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Metformin suppresses CRC growth by inducing apoptosis via ADORA1

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TABLE OF CONTENTS

1. ABSTRACT

Accumulating evidence suggests that the anti-diabetic drug, metformin, exerts anti-proliferative effects in many types of cancers. However, the function and mechanisms of metformin in human colorectal cancer (CRC) remain unknown. Here, we show that metformin induces growth inhibition and apoptosis through activating AMPK-mTOR pathway in human colorectal cancer cells. Notably, metformin treatment significantly up-regulated adenosine A1 receptor (ADORA1) expression in human colorectal cancer cells, while suppression of ADORA1 activity by its specific inhibitor rescued the growth inhibition induced by metformin. Moreover, ADORA1-mediated growth inhibition and apoptosis induced by metformin is AMPK-mTOR pathway dependent in human colorectal cancer cells. Taken together, these results indicate that metformin suppresses human colorectal cancer growth by inducing apoptosis via ADORA1, which provide evidence the anti-neoplastic effects of metformin in the treatment of human colorectal cancer.

2. INTRODUCTION

Colorectal carcinoma (CRC) is the second leading cause of cancer-related death worldwide. Despite the advances in treatment including surgery, radiotherapy and chemotherapy, the prognosis for patients with invasive CRC remains dismal. Thus, it is of great significance to look for new therapeutic strategies.

Metformin, an oral hypoglycemic agent, is widely used in the treatment of type 2 diabetes for more than 30 years (1, 2). Recently, increasing evidence has shown that meformin possesses strong anti-cancer effects (3-9). Metformin can enter into cancer cells via

OCT1 or combine with the insulin receptor to suppress mTOR pathway through AMPK activation in several types of tumors, which is believed to constitute the important and well known mechanism underlying the anti-cancer activities of metformin (10-14). Recently, Klander A *et al* (13) reported that metformin, independent of AMPK signaling, can also act through Rag GTPase to inhibit mTOR pathway. Thus, these results indicate that a rather complicated mechanism involve in the anti-cancer effects of metformin. However, the functions and molecular mechanisms of metformin in CRC remain unclear.

Adenosine receptors are G-protein coupled receptors family members that are classified into A_1, A_{2A}, A_{2B} and A_3 receptors. Adenosine A1 receptor (ADORA1) is known to inhibit the adenylate cyclase to decrease the level of Cyclic adenosine monophosphate (cAMP) and play a role in regulating cell metabolism and gene transcription (15). ADORA1 has highly affinity with adenosine, which is involved in proinflammatory (16-18) and anti-inflammatory effect (19-22). Recent studies have shown that ADORA1 is over-expressed in many types of malignancies including colon cancer (23), breast cancer (24), leukemia (25) and melanoma (26). ADORA1 binding with adenosine leads to growth retard and apoptosis in various types of tumor cells and play an important role in carcinogenesis (27). Recently, Saito *et al* (28) found that ADORA1 activation by adenosine induced the apoptosis and inhibited proliferation of human colon cancer cells. However, it is unknown that whether the anti-proliferative effects of metformin occur through ADORA1-associated mechanisms in human colorectal cancer cells.

In the present study, we determined the function and mechanism of metfomin in human colorectal cancer cells. We find that metformin induces growth inhibition and cell apoptosis in human colorectal cancer cells through AMPK-mTOR pathway. Notably, ADORA1 expression is markedly up-regulated in the colorectal cancer cells treated with metformin, and inhibition of ADORA1 activity by its specific inhibitor rescues the growth inhibition induced by metformin. Moreover, ADORA1-mediated growth inhibition and apoptosis induced by metformin is through AMPK-mTOR pathway in human colorectal cancer cells. These data collectively indicate metformin suppresses human colorectal cancer growth by inducing apoptosis via adenosine A1 receptor, which provide evidence the anti-neoplastic effects of metformin in the treatment of human colorectal cancer.

3. MATERIAL AND METHODS

3.1. Cell lines and culture

Two human colorectal cancer cell lines (SW480 and HCT116) were purchased from Shanghai Institues for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivatde fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified cell incubator at 37ºC with an atmosphere of 5% CO_2. The cells were incubated with various concentrations metformin for 48h, 72h and 96h. To evaluate the effect of the metformin, the cells were incubated with 1.0. mM metformin for 48 h after seeding 12h.

