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Research Article

Metformin Eliminates CD133^{high}/CD44^{high} Prostate Cancer Stem Cells Via Cell Cycle Arrest and Apoptosis

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Abstract

Objectives: Cancer stem cells (CSCs), a small subpopulation of tumors, are responsible for chemo-radioresistance, metastasis, and cancer recurrence. The main aim of the present study is to investigate the potential effects of metformin on prostate CSCs (PCSCs).

Methods: Flow cytometry was used to isolate cells with co-expression of CD133 and CD44. Sorted PCSCs were treated with different concentrations of metformin to determine the effects of metformin on cell viability using MTT assay. The association of cells exposure to metformin with apoptotic cell death and caspase activity, as well as cell cycle, were performed using the Muse Cell Analyzer.

Results: In our study, for the first time we demonstrated the anti-cancer effects of metformin on PCSCs. Our results revealed that treatment with metformin reduced cell viability in CD133^{high}/CD44^{high} cells in a dose- and time-dependent manner. Metformin significantly induced early apoptosis and triggered the activity of several caspases associated with the apoptotic process. Metformin significantly altered the cell cycle distribution in CD133^{high}/CD44^{high} cells, leading to G0/G1 phase arrest.

Conclusion: The results of the study revealed that metformin triggers cell death and apoptosis and modulates cell cycle distribution in CD133^{high}/CD44^{high} PCSCS. The present study raises the possibility that metformin is a potential anti-cancer agent for targeting PCSCs.

Keywords: Prostate cancer, cancer stem cell, metformin

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Prostate cancer (PCa) is the second most common malignancy among men.[1] Treatment for prostate cancer depends on the characteristics of the individual patient. Surgery, radiation therapy, and expectant management, including active surveillance and watchful waiting, are the main treatment options for PCa.[2] PCa is a heterogeneous disease, and the experience of disease recurrence and subsequent metastasis following current treatment protocols highlights the need for an in-depth understanding of the histopathology of the disease and effective approaches that can support current treatment alternatives.

A substantial body of research indicates that tumor heterogeneity due to the plasticity of CSCs presents difficulties in the clinical management of the disease.[3,4] After the first identification of CSCs in hematological malignancies, CSCs were distinguished in several solid cancers and gained

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interest. Even though CSCs are a small subpopulation of tumors, they are thought to be the cause of chemo-radio resistance, metastasis, and relapse.[5] Given these important developments and findings, there has been a marked increase in studies that aims to target CSCs and elucidate molecular characteristics of CSCs.[6] Considerable evidence demonstrates the critical roles of prostate CSCs (PCSCs) in the processes of cancer initiation, invasion, resistance to therapy, and metastasis.[7,8] In our previous studies, it has been reported that PCSC are more resistant to various anti-cancer drugs compared to the bulk population (non-CSCs).[9–11]

Metformin is an oral biguanide that is commonly used to treat type 2 diabetes mellitus. Its main benefits include lowering insulin resistance and inhibiting hepatic gluconeogenesis.[12] Following success in diabetes, possible implications of metformin in cancer have been investigated. Epidemiological studies demonstrated that patients receiving metformin had a lower risk of cancer.[12] Metformin's direct effects on cancer have been documented in an increasing number of studies. The mechanisms of action of metformin in cancer are by lowering systemic insulin levels and inhibiting the mitochondrial transport chain.[13] The results of considerable studies have revealed that metformin effectively targets and eliminates CSCs in the breast,[14] ovarian,[15] colorectal,[16] brain,[17] and pancreatic cancers.[18] Although there is an opinion that metformin may be a potential agent for targeting PCSCs,[19] the possible therapeutic effects of metformin on PCa CSC have not yet been determined. The goal of this study was to reveal metformin's anti-cancer effects on $CD133^{high}/CD44^{high}$ PCSCs and to provide new insights into mechanisms of action of metformin in PCSCs.

Methods

Cell Culture Conditions

The androgen-independent human prostate cell line PC3 is provided from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 (Gibco, Thermo Fisher Scientific, USA) cell culture media supplemented with 10% FBS (Biowest, France) and penicillin (100U/ml)/streptomycin (10µg) (Lonza, Switzerland).

