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**ORIGINAL ARTICLE**

# Metformin reduces the proliferation of prostate and liver cancer cell lines and alters the expression of DKK1 and GADD45A

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**ABSTRACT**

Although the antidiabetic drug metformin (MET) exhibits anticancer properties, its mechanisms of action in prostate and liver cancer remain unclear. This study aimed to investigate the antiproliferative effects of MET and its impact on key regulatory genes involved in the Wnt, one-carbon metabolism, and p53 signaling pathways in prostate (PC3) and liver (HepG2) cancer cells. Bioinformatic analysis of public RNA-seq datasets (C4-2 and Huh-7) was performed to identify differentially expressed genes following MET exposure. DKK1, MTHFD2, and GADD45A were selected for validation. PC3 and HepG2 cells were treated with MET (5–40 mM) for 24 hours. Cell viability was evaluated using the CCK-8 assay, and RT-qPCR was employed to quantify changes in gene expression. MET inhibited the proliferation of both cell lines in a dose-dependent manner. In MET-treated PC3 cells, DKK1 expression increased up to 2.08-fold, but decreased to 0.6-fold in HepG2 cells. MTHFD2 expression rose in both lines. GADD45A expression was consistently elevated. These results demonstrate that MET has a significant antiproliferative effect on prostate and liver cancer cells. The differential regulation of DKK1 and MTHFD2, along with the consistent upregulation of GADD45A, is associated with MET's antitumor activity. Although this study did not directly test the exact mechanistic causality, the findings suggest that these pathways may play essential roles in MET's action, particularly the activation of the p53 pathway through GADD45A. This study identifies key molecular associations and supports further investigation into MET's therapeutic potential by exploring these altered oncogenic pathways.

***Keywords:*** Metformin, Prostate Neoplasms, Liver Neoplasms, GADD45A protein, DKK1 protein, MTHFD2 protein

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## Introduction

Prostate cancer is among the common cancers in men, ranking third in incidence in many countries due to aging populations. Liver cancer, mainly HCC, is the second leading cause of cancer deaths worldwide. Although the antidiabetic drug metformin (MET) exhibits anticancer properties, its mechanisms of action in prostate and liver cancer remain not fully elucidated (1-5). MET, a widely used antidiabetic drug, has garnered attention as a promising anticancer agent in both prostate and liver cancers. Studies report its antiproliferative effects, with minimal side effects. In addition, the anti-inflammatory, antioxidant, and protective against radiation-induced tissue injury effects of metformin have also been demonstrated, which may contribute to its therapeutic benefits. Despite extensive research, the precise molecular mechanisms driving MET anticancer activity require further investigation to optimize its therapeutic potential (6-12).

At the molecular level, MET primarily activates AMPK, which subsequently inhibits the mTOR pathway, a key regulator of cell growth and proliferation, and ultimately leads to the activation of tumor suppressor pathways (13). In prostate cancer, reduced prevalence observed among T2DM patients taking MET may be linked to lowered testosterone and IGF-1 levels. PI3K and androgen receptor (AR) signaling pathways, both of which play significant roles in prostate tumorigenesis (14). A clinical study revealed that MET improved overall survival and clinical significance in PCa patients with diabetic and non-diabetic conditions (15). However, some studies report inconsistent findings concerning clinical outcomes following prostatectomy (16).

Beyond the activation of AMPK, MET affects other crucial oncogenic pathways, such as epigenetic mechanisms. For example, findings indicate the potential of MET to be a therapeutic agent by targeting microRNA methylation and influencing epigenetic regulation in breast cancer (17). It has been reported to cause Wnt/β-catenin overactivation in some cancers, potentially promoting tumor progression (18). Conversely, MET inhibits cell growth in androgen receptor-positive prostate cancer cells by targeting downstream genes, including tumor suppressors in the p53 pathway (19). Furthermore, MET may exert antiproliferative effects by interfering with one-carbon metabolism (folate cycle), critical for nucleotide biosynthesis and methylation reactions (20). However, MET's complex impact on mitochondrial carbon metabolism might, under some circumstances, support tumor cell survival (21). Overall, existing literature highlights the antitumor effects of MET.

