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Molecular Therapeutic Potency of Metformin by Targeting p53-Related Molecules in Mutant p53 Colon Cancer Cell Line

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Abstract

Colon cancer is a malignancy in gastrointestinal tract. It causes high mortality rate in global cancer population. However, chemotherapy as its first option therapy is still controversial due to its effectiveness and its adverse effects. Finding supportive and alternative drugs to cure cancer is one of focus in cancer research. A drug which also has anticancer effects is metformin. Metformin is a biguanide antidiabetic which show its potential anticancer benefit in metabolic-related cancers including colon cancer. To investigate anticancer potency of metformin in targeting p53-related molecules. Metformin treatment were divided into 4 groups by 0, 5, 10 and 20 mM concentrations and incubated in 37°C and 5% CO_2 condition for 48 hours. Immunohistochemistry were conducted to asses level of expression of Bax, p21, cyclin D1 and E2F1, respectively. Level of expression were measured by H-SCORE using percentage and intensity calculation. Comparisons of H-SCORE between groups were performed by ANOVA for parametrical data and Kruskal-Wallis for non-parametrical data. Growth inhibition were observed after metformin treatment. Metformin increases Bax expression significantly at all concentrations. p21 expression was also increased after metformin treatment but is not statistically significant. Subsequently, metformin decreases cyclin D1 expression at 10 and 20 mM concentration thus decreased E2F1 expression at 5 and 10 mM concentration. These data suggest that metformin may have potential therapeutic effects in mutant p53 colon cancer cell line by targeting p53-related molecules.

Keywords: Colon cancer, p53, Biguanide, Metformin, p53-mutant cell line

INTRODUCTION

Colon cancer is malignancy in the gastrointestinal tract which cause high mortality rates in global cancer population. Colon cancer is third most common cancer in males and the second in females. Colon cancer incidence was decreased in recent years in developed countries due to increase of patient treatment quality and early detection. Unfortunately, incidence of colon cancer in low risk area which include developing countries was increased.

Chemotherapy remains one of therapeutic option for colon cancer treatment. Chemotherapy act as adjuvant by reducing risk of relapse and patient death. Therefore, use of chemotherapy is still controversial due to its effectiveness and adverse effects. As reported by O'Connor, *et al*, no survival benefit were found in stage II colon cancer patients with poor prognostic features nor for no poor prognostic features after chemotherapy treatment. Moreover, some colon cancer chemotherapy regimen such as 5-fluorouracil (5-FU) could cause fatal adverse effect.

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Metformin is a biguanide which has potential anticancer effect. Landman, *et al* reported that use of metformin related to lower cancer risk in diabetes type II patient. More specific, Zhang, *et al*., reported use of metformin reduced risk of colon cancer significantly. Interestingly, metformin could has benefit to non-diabetic patients also as suggested by Hosono, *et al*.

In their study, metformin inhibited growth of colon cancer marker, aberrant crypt foci (ACF), consistently with decreased proliferating cell nuclear antigen index.

In vitro study could help to understand underlying molecular anticancer mechanism of metformin. Alimova, *et al*,. found that cytotoxic effect of metformin in breast cancer cell line influenced by cell cycle arrest and altered erbB2 receptor tyrosine kinase (RTK) pathways. Cytotoxic effect of metformin were also found in p53-deficient colon cancer cell line. Zakikhani, *et al*., reported inhibition of colon cancer cell line consistent with increased AMP-activated protein kinase (AMPK) activity.

In present study, we investigate potential anticancer of metformin in p53-deficient colon cancer cell line by assess the following p53-related molecules: p21 (a cyclin-dependent kinase inhibitor) and Bax (a pro-apoptotic molecule). We also assess an oncogenic molecules expression, cyclin D1 and E2F1 which is downstream target of cyclin D1.

MATERIALS AND METHODS

Antibodies.

Mouse monoclonal antibodies included anti-human cyclin D1 and anti-human E2F1 from Neomarker (CA, USA), anti-human p21 and anti-human Bax from Biocare (Concord, CA, USA).