3.2. Cell proliferation assay

The cell proliferation was assessed by WST (water-soluble tetrazolium salt) assay using the CCK-8 (Cell Counting Kit-8, Dojindo, Kumamoto, Japan) according to the manufacturer's-instruction. Briefly, cells were seeded at a density of 1×10^5 cells into 24-well plates. The cells were then treated with metformin, AMPK inhibitor or DPCPX. The plates were incubated for 48h, CCK-8 solution was added to each well. The number of viable cell was assessed by measurement of the absorbance at 450 nm with an Absorbance Microplate Reader (BioTek Instruments, USA). Cell survival for all experiments was expressed as the percentage of viable cells compared with the untreated cells.

3.3. Soft agar colony formation assay

The cells were treated with 1.0. mM metformin, then cells were resupended with 0.3.% soft agar in RPMI-1640 medium containing 10% FBS and layered onto 0.6.% solidified agar in RPMI-1640 medium containing 10% FBS in 6-well plates (density of 1×10^3 cells/well). The plates were incubated at 37ºC in a humidified atmosphere of 5% CO_2 for 10 days. Colonies containing at least 50 cells were counted.

3.4. Cell apoptosis analysis

Cells were seeded at a density of 1×10^5 cells into 6-well plates. The cells were then treated with 1.0. mM metformin or with 1.0. μM DPCPX for 48h. Each assay was performed in triplicate and repeated three times independently. Cell apoptosis rate was calculated by using Annexin V-FITC Apoptosis Detection kit I (BD pharmingen, USA), according to the manufacturer's protocol. Cells were harvested, washed in PBS and resuspend in 1×Bingding Buffer at concentration of 1×10^6 cells/ml, 5ul of Annexin V-FITC and 5ul of propidium iodide(PI) were added to 100ul cells suspension and incubated in dark for 15min. Then 400ul 1×Bingding Buffer were added. Finally, the apoptosis was analyzed by flow cytometry (BD FACSCalibur, USA) and Annexin V-FITC-positive were considered to be apoptosis.

3.5. Gene expression profiles chip analysis

Gene expression analysis was performed according to Affymetrix protocols, using the Affymetrix Gene Chip Human Genome U133 Plus 2.0. Array (Affymetrix, USA) and the kit for One-Cycle Target Labeling and Control Reagents. Briefly, we used a T7-oligo (dT) primer to produce double-stranded cDNA

from 5μl of total RNA. The cDNA was purified and biotin-labeled cRNA, and then transcription synthesized. Add the cRNA to the fragmentation buffer and heated for 35 min at 95°C. Hybridize the fragmented cRNA (15μg) with the Chip Array for 16 h at 45°C. Arrays were washed and stained with R-phycoerythrin streptavidin using the Gene Chip Fluidics Station 400. Finally, scanning the chip, and used the Affymetrix Microarray Suite (version 5.0.) to obtain the oligonucleotide hybridization data for gene expression value analysis.

3.6. Quantitative RT-PCR analysis

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA. USA) and concentration wes measured by spectrophotometer. Reverse transcription wes performed to synthesize cDNA using revorse transcription kit (Invitrogen, CA. USA). The reation was perfomed in an Applied Biosytems 7500 System with the mixture of cDNA, primers and Power SYBR Green PCR Master Mix(2×, applied Biosystems, Warrington, United Kingdom). Primers used included sequences 5′-CCACAGACCTACTTCCACACC-3′ (sense) and 5′-TACCGGAGAGGGATCTTGACC-3′ (antisense) for ADORA1, 5′-GGACCTGACCTGCCGTCTAG-3′ (sense) and 5′-GTAGCCCAGGATGCCCTTGA-3′ (antisense) for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Levels of RNA expression were determined using the 7500 Fast System SDS software version 1.3.1. (Applied Biosystems, USA) according to the $2^{-\Delta\Delta Ct}$ method. The results from 4-5 independent repeat assays, performed in different plates each using different cDNAs from the cultures analyzed, were averaged to produce a single mean quantity value for each mRNA.