However, evidence suggests that the MET role may be context-dependent, potentially promoting cancer development in specific cancer types or tumor microenvironments. In liver cancer, MET use correlates with improved survival among diabetic patients (22). MET has been shown to induce apoptosis in liver cancer cells via activation of the AMPK and p53 pathways (23), although some evidence suggests that AMPK-independent mechanisms, including the inhibition of lipogenesis, also contribute to its antitumor effects (24). Notably, MET efficacy against liver cancer stem cells appears limited (25). Additionally, contradictory roles of MET in regulating Wnt signaling have been observed across cancer types, reflecting context-dependent outcomes, with MET inhibiting metastatic transitions in colorectal cancer but potentially promoting survival in others (26, 27).

Taken together, current evidence identifies the Wnt/β-catenin pathway, one-carbon metabolism, and p53-associated stress signaling as principal molecular axes influenced by metformin in various cancers. However, these pathways have been mainly studied in isolation. What remains unclear—and represents a critical research gap—is how metformin may simultaneously and differentially modulate these three interconnected systems within specific cancer contexts. In particular, no study has examined the coordinated regulation of DKK1 (a Wnt inhibitor), MTHFD2 (a one-carbon metabolism enzyme), and GADD45A (a p53-responsive stress gene) in response to metformin exposure in prostate and liver cancer models. The present work addresses this gap by investigating metformin's antiproliferative action and its impact on the expression of these key regulatory genes in PC3 and HepG2 cells, thereby providing insight into its potentially context-dependent and dualistic role in cancer biology.

## Methods

**Bioinformatic Analysis:**

RNA seq data of prostate (GSE196343) and liver (GSE190076) cancer were downloaded from GEO

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(NCBI). The original study for prostate cancer used C4-2 cells treated with 5 mM MET for 24 hours, while for liver cancer, Huh-7 cells were treated with 25 mM MET for 48 hours. Each study used three replications for control and treatment groups. Quality control of raw RNA-seq reads was assessed using FASTQC software (version 0.12.0) and then aligned to the reference genome by Hisat2 (version 2.2.1). Next, aligned reads were used to count gene levels using the HTSeq-count tool (version 1.99.2). Finally, differentially expressed genes were determined using DESeq2 (version 4.3.1). All data analyses were conducted using the Linux Ubuntu system (version 22.04).

**Cell Culture and Treatmen**

PC3 (NCBI Code: C427) and HepG2 (NCBI Code: C158) Cells were purchased from the Pasteur Institute (Iran). Cells were cultured in RPMI and DMEM (Gibco, Manchester, UK), respectively, supplemented with 10% fetal bovine serum (Gibco, Manchester, UK), 2 mM L-Glutamine, 100 U/mL penicillin (Gibco, Manchester, UK), 100 U/mL streptomycin (Gibco, Manchester, UK) in a 5% atmosphere at 37° C. After reaching passage 4, cells were grown to 70-90% confluency before use in subsequent experiments. The culture medium was replaced with fresh medium containing various concentrations of MET (Fakher Pharmaceutical Company, purity 98.5%). Working solutions (5, 10, 20, and 40 mM) were prepared by diluting a 1000 mM stock solution of MET. After 24 hours of incubation at 37°C, RNA was extracted and stored at -70°C.

**Proliferation Assay**

CCK-8 (Dojindo, Tokyo, Japan, Cat. No: 96992) was used to measure cell viability under MET treatment. Briefly, $5 \times 10^3$ cells in 100µL medium supplemented with 10% FBS were seeded per well in a 96-well plate and incubated at 37° C for 24 hours, followed by treatment with MET concentrations. After 24 hours, 10 µL CCK-8 solution was added to each well and incubated for 4 hours. Finally, the absorbance was calculated at 450 nm using a Nanodrop spectrophotometer (Synergy HT; BioTek, USA).

**RNA Extraction and cDNA Synthesis:**

For RNA extraction testing, $5 \times 10^5$ cells were seeded per well in a 6-well plate with 2 ml of culture medium supplemented with 10% FBS and incubated at 37° C for 24 hours. Cells were then treated with varying concentrations of metformin, and after 24 hours of treatment, RNA extraction was performed using the FavorPrepTM Blood/Cultured Cell Total RNA Mini Kit (FAVORGEN BIOTECH, Cat. No: FABRK 000-Mini).

