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ORIGINAL ARTICLE

Downregulation of tumour biomarkers in colon cancer cells with IRNA PFK-1 plus metformin

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Summary

Purpose: *Metformin has been widely used for the treatment of Type 2 Diabetes Mellitus (T2DM), hyperglycemia and polycystic ovarian syndrome. Recent studies have suggested the potential of this substance as a cancer chemopreventive agent. We evaluated the antitumoral effect of iRNA-PFK-1 and the combined therapy iRNA-PFK-1 + metformin in RKO p53-positive cells.*

Methods: *mRNA levels of tumor suppressor genes AMPK, APC, and c-MYC, KRAS oncogenes were measured by qRT-PCR in RKO cells treated with 25 μM metformin alone or combined with iRNA-PFK-1, to evaluate the effect of both treatments.*

Results: *At 72 h after treatment with either 25 μM metformin, 150 nM iRNA-PFK-1, or the combined treatment, the transcriptional levels of these biomarkers were decreased by ~73% ($p<0.05$), ~99.9%, ($p<0.01$), and ~76% ($p<0.05$), respectively.*

Conclusion: *These in vitro results support the potential therapeutic role of metformin and PFK-1 in the treatment of colon cancer via down-modulation of the expression of several important cancer biomarkers.*

Key words: *iRNA-PFK-1, metformin, colon cancer, biomarkers*

Introduction

Colorectal cancer (CRC) was the second leading cause of worldwide cancer-related death in 2018 [1]. That year, an estimated 1.8 million new CRC cases were reported globally [2]. In Mexico, CRC was the fifth leading cause of cancer-related death, accounting for 7.2% of deaths by cancer [3], or ~4,300 deaths in that country per year [4,5].

Although poorly studied, one potential treatment for CRC is metformin (dimethylbiguanide 1,1-hydrochloride), a semisynthetic biguanide with two methyl groups attached to the nitrogen nucleus biguanide. Metformin, obtained from the plant galegin (*Galega officinalis*), is an insulin-sensitizing drug that has been widely used for the treatment of T2DM, hyperglycemia and polycystic ovarian syndrome [6-8]. Metformin increases glucose uptake and utilization by muscle tissue, and suppresses gluconeogenesis and glycogenolysis in the liver, thus decreasing basal glucose production [9]. These properties, coupled with its safety and low cost, are the primary reasons why metformin is the primary choice for the treatment of T2DM [10]. Interestingly, patients with T2DM who have been treated with metformin are reported to have lower cancer incidence than those undergoing treatment with other anti-diabetic medications [11-15].

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Recent studies suggest metformin is a potential chemopreventive agent that acts through suppression of cell hyperproliferation [16,17]. Research indicates that the hypoinsulinemic and hypoglycemic capacity of metformin inhibits various cancers associated with hyperinsulinemia, such as those in colon, pancreas, endometrium, liver, and breast [18-25]. Metformin can exert its antiproliferative effect through two distinct mechanisms, indirect and direct. The first one, or insulin-dependent, pathway is based on metformin's ability to activate AMP-activated protein kinase (AMPK), which prevents transcription of genes responsible for glycogenesis in liver cells [26]. As an effect, muscle cells uptake glucose, reducing in turn glucose and insulin concentration in blood. Tumor growth and proliferation is, in part, due to increased insulin receptors in neoplastic cells; thus, decreasing the amount of insulin in blood decreases the mitogenic effects of insulin. The direct mechanism, also known as insulin-independent, is an effect of the metformin's activation of AMPK through Serine/Threonine Kinase 11 (LKB1) [27]. AMPK activates TSC complex subunit 2 gene (TSC2), a tumor suppressor. TSC2 decreases mTOR activity, reducing in turn protein synthesis and proliferation. Interestingly, novel research suggests that the antitumor action of metformin is carried out independently of AMPK and LKB1 [28,29]. In two pre-clinical studies, metformin suppressed the activity of mTOR in an AMPK-independent fashion, but dependent upon GTPase RAG [28].

One of the metabolic properties of tumor cells is that they support their proliferative processes through aerobic glycolysis, mediated among other enzymes, by phosphofructokinase-1 (PFK-1). Cells in a proliferative state exhibit an increased expression of phosphofructokinase-2 to synthesize fructose-2,6-bisphosphate, which is necessary to allosterically activate PFK-1, maintaining in turn a glycolytic flux that ensures high levels of ATP necessary for proliferation [30-34]. Glucose that is not used by cells for aerobic glycolysis undergoes oxidative phosphorylation to produce fatty acids and nonessential amino acids, metabolites required by cells for continued proliferation. In cancer cells, a change in glucose metabolism marked by an increased rate of aerobic glycolysis tied to lactic acid fermentation in the cytosol, the Warburg effect, is promoted by oncogenes and inhibited by tumor suppressor genes [35].

