S6K (2590), p-4E-BP1 (2555), p-AMPKα (2535), p-p70S6K (2920), p-S6K1 (2922), p-4E-BP1 (2590). 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) and quantified by integral optical density (IOD) using Image-Pro Plus Software. The data were 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 analysis) between groups were examined for statistical significance using one-way ANOVA. The incidence of colorectal noninvasive adenocarcinomas was compared by Fisher exact test. Statistical significance was set at α = 0.05 (two sided).

Results
DMH induced colon neoplasia in rats
General observation. Compared with the 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/d) group, whereas it was significantly decreased in the metformin high-dose (360 mg/kg/d) group, likely due to the well-known 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 the DMH control group (Supplementary Table S3). 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 (Supplementary Table S3). 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).

Table 1. The number of tumor, AC, and ACF per colon in different groups of rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total number of tumors/colon</th>
<th>Total number of AC/colon</th>
<th>Total number of ACF/colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Normal</td>
<td>0.0 ± 0.0</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>2. DMH control</td>
<td>2.8 ± 0.8</td>
<td>242.90 ± 31.64</td>
<td>141.3 ± 15.2</td>
</tr>
<tr>
<td>3. VD3-L dose (30 IU/kg)</td>
<td>2.3 ± 0.5</td>
<td>219.70 ± 25.26</td>
<td>125.6 ± 12.18</td>
</tr>
<tr>
<td>4. VD3-M dose (100 IU/kg)</td>
<td>1.7 ± 0.7b</td>
<td>178.50 ± 22.18</td>
<td>104.7 ± 14.3b</td>
</tr>
<tr>
<td>5. VD3-H dose (300 IU/kg)</td>
<td>1.4 ± 0.8b</td>
<td>145.50 ± 10.11b</td>
<td>91.5 ± 7.2b</td>
</tr>
<tr>
<td>6. Met-L dose (40 mg/kg)</td>
<td>2.0 ± 0.7*</td>
<td>189.90 ± 21.35b</td>
<td>110.3 ± 13.0b</td>
</tr>
<tr>
<td>7. Met-M dose (120 mg/kg)</td>
<td>1.8 ± 0.4b</td>
<td>165.60 ± 20.18</td>
<td>98.5 ± 11.7b</td>
</tr>
<tr>
<td>8. Met-H dose (360 mg/kg)</td>
<td>1.6 ± 0.5b</td>
<td>139.90 ± 22.43</td>
<td>89.0 ± 12.3b</td>
</tr>
<tr>
<td>9. VD3-M + Met-L dose (100 IU/kg + 40 mg/kg)</td>
<td>1.5 ± 0.5b</td>
<td>151.70 ± 10.9b</td>
<td>93.4 ± 6.9b</td>
</tr>
<tr>
<td>10. VD3-L + Met-M dose (30 IU/kg + 120 mg/kg)</td>
<td>1.6 ± 0.5b</td>
<td>148.60 ± 20.98b</td>
<td>90.9 ± 9.7p</td>
</tr>
<tr>
<td>11. VD3-M + Met-M dose (100 IU/kg + 120 mg/kg)</td>
<td>0.9 ± 0.2bc</td>
<td>112.70 ± 8.58bc</td>
<td>71.7 ± 5.014b</td>
</tr>
</tbody>
</table>

NOTE: All data were expressed as mean ± SD, n = 10 per group. Abbreviations: H, high; Met, metformin; L, low; M, medium; VD3, vitamin D3. *p < 0.05, **p < 0.01, and ***p < 0.001 versus D3 control group. $p < 0.05 and $p < 0.01 versus DMH control group. $p < 0.05 and $p < 0.01 versus VD3-M group. $p < 0.01 and $p < 0.001 versus Met-M group.
Figure 1.
Effects of vitamin D3 and/or metformin on colon tumor formation. A, representative 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 mean ± SD, n = 20–25 per group. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus DMH + DSS group; †, P < 0.05 versus VD3-M group; ††, P < 0.01; †††, P < 0.001 versus Met-M group. D, total numbers of ACF and ACF of the mice. (Continued on the following page.)
ACF analysis. Multiple ACF (Fig. 1A) was 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 (Supplementary Table S4) 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 (Supplementary Table S4). As shown in Fig. 1B and 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 Supplementary Table S5).

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 the 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 (Fig. 1D, raw data were shown in Supplementary Table S6). However, when compared with either vitamin D3 or metformin treatment alone, the combination treated showed no difference (Fig. 1D).

