

# Antitumor activity of Metformin through p53 and Cyclin D1 in the Urothelial Cell Carcinoma

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RESEARCH ARTICLE

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**Antitumor activity of Metformin through p53 and Cyclin D1 in the Urothelial Cell Carcinoma**

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**ABSTRACT:**

Bladder cancer is considered as one of the main drivers of cancer related mortality in adult men. Data from Global Cancer Statistics 2018 (GLOBOCAN) showed that the bladder cancer was included among the Top 10 cancer incidence in worldwide. Meanwhile, metformin, an antidiabetic agent, is believed to be able to impede the varying cancer cells expansion. Many examinations had displayed that metformin interferes via the AMPK/mTOR axis pathway, thereby suppressing tumor growth. AMPK activation can also increase stromal cell survival through p53 activation. Metformin also disrupts the cell cycle by decreasing the cyclin D1 protein in cancer cells. The human cell line 5637 was treated with metformin 15 mM, examined for cyclin D1 and p53 by immunohistochemical staining and assessed for the viability of cancer cells. The Statistic test was utilized to make a comparison of tumor viabilities and other variables. No significant differences were found in the expression of wild type p53 and cyclin D1 but significant differences were observed in the viability between the control and metformin groups. We have proven in our study that the anti-tumor effect of metformin in reducing the viability of urothelial carcinoma tumor cells not only through p53 and cyclin D1.

**KEYWORDS:** Bladder cancer, p53, cyclin D1, viability.

**INTRODUCTION:**

As the most prevalent disease of the urinary tract, the bladder carcinoma is recognized as one of the primary determinants of cancer death in adult men. Based on the data from Global Cancer Statistics 2018 (GLOBOCAN), the bladder cancer is among the Top 10 cancers which the highest annual prevalence around the world reaching to around 549,000 cases and 200,000 deaths<sup>1</sup>. The current standard therapy for advanced-bladder carcinoma is cisplatin-based chemotherapy, but the response is short-lived and the tumor is often resistant, therefore new effective therapeutic agents are needed<sup>2</sup>.

Several drugs have been investigated to be targeted therapy, including: anti-EGFR, anti-VGEF and others<sup>3</sup>. Immunotherapy as a new landscape has also been recommended by most guidelines for the management of advance-bladder cancer.

Metformin (N', N'-dimethylbiguanide) is a biguanide antidiabetic remedy from an oral hypoglycemic agent and is widely used by more than 100 million people worldwide for the medication of T2DM<sup>4</sup>. Well-known as for an antidiabetic agent, the proven effectiveness of Metformin has become popular in impeding the expansion of different cancer cells both *in vitro* and *in vivo* studies, including pancreatic cancer<sup>5,6</sup>, prostate cancer<sup>7,8</sup>, ovarian cancer<sup>9</sup> and breast cancer<sup>10</sup>. Several epidemiological, animal, and cellular studies on metformin as an antineoplastic have positively

uncovered that metformin directly affects the cancer cells expansion<sup>11</sup>.

Most previous investigation exhibited that the capability of Metformin in interfering the metabolism of cellular energy via the AMPK/mTOR axis pathway leading to the suppressed expansion of tumor<sup>11</sup>. The proliferating cancer cells exhibit very different metabolisms from the usual differentiated cells. The high cell multiplication was supported by the consumption and diversion of additional nutrients by the cells of the cancer cells triggering the mandatory preparation of metabolic pathways for a balance of biosynthetic processes and an adequate production of ATP in strengthening the cell development and survival. All cancer cells depend on these metabolic changes, so this pathway is an attractive therapeutic target<sup>12</sup> and is still controversial.

Furthermore, by having a contribution as the inhibitor of mitochondrial complex I in the liver, Metformin tampers the creation of ATP leading to an energy stress, raised AMP-activated protein kinase (AMPK) and hindrance of gluconeogenesis, which then drops both blood glucose levels and insulin but raises the insulin sensitivity<sup>12</sup>. AMPK activation can stimulate cell apoptosis by modulating downstream targets including p53 and inhibition of mTOR complex 1 (mTORC1)<sup>13</sup>. AMPK activation can also increase stromal cell survival through p53 activation because p53-depleted cells will decrease SCO2 activity and reduce mitochondrial oxidative phosphorylation efficiency<sup>14</sup>. Metformin also induces apoptosis which is a suicidal self-defense mechanism, and disrupts the cell cycle in G0/G1 or S phase stops temporarily at the cellular level, by decreasing the cyclin D1 protein in cancer cells<sup>15</sup>.