Relevant to cancer biology is RAS oncogene, responsible for cell proliferation via the activation of various effector routes, such as the P13K/Akt pathway [36,37]. This pathway regulates glycolysis in different ways, one of which is the translocation of glucose transporter 1 into the cell surface to stimulate the activity of phosphofructokinase enzyme [38]. The mTOR pathway, located downstream of Akt, is involved in protein translation and activation of glycolysis, similar to the MYC oncogene [39]. MYC catabolizes glutamine for the synthesis of proteins and nucleotides [40].

An important biomarker of CRC is AMPK [41]. AMPK is an enzyme with central roles in the detection of cellular metabolism and in energy homeostasis; it interacts with various pathways (such as p53 and mTOR) [42]. Another gene linked to CRC is adenomatous polyposis coli (APC), a tumor suppressor that acts by slowing the activity of β-catenin [43,44]. The β-catenin/Tcf complex acts as a transcription factor of proto-oncogenes, like WISP-1, cyclin D1 and c-MYC [45]. Mutations in APC cause familial adenomatous polyposis, a disease that can result in the development of CRC [46].

To determine the independent and combined effect of metformin with/without iRNA-PFK-1 in colon carcinoma cell line RKO in this study we quantified RNA levels of tumor suppressing genes AMPK and APC, and oncogenes c-MYC and KRAS.

Methods

Cell culture and treatment with metformin

RKO cells were maintained in advanced Dulbecco's Modified Eagle's Medium (ADMEM) (GIBCO-BRL; Grand Island, NY, USA) supplemented with 2 % fetal bovine serum (FBS) (GIBCO-BRL; Grand Island, NY, USA), 1 % nonessential amino acids (GIBCO-BRL; Grand Island, NY, USA), 200 UI of penicillin G (GIBCO-BRL; Grand Island, NY, USA) and 200 mg of streptomycin (GIBCO-BRL; Grand Island, NY, USA) per mL at 37°C. Cells were washed with 5 mL or 2 mL sterile 1X phosphate buffered saline (PBS) (GIBCO-BRL; Grand Island, NY, USA) when using 75 cm^2 or 25 cm^2 cell culture flasks, respectively. Once cells reached confluence, the cell monolayer was detached from the bottle by adding 1 mL of trypsin (GIBCO-BRL; Grand Island, NY, USA), incubated at 37°C for 5-10 min. Then, trypsin was neutralized by adding an equal volume (i.e., 1 mL) of ADMEM supplemented with FBS and the entire mix was transferred to a 15 mL conical centrifuge tube. Subsequently, cells were centrifuged at 1,000 rpm for 5 min at room temperature. The supernatant (media with trypsin) was removed, and 2-4 mL of culture media were added to the pellet, which was re-suspended by gently pipetting up and down. A previously calculated volume of cells was seeded in a 75 cm^2 cell culture flask containing 10 mL of ADMEM (2% FBS, 1% antibiotic penicillin/streptomycin (GIBCO-BRL; Grand Island, NY, USA), 1% glutamine (GIBCO-BRL; Grand Island, NY, USA), 1% essential amino acids (GIBCO-BRL; Grand Island, NY, USA) or to a 25 cm^2 bottle with 5 mL of supplemented ADMEM media. The cells were incubated at 37°C with 5% CO_2 until they again reached 90% confluence. For metformin treatments,

40,000 cells were seeded in 24-well plates with DMEM, 2% FBS and 25 μM metformin (Sigma-Aldrich; St. Louis, MO, USA).

Transfection of iRNA-PFK-1

The iRNA PFK1 (UUAGUCCCAAGUUUAGAGC), was transfected at a final concentration of 150 nM using siPORT-Neo-FX transfection agent (Ambion; Waltham, MA, USA). We used 30 nM iRNA-GAPDH and 30 nM iRNA-nonsense as positive and negative control, respectively (Ambion; Waltham, MA, USA). Transfected cells were incubated for 24, 48 and 72 h, after which they were harvested, total cellular RNA was extracted (see Total RNA isolation, below), and RNA analyzed by qRT-PCR. All determinations were performed in triplicate.

Total RNA isolation

Total RNA was extracted from RKO cells using TRIzol (Invitrogen; Waltham, MA, USA) as per the manufacturer's instructions. Briefly, the contents of each well were mixed with TRIzol and transferred to a 1.5 mL microfuge tube. The mixture was incubated for 5 min at room temperature to dissociate nucleoprotein complexes completely. To promote phase separation, we added 40 μL of chloroform (Sigma-Aldrich; St. Louis, MO, USA) for every 200 μL of TRIzol used for cell lysis. This mix was mixed by inversion for 15 s, incubated on ice, and centrifuged for 15 min at 13,000 rpm at 4°C. The resulting aqueous phase was recovered, mixed with 100 μL isopropanol (Sigma-Aldrich; St. Louis, MO, USA) and incubated at -80°C for 1 h or overnight. After incubation, this mixture was centrifuged at 13,000 rpm for 15 min at 4°C. Subsequently, the supernatant was removed, and the pellet washed with 200 μL of 70% ethanol (Sigma-Aldrich; St. Louis, MO, USA) (prepared with DEPC water), then centrifuged at 13,000 rpm for 5 min at 4°C. Finally, the resulting ethanol layer was carefully removed using a micropipette, and the pellet was re-suspended in 20 μL of water with 0.3 μL DEPC RNaseOUT (Invitrogen; Waltham, MA, USA). Total RNA samples were stored at -80°C until further use.

