https://doi.org/10.31925/farmacia.2024.1.5 **ORIGINAL ARTICLE**

INVESTIGATION OF THE EFFECTS OF METFORMIN ON THE miR-21/PTEN/Akt PATHWAY IN HT-29 HUMAN COLORECTAL ADENOCARCINOMA CELL AND HUVEC CO-CULTURE

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Manuscript received: November 2023

Abstract

Colon adenocarcinoma is a major cause of cancer mortality worldwide. Type 2 diabetic people have an increased risk of developing colorectal cancer compared with nondiabetic people. There are studies showing that metformin inhibits angiogenesis, which is an important stage in cancer metastasis. Our aim was to identify the effects of metformin on miR-21, PTEN, and Akt gene expressions associated with angiogenesis in co-culture conditions established with human colorectal adenocarcinoma cells (HT-29) and human umbilical vein endothelial (HUVEC) cells. Cytotoxicity was evaluated *via* MTT assay, and PTEN, Akt, and miR-21 expressions were measured by real-time polymerase chain reaction in HUVEC cells under the effects of HT29 cells. Cell viability decreased with increasing doses of metformin, especially in the 160 µg/mL metformin treatment group. According to real-time PCR results, PTEN was significantly upregulated in 80 and 160 µg/mL metformin-treated cells, and Akt, and miR-21 expressions were downregulated significantly in all metformin treatment groups. An inverse relation was found between PTEN, Akt and miR-21 levels in HUVEC cells under HT29-HUVEC co-culture conditions. Increased PTEN signalling was associated with the prevention of angiogenesis through reducing cell proliferation and migration. The miR-21/PTEN/Akt signalling pathway may have a crucial role in the molecular mechanism of metformin's antiangiogenic effect.

Rezumat

Adenocarcinomul de colon reprezintă o cauză majoră a mortalității la nivel mondial. Persoanele cu diabet de tip 2 au un risc crescut de a dezvolta cancer colorectal comparativ cu persoanele non-diabetice. Există studii care arată că metforminul inhibă angiogeneza, o etapă importantă în metastaza cancerului. Scopul studiului a fost de a identifica efectele metforminului asupra expresiilor genelor miR-21, PTEN și Akt, asociate cu angiogeneza în condiții de co-cultură stabilite cu celule de adenocarcinom colorectal uman (HT-29) și celule endoteliale venoase ombilicale umane (HUVEC). Citotoxicitatea a fost evaluată prin testul MTT, iar expresiile PTEN, Akt și miR-21 au fost măsurate prin reacția în lanț a polimerazei în timp real în celulele HUVEC sub efectele celulelor HT29. Viabilitatea celulară a scăzut odată cu creșterea dozelor de metformin, în special în grupul de tratament cu metformin de 160 µg/mL. Conform rezultatelor RT-PCR, PTEN a fost semnificativ supraregulat în celulele tratate cu metformin de 80 și 160 µg/mL, iar expresiile Akt și miR-21 au fost semnificativ subregulate în toate grupurile de tratament cu metformin. O relație inversă a fost găsită între nivelurile PTEN, Akt și miR-21 în celulele HUVEC în condițiile de co-cultură HT29-HUVEC. Creșterea semnalizării PTEN a fost asociată cu prevenirea angiogenezei prin reducerea proliferării și migrației celulare. Calea de semnalizare miR-21/PTEN/Akt ar putea avea un rol crucial în mecanismul molecular al efectului antiangiogenic al metforminului.

Keywords: Akt, HUVEC. HT-29, PTEN, miR-21, metformin

Introduction

The data provided by GLOBOCAN in 2018 indicate colorectal cancer as the third most common cancer in the world, and its mortality is ranked second of all cancer deaths worldwide [1]. Type 2 diabetic patients are characterised by a higher risk of developing colorectal

cancer compared with nondiabetic subjects. The risk is augmented in patients also suffering from obesity [2]. Metformin, an inexpensive and well-tolerated oral agent, is commonly used for the treatment of type 2 diabetes, and its use is associated with decreased mortality and complications by approximately 30% compared to other antidiabetic drugs [3]. The results of studies on metformin and its effect on cancer development are contradictory; several cohort studies and meta-analyses were performed to clarify the association of metformin usage effects on cancer, and they reported variable results as decreased risk, increased risk, or no association [4-6]. *In vitro* and *in vivo* trials have shown that metformin plays a major role in regulating cell proliferation, apoptosis, and the growth of experimental tumours which are important for cancer progression [7, 8].