Reagents

Metformin was obtained from Dr. Endang Lukitaningsih, Faculty of Pharmacy, Gadjah Mada University, Indonesia. It was used across a range of concentrations at 5, 10 and 20 mM diluted in media.

Cell line and culture conditions

The human colon cancer line WiDr was obtained from Parasitology Laboratory of Gadjah Mada University in Indonesia. Cell line was maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) cultured at 37°C humidified atmosphere containing 95% air and 5% CO_2. Cells were passaged by 0.25% Trypsin-EDTA when they reached 80% confluence. Chemicals were purchased from Neomarker (CA, USA).

Cell proliferation assay

Cells were plated onto 96-well plates at 3.5 x 10^4 cells well. After 24 hours in culture, the medium was removed and replaced by medium with (treatment) or without (control) metformin for 48 hours. The effect of metformin on WiDr cell line was evaluated by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), wells were read at 490 nM with a microplate reader. The percentages of surviving cells from each group relative to control defined as 100% survival.

Immunohistochemistry

Protein expression of Bax, p21, cyclin D1, and E2F1 was assessed by using immunohistochemistry staining against monoclonal antibodies anti-human Bax, p21, cyclin D1, and E2F1. Methanol-fixed cells were stained with hematoxylin and eosin evaluated microscopically. After Bax, p21, cyclin D1, and E2F1 primary antibodies, the secondary antibody was anti-mouse and third step was ABC complex (Streptavidin-Biotin peroxidase) and DAB (3,3'-diaminobenzidine) chromogen. Scores of immunostaining were calculated by multiplying the percentage of labeled cells with the intensity (1+,2+,3+) of staining for a total score gain from 0 to 300. Thus the formula could be aplied by:

H-SCORE= (3 x % of strong intensity cells) + (2 x % of moderate intensity cells) + (1

x weak intensity cells) + (0 x negative intensity cells).

Statistical analysis

=Values are expressed as the mean ± SD in the bar graphs. The significance of the difference between the control and each experimental test condition was analyzed by ANOVA and Kruskall-Wallis test and $p<0.05$ was considered statistically significant.

RESULTS

Metformin induces growth inhibition of colon cancer cell line

As shown in Fig. 1, metformin inhibits growth of WiDr cell line in a dose dependent manner. Three metformin concentrations (5 mM, 10 mM, and 20 mM) significantly reduced WiDr cell line viability.

Metformin increases apoptotic activity by inducing Bax expression levels

Apoptotic activity of WiDr cell line was significantly increased after metformin treatment as shown by Fig. 2 increased Bax expression levels. Bax was increased at 5 and 10 mM metformin concentrations. Maximum Bax expression levels were observed at 20 mM concentration.

Metformin inhibits cell cycle-related molecule and increases its inhibitor

Metformin shown to influence cell cycle activity by targeting cyclin D1 and p21 (Fig. 3). Metformin significantly inhibited cyclin D1 expression at 10 mM and 20 mM. Decreased cyclin D1 expression was also found at 5 mM metformin but is not statistically significant. Consistent with decreased cyclin D1 expression, metformin induces p21 expression but is not statistically significant between groups.