3.7. Western blot analysis

RIPA Buffer containing protease inhibitor cocktail (Pierce, USA) was used to lyse the cells. BCA Protein Assay Kit (Pierce, USA) was used to determine protein concentration. An equal amount of protein (50ug) from each condition was subjected to 12.5.% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF (polyvinylidene fluoride) membranes. Then the membranes were blocked using TBST (TBS + 0.1.% Tween-20) containing 5% not-fat milk for 2h. Western blotting carried out using primary antibodies (mouse polyclonal anti-GAPDH antibody 1:5000 (Cell Signaling Technology, USA), rabbit monoclonal anti-p-AMPK (Thr172) antibody 1:1000 (Cell Signaling Technology, USA), rabbit monoclonal anti-p-mTOR (Ser 2448) antibody 1:1000 (Cell Signaling Technology, USA), rabbit monoclonal anti-ADORA1 antibody 1:5000 (Abcam Company, USA), rabbit polyclonal anti-Caspase3 antibody 1:500 (Santa Cruz Biotechnology, USA), rabbit polyclonal anti-Bcl2 antibody 1:500 (Santa Cruz Biotechnology, USA), rabbit polyclonal anti-Caspase9 antibody 1:1000 (NeoMarkers Fremont, USA) overnight at 4ºC. The membranes were then washed with TBST and incubated with fluorescence-labeled secondary antibody (1:20000) for 2h at room temperature. LI-COR Odyssey Infrared fuorescence scanner was use to capture the images.

3.8. Statistical analysis

Data analyses were carried out using SPSS V17.0. software with the Student′s t-test. Results are presented as mean ± SE. Statistical significance was determined at $p < 0.0.5$.

4. RESULTS

4.1. Metformin suppresses the proliferation of human colorectal cancer cells

To determine whether metformin could affect human colorectal cancer cell growth, SW480 and HCT116 cells were treated with different concentration of metformin for indicated time. As shown in Figure 1A and B, metformin suppressed the proliferation of SW480 and HCT116 cells in dose and time-dependent manner. The maximum inhibition was observed in 96 hour at a high concentration of 10.0. mM metformin, which suppressed cell proliferation by 85.3.6% and 63.0.0% in SW480 and HCT116 cells, respectively (*P<0.0.5). To further characterize the effect of metformin on human colorectal cancer cell growth, soft-agar colony formation assay was performed. We found that the number of colonies from metformin-treated SW480 cells was obviously less than untreated cells (Figure 1C,E), which the colonies number of metformin-treated group was only 55.0.% of untreated group (*P<0.0.5). Similar results were observed in the HCT116 cells (Figure 1D,E), which the colonies number of metformin-treated group was only 53.3.% of untreated group (*P<0.0.5). Thus, these results suggested that metformin suppresses the growth of human colorectal cancer cells.

4.2. Metformin induces apoptosis in human colorectal cancer cells

To elucidate the mechanism of metformin's growth inhibitory effects on human colorectal cancer cells, we analyzed cell apoptosis after treatment with metformin treatment. Both SW480 and HCT116 cells were treated with 1.0. mM metformin for 48h, and apoptosis of cells was analyzed by flow cytometry and western blot. As shown in Figure 2A, B and C, the apoptotic rate was significantly increased in both metformin-treated SW480 and HCT1160 cells (*P<0.0.5). In agree with the results of apoptosis analyzed by flow cytometry, we also found that the expression of Caspase-3 and Caspase-9 were up-regulated, while the expression of Bcl-2 was down-regulated after treatment with metformin by western blot (Figure 2D).

4.3. Metformin treatment up-regulates ADORA1 expression in human colorectal cancer cells

To define the mechanism underlying the anti-tumor prosperities of metformin in human colorectal