Histopathological analyses. Histopathological analyses at the end of 20 weeks revealed that all tumors in mice were adenomas or noninvasive adenocarcinomas with the lesion limited to the mucosa. No invasive adenocarcinomas beyond the mucosa were observed (Fig. 1E). Compared with the DMH-DSS control group, the incidence of noninvasive 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 (Fig. 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. To evaluate the effects of vitamin D3 and metformin on insulin signaling pathway, we measured the serum levels of glucose, insulin, IGFI, IGFBP-1, IGFBP-3 (Fig. 2A–E, raw data were shown in Supplementary Tables S7–S11), and colonic tissue phosphorylation of Akt in mice (Fig. 2F, raw data were shown in Supplementary Table S12). DMH-DSS-treated mice showed significant decreases in serum levels of insulin, IGFI, and IGFBP3 as compared with the untreated mice (Fig. 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, IGFI, or IGFBP-1 (Fig. 2A–D). There was, however, a significant elevation of serum IGFBP-3 in the combination group (Fig. 2E). Phosphorylation of Akt was significantly reduced in the metformin group, but it was not further decreased in the combination group (Fig. 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 (Fig. 3A, raw data were shown in Supplementary Table S12). Compared with the normal untreated group, the phosphorylation of AMPK in the DMH-DSS-treated control group was decreased, whereas the phosphorylation of its downstream targets, including mTOR, P70S6K, and S6P, was 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 (Fig. 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 (Fig. 3C and 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 (Fig. 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 the VDR/β-catenin pathway was further investigated by SDS/PAGE and Western blot analysis (Fig. 4A, raw data were shown in Supplementary Table S13). Compared with the untreated control group, the expression of VDR in the DMHþDSS-treated group decreased, whereas the protein expressions of β-catenin, c-Myc, and Cyclin D1 increased significantly, demonstrating reduced VDR/β-catenin signaling in the DMHþDSS-induced colon neoplasia mouse model.

Compared with the DMHþDSS-treated group, the expression of CYP27B1 increased significantly in the vitamin D3-treated groups (Fig. 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 (Fig. 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 (Fig. 4D and 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 DMHþDSS-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 (Fig. 4F).

Discussion

Vitamin D3 and metformin both have been shown to have chemopreventive effects against colorectal neoplasia in previous studies (15, 16, 27, 28). Using either a DMH-induced colon carcinogenesis rat model or a DMHþDSS-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 DMHþDSS-induced colitis-associated colon neoplasia mouse model.

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**Table 2. The incidence of noninvasive adenocarcinoma in a mouse colorectal neoplasia model**

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>n</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Normal</td>
<td>12</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>2. DMH + DSS control</td>
<td>10</td>
<td>4</td>
<td>40%</td>
</tr>
<tr>
<td>3. VD3-M dose (200 IU/kg)</td>
<td>9</td>
<td>1</td>
<td>11%</td>
</tr>
<tr>
<td>4. Met-M dose (240 mg/kg)</td>
<td>9</td>
<td>1</td>
<td>11%</td>
</tr>
<tr>
<td>5. VD3-M + Met-M dose (200 IU/kg + 240 mg/kg)</td>
<td>11</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

NOTE: Incidence: (n/N) × 100.
Abbreviations: Met, metformin; M, medium; N, total number of mice; n, number of mice having noninvasive adenocarcinoma.

*P < 0.05 versus the control group.

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![Figure 2](image-url)
Metformin is a widely used antihyperglycemic 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 found 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 antitumor effect is not always correlated with decreased insulin resistance in an azoxymethane-induced colorectal tumor mouse model (10, 11).

IGFI shares a common receptor IR/IGFIR with insulin, and has been associated with increased risks of colorectal and other cancers (36). IGFBPs attenuate the carcinogenic effects of IGFI by binding to IGFI, thus limiting the binding of free fraction IGFI to IR/IGFIR (37). Some IGFBPs, especially IGFBP-3, have also been shown to inhibit proliferation and induce apoptosis of several cancer cells independent of IGFI (38, 39). In the present study, we found that metformin alone or combined use with vitamin D3 did not affect serum levels of IGFI 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 IGFI. 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(OH)2D3, the active metabolite of vitamin D3, is synthesized by the enzyme 25-hydroxyvitamin D3 1α-hydroxylase (CYP27B1; ref. 40), and is degraded by the enzyme 25-hydroxyvitamin D(3) 24-hydroxylase (CYP24A1; ref. 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 CYP27B1. These results suggest that metformin enhances the chemopreventive effects of vitamin D3 by inhibiting its degradation and promoting its synthesis and binding to VDR. The 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 coactivator of T-cell factor/lymphoid enhancer factor, 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 APCMin/þ mice (45–47). In this study, protein expression of β-catenin and c-Myc in colorectal tumors was reduced by vitamin D3 alone and was 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. 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 antidiabetic drug, respectively; therefore their combined use might be a safe and promising strategy for the chemoprevention of colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Li, Q.-L. Wang, X. Liu, S.-H. Dong, H.-X. Li, N.A. Berger, L. Li

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