Indeed, those aforementioned descriptions indicate that metformin is able to be the prospective nominee for the latest remedial agents for the bladder carcinoma. However, it is not yet fully clear on how the anticancer features of metformin work on bladder carcinoma including the duration of administration along with its precise mechanism. Thus, it needs to be investigated in cell line 5637 bladder carcinoma which serves the purpose of this investigation.

#### MATERIAL AND METHODS:

In producing 1 M stock solution, this investigation used Metformin (1,1-dimethylbiguanide hydrochloride) produced by Tokyo Chemical Industry (TCI) Co., Ltd. By using the purified water, it was then diluted and then stored at -20°C. The concentrations of 15mM were utilized and diluted in culture media.

This study used the human cell line 5637, which was Transitional cell (Urothelial) Carcinoma grade II and

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purchased from American Type Culture Collection (ATCC® HTB-9TM) Manassas Virginia. The maintenance of cell lines was performed in RPMI 1640 by employing 10% fetal bovine serum (FBS) (Invitrogen Corp., Grand Island, NY) and 1% of penicillin-streptomycin which then cultured at 37°C with 5% CO<sub>2</sub> contained humidified atmosphere. The trypsinization of the cells was conducted upon confluency and the propagation to passage 2 was done before being sub-cultured for further experiments into 6 well plates. Finally, 0.25% trypsin-EDTA was utilized to passage the cells when ~80% confluence was accomplished.

Eighteenth well plates were prepared for the seeding process of the cells at 1-1.5 x 10<sup>6</sup> cells and then the incubation of the seeding cells was performed in medium containing with 10% FBS. The treatment of the cells was done with Metformin at the concentration of 15mM after seeding. The addition of MTT was completed for each well and the incubation was proceeded for 4 hours at 37°C and analyzed after 24 hr. The Automated Cell Counter TC210TM (Bio-Rad Laboratories, Inc.) was utilized for estimating the level of absorbance. By using a hemocytometer under an inverted microscope (CKX53, Olympus) the calculation of viable cells was implemented.

A smear slide was utilized in order to minimize the nonspecific staining caused by peroxidized block, while hydrogen peroxide was used to incubate the slides for 10-15 minutes. Slides were incubated overnight with monoclonal antibodies for Cyclin D1 (dilutions 1:200; Diagnostic BioSystems) and p53 (dilutions 1:100; Biocare Medical), and washed in phosphates buffer saline. It was followed by for 10 minutes a secondary antibody (Biogear Universal HRP Excell Stain System – Biogear, BDK-HES125) at room temperature and DAB chromogen for 5-15 minutes. Slides then were counterstained with Meyer's hematoxylin and dehydrated with 95% alcohol. We use human Breast carcinoma tissue as a positive control for cyclin D1 and p53.

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The expression of cyclin D1 and p53 was evaluated in the percentage of nuclear tumor cell positively stained by two pathologists using Olympus CX41RF light microscopes in the blinded fashion and documented using Olympus DP2-BSW.

The mean ± SD displayed the figures extracted from the statistical analysis and analysis using again with One-Sample Kolmogorov-Smirnov Test was done after that. Meanwhile, Kruskal-Wallis test was employed to make the comparison between the tumor viabilities (metformin treated versus control) and another variable.

## RESULTS AND DISCUSSION:

After the treatment administration in 5637 cells with Metformin at 15mM concentration during 24h assessment of its effects, it was acquired that Metformin displayed the capability in the inhibition of cell growth of Transitional (Urothelial) Carcinoma cells. The expansion of 5637 line cells was fundamentally impeded by Metformin. Metformin restrained the viabilities of 5637 line cells by 32% at 15mM concentration.

### Metformin effect on p53 wild expression in Urothelial Carcinoma Cells:

A display of significant different was not seen in the expression of wild type p53 between K0 and the treatment group with Metformin 15mM 24 h (p=0.809). And the wild type p53 expression between K24 and the treatment group with Metformin 15mM 24 h (p=0.688) did not express any significant different.