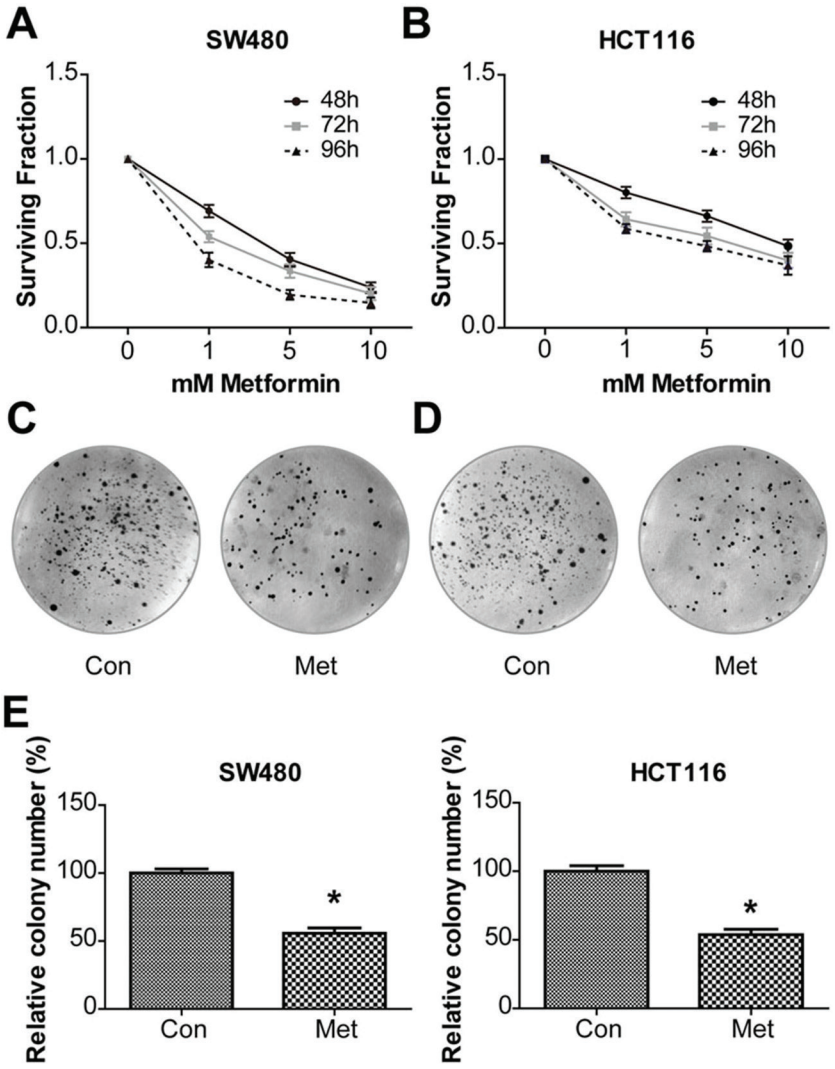

Figure 1. Metformin suppresses cell proliferation and colony formation of human colorectal cancer cells. (A)-(B) SW480 and HCT116 cells were incubated with 1.0. mM, 5.0. mM, 10.0. mM metformin for 48 h, 72 h, 96 h, and the cell proliferation was determined by CCK8 assay. (C)-(D) SW480 and HCT116 cells were incubated with 1.0. mM metformin after seeding 12h. The effect of metformin on cells proliferation was assessed by the colony formation assay on the 10th day after seeding. (E). Colonies containing 50 or more cells was counted. Data are representative of three independent experiments. *P < 0.0.5. Con, control; Met, metformin.

cancer, we performed gene expression profiling analysis in both SW480 and HCT116 cells treated with or without metformin. We analyzed the differentially expressed genes by Gene Ontology (GO) analysis, and found that ADORA1 expression was significantly up-regulated in human colorectal cancer cells after treatment with metformin (Table 1). To validate the upregulation of ADORA1 expression induced by metformin in human colorectal cancer cells, we then detected the mRNA and protein expression of ADORA1 by RT-PCR and Western blotting. As shown in Fig 3A, the mRNA expression of ADORA1 significantly increased in metformin-treated group compared with untreated group (*P<0.0.5), which was consistent with the results of microarray analysis.

Table 1. The fold change of ADORA1 expression in gene expression profiling analysis in metformin group compared with control group of human colonrectal cancer cell lines

Gene Symbol	Cell line	Met group (FC)
ADORA1	SW480	8.3.7451128
ADORA1	HCT116	7.5.9828065

Similarly, the increase in protein expression of ADORA1 was also confirmed by western blotting (Fig 3B). These results indicate that metformin treatment induces the increase of ADORA1 expression in human colorectal cancer cells.