Reverse transcription (RT)

We prepared "Mix 1" by resuspending 5 μL (~1000 ng) of isolated RNA into 5.5 μL of DEPC H_2O, adding 1 μL of 'Random' primers (Invitrogen; Waltham, MA, USA). Using a thermocycler, Mix 1 was incubated at 72°C for 10 min and subsequently placed on ice for 3 min. Simultaneously, we prepared "Mix 2" by adding 2 mL of 0.1 M DTT (Sigma-Aldrich; St. Louis, MO, USA), 0.5 μL of 40 U/μL RNaseOUT, 1 μL of dNTP's (Invitrogen; Waltham, MA, USA), and 1 μL of 200 U/μL M-MLV (Invitrogen; Waltham, MA, USA) into 4 μL of 5X RT Buffer. Upon completion of "Mix 1" incubation, we added 8.5 μL of "Mix 1" to the entire volume of "Mix 2"and further incubated the mixture with the following conditions: 10 min at 25°C, 1 h at 37°C, 5 min at 94°C, then 30 min at 4°C. The resulting cDNA was stored at -20°C until use.

Real time polymerase chain reaction (qPCR) for targeted genes

The reaction components were as follows (in a final volume of 20 μL): 10 μL of 2X SybrGreen (Invitrogen; Waltham, MA, USA), 7 μL of DEPC water, 1 μL of the forward primer (40 mM PFK1 primers, FW: 5'GAGTGACTTGTTGAGTGACCTCCAGAAA 3'and RV: 5'CACAATGTTCAGGTAGCTGGACTTCG 3'; KRAS, FW: 5' GGGGAGGGCTTTCTTTGTGTA 3' and RV: 5' GTCCTGAGCCTGTTTTGTGTC 3'; APC, FW: 5' TTATGGAAGCCGGGAAGGA 3' and RV: 5' TGGAAATGAACCCATAGGAACAG 3' ; c-MYC, FW: 5'TCAAGAGGCGAACACACAAC 3'and RV: 5'-GGCCTTTTCATTGTTTTCCA-3'; AMPK, FW:

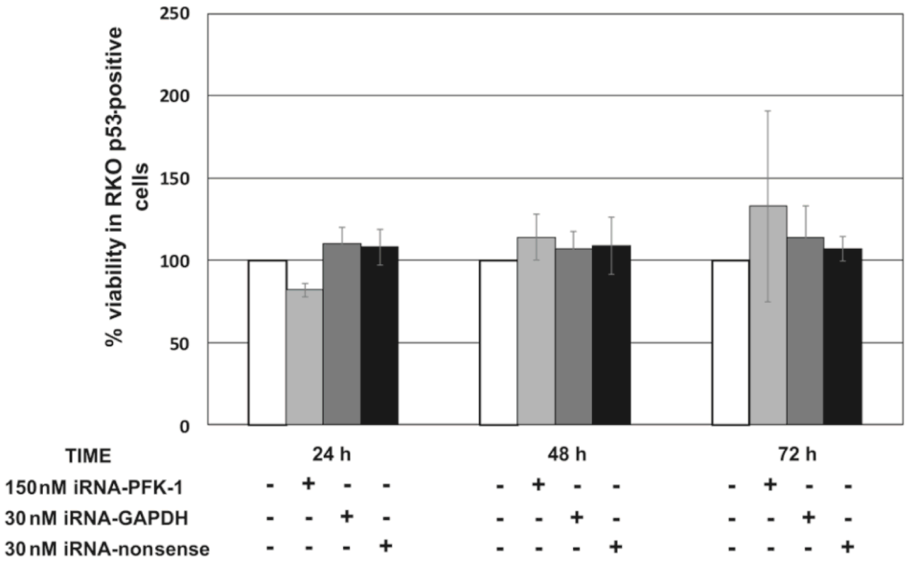

Figure 1. Cell viability assay in RKO cell line post-transfection with iRNA-PFK-1, iRNA-GAPDH, iRNA-non sense. A cell viability assay using Alamar Blue (Invitrogen; Waltham, MA, USA) (λ=570/600 nm) was performed in the RKO cell line 24, 48 and 72 h post-transfection with iRNA-PFK-1 (150 nM), iRNA-GAPDH (30 nM) and iRNA-non sense (30 nM). No significant difference in cell viability was observed after transfection with any of the treatments (n=3, SD).