Briefly, cells were harvested, then 350 µl of FARB Buffer and 3.5 µl of ß-Mercaptoethanol were added to the pellet. This mixture was transferred to a filter column within the collection tube. After centrifugation, the supernatant was moved to a new microcentrifuge tube where an equal volume of 70% ethanol (RNase-free) was added and mixed thoroughly by vortexing.

The sample was then applied to the FARB Mini Column and washed twice. RNA was finally eluted by adding 50 µL of RNase-free double-distilled water directly onto the membrane, followed by a high-speed centrifugation. The purified RNA samples were stored at -70°C until further use. For cDNA synthesis, the Yekta Tajhiz Azma cDNA Synthesis Kit (Iran, Cat No: YT4500) was employed. In this process, 500 ng of the extracted RNA was combined with 1 µL of random hexamer primers in 13.5 µL of DEPC-treated water and incubated at 70°C for 5 minutes. Subsequently, 4 µL of 5x first-strand buffer, 1 µL of 10 mM dNTPs, 0.5 µL of RNasin (40U/µl), and 1 µL of M-MLV reverse transcriptase were added. The mixture was incubated at 37°C for 60 minutes to allow reverse transcription. The resulting cDNA was stored at -20°C for later applications.

**RT-qPCR**

For the RT-qPCR assay, primers were designed by Gene Runner software and listed in Table 1. YTA qPCR Probe MasterMix (Yekta Tajhiz Azma, Iran, Cat No: YT2505) was used in RT-qPCR. A total of 20µL volume of the reaction solution was comprised of 2×qPCR Mastermix (10µL), for each forward and reverse primer (10µM; 1µL), cDNA (2µL), and 6µL ddH2O. After preparing the reaction solution, the cycling temperature condition was followed based on the manufacturer's protocol.

To ensure Primer specificity and temperature setup first, a temperature gradient was performed for each primer pair, and 2% gel electrophoresis was performed on selected PCR products to confirm the presence of a single amplicon of the expected size (Table 1).

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Representative gel images are presented in Supplementary Figure 1. To ensure the reliability of RT-qPCR data, Standard curves were generated for each target gene (DKK1, MTHFD2, GADD45A) and the reference gene (β-ACTB) using a 5-point 10-fold serial dilution of cDNA from representative samples. The PCR amplification efficiency (E) for each primer pair was calculated from the slope of the standard curve. All primer efficiencies ranged between 90% and 110%. Detailed standard curves and efficiency values for each primer pair and melt curves are provided in Supplementary Figure1.

**Statistical Analysis**

Relative gene expression was quantified using the Pfaffl method, incorporating primer-specific amplification efficiencies. Before applying statistical tests, data distributions were evaluated using the Shapiro–Wilk test to assess normality and Levene's test to confirm homogeneity of variances. Comparisons between two independent groups were performed using an unpaired, two-tailed Student's t-test.

For comparisons across more than two groups, one-way ANOVA was used, followed by post hoc multiple-comparison tests when appropriate. RNA-seq differential expression analysis was conducted using DESeq2 with Benjamini–Hochberg false discovery rate (FDR) correction; adjusted p-values (padj < 0.05) were considered statistically significant. All statistical analyses were performed in R (version 4.3.0), and graphs were generated using GraphPad Prism (version 7.0.0). For RT-qPCR experiments, statistical significance was defined as $p < 0.001$. Fold-change values were interpreted separately as biological effect size, and changes with both $p < 0.001$ and $|\text{fold change}| > 0.5$ were considered biologically meaningful. Error bars in all graphical presentations represent the standard deviation (SD) of biological replicates (n = X per group).

## Results

**Differential Gene Expression Analysis Reveals Shared MET-Responsive Genes:**

Bioinformatic analysis identified 5,047 differentially expressed genes in liver cancer cells (Huh-7) and 5,945 in prostate cancer cells, with 2,335 genes commonly altered by MET treatment across both datasets (Figure 1). From this subset, three genes, DKK1, MTHFD2, and GADD45A, were selected for further validation (Figure 2).