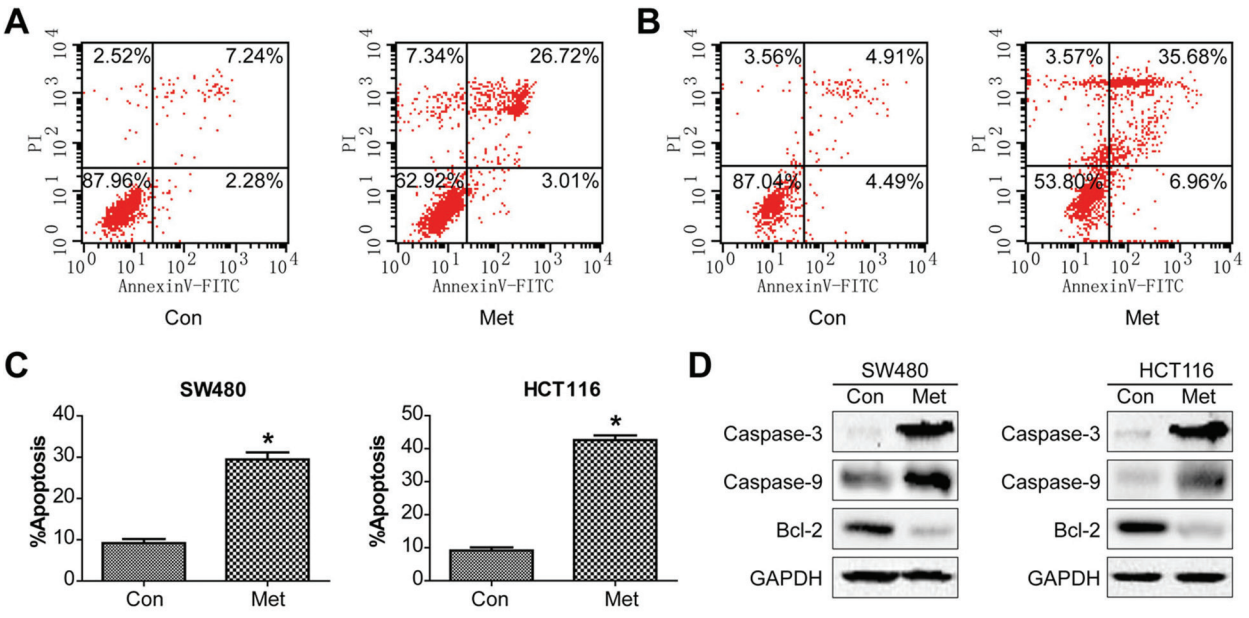

Figure 2. Metformin induces apoptosis in human colorectal cancer cells. (A)-(B) Representative histograms depicting apoptosis of SW480 and HCT116 cells treated with 1.0. mM metformin for 48 h. Cells staining positive for Annexin V-FITC (x-axis) were considered to be apoptosis. (C) The percentage of apoptotic cells staining positive for Annexin V are indicated. (D) Expression analyses of the Caspase-3, Caspase-9 and Bcl-2 protein in SW480 or HCT116 by treated with metformin for 48h. Data are representative of three independent experiments. *P < 0.0.5. Con, control; Met, metformin.

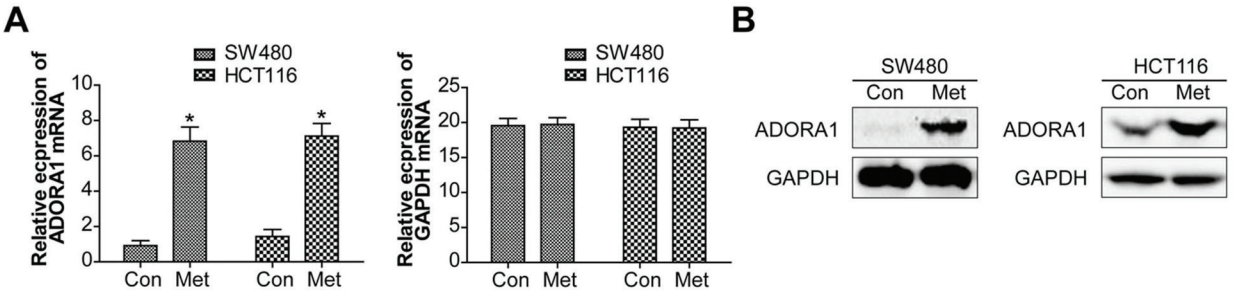

Figure 3. Metformin treatment up-regulates ADORA1 expression in human colorectal cancer cells. (A) qRT-PCR for ADORA1 mRNA was performed in SW480 and HCT116 cells treated with metformin for 48h after seeding 12h. (B) ADORA1 protein expression in SW480 and HCT116 treated with metformin was analyzed by western blot. Results shown are representative of three independent experiments. Con, control; Met, metformin.