Isolation of $CD133^{high}/CD44^{high}$ PC3 CSCs

Cell surface markers CD133 and CD44 were used for the isolation of PC3 CSCs. Cells were sorted according to the protocols described in previous studies.[9–11] Briefly, 1x106 cells were incubated with antibodies containing PE-labeled CD133 (clone AC133/1) and FITC-labeled CD44 (Clone G44-26) for 20 minutes at +4°C.[9–11] After washing with FACS washing solution, $CD133^{high}/CD44^{high}$ population was sorted using the FACSAria flow cytometer.

Cell Viability Assay

Cell viability assay was performed to determine the half-maximum inhibitory concentrations (IC50) of metformin (Sigma-Aldrich, USA). MTT (Merck, USA) metod was used to obtain the IC50 value. Exponentially growing cells were adjusted to 1x104 and placed in 96-well for 12 h to acquire morphological characteristics. Then, the cells were treated with different concentrations of metformin (1-10 mM) for 24, 48, and 72 hours. After the incubation period, 20 µl of MTT was added to each well and incubated for 4 h at 37°C, and to dissolve the reduced formazan products 100 µl of SDS was applied. In the final step, the absorbance was measured at 570 nm with a microplate-reader. To confirm the IC50 value obtained based on the MTT result, Muse Count and Viability Kit was used as in previous studies.[9–11]

Determination of Apoptosis by Annexin V assay

Muse Annexin V and Cell Death Kit (MCH100105, Milipore) was used to detect apoptotic cell death in cells due to treatment with metformin. For this, cells (1x104cells/well) were seeded on a 6-well plate and incubated with 7.5 mM dose of metformin. At the end of the incubation period, 100 µL of cell suspension and 100 µL of Muse Annexin V and cell death kit solution were mixed and incubated for 20 minutes at room temperature in the dark. Live, dead, early apoptosis and late apoptosis cell populations were determined by analyzing with Muse Cell Analyzer (Millipore, USA).

Determination of Multicaspase Activity

Caspase activity was performed with the Muse Multi Caspase assay kit (MCH100109, Merk Millipore, USA). $CD133^{high}/CD44^{high}$ cells were seeded on 6-well plate. The cells were treated with metformin (7.5 mM) and collected using 0.25 % trypsin-EDTA solution. Afterwards, the cells were collected and 50 µL of these cells were taken and added to 5 µL of Muse Multi caspase working solution reagent and incubated for 30 minutes. Subsequently, each sample was mixed with 7-AAD (150 µL) dye working solution and incubated for 5 minutes. Cell populations defined as live cells, dead cells, caspase+ cells saspase+/dead cells were evaluated via Muse Cell analyzer (Millipore, USA).

Cell Cycle Analyses

The Muse™ Cell Cycle Kit (MCH100106, Milipore) was used to determine whether metformin induced cell death due to cell arrest. Briefly, cells were treated with 7.5 mM dose of metformin. Following this step, cells were collected and 1 ml of these cells (1x106 cells) was taken and mixed by slow-

ly adding cold 70% ethanol to it and incubated at -20°C for approximately 3 hours. At the end of the incubation period, the cells were washed with PBS, and 200 µl of these cells were taken and mixed with 200 µl of muse cell cycle reagent and incubated for 30 minutes at room temperature in the dark. The G0/G1, S, and G2/M phase ratios of the cells in the sample were determined using the Muse cell analyzer (Millipore, USA).

Statistical Analysis

One-way ANOVA was used to compare the statistical differences of more than two groups. Multiple comparisons were confirmed using Dunnett's multiple comparisons test. The comparisons involving two groups were analyzed using Student's t-test. Values equal to or below 0.05 ($p<0.05$) were considered significant. Statistical analyzes were performed using the GraphPad Prism version 8.02, GraphPad Software, Inc., California, USA).

Results

Isolation of CD133^{high}/CD44^{high} Population in PC3 Cells

We used flow cytometry to determine the distribution of CD44 and CD133 expression in the PC3 prostate cancer cell line and to select appropriate gates for sorting (Fig. 1). The CD133^{high}/CD44^{high} phenotype represents a minor population within PC3 cells. The CD133^{high}/CD44^{high} cell population was approximately 2.7%, while the bulk population (non-CSCs) ratio was 97.3%.

