European Review for Medical and Pharmacological Sciences 2014; 18: 1602-1609

The relationship between anticancer effect of metformin and the transcriptional regulation of certain genes (CHOP, CAV-1, HO-1, SGK-1 and Par-4) on MCF-7 cell line

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Abstract. – BACKGROUND: 1,1-dimethylbiguanide hydrochloride (biguanide metformin) is a hypoglycemic agent that is widely used in the treatment of Type 2 diabetes. Use of metformin was found to be associated with the lower risk of cancer. It is suggested that metformin has an anticancer and antiproliferative effect and affects the apoptosis by activating the AMPK and inhibiting the mammalian target of rapamycin (mTOR). Although the effects of metformin treatment of various types of cancers are defined with many mechanisms, the literature provides only sufficient information about how it affects the SGK-1, Par-4 and Cav-1 mRNA expressions and the impact of this effect on cytotoxicity. The breast cancer is globally one of the most important causes of cancer-related mortality for women. We, therefore investigated the possible effects of metmorfin on proliferation, cytotoxicity and some unfolded protein response (UPR) genes in the breast cancer cells (MCF-7).

MATERIALS AND METHODS: We administrated 0.31 mM, 2.5 mM and 10 mM of metformin alone and in combination with 2-DG to the MCF-7 cells and monitored the cell viability and proliferation with real-time cell analyzer system for 48 hours. We also measured CHOP, Cav-1, HO-1, SGK-1 and Par-4 genes mRNA expression levels using Real Time-Polymerase Chain Reaction (RT-PCR). We considered the GAPDH gene as reference gene and the control groups as calibrator. We performed an analysis for relative gene expressions of the study groups.

RESULTS: Metformin caused transcriptional regulation of UPR and tumor-related genes in MCF-7 cells and inhibited the proliferation depending on the dose, resulting in cytotoxic effect.

CONCLUSIONS: We consider that administration of metformin with chemotherapeutic agents could be an effective method in treatment of breast cancer through mechanisms such as reduced resistance to chemotherapy and increased cytotoxic activity.

Key Words:
Metformin, Cytotoxicite; SGK1, Breast cancer cells.

Introduction

Breast cancer is the most common type of cancer in American and northwest European women. Approximately one-third of the patients with breast cancer die due to metastasis[1]. Breast cancer (MCF-7) cells were obtained from pleural effusion of patients with metastatic breast cancer[2].

1,1-dimethylbiguanide hydrochloride (biguanide metformin) is a hypoglycemic agent that is widely used in the treatment of Type 2 diabetes[3]. The basic metabolic effect of metformin involves inhibiting gluconeogenesis in the liver to reduce blood glucose concentration[4]. Metformin suppresses production of endogenous glucose while increasing sensitivity to peripheral insulin[5].

Caloric limitation or use of metformin lead to energy stress, resulting in increased AMP/ATP[6]. AMP activated protein kinase (AMPK), which regulates the cellular homeostasis, is activated in response to energy stress[7]. Playing a role in regulation of glucose and fatty acids, AMPK is a serine/threonine kinase and functions as an intracellular energy sensor[8]. The explicit effect of AMPK activation is that it increases oxidation of hepatic fatty acids, and ketogenesis and inhibits the synthesis of cholesterol, lipogenesis, and synthesis of triglycerides. It enhances fatty acids oxidation of skeletal muscle as well as glucose intake of muscle cells, and regulates the insulin secretion of pancreatic cells[9].

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The recent studies have revealed that use of metformin in the patients with Type 2 diabetes is associated with lower risk of cancer[10]. It is suggested that metformin acts by affecting the anticancer effect, cell proliferation, and apoptosis[11,12]. Activation of AMPK and inhibition of mammalian target of rapamycin (mTOR) are considered to be responsible for the basic antiproliferative effect of metformin[13]. Additionally, metformin activates the PERK-ATF4 pathway, resulting in endoplasmic reticulum stress signaling (ERSS), and upregulates the *CHOP* mRNA level[14].

2-deoxyglucose (2-DG) is a synthetic glucose analog which inhibits the glycolysis and blocks the cell growth. Glucose deprivation is a condition occurring in the center of solid tumors and activates the unfolded protein response (UPR) in the cells[15]. 2-DG stimulates the ER stress through inhibition of protein glicosylation[16].

Metformin inhibits glucose starvation or 2-DG, and production of ATP from glycolysis while inhibiting mitochondrial complex-1. The combination of 2 energetic stresses results in serious energetic stress and strong apoptotic response[17]. It is reported that treatment of breast cancer cells with metformin alone leads to G0/G1 arrest in the cells without increasing the apoptosis[17], or glucose deprivation alone results in very little amount of apoptosis but the combination of glucose deprivation and metformin increases the apoptosis[17].