4.4. Inhibition of ADORA1 rescues the growth inhibition induced by metformin in human colorectal cancer cells

To determine whether induction of ADORA1 is required for the growth-inhibition effects of metformin on human colorectal cancer cells, we exposed SW480 and HCT1160 cells with the combination of metformin and ADORA1 specific inhibitor DPCPX, and then analyzed cell colony formation and apoptosis. As expected, metformin suppressed the colony formation of both SW480 and HCT116 cells, however, the number of colonies in the presence of the combination of metformin and DPCPX was obviously more than that treated with metformin alone (fig 4A,B and C). Consistent with the results shown above, metformin induced the apoptosis of human colorectal cancer cells, however, addition of ADORA1 specific inhibitor DPCPX inhibited the apoptosis-promoting activity of metformin on both SW480 and HCT1160 cells (fig 4D, E and F). Take together, these results suggested the growth-inhibition function of metformin on human colorectal cancer cells is at least partially through inducing ADORA1 expression.

4.5. ADORA1 mediates the growth inhibition and apoptosis induced by metformin in an AMPK-mTOR pathway dependent manner

We next determined the potential mechanism by which ADORA1 induction by metformin triggered growth inhibition and apoptosis in human colorectal

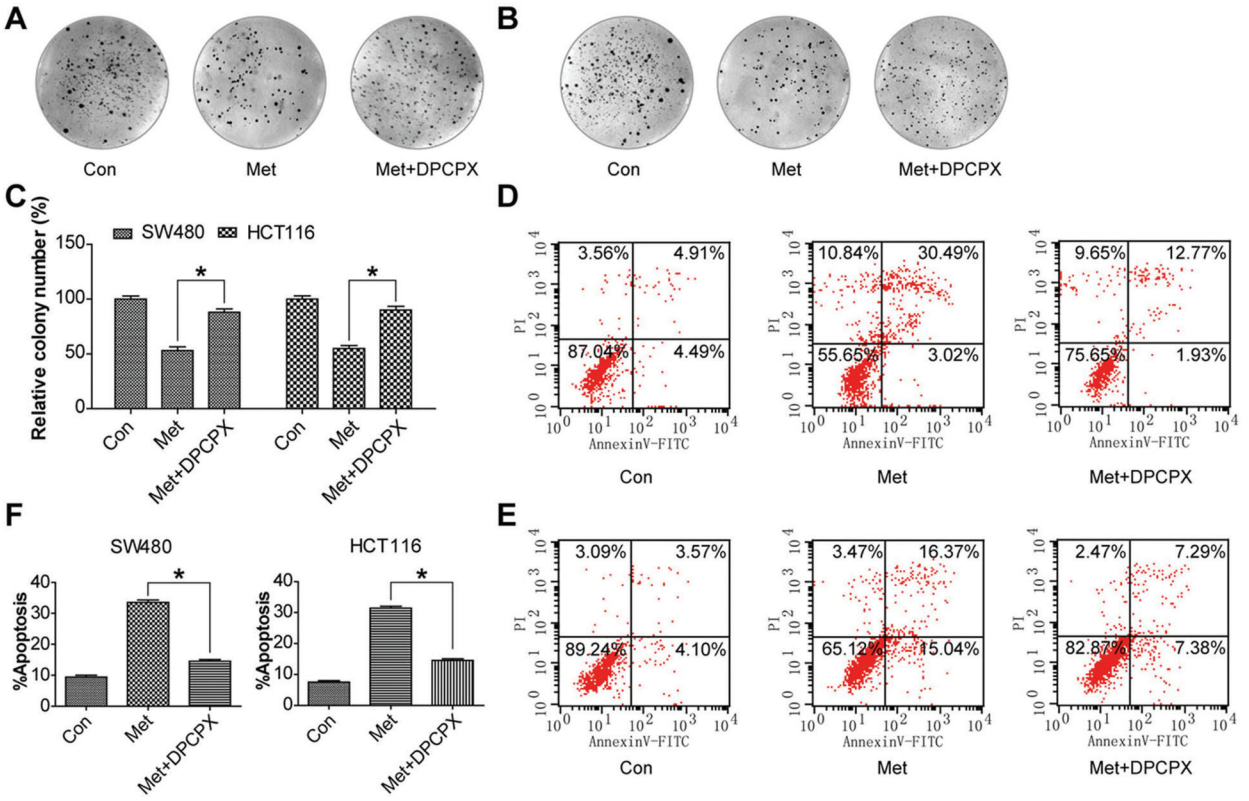

Figure 4. Inhibition of ADORA1 rescues the growth inhibition induced by metformin in human colorectal cancer cells. (A)-(B) SW480 and HCT116 cells were incubated with 1.0. mM metformin alone or in combination with 1.0. μM DPCPX. The number of colonies was counted on the 10th day after seeding. (C) Colonies containing 50 or more cells were counted. Results are means of three independent experiments ± S.D.*P<0.0.5, compared with metformin treatment alone. (D)-(E) Dot plot depicting apoptosis of SW480 and HCT116 cells treated with 1.0. mM metformin alone or in combination with 1.0. μM DPCPX for 48 h. Cells staining positive for Annexin V-FITC (x-axis) were considered to be apoptosis. (F). The percentage of cells staining positive for Annexin V are indicated. Results are means of three independent experiments ± S.D. *P<0.0.5, compared with control group. Con, control; Met, metformin; Met + DPCPX, metformin combined with DPCPX.