**Table 1: The p53 wild and Cyclin D1 expression in cell line 5637 Transitional (Urothelial) Cell Carcinoma of Bladder**

Group	Mean ± SD
<b>p53 wild Expression</b>	p=0.682
Control Cell K0	16.83 ± 12.57
Control Cell 24 H	16.00 ± 17.36
Metformin 15 mM 24 H	14.17 ± 11.25
<b>Cyclin D1 Expression</b>	p=0.091
Control Cell K0	11.67 ± 14.02
Control Cell 24 H	2.50 ± 2.74
Metformin 15 mM 24 H	4.17 ± 3.76
<b>Viability (%)</b>	p=0.003*
Control Cell K0	91.00 ± 7.56
Control Cell 24 H	84.33 ± 8.58
Metformin 15 mM 24 H	59.25 ± 3.20

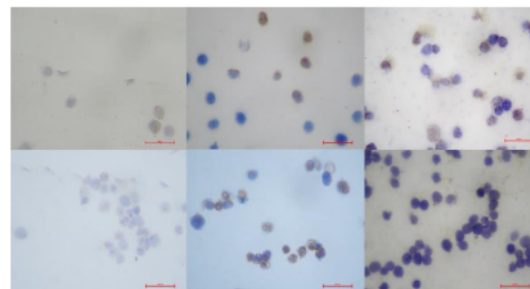
\* $\alpha < 0.05$ , considered as significant

### Metformin effect on cyclin D1 expression in Urothelial Carcinoma cells

The implication of metformin on the progression of cell cycle was implemented using cyclin D1 expression analysis. The 5637-line cells were smeared and stained by the immunohistochemical method using cyclin D1. The cyclin D1 expression between K0 and the treatment group with Metformin 15mM 24 h (down) (p=0.179) did not reveal any significant difference. And the cyclin D1 between K24 and the treatment group with Metformin 15mM 24 h (up) (p=0.423) also did not display any significant difference.

### Metformin is effective to inhibit Urothelial Carcinoma cell viability

A significant difference was presented in the viability between K0 and the treatment group with Metformin 15 mM 24 h (p=0.004)\*. And the viability between K24 group and the Metformin 15mM 24 h group (p=0.004)\* exhibited significant difference.



**Figure 1: The p53 wild (Upper) and Cyclin D1(lower) expression in nuclei of cell line 5637 Transitional (Urothelial) Cell Carcinoma of Bladder (K0(left), K24(middle) and Metformin 15mM 24 hr (right). All figures captured in 400x magnification.**

### Metformin effect on p53 wild expression in Transitional (Urothelial) Carcinoma cells:

Some literature states that metformin may increase the expression of wild p53, this examination revealed different outcomes, a decreasing wild p53 expression although it was not significant when metformin 15mM given, compared to K0 or K24 hours. These results indicate that metformin does not always affect cancer cells through p53. There seemed to be no difference between the cancer cell group of metformin and the control group, either K0 or 24 hours control. However, the administration of metformin succeeded in reducing the viability of cancer cells significantly.

There was no difference in p53 expression which indicates a difference results of from the previous studies which stated that p53 family proteins' activities and expression can be controlled by Metformin in the suppression of tumorigenesis. The interaction between AMPK, metformin, and p53 family proteins has fundamental contribution to the anticancer features of metformin. The induction of phosphorylation of p53 on serine 15 is done by an activated AMPK and makes the arrest of cell-cycle<sup>16</sup>. Several previous investigations have proven that p53 does have involvement in the anti-cancer features and activities of Metformin<sup>4,17</sup>. AMPK will be triggered by Metformin employing the induction of p53 phosphorylation, invasion activation and metastasis hindrance in the melanoma<sup>16,17</sup>

The control of expression and phosphorylation in p53 is conducted by AMPK and while the involvement of p53 is spotted in cell metabolism. In G0/G1 phase cell cycle will be blocked by Metformin leading to the noticeable drop on the G1, cyclins expression (including cyclin D1) and no transformation of p53 status in many different cancers<sup>4,18-20</sup>. The inhibition of the cancer cell expansion by Metformin was heavily involved with p53 activity<sup>17,21-23</sup>. The determination of Metformin effect may be driven by the given dosage and the p53 leading to the antitumor features of Metformin itself. Correspondingly, previous investigation on hepatoma cells by Yi *et al.*

revealed that Metformin at low dosage may influence p53-dependent senescence, while at the high concentration, it can affect the apoptosis in cell<sup>20,24</sup>

However, it was proven by several investigations that Metformin does have the capability in the inhibition of cancer cells' viability lacking with p53<sup>14,25</sup>. Therefore, these indications underline the ability of metformin as the inhibitor for cancer cells' expansion and survival in both p53-dependent and p53-independent ways<sup>16</sup>.