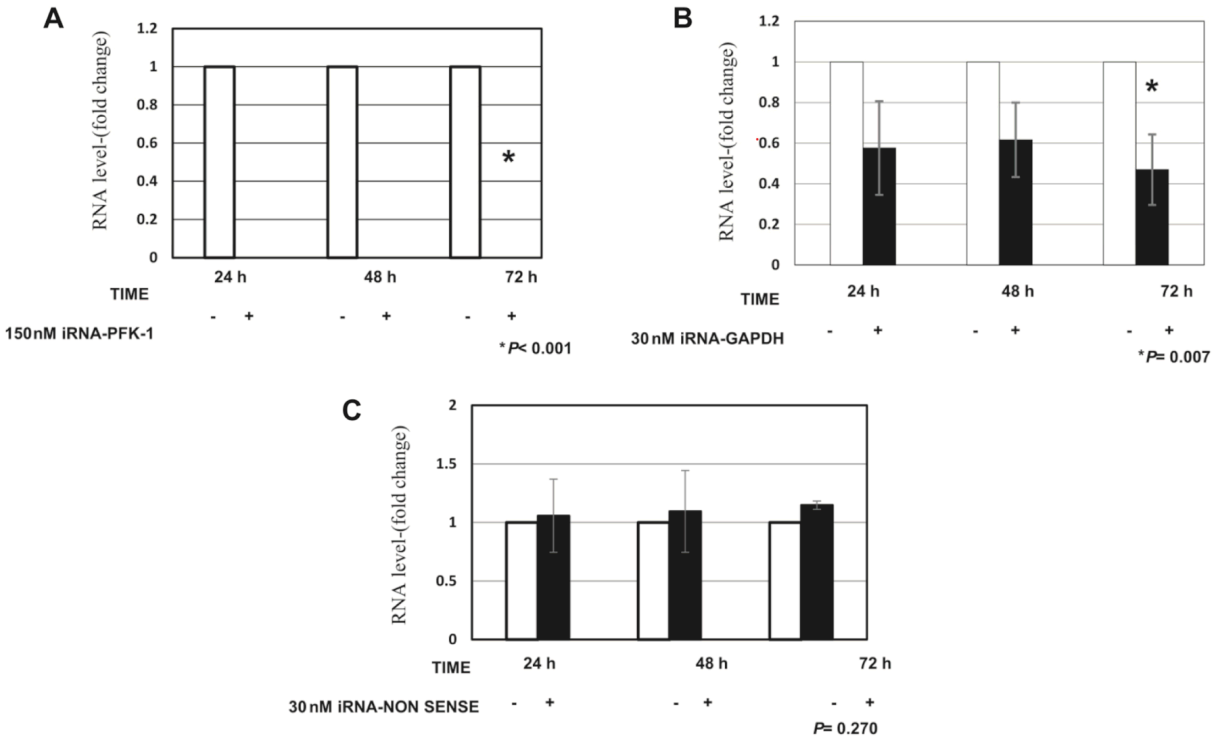

Figure 2. Effect of iRNA-PFK-1, iRNA-GAPDH and iRNA-non sense on the expression levels of PFK-1 in RKO P53-positive cells. **A:** 72 h after transfection with iRNA-PFK-1 (150 mM), a decrease of ~99% in the RNA levels of PFK-1 was observed, p=0.05. **B:** 72 h after transfection with iRNA-GAPDH (30 nM), a decrease of ~54% in the RNA levels of GAPDH was observed, p=0.05. **C:** 72 h after transfection with iRNA-NON SENSE (30 nM), an increase of ~14% in the RNA levels of GAPDH was observed, p=0.270. All results were normalized with GAPDH or actin.