**MET Treatment Inhibits Cell Proliferation in PC3 and HepG2 Cells:**

MET treatment significantly reduced cell viability in both PC3 and HepG2 cell lines in a dose-dependent manner. In PC3 cells, viability decreased considerably starting at 10 mM MET, with percentages of 80.7% ± 2.8 at 10 mM ($p < 0.0208$), 70.1% ± 0.3 at 20 mM ($p < 0.0001$), 54.3% ± 0.27 at 40 mM ($p < 0.0001$), and 31.74% ± 0.52 at 80 mM ($p < 0.0001$). In HepG2 cells, viability decreased at all tested concentrations (5–80 mM), with percentages of 91.86% ± 1.45 at 5 mM ($p < 0.0387$), 79.1% ± 1.54 at 10 mM ($p < 0.0059$), 67.73% ± 2.3 at 20 mM ($p < 0.0052$), 49.33% ± 0.67 at 40 mM ($p < 0.0001$), and 26.6% ± 1.19 at 80 mM ($p < 0.0001$) (Figure 3)

**Table 1. Primer sequences for RT-qPCR**

| primer | Accession codes | Forward Sequence (5' to 3') | Reverse Sequence (5' to 3') | Amplicon Size (bp) |
|---|---|---|---|---|
| β-ACTB | NM_001101 | AGATCATTGCTCCTCCTGAG | AGTCATAGTCCGCCTAGAAG | 159 |
| DKK1 | NM_012242 | TGTATCACACCAAAGGACAAG | TCTTTCAGGACAGGTTTACAG | 115 |
| GADD45A | NM_001924<br>NM_001199741<br>NM_001199742 | ACAAAGAACCATGCAGGAAG | AATACCCAAACTATGGCTGC | 110 |
| MTHFD2 | NM_006636<br>NM_001410192 | TCAGTTGCCTCTTCCAGAGC | TGGAGTAGCCGGTAACATGG | 139 |

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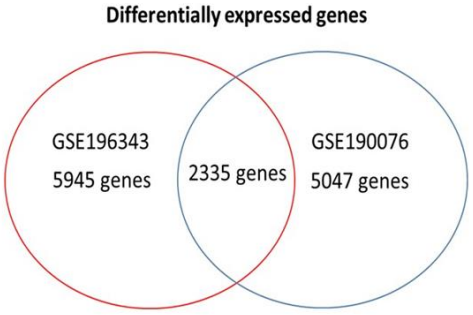


**Figure 1. Shared metformin-responsive genes between C4-2 and Huh-7 RNA-seq datasets. Differential expression analysis of MET-treated prostate (C4-2) and liver (Huh-7) RNA-seq datasets identified 5,945 altered genes in C4-2 and 5,047 in Huh-7 cells. The Venn diagram shows 2,335 genes commonly regulated in both datasets, representing MET-responsive transcripts. These shared genes formed the basis for the selection of DKK1, MTHFD2, and GADD45A for experimental validation. Significance for RNA-seq data was determined using DESeq2 with Benjamini–Hochberg FDR correction (adjusted p < 0.05).**