Metformin Inhibited the Viability of CD133^{high}/CD44^{high} Cells

To evaluate the effects of metformin on cell viability, we treated cells with different concentraions of metformin (1-10mM) for 24, 48, and 72 hours. Compared to control group, MTT assay results showed that metformin decreased cell viability in a dose and time-dependent manner ($p<0.001$) (Fig. 2). The IC50 values were 28.44, 15.31, and 7.72 mM for 24, 48 and 72h, respectively. Based on the results from MTT, a dose of 7.5 mM metformin for 72h was used for further analysis. Moreover, the effects of 7.5 mM dose of metformin on cell viability were confirmed with the Muse Count and Viability Kit using Muse Cell Analyzer (Fig. 2).

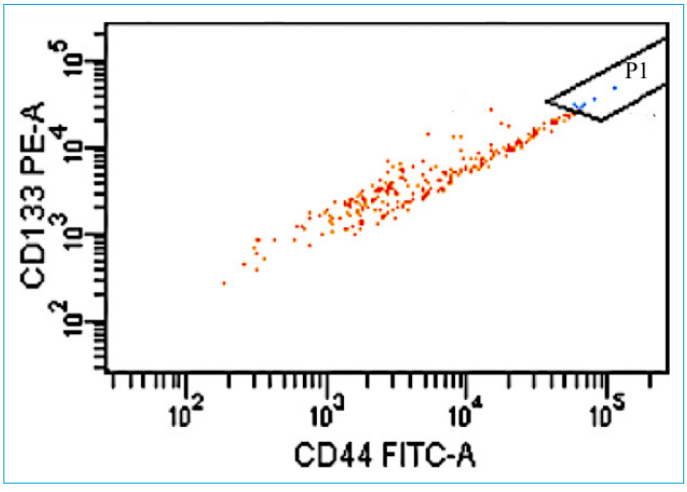

Figure 1. Isolation of CD133^{high}/CD44^{high} cell population by flow cytometry. Cells were stained using antibodies CD133-PE and CD44-FITC and sorted. P1 represents the CD133^{high}/CD44^{high} cell population.

Metformin Induced Apoptosis CD133^{high}/CD44^{high} Cells

The Annexin V & Dead Cell Kit was used to determine whether the inhibitory effect of exposure to metformin on CD133^{high}/CD44^{high} cell viability was accompanied by induction of apoptotic cell death. As seen in Figure 3, exposure to 7.5 mM dose of metformin resulted in a significant increase in apoptosis compared to control ($p<0.001$). Early and late apoptosis were approximately 33.48% ($p<0.0001$) and 6.28% ($p<0.001$), respectively (Fig. 3). These results reveal that metformin specifically induces early apoptosis in PC3 CSCs.

Metformin Triggered Apoptosis Through the Activity of Caspases

Caspases are essential in different stages of apoptosis in response to different proapoptotic stimuli, leading to the progression of the apoptotic process. Analyzes were performed with the multiple caspase kit to reveal the roles of caspases in metformin-induced apoptotic cell death. As