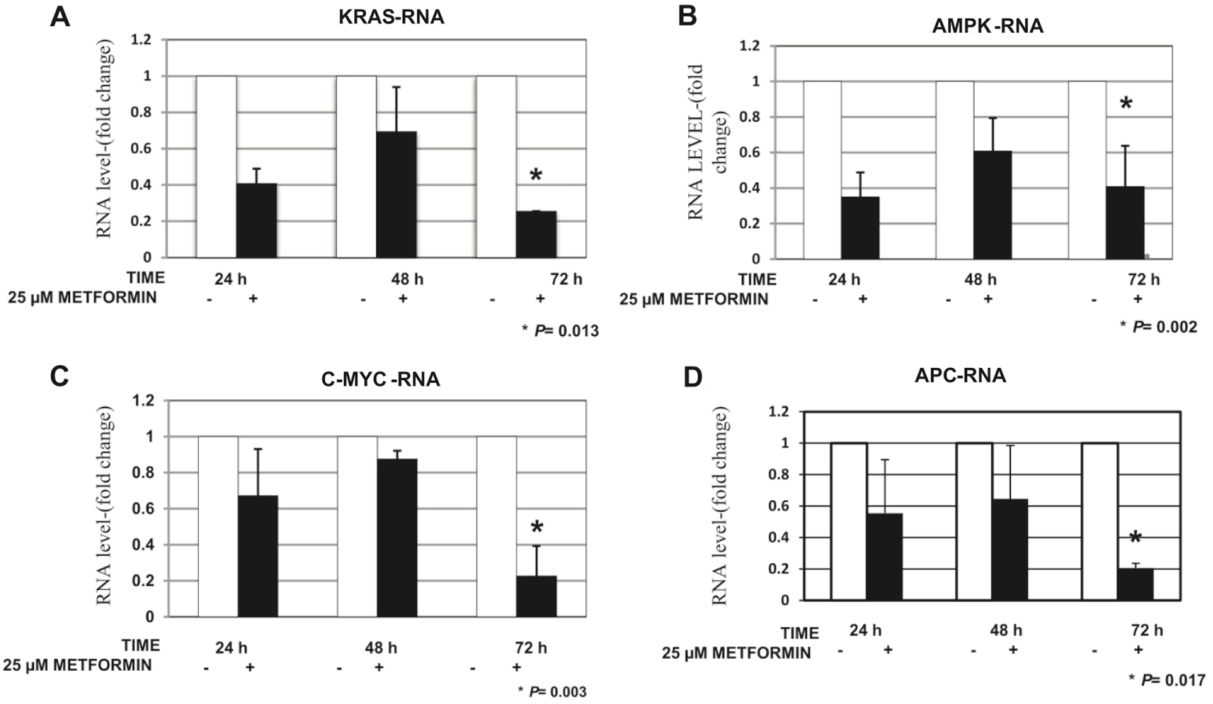

Figure 3. Effect of metformin on the expression levels of KRAS, AMPK, c-MYC and APC in RKO P53 positive cells. **A:** A decrease of ~75% in the expression levels of KRAS was observed 72 h after treatment with metformin (25 µM), p=0.013. **B:** A decrease of ~60% in the expression levels of AMPK was observed 72 h after treatment with metformin (25 µM), p=0.002. **C:** A decrease of ~78% in the expression levels of c-MYC was observed 72 h after treatment with metformin (25 µM), p=0.003. **D:** A decrease of ~80% in the expression levels of APC was observed 72 h after treatment with metformin (25 µM), p=0.017. All results were normalized with GAPDH.