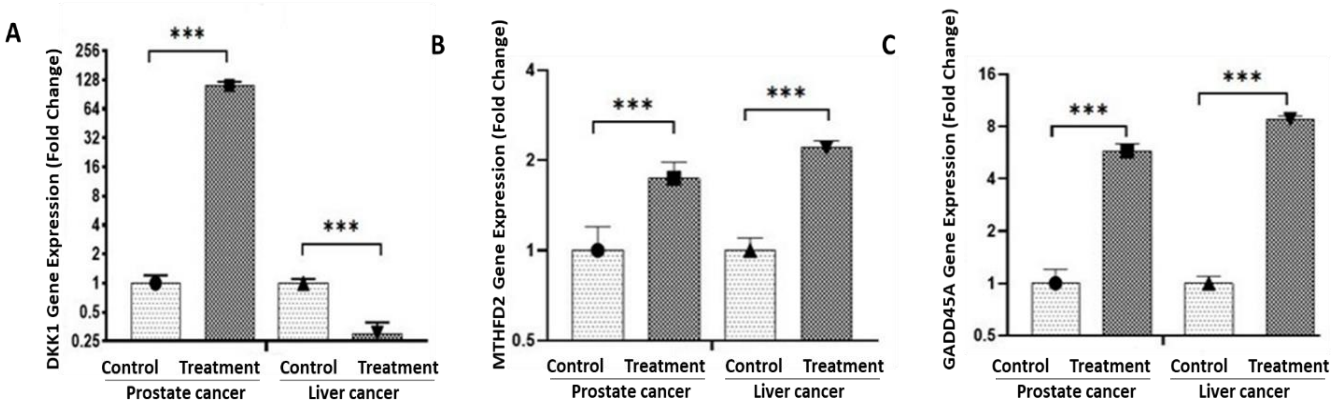


**Figure 2. RNA-seq–derived expression profiles of DKK1, MTHFD2, and GADD45A following MET exposure. (A–C) Expression changes in DKK1, MTHFD2, and GADD45A derived from MET-treated prostate and liver RNA-seq datasets. MET increased DKK1 expression in prostate cells but decreased it in liver cells, while MTHFD2 and GADD45A were consistently upregulated in both datasets. Statistical significance was based on DESeq2 FDR-adjusted p-values (***padj < 0.0001).**

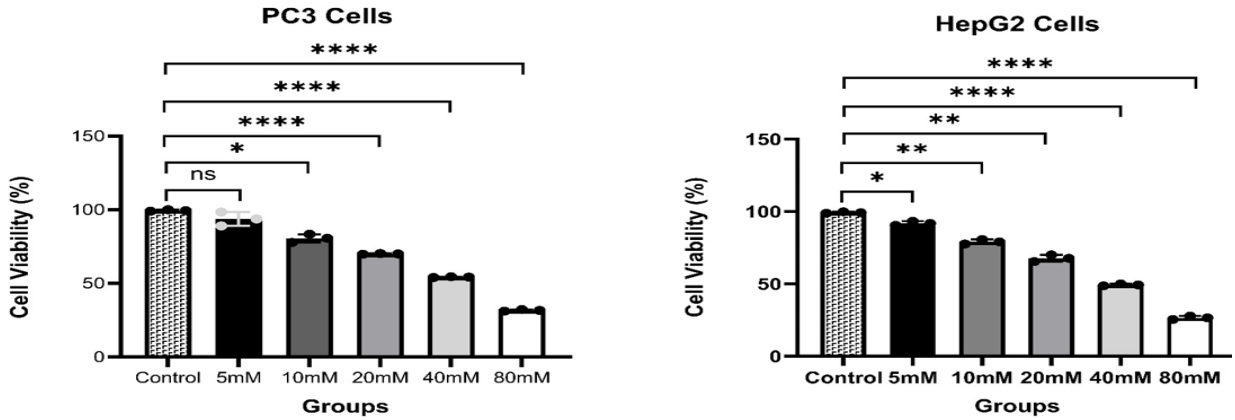


**Figure 3. Effects of metformin on cell viability in PC3 and HepG2 cells. PC3 and HepG2 cells were treated with MET (5–80 mM) for 24 h, and cell viability was measured using the CCK-8 assay. Data are presented as mean ± SD of n = 3 biological replicates. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test, comparing each treatment to its respective untreated control. Significance levels: ns (p > 0.05), *p < 0.05, **p < 0.006, ***p < 0.0004, ****p < 0.0001. Error bars represent SD.**

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**MET Differentially Regulates DKK1, MTHFD2, and GADD45A Expression in Prostate and Liver Cancer Cells**

RT-qPCR analysis confirmed the bioinformatic findings by quantifying mRNA expression of DKK1, MTHFD2, and GADD45A under MET treatment in both cell lines. In prostate cancer cells, DKK1 expression significantly increased with fold changes of 0.97 ± 0.08, 1.2 ± 0.07, 1.37 ± 0.11, and 2.08 ± 0.03 at 5, 10, 20, and 40 mM MET, respectively ($p < 0.05$, n=3) (Figure 4A). Conversely, DKK1 was significantly downregulated in liver cancer cells, showing fold changes of 0.47 ± 0.01, 0.65 ± 0.016, 0.6 ± 0.05, and 0.65 ± 0.12 across the same concentrations ($p < 0.05$, n=3) (Figure 4B). Both GADD45A and MTHFD2 showed notable upregulation at all tested concentrations in prostate and liver cancer cells. GADD45A fold changes ranged from 1.40 ± 0.01 to 5.1 ± 0.059 in prostate and 1.18 ± 0.2 to 3.78 ± 0.22 in liver cells ($p < 0.05$, n=3) (Figures 4C-D). MTHFD2 increased from 1.34 ± 0.09 to 4.2 ± 0.32 in prostate and 1.56 ± 0.07 to 5.33 ± 0.14 in liver cells ($p < 0.05$, n=3) (Figures 4E-F).