cancer cells. Previous studies have shown that metformin activates AMPK to phosphorylate mTOR and exerts its anti-proliferative function. We therefore determined whether AMPK participates in the growth inhibition effects mediated by ADORA1 after metformin treatment. As shown in fig 5A, metformin increased ADORA1 expression and AMPK phosphorylation, but decreased mTOR phosphorylation in both SW480 and HCT116 cells. However, AMPK inhibition by specific inhibitor Compound C (CC) markedly suppressed AMPK phosphorylation and increased mTOR phosphorylation (Fig 5A). Moreover, specific inhibiton of ADORA1 activity by DPCPX also markedly suppressed AMPK phosphorylation and increased mTOR phosphorylation induced by metformin. Meanwhile, inactivation of ADORA1 inhibited metformin-mediated growth inhibition, as evidenced by restored proliferation (Fig 4) and altered expression of apoptosis related protein such as increased expression of caspase 3 and caspase 9, decreased expression of Bcl-2 (Fig 5B and C). Together, ADORA1 mediates the growth inhibition and apoptosis induced by metformin in an AMPK-mTOR pathway dependent manner in human colonrectal cancer cells.

5. DISCUSSION

In the present study, we investigate the role of metformin and its molecular mechanisms in human colorectal cancer. We find the growth-inhibition effects of metformin on human colorectal cancer cells and also propose a novel mechanism in which metformin induces ADORA1 expression and subsequently promotes apoptosis via APMK-mTOR pathway.

Metformin is one of the most widely used anti-diabetic drugs prescribed for type 2 diabetes. Recently, it has been found that metformin also has potential anti-tumor effects in various types of cancer including lung cancer (3), hepatoma (4), breast cancer (7) and leukemia (9, 14). Consistent with previous reports, we find that metformin significantly suppresses the proliferation and colon formation of human colorectal cancer cells, which the growth-inhibitory effect is attribute to the apoptosis induced by metformin treatment. Increasing evidence indicates that the mTOR plays important role in the survival and proliferation of cancer cells (30-33). Metformin has been shown to inactivate