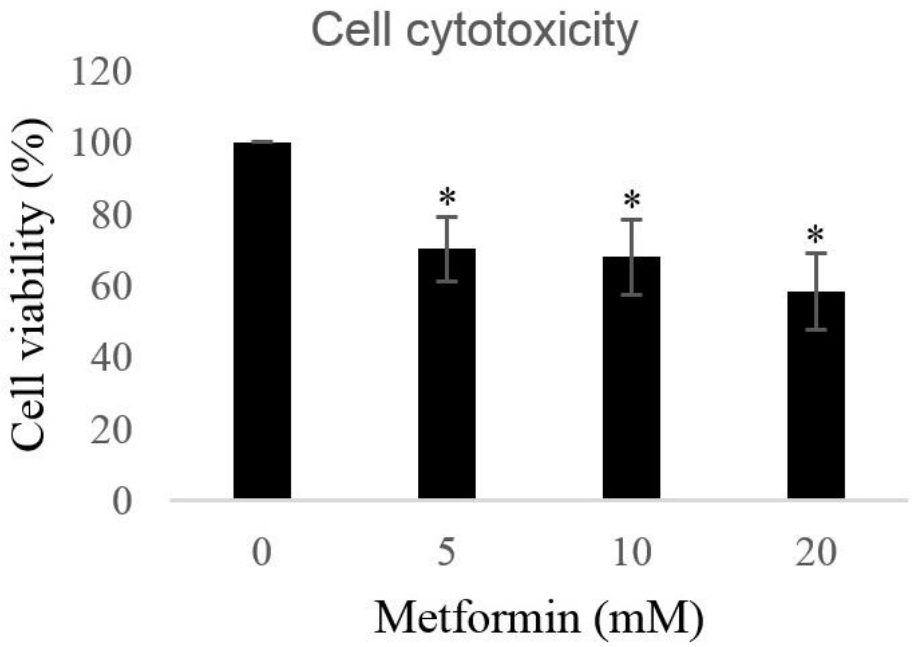

Figure 1. Metformin induces growth inhibition of colon cancer cell line. WiDr cell line were seeded onto 96-well plates and treated by metformin followed by incubation at 37°C with 5% CO_2. The effects of metformin treatment were assessed by MTT assay after 24 hours. Data presented as mean ± standard deviation. *$p<0.05$ versus control.

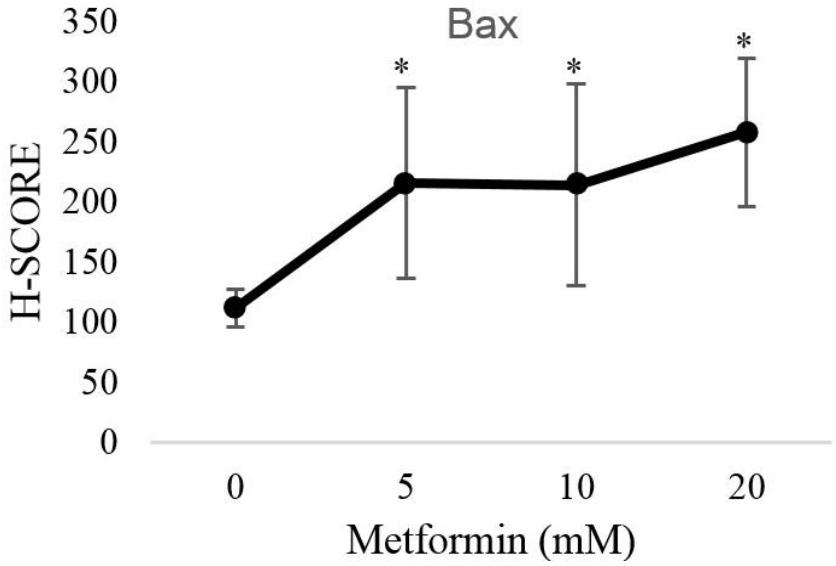

Figure 2. Metformin increases Bax expression levels. After metformin treatment, IHC was conducted to assess Bax expression. Cell percentage and intensity were calculated to measure H-SCORE. Data presented as mean ± standard deviation. *p<0.05 versus control.