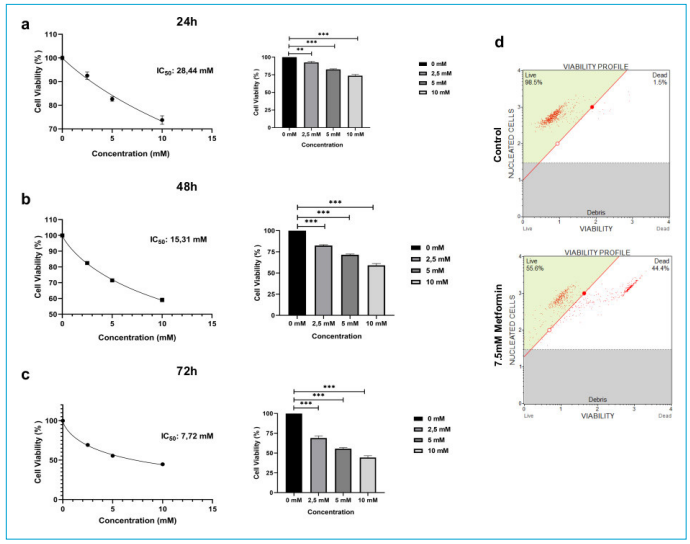

Figure 2. Effects of metformin on CD133^{high}/CD44^{high} cells. CD133^{high}/CD44^{high} cells were treated with different doses of metformin (0, 2.5, 5 and 10 mM) for 24 **(a)**, 48 **(b)** and 72h **(c)**, and cell viability was determined by MTT assay. **(d)** The effect of metfromin on cell viability was confirmed by Muse Count and Viability (**p<0.01, ***p<0.001)

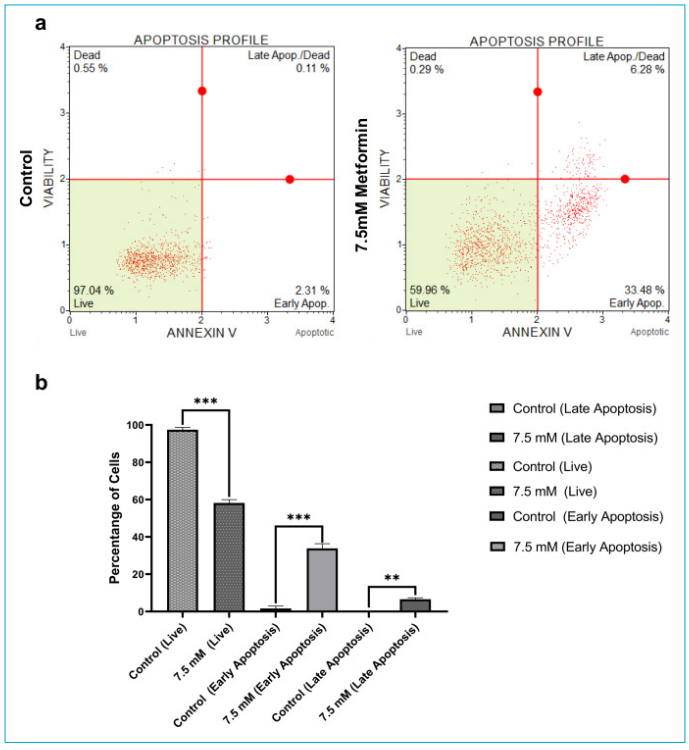

Figure 3. (a) Effects of metformin on apoptotic cell death. CD133^{high}/CD44^{high} cells were exposed to 7.5 mM dose of metformin for 72 h. The scatter plots of analyzes with the Muse Annexin V Dead Cell Kit showed the percentages of apoptotic cells after treatment with metformin. All experiments were performed in triplicate and **(b)** The graph represents the percentages of apoptotic cells. **p<0.001 and ***p<0.0001 indicate significant difference.