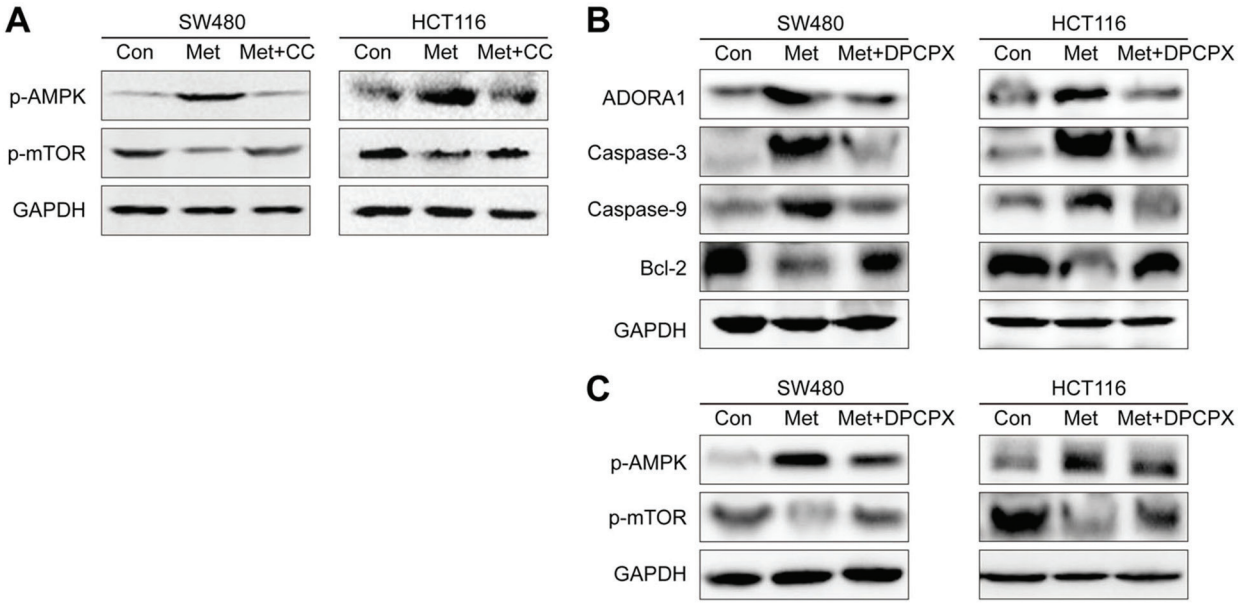

Figure 5. ADORA1 mediates the growth inhibition and apoptosis induced by metformin in an AMPK-mTOR pathway dependent manner. (A) The phosphorylation of AMPK and mTOR in SW480 and HCT116 cells was determined by western blot after incubated with 1.0. mM metformin or in combination with 20.0. μM CC. A reproducible result was obtained in three independent experiments. (B)-(C) The expression of p-AMPK, p-mTOR, ADORA1, caspase-3, caspase-9 and Bcl-2 in SW480 and HCT116 cells was detected by western blot after incubated with 1.0. mM metformin or in combination with 1.0. μM DPCPX. A reproducible result was obtained in three independent experiments. Con, control; Met, metformin; Met + CC, metformin combined with Compound C; Met + DPCPX, metformin combined with 8-cyclopentyl-1,3-dihydropylxanthine.

mTOR via activation of AMPK, thereby causing cell cycle arrest, apoptosis and inhibition of tumorigenesis (33-37). In human colorectal cancer, the anti-tumor effects of metformin are indeed mediated by activation of AMPK and suppression of mTOR, leading to increasing apoptosis of human colorectal cancer cells.

Despite these common mechanisms mentioned above, whether other unknown mechanisms are also involved in anti-tumor effects of metformin in human colorectal cancer. To better define the mechanism underlying the anti-tumor prosperities of metformin, we analyzed the differential expression profile of human colorectal cancer cells treated with or without metformin. Among many differentially expressed genes, we find ADORA1 that is reported to induce cancer cell apoptosis is significantly up-regulated. ADORA1 is one subtypes of four adenosine receptors (A_1, A_{2a}, A_{2b}, A_3) (38). Previous studies have shown that ADORA1 activation inhibits cell proliferation and promotes apoptosis in tumor cells (27). Adenosine binding with ADORA1 induces cell apoptosis and inhibits growth of human colon cancer, and ADORA1 agonists can activate caspase-3 and caspase-9 in RCR-1 astrocytoma cells. Here, we first report that metformin inhibits cell proliferation and promotes cell apoptosis via induction of ADORA1 in human colorectal cancer. In the present study, we find that metformin treatment up-regulates ADORA1 expression in human colorectal cancer cells, and inhibition of ADORA1 activity by its specific inhibitor DPCPX rescues metformin-mediated growth inhibition, as evidenced by restored proliferation and altered expression of apoptosis related protein such as increases of caspase-3 and caspase-9, decreases of Bcl-2. Moreover, specific inhibition of ADORA1 activity also markedly suppressed p-AMPK and increased p-mTOR induced by metformin. Thus, our data suggest that ADORA1 mediates the growth inhibition and apoptosis induced by metformin in an AMPK-mTOR pathway dependent manner in human colorectal cancer cells.

In conclusion, metformin exerts anti-tumor effects on human colorectal cancer cells by up-regulation of ADORA1 with downstream induction of growth-inhibition and apoptosis. Thus, our results provide evidence for mechanism and the therapeutic effectiveness of metformin in the treatment of human colorectal cancer.

6. ACKNOWLEDGEMENTS

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