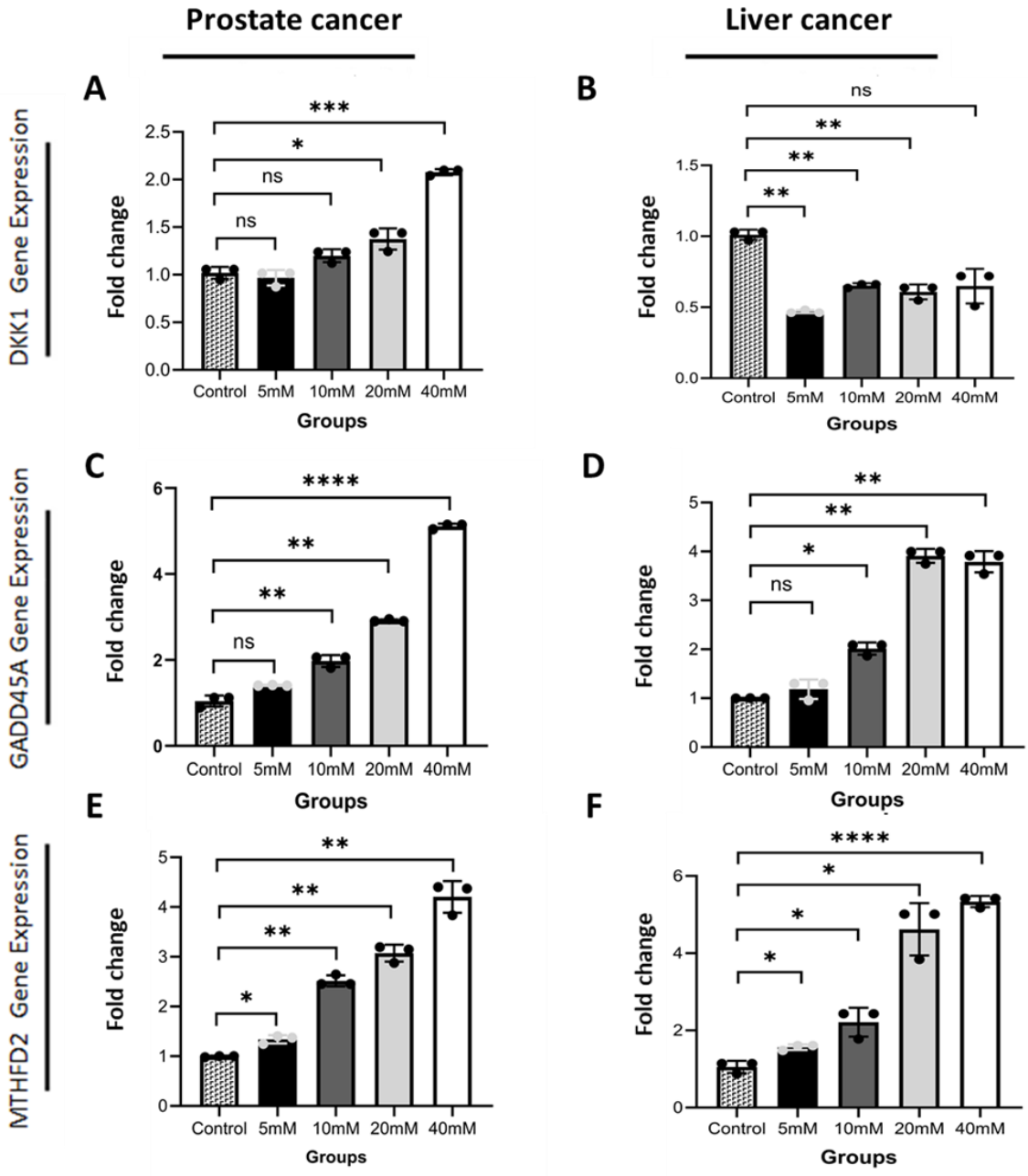


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**Figure 4. RT-qPCR validation of MET-regulated genes in PC3 and HepG2 cells. MET increased DKK1 expression in PC3 cells but decreased it in HepG2 cells (Figure A–B). GADD45A expression was upregulated across all MET concentrations in both cell lines (Figure C–D). MTHFD2 expression was also consistently increased following MET treatment (Figure E–F). Data represent mean ± SD of n = 3 biological replicates, and relative expression was calculated using the Pfaffl method incorporating primer efficiencies. Statistical significance was assessed using one-way ANOVA followed by post hoc testing, with *p < 0.05 considered significant. Error bars represent SD.**

## Discussion

Liver and prostate cancers are among the most prevalent malignancies worldwide, representing significant challenges in oncology due to their heterogeneity, late diagnosis, and variable therapeutic responses. Despite advances in treatment, prognosis remains poor for many patients, highlighting the urgent need for more effective and personalized therapeutic strategies.