**Table 2: The p value of p53 wild and Cyclin D1 in cell line 5637 Transitional (Urothelial) cell Carcinoma of Bladder**

Group	p value
<b>p53 wild</b>	
Control Cell K0 - Metformin 15 mM 24 H	0.809
Control Cell 24 H- Metformin 15 mM 24 H	0.688
<b>Cyclin D1</b>	
Control Cell K0 - Metformin 15 mM 24 H	0.179
Control Cell 24 H- Metformin 15 mM 24 H	0.423
<b>Viability</b>	
Control Cell K0 - Metformin 15 mM 24 H	0.004*
Control Cell 24 H- Metformin 15 mM 24 H	0.004*

\* $\alpha < 0.05$ , considered as significant

#### Metformin effect on D1 expression in Transitional (Urothelial) Carcinoma cells

The effect of Metformin in cyclin D1 was not proven in this study because cyclin D1 expression did not disclose any significant difference between control and treatment group with 15mM Metformin 24h, however, it was revealed that metformin is able to weaken the cancer cells viability.

It expressed that Metformin is able to weaken cancer cells' viability without going through Cyclin D1. Cyclin D1 is not the only pathway for metformin to decrease cancer cell viability. Metformin can reduce the viability of cancer cells through other pathways, including the AMPK pathway, IGF1R and the effect of metformin through IGF1R is greater, so even though cyclin D does not decrease the viability of cancer cells can still be lowered.

The current investigation examines the over expression of cyclin D1 (CCND1) as the significant regulator in the arrest of G1-cell cycle. The occurrence of D1 over expression is closely connected with the weak prognosis along with the chemoresistance in ovarian cancer and the possibility in inducting the arrest of cell cycle by metformin was suggested by many experimental evidences<sup>26</sup>. Furthermore, by dropping the protein levels of cyclin D1, Metformin successfully shrinks the development of the tumor<sup>9</sup>. The rise in AMP: ATP ratio happens when the chain of respiratory complex I is being inhibited by Metformin. Metformin is capable in giving the secondary effects of ATP depletion rather than to the initiation of AMPK. Thus, in the prostate

cancer treatment, the efficiency of metformin in inhibiting the expansion of cancer cell and the development of tumor may entail crucial consequences<sup>9</sup>.

In this study, Cyclin D1 did not decrease due to metformin administration, although the viability of cancer cells decreased. It shows that the administration of metformin does not always affect cyclin D1 and the decrease in cancer cell viability is not only due to the falling cyclin D1 and the rising p53 along with the effect of metformin on AMPK and IGF 1 R. The action of metformin on cancer cells currently found is through AMPK which is a regulatory source energy/metabolism. Activated AMPK will affect several pathways, including Cyclin D1, p53, mTOR.

An examination by Gwak et al revealed that the contribution in cell cycle arrest by AMPK through cyclin D1 has not been clear yet. This current investigation followed this up by analyzing AMPK signaling in determining AMPK contribution in cyclin D1 deregulation which was induced with Metformin in the ovarian cancer cells. Indeed, cyclin D1 becomes the target of Metformin in diminishing the expansion of cancer cell along with a little or no effect on the normal cells bringing a foolproof evidence for a signaling node to govern cyclin D1 as the main aim for cancer therapy<sup>26</sup>.

This study proves that the action of metformin reduces cancer cell viability not only through p53 and cyclin D1, but through IGF1R and AMPK to mTOR. As we know, most studies have shown that metformin interferes with cellular energy metabolism via the AMPK/mTOR axis pathway, thereby suppressing tumor growth<sup>11</sup>. Proliferating cancer cells exhibit very different metabolisms from normally differentiated cells. Additional nutrients are being consumed by cancer cells which then divert them in supporting the high cell expansion, consequently, a balance of biosynthetic process have to be prepared by metabolic pathways along with adequate production of ATP in reinforcing the development and continuation of the cell<sup>11</sup>

Several studies expressed the effects of Metformin in contributing to the decrease of viability via IGF-1R since IGF-1R can enhance the development and progression of cancer. The improved activation of IGF1R by GH status IGF-1 or other procedures are able to build an anti-apoptotic environment promoting cell survival and malignant changes. Similarly, IGF1R signaling pathway can be employed and initiated oncogenes like HBx and Ewing Sarcoma fusion protein through accelerating the gene transition of IGF-1R while losing the tumor suppressor genes like BRCA1, p53 or WT1 causing the overexpression in IGF-1R along with

the transcriptional control loss<sup>27</sup>. However, Any insulin/IGF-I responses can be improved by hyperglycemic conditions leading to the transformed activation profile of AMPK as this conditions are also able to avoid the treatment of metformin to become fully effective in restraining the growth promoting signals in pancreatic cancer cells<sup>28</sup>.