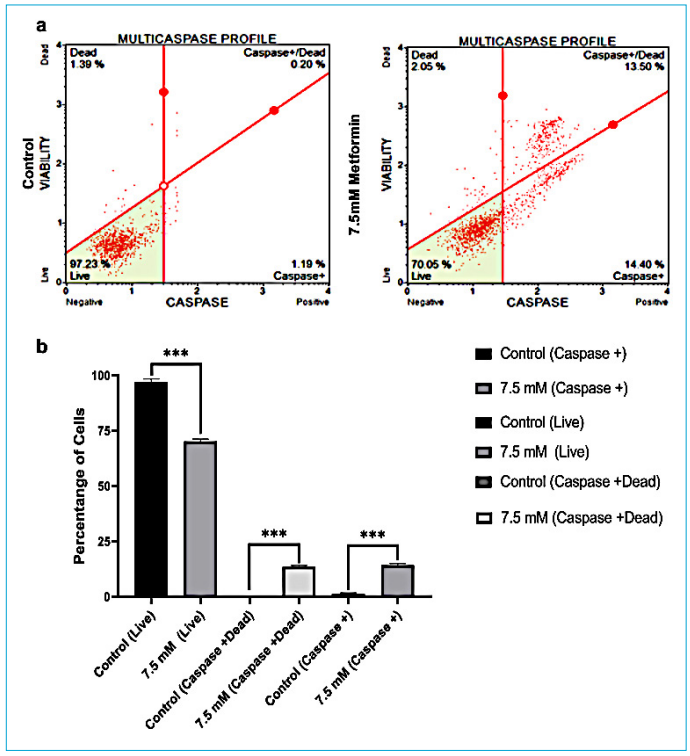

Figure 4. (a) Effects of metformin on caspases activity. CD133^{high}/CD44^{high} cells were exposed to 7.5 mM dose of metformin for 72 h and multicaspases were evaluated via Muse multicaspase kit. The plot represents the percentages of four different populations: Live, Caspase(+), Caspase(+)/Dead and Dead cells. **(b)** The graph represents the percentages of live, caspase(+) and Caspase(+)Dead cells. All experiments were performed in triplicate. ***p<0.0001 indicate significant difference.

seen in Figure 4, four different cell populations were distinguished: live cells (both caspase and 7-AAD negative), dead cells (Caspase negative and 7-ADD positive), Caspase+ cells exhibiting Pan caspase activity (Caspase positive, 7-AAD) negative), Caspase+/Dead cells (Caspase positive, 7-ADD positive) and the percentage of these populations were 70.05%, 2.05%, 14.10% and 13.50%, respectively. The obtained data indicate that metformin induces apoptosis in PC3 CSCs in a caspase-dependent manner.

Metformin Caused Cell Cycle Arrest in CD133^{high}/CD44^{high} Cells

To investigate the effects of metformin on the cell cycle, cells were treated with a 7.5 mM dose of metformin. The phase distributions in the cell cycle were determined using the Muse Cell Cycle Kit. After 72 h treatment, the results obtained indicate that metformin induces cell cycle arrest at the G0/G1 phase (p< 0.001) (Fig. 5).

Discussion

Recently, approaches in cancer treatments have drawn striking attention to the importance of selective targeting of CSCs. In this study, we revealed that metformin has cytotoxic effects on CD133^{high}/CD44^{high} PCSCs. Additionally, as a secondary objective, we aimed to provide clues regarding the possible mechanisms of action of metformin. Our findings showed that metformin induces apoptosis in PCSCs due to the involvement of caspase activity and leads to cell cycle arrest.

Since the prostate is a complex and heterogeneous organ at the cellular level, the isolation of CSCs within the structure is of critical importance. We used the surface markers CD133 and CD44 for the isolation of CSCs in the PC3 cell line. PCSCs were first isolated by Collins et al. according to their CD44+α2β1hiCD133+ properties, and these cells have been reported to have a high potential for self-renewal and differentiation into heterogeneous cell lines.[7] The main reason for choosing these two markers is that elevated CD133 and CD44 expression are linked to drug resistance. A strong relationship between high CD133 and CD44 expressions and drug resistance in PCa has been reported in the literature.[11,20,21]