In this study, we explored the impact of MET, a widely used antidiabetic drug with emerging anticancer properties, on prostate and liver cancer cells using a combination of bioinformatic analysis and in vitro validation. The anticancer effects of MET are well-documented, yet the precise mechanisms and their context-dependency remain subjects of active research and, at times, conflicting reports in the literature. For instance, while MET is widely known to exert its anticancer effects through AMPK activation, evidence also points to AMPK-independent mechanisms (21).

Similarly, MET's influence on critical pathways like Wnt signaling appears divergent: some studies report Wnt inhibition, while others suggest MET can promote Wnt activity or progression in specific cancer types or contexts (15, 23, 24). Such contradictory findings, which highlight MET's ability to act both as a tumor suppressor and, under specific conditions, potentially contribute to tumor cell survival (18, 23), underscore that MET's effects are highly context-dependent, influenced by factors such as cancer type, cellular energetic state, and specific genetic alterations. This inherent complexity and the presence of conflicting evidence directly justify the comparative approach taken in our study.

By simultaneously investigating MET's effects on prostate and liver cancer cells, and focusing on a tripartite evaluation of DKK1, MTHFD2, and GADD45A, we aimed to gain a more nuanced understanding of both its differential and convergent mechanisms. However, it is crucial to emphasize that additional mechanistic tests are necessary to resolve these discrepancies fully and to definitively establish the specific cellular and molecular contexts that dictate MET's varied responses.

Metformin significantly reduced proliferation in both prostate (PC3) and liver (HepG2) cancer cells, consistent with prior reports in these tumor types (28-31). PC3 cells exhibited measurable inhibition beginning at 10 mM, indicating lower sensitivity compared with HepG2 cells, which responded at all tested concentrations. These differences are in line with previous studies (32) showing cell line–specific variability in metformin responsiveness and likely reflect underlying metabolic and genetic distinctions. Overall, the proliferation data provide a foundation for interpreting the gene-specific effects described in subsequent sections.

A notable finding of this study is the contrasting, cell-type-specific regulation of DKK1 by metformin. In HepG2 liver cancer cells, metformin was found to suppress DKK1 expression, which aligns with bioinformatic data from Huh-7 cells and supports the established role of DKK1 downregulation in inhibiting the proliferation of liver cancer (33, 34). This indicates a potential mechanism whereby metformin's antiproliferative effects in liver cancer might be linked to the suppression of the Wnt signaling pathway through DKK1 downregulation. However, establishing a direct causal relationship would necessitate further functional validation.

However, the results in prostate cancer were strikingly different. While the bioinformatic analysis of AR-positive C4-2 cells showed a dramatic 120-fold upregulation of DKK1, our in vitro experiments on AR-negative PC3 cells showed a more modest, though significant, increase only at higher concentrations. This discrepancy between cell lines is a critical point of analysis. Although the RNA-seq data were generated from AR-positive C4-2 cells, PC3 cells were intentionally selected for experimental validation because they represent an AR-negative, castration-

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resistant prostate cancer model with significant clinical relevance.

This choice allowed us to evaluate metformin's effects in a fundamentally different androgen-signaling environment and to determine whether the transcriptomic patterns observed in C4-2 cells are conserved or altered in an AR-independent context. This would explain the varied responses and suggests that AR status could be a biomarker for predicting patient response to metformin treatment. Our results support the hypothesis that metformin's regulation of DKK1 in prostate cancer may be AR-dependent, which could explain the marked DKK1 upregulation observed in C4-2 cells versus the more modest response in AR-negative PC3 cells.

This raises the possibility that AR status influences metformin's impact on Wnt signaling and may serve as a biomarker for predicting therapeutic response. To fully delineate these mechanisms, future studies should systematically compare AR-positive (LNCaP, VCaP, C4-2) and AR-negative (PC3) cell lines to directly assess AR-dependent versus AR-independent effects on DKK1 and related Wnt pathway components.