In determining the resistance of anticancer therapies like HER2 targeted therapy, radiotherapy and hormonal therapy, Up-regulation of IGF1R signaling was developed<sup>27</sup>. Other study also displayed that metformin treatment successfully lessens insulin receptor/IGF I phosphorylation which can be triggered by the changes in its ligand levels. IGF1R and IR expressions were downregulated by Metformin through dropping the promoter activity of these receptor genes. By using the phosphorylation of IRS-1 in the inhibitory region (Ser789), the insulin signaling can be damaged to avoid the signal transduction to PI3K/AKT. Furthermore, the capability of metformin in decreasing the levels of insulin, in part its anticancer feature, may further describe the supporting evidence on the activities of body's additional mechanism<sup>29</sup>.

Both insulin and IGFs become the fundamental metabolism and growth regulators. Based on many previous studies, it was revealed that both insulin and IGFs have a contribution in the expansion and growth of cancer through the activation of signaling pathways connected with the cell development and proliferation<sup>30</sup>. Furthermore, both tumor formation and metastasis can be influenced by the overexpression of IGF-1R<sup>31,32</sup>. Similarly, IGF-1R overexpression is able to activate endometrial hyperplasia and has contribution in the development of type I epithelial cell by triggering PI3K/Akt/mTOR signaling in endometrial cancer cells<sup>33,34</sup>.

The suggestion that Metformin is able to implement its anticancer benefits by dropping IGF-1 levels comes from the current documentation. Memmott *et al.* examined that by restricting the tumor's expansion and development in a tobacco carcinogen-induced lung cancer model in A/J mice, metformin accomplished it by dropping the circulating levels of insulin and IGF-1<sup>35</sup>. The confirmation by Malaguarnera *et al.* also revealed that by decreasing the clonogenic capacity, the cell development and invasion, metformin has prevented the androgen-mediated up-regulation of IGF-1R<sup>36</sup>. In addition, emerging investigations also displayed that metformin-mediated activation of AMPK improved the phosphorylation of IRS-1 weakening IGF-1-induced initiation of Akt/TSC1/mTOR<sup>28,37,38</sup>. The crosswalk interruption between insulin receptor/IGF-1R and G protein-coupled receptor (GPCR) signaling via metformin-induced initiation of AMPK could be an

optional method related to IGF-1<sup>37,39,40</sup>. Metformin can block androgen induced IGF-1R up-regulation and IGF-1-mediated biological effects<sup>36</sup>.

Several studies on IGF1R stated, IGF1R overexpression in most of urothelial carcinoma and this makes the presence of overexpression in urothelial carcinoma causing IGF1R to be investigated and can be considered as the urothelial carcinoma therapeutic target<sup>41</sup>. Indeed, some animal and subclinical investigations demonstrated that the combination of metformin and IGF-1R axis inhibitors are proven to be feasible in the endometrial cancer's treatment<sup>42</sup>. In some other investigation, the crosstalk between insulin/IGF-1 receptor and GPCR signaling systems can be prevented on Ca<sup>2+</sup> mobilization, mTORC1 activation, DNA synthesis and proliferation in a variety of pancreatic cancer cell lines. The disruption of crosstalk between insulin/IGF-1 and GPCR signaling systems was accomplished by Metformin through AMPK in human pancreatic cancer cells<sup>39</sup>. The ability of metformin in triggering AMPK or impeding the downstream growth factor by signaling completed by mTOR inhibition becomes the foundation of metformin's biological consequence on cancer cells. The indirect effects of Metformin were found on the IGF and JNK/p38 MAPK pathways; other possible mechanisms which include the inhibition of HER2 and NF-κB signaling pathways<sup>40</sup>.

#### CONCLUSION:

The reduction of urothelial carcinoma tumor cells' viability was the result of Metformin's anti-tumor features which are not only accomplished through p53 and cyclin D1.

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#### CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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