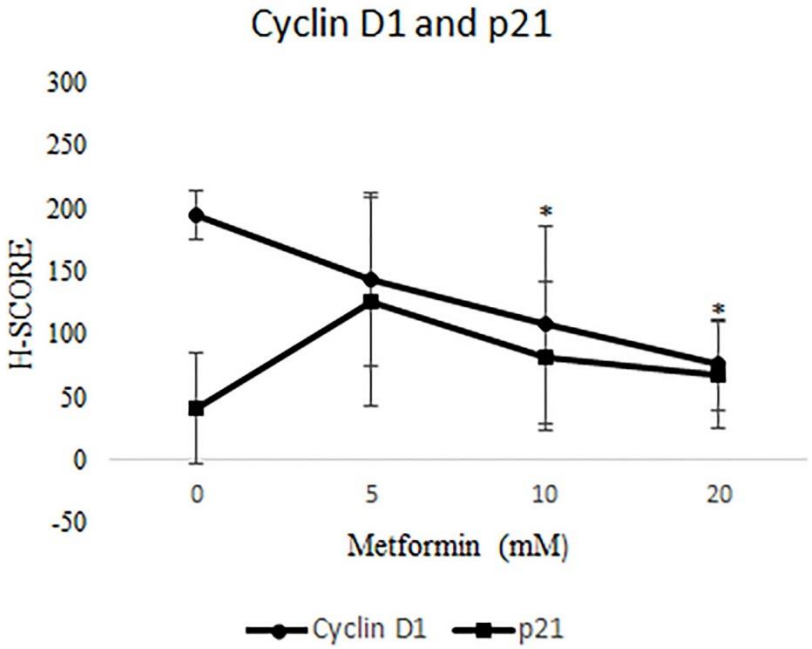

Figure 3. Metformin alters cyclin D1 and p21 expression levels. Metformin decreases cyclin D1 expression levels. p21 expression levels was increased after metformin treatment but is not statistically significant. Data presented as mean ± standard deviation. *p<0.05 versus control.

Metformin also altered E2F1 expression levels. Significant increased E2F1 expressions were observed at 5 mM and 10 mM metformin. Metformin sharply reduced E2F1 expression at 5 mM. Moderate reduction of E2F1 was found at 10 mM metformin. Metformin also reduced at 20 mM metformin but is not statistically significant (Fig. 4).

DISCUSSION

p53 has pivotal role in cancer development by controlling cell homeostasis. It regulates cell growth and cell senescence by apoptosis, cell cycle, and DNA repair regulatory mechanism. Dysregulation of p53 and its related molecules functions causes uncontrolled cell growth leading to malignancy. Mutation in p53 gene has been reported in half of colorectal cancer case.

p53 controls intrinsic apoptosis pathway by activating pro apoptosis activities. By targeting several genes such as Bax, noxa and puma, p53 could induce cytochorme-c release into cytoplasm from mitochondria. This processes lead to protease cascade which end to cell execution by caspase-3. p53 contribution in extrinsic apoptosis pathway was also reported. p53 regulates extrinsic apoptosis pathway related molecules such as Fas ligand which subsequently induce apoptosis.

Cell cycle is a complex system which regulate cell proliferation. Cell cycle controlled by interaction of several molecules such as cyclins and cyclin dependent kinases (CDKs). For example, cyclin D1 promotes cell progression in G1 phase when cyclin E promotes progression in mid late G1 phase and early S phase. This processes induced by formation complex with CDKs. CDK4 and CDK6 activity relates to cyclin D1 and CDK2 relates to cyclin E function. Moreover, cyclins-CDKs complexes could phosphorylate Rb-E2F1 complexes protein which lead to E2F1 release. E2F is a family molecules which responsible to up regulation of cancer promoting genes such as CCNE, CCNA and Myc.

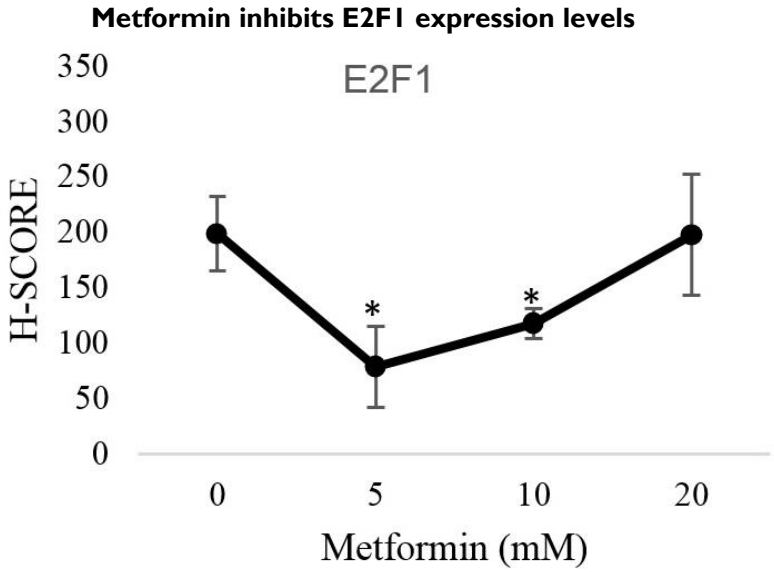

Figure 4. Metformin decreases E2F1 expression levels. At 5 mM and 10 mM concentrations, metformin significantly decreases E2F1 expression levels. Data presented as mean ± standard deviation. * $p<0.05$ versus control.