Metformin's molecular mechanisms depend on the administered doses and the treatment durations and are associated with AMP-activated protein kinase (AMPK) and phosphatase and tensin homolog (PTEN) [5, 9, 10]. PTEN negatively regulates the PI3K/Akt/mTOR pathway by dephosphorylating phosphatidylinositol 3,4,5-triphosphate (PIP3), the direct activator of Akt [11]. PTEN is a tumour suppressor gene that inhibits the mechanistic target of the rapamycin (mTOR) pathway, and it is negatively regulated by miR-21 [12, 13]. MiR-21 is a putative oncogene that can be a characteristic for various cancer cells; its expression in colorectal cancer is significantly higher compared to the normal tissues and negatively correlated with the survival time of the patients [14].

Tumour angiogenesis, which means the formation of new vessels from pre-existing small vessels, is also important in order to supply the oxygen and nutrients required for tumour progression. A large number of factors, like the vascular endothelial growth factor (VEGF), the fibroblast growth factor (FGF), or the platelet-derived growth factor (PDGF), can initiate or stimulate the angiogenesis process [15]. A reduced PTEN expression in the endothelial cells or the activation of PI3K can promote angiogenesis through increased cell proliferation, survival, and migration [16]. The clinical observations of diabetic patients treated with metformin indicated fewer vascular complications, highlighting its potential to treat vascular dysfunction not only in diabetes, but also in other diseases associated with vascular dysfunction [17]. The antiangiogenic effects are produced by the inhibition of the VEGF-dependent activation of the endothelial cells and are not limited to diabetes [18]. A study of the metformin effect on HUVEC cells revealed an additional mechanism in which metformin AMPK activation induces upregulation of Smurf1, leading to the proteosomal degradation of activin receptor-like kinase 1 (ALK1) [19].

The aim of the present study was to investigate the mechanisms by which metformin could prevent angiogenesis in colon adenocarcinoma using *in vitro* models based on a HT29 and HUVEC co-culture.

Materials and Methods

Chemicals

Metformin (1,1-dimethylbiguanide hydrochloride, CAS Number 1115-70-4) and other chemicals were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

Cell lines and co-culture

Two human cell lineages, HT-29 and HUVEC were used. Human colorectal adenocarcinoma cell line HT-29 (ATCC HTB-38) and human primary umbilical vein endothelial cells HUVEC (ATCC PCS-100-010™) cells were purchased from American Type Culture Collection (ATCC). Both sets of cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Carlsbad, USA) containing 10% foetal bovine serum (FBS; Gibco). For the co-culture model, endothelial cells were seeded as a monolayer (1.5 x 104 cells/cm^2/well, (96% CO_2, 37°C) in 24-well plates, and colorectal adenocarcinoma cells were cultured in transwell inserts (0.4 μm pores; Corning Inc., Lowell, USA), which were placed in the same wells containing the endothelial cells. Interactions between the two cell types involved paracrine signalling, thereby simulating the *in vivo* situation (Figure 1). All doses of metformin were dissolved in 1 mL DMEM (100 μL/well, n = 6). After treatments using this co-culture system, both HT-29 and HUVEC cells were physically separated, and HUVEC cell components were examined for cell viability and gene expression levels of miR-21, PTEN and Akt.