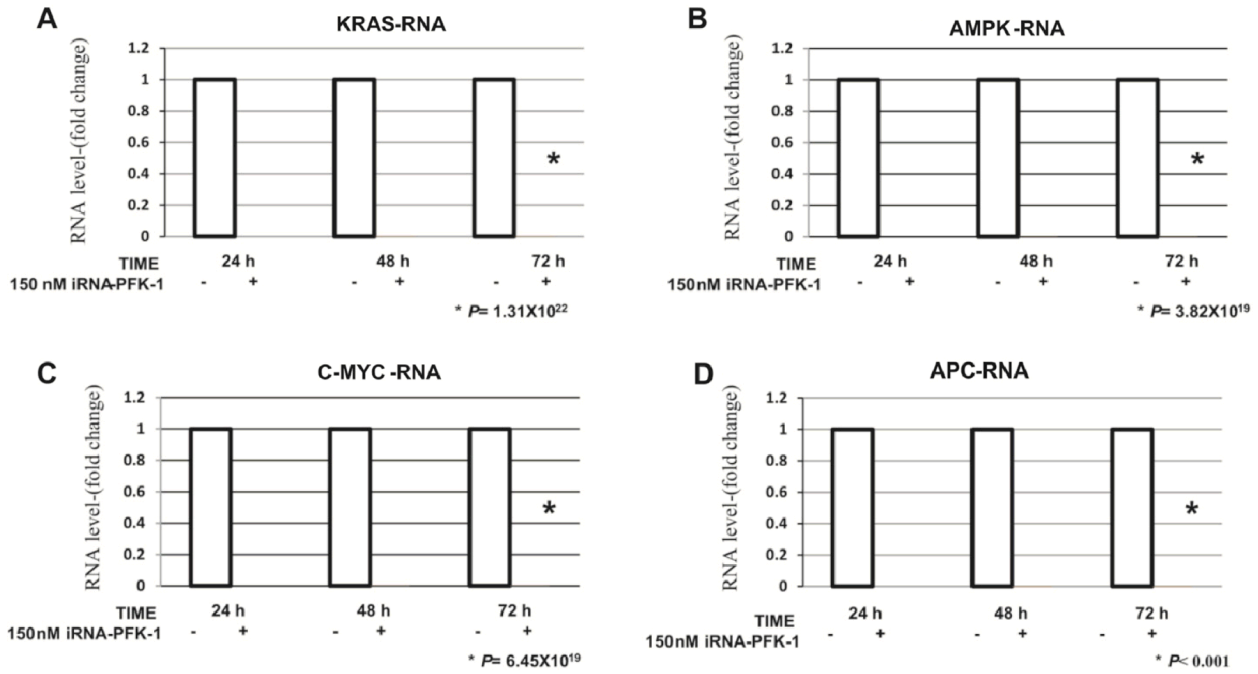

Figure 4. Effect of iRNA-PFK-1 on the expression levels of KRAS, AMPK, c-MYC and APC in RKO P53 positive cells. **A:** 72 h after transfection with iRNA-PFK-1 (150 nM), a decrease of ~99 % in the RNA levels of KRAS was observed, $p=1.3\times10^{-22}$. **B:** 72 h after transfection with iRNA-PFK-1 (150 nM), a decrease of ~99 % in the RNA levels of AMPK was observed, $p=3.82\times10^{-19}$. **C:** 72 h after transfection with iRNA-PFK-1 (150 nM), a decrease of ~99% in the RNA levels of c-MYC was observed, $p=6.45\times10^{-19}$. **D:** 72 h after transfection with iRNA-PFK-1 (150 nM), a decrease of ~99% of the RNA levels of APC was observed, $p < 0.001$.

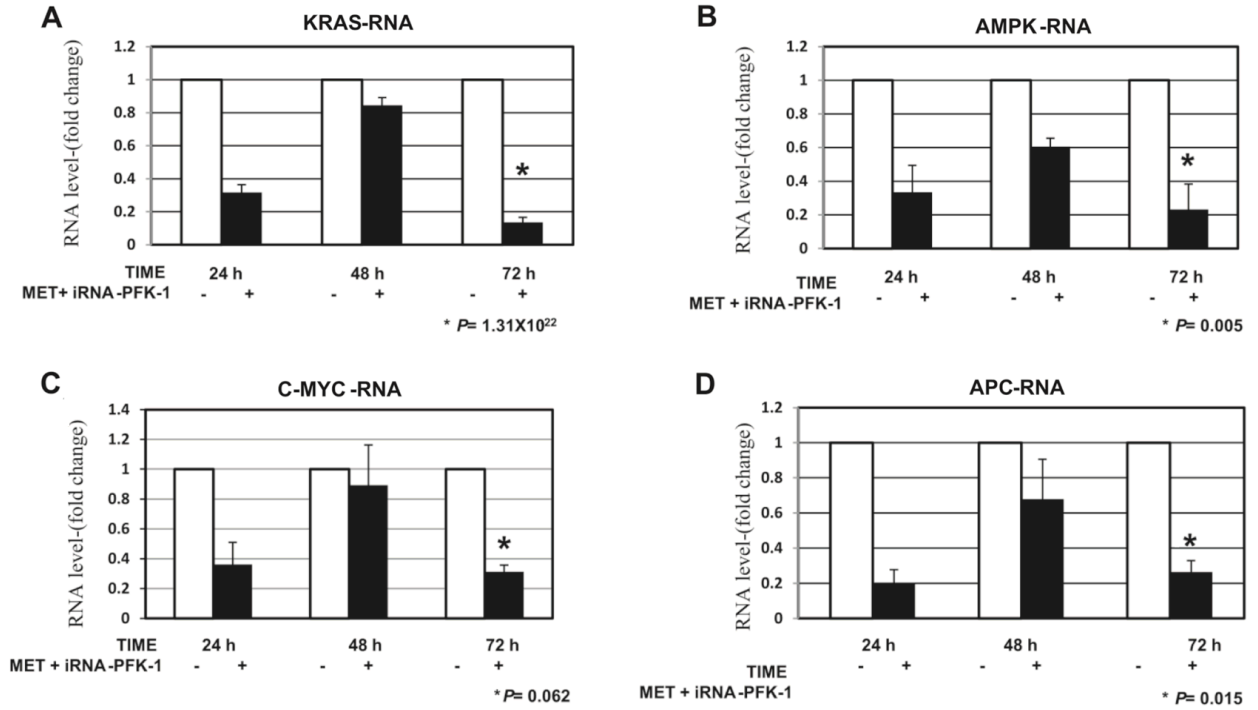

Figure 5. Effect of the combined therapy of metformin and iRNA-PFK-1 on the expression levels of KRAS, AMPK, c-MYC and APC in RKO P53 positive cells. **A:** 72 h after transfection with iRNA-PFK-1 (150 nM) and metformin (25 μM), a decrease of ~87% in the RNA levels of KRAS was observed, $p=1.31\times10^{-22}$. **B:** 72 h after transfection with iRNA-PFK-1 (150 nM) and metformin (25 μM), a decrease of ~77% in the RNA levels of AMPK was observed, p=0.005. **C:** 72 h after transfection with iRNA-PFK-1 (150 nM) and metformin (25 μM), a decrease of ~78% in the RNA levels of AMPK was observed, p=0.062. **D:** 72 h after transfection with iRNA-PFK-1 (150 nM) and metformin (25 μM), a decrease of ~74% in the RNA levels of APC was observed, p=0.015.