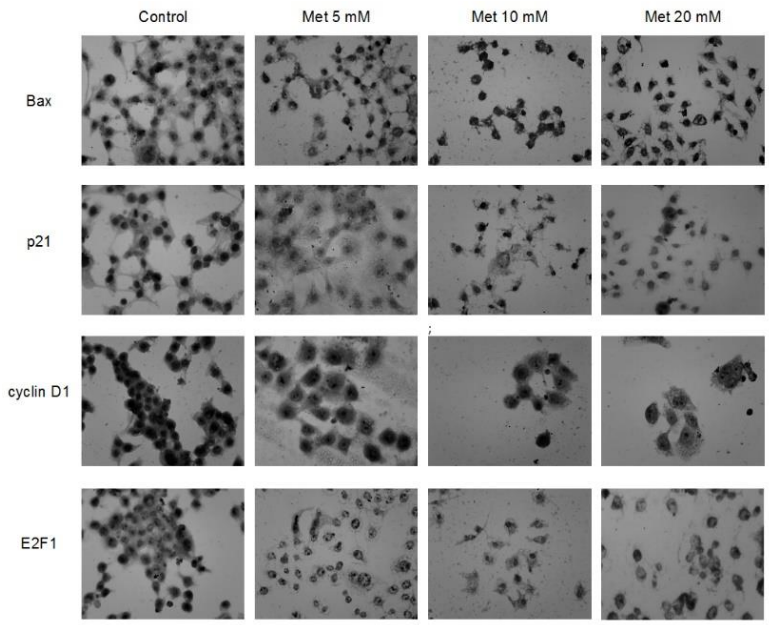

Figure 5. Metformin alters p53-related molecules in WiDr cell line. Representative image (magnification x 400) of IHC analysis after metformin treatment in WiDr cell line.

p21 is a p53 related protein which also regulate cell cycle. Harper and colleagues reported that p21 is potent inhibitor of CDKs such as CDK2, CDK4, and CDK6. Impairment of cyclins and CDKs complexes result in cell cycle arrest.

In our study, we used WiDr cell line to evaluate effects of metformin in p53 deficient colon cancer cell line. We reported that metformin induced Bax expression significantly (Fig. 2). Our finding consistent to previous study by Buzzai and colleagues. They reported that metformin induced Bax expression in p53 deficient colon cancer cell line. As per Suzuki, *et al*. report, Bax could be induced by p19 in a p53 independent pathway.

In this study, metformin induced p21 expression but is not statistically significant (Fig. 3). Takhahasi and colleagues also found that increase of p21 expression by metformin could be in a functional p53 independent pathway. p21 which is normally transcriptional target by p53 could be induced by others mechanisms such as post transcriptional and post translational mechanism.

These events followed by decrease of cyclin D1 (Fig. 3) and and its downstream target, E2F1 (Fig. 4), significantly. Metformin is a LKB1/AMPK pathway activator drug. Activated AMPK could alter its downstream target including cyclin D1 (20). Zhuang and Miskimins Reported that metformin induced AMPK activity which subsequently decreased cyclin D1 expressions. Decrease of cyclin D1 expression would also reduce Rb-E2F1 complexes phosphorylation thus decrease E2F1 release and activity.

CONCLUSIONS

Our study supports previous studies which evaluate effect of metformin for cancer treatment *in vitro*. Moreover, its results also indicates metformin treatment could influence p53 related molecules in p53 mutant colon cancer cell line. Thus metformin treatment might beneficial for p53 mutated colon cancer patients.

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