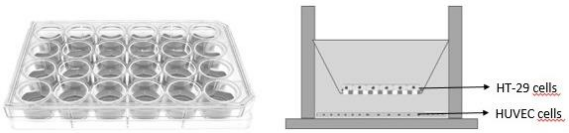

Figure 1.
Schematic representation of the co-culture model with colorectal adenocarcinoma cells plated on the insert and endothelial cells in the lower well

MTT assay

The MTT assay was carried out by a commercially available kit (Sigma Alderich, USA). The colorimetric measurement of the absorbance of formazan molecules produced by the reduction of yellow MTT tetrazolium salt by cellular enzymes is an indicator of cells' viability. MTT (10 μL) was added to the HUVEC cells containing wells. and incubated (5% CO_2, 37°C) for 4 hours. Then, the medium was discarded, and 100 μL of dimethyl-sulfoxide (Sigma, USA) was added to each well. The optical density was determined at 570 nm using an Epoch™ Microplate Spectrophotometer reader (BioTek Instruments, Winooski, USA), and the cell viability (%) was calculated [20, 21].

Real-Time PCR Analysis

The total RNA was isolated from HUVEC cells in a QIAcube (Qiagen) using a RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. The RNA samples were reverse-transcribed into complementary

DNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The relative PTEN, AKT, and miR-21 expression analyses were performed with StepOnePlus Real-Time PCR System technology (Applied Biosystems) using cDNA synthesised from co-culture RNA, as previously described. All primers and probes used were purchased as TaqMan Gene Expression Assays (Applied Biosystems), and they are listed in Table I [22]. The expression data for β-actin was used as the endogenous control.

Tabel I.

Primer sequences of miR21, PTEN, Akt and β-actin

Gene	Sequence	References
miR21	Forward 5'-3': TAGCTTATCAGACTGATGTTGA Reverse 5'-3': GCCAGCACAGAATTAATACGAC	m04244285
Pten	Forward 5'-3': AGAACAAGATGCTAAAAAGGACAA Reverse 5'-3': TGTCAGGGTGAGCACAAGAT	m00477208
Akt1	Forward 5'-3': GTGGCAAGATGTGTATGAG Reverse 5'-3': CTGGCTGAGTAGGAGAAC	m00583646
B-actin	Forward 5'-3': GCACCACACCTTCTACAATG Reverse 5'-3': TGCTTGCTGATCCACATCTG	Hs99999903

Statistical analysis

Values are expressed as the mean ± standard deviation (SD). The MTT assay and the real-time polymerase chain reaction (PCR) results were analysed using the one-way analysis of variance post hoc Duncan test. SPSS-18 for Windows was used for all statistical analyses. The p values less than 0.05 were considered significant.

Results and Discussion

Metformin's effects on HUVEC cell viability in HT29-HUVEC co-culture

As shown in Figure 2, 160 µg/ml metformin significantly decreased the number of HT29-HUVEC cells by approx. 20% after 24 hours of treatment. The total number of cells *per* well is indicated in the method section. Figure 2 displays the data in terms of optical density, and the cells were not subjected to additional passages or counting. The effect of metformin on cell viability is seen even at the lowest dose. The effect of metformin increased as its dose decreased.

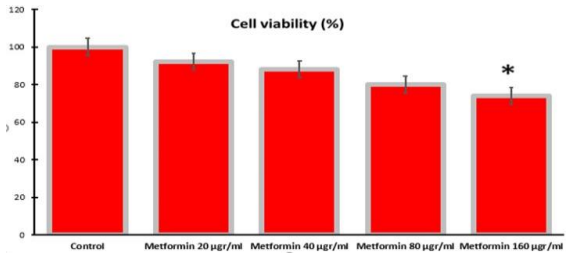

Figure 2.

Effect of metformin treatment on the proliferation of HUVEC as determined by the MTT assay
HT-29 and HUVEC cells were co-cultured in the presence of varying concentrations of metformin for 24 hours The results are shown as the mean ± SD (*p < 0.05; **p < 0.01)

Real-time PCR results

The tumour suppressor gene PTEN was significantly upregulated after exposure to metformin 80 µg/mL and 160 µg/mL when compared to the control group. The expression was 2.9 fold higher for 80 µg/mL and 4.2 fold higher for 160 µg/mL (Figure 3, p < 0.01).