5' GACTGCTACTCCACAGAGATCG 3' and RV: 5' TCAGCATCTGAATCACTCCTTT 3'), 1 μL of the reverse primer (40 mM PFK1, KRAS, APC, c-MYC or AMPK), and 1 μL of 10 ng/μL cDNA. Note: for the GAPDH primers, FW: 5' AGGGCTGCTTTTAACTCTGGT 3' and RV: 5' CCCCACTTGATTTTGGAGGGA 3' , 1 μL of 5 mM (instead of 40 mM) was used for both the forward and reverse primers (Invitrogen; Waltham, MA, USA). The reaction conditions were as follows: 2 min at 50°C, 10 min at 95°C, then 45 cycles of 15 sec at 95°C and 1 min at 60°C.

Statistics

All variables were analyzed in triplicate and values were expressed as means ± SD. One-way analysis of variance (ANOVA) and Dunnett's test were used to determine statistically significant differences among means. The differences were considered significant if $p \leq 0.05$.

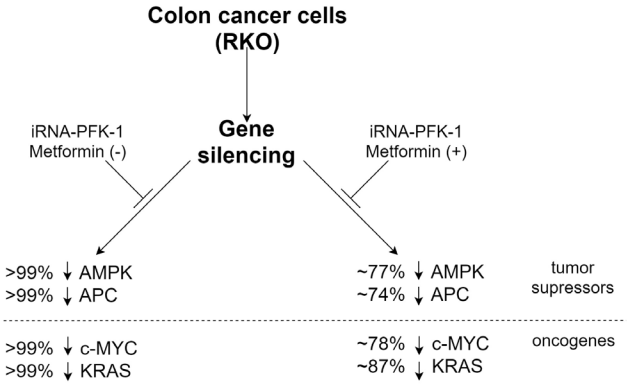

Figure 6. Inhibition of oncogenes and tumor suppressor genes after treatment with iRNA-PFK-1 and iRNA-PFK-1 + Metformin.

Results

Cell viability and iRNA transfection controls

In this study, we used RKO cells (colon cancer p53-positive) to evaluate monotherapy with metformin or iRNA-PFK-1, as well as the combined therapy with both metformin and iRNA-PFK-1. First, we carried out cell viability tests in response to different iRNA treatments (150 nM PFK1, 30 nM GAPDH or 30 nM nonsense) (Figure 1) and in response to a non-cytotoxic concentration of metformin (25 μM), as previously reported. No significant difference in cell viability was observed after any of the treatments (Figure 1). We then confirmed the effect of each iRNA on the expression levels of PFK-1 (~99%, $p<0.001$) at 72 h (Figure 2A), using iRNA-GAPDH as a positive control (72 h post-treatment, GAPDH was reduced to ~50%, $p=0.007$) (Figure 2B) and iRNA-nonsense as a negative control for specificity (no difference was observed for GAPDH between the control and the treatment, $p=0.270$) at 72 h (Figure 2C).

Metformin in monotherapy

Seventy-two h post-treatment with metformin, we observed a decrease in gene expression levels of oncologic markers KRAS (~75%, $p=0.013$), AMPK (~60%; $p=0.002$), cMYC (~68%, $p=0.003$) and APC (~80%, $p=0.017$), when compared to untreated cells (Figure 3). All results were normalized with GAPDH.

iRNA-PFK-1 cell line treatment

After 72 h of iRNA-PFK-1 transfection, we observed a ~99% ($p<0.001$) decrease in gene expression levels of KRAS, cMYC, AMPK and APC genes (Figure 4). These results suggest the inactivation of oncologic biomarkers through iRNA-PFK-1. All results were normalized with GAPDH.

iRNA-PFK-1 + Metformin therapy

Inhibition of RNA levels with the combined therapy of each of the studied genes at 72 h were: KRAS (~87%, $p=1.31\times10^{-22}$), AMPK (~77%; $p=0.005$), cMYC (~78%, $p=0.062$) and APC (~74%, $p=0.015$) (Figure 5). All results were normalized with GAPDH.

Discussion

Currently, there are different therapies for colon cancer, the most used one being 5-FU in combination with other medications like irinotecan and oxaliplatin. However, only 40-50% of CRC patients respond to this approach. To achieve a better response in the control of this pathology, alternative therapies are actively being sought [47-49]. In this article, we show one such potential approach with metformin, iRNA-PFK-1, or a combination of both.

Metformin, the most commonly prescribed drug for T2DM, has repeatedly been found to be associated with a decreased risk of developing various types of cancer, specifically pancreatic, colon and hepatocellular carcinomas, since this drug is associated with inhibition of cancer cell growth and proliferation [50]. A meta-analysis focusing in CRC revealed that metformin was associated to a 36% reduced mortality risk, while there was no evidence that sulfonylureas affected the risk of cancer in any tissue [51]. Our study supports the anti-cancer role of metformin, as at 72 h post-metformin treatment (25 μM), the transcriptional levels of several well studied cancer biomarkers, i.e., KRAS, c-MYC, APC and AMPK, were significantly decreased by ~75, ~78%, ~80% and ~60%, respectively, (p values < 0.05), as shown in Figure 3.