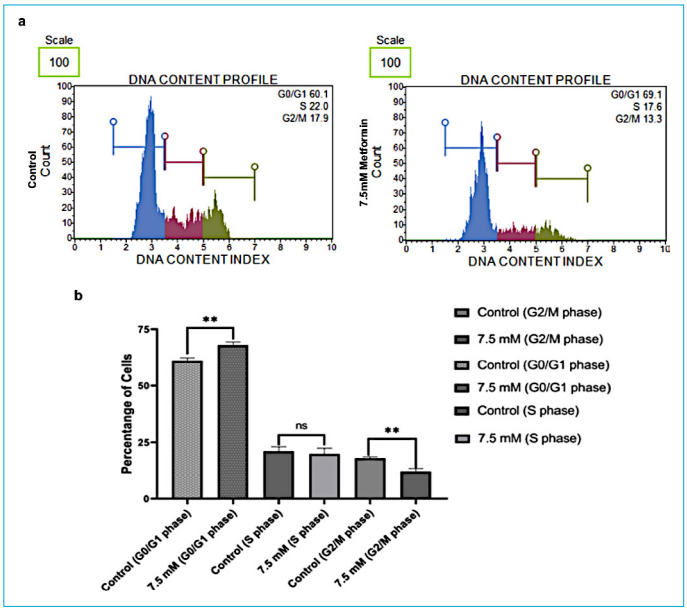

Figure 5. (a) Cell cycle analysis of the cells treated with metformin. CD133^{high}/CD44^{high} cells were treated with 7.5 mM dose of metformin for 72 h. Histograms represent the percentages of cells in the G0/G1, S, and G2/M phases of the cell cycle. **(b)** The graph represents the percentages of G0/G1, S and G2/M phases. The experiments carried out were in triplicate. **p< 0.01 indicates a significant difference, ns not significant.

In the study, in which data obtained from retrospective epidemiological and clinical studies in PCa were evaluated, it was noted that metformin may be an effective agent in the treatment of PCa.[13] Several reports investigating the effects of metformin in PCa cell lines showed that metformin inhibited the proliferative activities of PCa through various mechanisms.[22–26] The anti-cancer effects of metformin on PCa cells and the scientific evidence presented in the literature that it targets the stem cell population in other cancers led to the development of the hypothesis that metformin would be effective in PCSCs. Consistent with the literature, this study found that metformin inhibited the proliferative activity of PCSCs.

Studies on the underlying mechanisms of metformin, which has proven to be anti-proliferative in various cancers, show that it usually eliminates cancer cells through apoptosis and cell cycle arrest.[22–26] Consistent with the literature, the results of our study showed that metformin have antiproliferative effects in CD133^{high}/CD44^{high} PCSCs, associated with induction of caspase-mediated apoptosis and cell cycle arrest. In Annexin V assay findings, it was found that metformin triggered particularly early apoptotic cell death (approximately 33.48%) in PCSCs. The number of pathways related to cell death mechanisms triggered by anti-cancer drugs is increasing in the light of new studies, and these pathways overlap with each other at some points. Apoptosis occurs in a caspase-dependent and caspase-independent manner.[27,28] We evaluated multicaspase activity to determine whether the metformin-induced apoptotic pathway is dependent on caspase activity. Our findings showed that metformin-mediated apoptosis is dependent on caspase activity. Several studies have reported that metformin-mediated apoptosis could be both caspase-dependent and caspase-independent.[29–31] Another important finding regarding the mechanism of action of metformin was the finding that its effect on the cell cycle was achieved by leading to G0/G1 phase arrest. Similarly, a study from Sahra et al. reports that metformin induced G0/G1 arrest in DU145, PC-3 and LNCaP prostate cancer cells, accompanied by a strong decrease of cyclin D1.[26]

Ongoing studies in the field of cancer draw attention to the critical roles of CSCs in tumor initiation, resistance to treatment, cancer recurrence and metastasis.[6,8,19] At this point, targeting the CSCs population of anti-cancer drugs seems to be the key to obtaining effective outcomes in cancer treatment. A number of studies have reported potential roles for metformin-mediated targeting and elimination of CSCs.[15–18] Herein, this is the first study that demonstrated anti-proliferative effects of metformin on CD133^{high}/CD44^{high} PCSCs.

Conclusion

Although there have been great developments in the diagnosis and treatment of cancer, unfortunately, the desired concrete success has not been achieved yet. The point we have reached in the steps taken in the cancer treatment journey draws attention to the need to target CSCs, which are defined as the root of cancer, for an effective cancer treatment strategy. Our study demonstrated that treatment of cells exhibiting the CD133^{high}/CD44^{high} phenotype with metformin inhibits cell proliferation through mechanisms accompanied by caspase-mediated induction of apoptosis and cell cycle arrest in vitro. Future studies are required to elucidate the effects of metformin on PCSCs in vivo and preclinical studies, as well as to understand the anti-cancer mechanisms of metformin.

Disclosures

Peer-review: Externally peer-reviewed.

Conflict of Interest: No potential conflicts of interest were disclosed.

Authorship Contributions: Concept – E.A.; Design – E.A.; Supervision – E.A., G.O.; Data collection &/or processing – E.A.; Analysis and/or interpretation – E.A., M.C.; Writing – E.A., M.G.; Critical review – E.A, M.C., M.G., G.O.

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