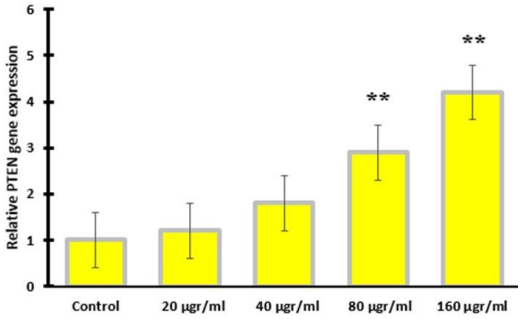

Figure 3.

Gene expression levels in terms of fold change of the PTEN in HUVEC cells after co-culture with HT-29 cells
The results are shown as the mean ± SD (*p < 0.05; **p < 0.01)

All the metformin-treated cell cultures showed an increase in Akt expression levels compared to the control group. The lowest concentration of metformin showed the highest Akt expression, while the highest dose of metformin, 160 µg/mL, showed the lowest increase in Akt expression levels. The Akt expression level augmentation compared to the control group was 9.8 folds for the 20 µg/mL concentration, 5.8 folds for 40 µg/mL, 3.9 folds for 80 µg/mL, and 3.1 folds for the 160 µg/mL concentration (Figure 4, p < 0.01). The increment ratios follow a linear relationship negatively dependent of the logarithm on metformin concentration (R^2 = 0.903).

The expression of miR-21 changed following a similar pattern with that of Akt, with the cells exposed to 20 µg/mL of metformin exhibiting the highest rise in expression levels compared to all groups. All tested concentrations presented a significant augmentation of miR-21 expression. The increment ratio was 7.9 folds for 20 µg/mL, 6.5 folds for 40 µg/mL, 3.4 folds for 80 µg/mL, and 2.9 folds for the 160 µg/mL (Figure 5, p < 0.01). These values follow a linear relationship negatively dependent of the logarithm on metformin concentration (R^2 = 0.936).

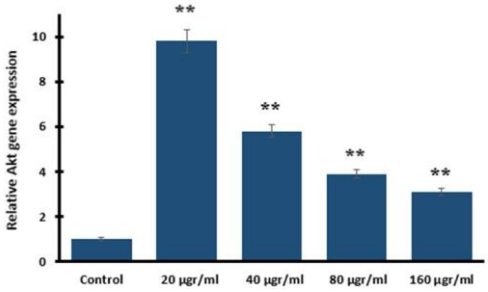

Figure 4.

Gene expression levels in terms of fold change of the Akt in HUVEC cells after co-culture with HT-29 cells
The results are shown as the mean ± SD (*p < 0.05; **p < 0.01)

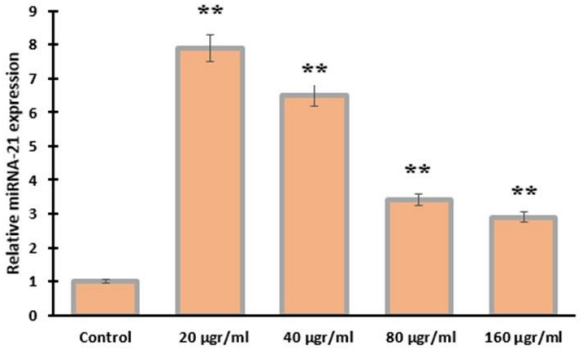

Figure 5.

Gene expression levels in terms of fold change of the miR-21 in HUVEC cells after co-culture with HT-29 cells
The results are shown as the mean ± SD (*p < 0.05; **p < 0.01)

The main function of PTEN is to counteract the PI3K function by converting PIP3 to phosphatidylinositol 4,5-bisphosphate (PIP2). PIP3 is essential for the activation of Akt through phosphorylation at Ser473. The cells that have impaired PTEN activity have a hyperactive Akt function that translates into oncogenesis, excessive cell proliferation, apoptosis, suppression, and metastasis [23]. Additionally, this pathway plays a key role in angiogenesis in normal processes and also in cancer-related angiogenesis. The PTEN/Akt pathway causes vessel endothelial cell proliferation during oncogenesis for the purpose of cancer cell nutrition in accordance with VEGF secretion [24].