With respect to cancer and cell proliferation, metformin exerts both indirect (insulin-depend-

ent) and direct (insulin-independent) actions at the cellular level. The insulin-independent effect is mediated by the activation of AMPK, which leads to reduction of mTOR activity, causing reduction of protein synthesis and cell growth [29]. Since insulin has been shown to have mitogenic activity that induces cell replication, the insulin-dependent effect of metformin is mediated by its blood glucose lowering ability and subsequent reduction of insulin levels [52]. Unlike metformin, the therapeutic administration of insulin leads to the regulation of glucose uptake, mediated by the PI-3-kinase pathway (related to the translocation of the GLUT 4 glucose transporter to the cell membrane), and triggers mitogenic events regulated through RAS signaling [53]. Growing evidence suggests that insulin and IGF receptors mediate their effects on regulating cell proliferation, apoptosis, and glucose control, among others. Thus, the carcinogenic effects of insulin are associated with increased cell proliferation and suppression of apoptosis [54].

Increased glucose metabolism is one of the distinguishing features between normal cells and cancer cells. Therefore, some of the newly developed chemotherapeutic agents target glycolytic enzymes that are overexpressed in cancer [55]. One new technology that aims to regulate gene expression is gene therapy. Gene therapy has emerged as a new therapeutic option that allows the treatment of a disease at its genetic roots, by counteracting or replacing a malfunctioning gene within the cells by the transfer of genetic material using vector technology [56]. One type of genetic material that can be used in gene therapy is small interfering RNA (siRNA), a class of double stranded RNA (dsRNA) with a length of 20-25 base pairs that participates in the RNA interference (iRNA) pathway.

The power of iRNA lies in the ability to artificially regulate genes by designing siRNAs based on the mRNA sequence of the gene(s) of interest. These siRNAs have the ability to silence genes involved in the pathogenesis of various diseases associated with a known genetic background, such as genetic defects, autoimmune diseases and cancer [57]. Even though cancer gene therapy has not yet been used in clinical practice, basic and clinical advances have been reported and gene therapy is emerging as a new therapeutic approach for the treatment of certain malignancies [58].

Phosphofructokinase-1 (PFK-1) is an enzyme that participates in the glycolytic pathway, catalyzing the transfer of phosphate groups from ATP to fructose-6-phosphate to form fructose 1,6-bisphosphate. The catalytic action of PFK-1 is the first point of commitment of glucose to the glycolytic pathway, and fructose 1,6-bisphosphate is destined to this pathway, unlike previous products of glycolysis (glucose 6-phosphate and fructose 6-phosphate) that might participate in other metabolic pathways [59]. The hydrolysis of ATP involved in the catalytic action of this enzyme renders this glycolytic step essentially irreversible. Since cancer cells are highly dependent on glucose, and glycolytic enzymes like PFK-1 have been shown to be overexpressed during this disease [60] the down-regulation of these enzymes through iRNA emerges as an attractive therapy [61]. In our model, gene silencing with 150 nM iRNA-PFK-1 also caused an inhibition of RNA levels of all of the studied cancer biomarkers (by ~99.9%, $p < 0.01$) (Figure 4) and this is probably attributed to the energy decline in our model of study. Because PFK-1 functions as the gatekeeper to glycolysis, it is subject of complex allosteric regulation: its catalytic activity is enhanced when ATP cellular levels diminish or when ADP and AMP intracellular levels increase. In our study, when performing the combined therapy with metformin + iRNA-PFK-1, a reduction in RNA levels at 72 h was also observed for KRAS, AMPK, c-MYC and APC (by ~76%, $p < 0.05$). Our results support the potential therapeutic role of metformin and PFK-1 in the treatment of colon cancer via *in vitro* modulation of the subexpression of several important cancer biomarkers.

Conclusions

Our study supports the anti-cancer role of metformin due to the decreased expression levels of cancer biomarkers KRAS, c-MYC, APC and AMPK in RKO cells 72 h after treatment with metformin. Also, in these cells, gene silencing with iRNA-PFK-1 caused an inhibition of gene expression levels of all the cancer biomarkers studied, supporting the fact that PFK-1 is a big regulator of the glycolytic pathway, which is overexpressed in cancer cells. Likewise, the use of a combined therapy of metformin and iRNA-PFK-1 also showed a reduction of the expression of such cancer biomarkers, as shown in Figure 6.

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Authors' contributions

CPRI conceived and designed the study. CPRI, EARS, EFC and LSL performed the experi-

ments. CPRI and MSS interpreted the results of the experiments and analyzed the data. CPRI, EARS, EFC, LSL, MGSS, ECA, DAON, MSS and AMRE drafted the manuscript. CPRI, EARS and MSS prepared the figures. CPRI, EARS, EFC, LSL, MGSS, ECA, DAON, MSS and AMRE edited and revised the manuscript. CPRI, EARS, EFC, LSL, MGSS, ECA, DAON, MSS and AMRE approved the final version of manuscript to be published.

Conflict of interests

The authors declare no conflict of interests.

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