In contrast to the cell-type-specific behavior of DKK1, metformin consistently increased GADD45A expression in both prostate and liver cancer models. This uniform response suggests that activation of p53-associated stress signaling may represent a shared, convergent mechanism underlying metformin's antiproliferative effects.

The established role of GADD45A in mediating p53-driven cell-cycle arrest and apoptosis (35, 36) supports this interpretation. Although our findings indicate a strong association between metformin exposure and GADD45A induction, definitive evidence of a causal role will require targeted functional studies, such as GADD45A knockdown or pathway inhibition assays.

The observed upregulation of MTHFD2, despite metformin's apparent inhibitory effect on cell proliferation, is particularly intriguing. As MTHFD2 is generally linked to tumor growth and metabolic rewiring, its induction may reflect a compensatory metabolic response or stress-adaptation mechanism rather than a pro-tumorigenic shift. This paradox highlights the complexity of metformin's influence on one-carbon metabolism and underscores the need for further mechanistic investigation. One of the most counterintuitive findings was the upregulation of MTHFD2, a known tumor-promoting enzyme, in both cell lines following metformin treatment. This is paradoxical, given that metformin dramatically inhibited cell proliferation.

This result strongly suggests that metformin's anticancer mechanisms can override the oncogenic signals from specific metabolic pathways like one-carbon metabolism. As others have shown, metformin mitigates PI3K/AKT signaling (37), which is a central upstream regulator of cell growth.

We interpret the MTHFD2 upregulation not as an insignificant side effect, but as evidence of a cellular stress response that is ultimately overcome by metformin's dominant inhibitory effect on primary oncogenic drivers like the AKT pathway. This highlights the hierarchical nature of metformin action and cautions against using single metabolic markers like MTHFD2 to predict its efficacy. This suggests that MET's primary antiproliferative effects may operate through pathways that override the observed MTHFD2 upregulation.

Furthermore, the inherent molecular and genetic heterogeneity between different cell lines, even within the same cancer type (e.g., C4-2 vs. PC3 for prostate cancer, or Huh-7 vs. HepG2 for liver cancer), can lead to variations in drug response. While our study aimed to validate key findings in established models, acknowledging these differences is crucial for interpreting the generalizability of our results.

The observed differential effects, particularly with DKK1, underscore the importance of cell context-specificity and genetic background in mediating metformin's anticancer actions. This highlights the necessity for further investigation across a broader panel of cell lines and, ultimately, in vivo models and patient-derived samples to fully elucidate MET's therapeutic potential across the spectrum of prostate and liver cancers.

Although MET has been widely associated with AMPK activation, mTOR suppression, and p53–GADD45A signaling, our findings do not directly demonstrate activation of these pathways, as no protein-level or phosphorylation analyses were performed. Therefore, interpretations involving AMPK/mTOR/p53 should be considered hypothesis-generating rather than mechanistic conclusions.

In conclusion, this study highlights MET's multi-faceted effects on key genes (DKK1, MTHFD2,

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GADD45A) across prostate and liver cancers and its potential as an antitumor agent modulating vital signaling pathways. The complex effects of MET on DKK1, MTHFD2, and GADD45A, as a potential mediator of MET, merit further investigation. In vivo studies and evaluation at clinically relevant exposures are essential to fully understand the role of MET and its therapeutic potential in oncology.

**Limitations**

The study reveals new insights into how MET affects prostate and liver cancer cells differently, but it has several limitations. Primarily, it relies on in vitro cancer cell lines, which do not fully mimic the complexity of tumors in living organisms, highlighting the need for in vivo and patient-derived validations to ensure clinical relevance. Additionally, the analysis focused on mRNA expression of a few genes without confirming protein-level changes, which could better reflect functional impacts. The work also lacks complementary assays assessing specific cell death mechanisms and biochemical validation of pathway activation, including Western blot analysis of phospho-AMPK, total AMPK, phospho-mTOR, or p53 protein levels. Additionally, pathway perturbation experiments using pharmacologic modulators or genetic approaches were not performed. These experiments will be necessary to determine whether the observed transcriptional changes are mediated through AMPK-dependent or independent mechanisms.

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