MicroRNAs are small RNA structures that have been an important research focus in recent years and play a central role in the regulation of many cellular functions. They play a role in both normal cellular processes and cancer development. More than 500 microRNAs have been reported today. miR-21 is one of the important microRNAs that play an important role in oncogenesis, regulating cell proliferation, angiogenesis related to tumour cell nutrition, and metastasis. The miR-21 is a major regulator of the PTEN/Akt pathway, and these three important molecules progress carcinogenesis. PTEN is a tumour suppressor molecule that inhibits Akt. Akt inhibition results from cell proliferation inhibition. On the other hand, miR-21 supresses the PTEN, which in turn causes activation of the Akt pathway that results in an increase in cell proliferation. miR-21 regulation *via* exogenous compounds is important for controlling tumour progress and inhibiting tumour cell-related angiogenesis induction [25, 26].

According to the guidelines of the American Diabetes Association and the European Association for the Study of Diabetes, metformin is a first-line drug to treat type 2 diabetes patients. Metformin exerts its glucose-lowering effect by interrupting glucose uptake from the gastro-intestinal tract, increasing glucose uptake into cells, and increasing insulin sensitivity [27, 28]. Also, metformin activates one of the important energy metabolism regulator molecules, AMPK and causes inhibition of gluconeogenesis. Besides its success in diabetes treatment, in several different studies, it has been shown that metformin could have antitumour, antiaging, cardiovascular and neuroprotective effects. Additionally, it could be an effective treatment for polycystic ovary syndrome [29, 30].

The objective of this study was to investigate the anti-angiogenic effects of metformin treatment in HT-29/HUVEC co-culture conditions. The results of our study indicated that metformin administration resulted in a significant dose-dependent decrease in miR21 expression levels in HUVEC cells that were co-cultured with HT29 cells. The reduction of miR21 levels is correlated with an increase in PTEN levels, as PTEN expression is controlled by miR21. The increase of PTEN levels is corroborated by the decrease in Akt levels. These results suggest that metformin affects the miR21-PTEN-Akt pathway, which is associated with angiogenesis in HUVEC cells under the influence of HT29 cells, and therefore reduces angiogenesis-related cell proliferation. Luo *et al.* demonstrated that miR-21 expression levels affect the PTEN response to metformin administration in the angiogenesis process. They showed that 1 mM, 5 mM, 10 mM, 20 mM, and 50 mM metformin exposure downregulated miR-21 expression in a concentration-dependent manner, and metformin exposure exerted antiproliferative effects in HUVEC cells [31].

Metformin demonstrated an endothelial protective effect under high glucose-dependent conditions by inhibiting the autophagy mechanism, a process related to AMPK activation [32]. In another study, metformin usage in pregnancy ameliorates endothelial cell impairment *in vivo* and *in vitro via* p65 regulation [33]. According to these results, the effect of metformin application on angiogenesis in different pathogenic conditions may show duality, and it tries to protect the system with a mechanism in favour of the organism.

Conclusions

In our study, we investigated the effect of metformin on the miR-21-PTEN-Akt pathway in the HUVEC cell line under the influence of HT29 cells using the co-culture method. This study is the first in the literature investigating the proliferation of HUVEC cells co-cultured with HT-29 colorectal carcinoma cells. The

study was aimed at modelling the development of angiogenesis in colorectal cancers and investigating the effect of metformin on angiogenesis. Metformin administration in doses of 20 μg/mL up to 160 μg/mL suppressed angiogenesis by targeting miR21. There is a need for detailed studies showing the expression levels of other angiogenesis-related molecules in the co-culture model with HUVEC and in angiogenesis studies, as well as the migration of HUVEC cells.

Conflict of interest

The authors declare no conflict of interest.

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