We investigated how metformin affects, through cytotoxic activity in MCF-7 cells, the expression levels of CCAAT/enhance binding protein (C/EBP) homolog protein (CHOP)[18] which is included in the unfolded protein response (UPR) genes, heme oxygenase1 gene (HMOX-1)[19], Prostate apoptosis response (Par-4) playing role in the apoptotic activity[20], Caveolin-1 (Cav-1)[21] which plays a critical role in regulation of AMPK[22] and biological function of insulin-like growth factor receptor (IGF-IR), and serum- and glucocorticoid-inducible kinase 1 (SGK1)[23] that has a role in inhibition of apoptosis. We combined various doses of metformin with 3 mM 2-DG to imitate the micro environment surrounding the solid tumors.

Materials and Methods

The MCF-7 cells used in this study were acquired from the T.R. Ministry of Agriculture and Rural Areas, Foot and Mouth Diseases Institute. MCF-7 cells were cultured in the flasks of 25 cm^2 at 37°C in 5% on CO_2 with RPMI 1640 medium (Biological Industries) containing 10% of fetal bovine serum (Sigma Aldrich), 1% of Na-pyruvate (Biological Industries, Beit Haemek, Israel), 100U penicillin/0.1 mg streptomycin (Sigma Aldrich, St Louis, MO, USA), and HEPES 25 mM/500 mL. The complete medium was removed when the cells became confluent by 70%-80% and washed with phosphate buffered saline (PBS). After removing PBS, the cells were removed by trypsin-ethylenediaminetetraacetic acid (EDTA; 0.25% trypsin, 0.02% EDTA, Biological Industries). The cells were counted then cultured into an e-plate with each well containing $9x10^3$ cells for the proliferation assay. The cells were also cultured into 8 flasks of 25 cm^2 for gene expression.

Study Groups

There were 8 groups in the study: (1) CONT, (2) 2-DG, (3) MET-A, (4) MET-B, (5) MET-C, (6) METDG-A, (7) METDG-B, and (8) METDG-C (Table I). The doses of metformin were determined considering the IC_{50} level at the 48th hour. The above groups were individually planned for Real Time Cell Analyzer (RTCA) and RT-PCR. Predetermined doses of 2-DG and metformin as well as combinations of metformin with 2-DG were administrated to the cells in the e-plates for RTCA following a 24-hour plating process. The control group only received complete medium. A triplicate method is used for all of the groups. A measurement was obtained every 30 minutes, and the cells were monitored for 48 hours with RTCA (xCELLigence). The medium was then replaced at the 24th hour.

MCF-7 cells were cultured in the flasks of 25 $cm^{2'}$ at 37°C in a medium with 5% of CO_2 for RT-PCR assay. When the cells became confluent by 50-55%, the doses planned in the study group were administrated into the complete medium for 48 hours. The complete medium in the flasks were removed after the 48th hour. The cells were removed by trypsin-EDTA after washing with PBS and added some amount of RPMI-1640 medium. The supernatant was removed after centrifuging at 1000 g for 5 minutes. RNA was isolated using High Pure PCR RNA Isolation Kit. The acquired RNAs were converted into cDNA using Transcriptor First Strand cDNA Synthesis Kit. The primaries and probes were designed by Universal Probe Library (UPL) program (Table II). The levels of gene expression of CHOP,

Table I. Eight separate groups were created for the study.

	A	B	C	
MET	Metformin 0.31 mM	Metformin 2.5 mM	Metformin 10 mM	
METDG	Metformin 0.31 mM + 2-DG	Metformin 2.5 mM + 2-DG	Metformin 10 mM + 2-DG	
2-DG				2-Deoxy Glucose 3 mM
CONT				Control

HMOX-1, CAV-1, SGK-1 and Par-4 were measured using quantitative RT-PCR (LightCycler 480 II) device. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was considered to be the reference gene, and the control groups were considered to be the calibrator.

Statistical Analysis

The relative gene expression analysis of the study groups was performed using QIAGEN 2009 relative expression software (REST) program. $p < 0.5$ was considered significant.

Results

Calculation of IC_{50} Value of Metformin

MCF-7 cells were cultured in an e-plate with $9x10^3$ cell/well in the 100 µL complete medium then placed in the RTCA. Following a 24-hour plating process, a complete medium was applied, containing metformin of 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.315, 0.156, 0.078 0.039, and 0.0195 mM. IC_{50} was determined at the 48th hour by sigmoidal dose-response. The calculation at the 48th hour was IC50/EC50: $4.27*10^{-6}$ M (Square R: 8,7803 e-001).

RTCA

MET-A administrated group showed similar cell proliferation to the control group for 48 hours whereas METDG-A administrated group had a cytostatic effect for 48 hours. MET-B administrated group had a mild proliferation up to the 34th hour then showed a cytostatic effect. METDG-B administrated group had a cytostatic effect following administration, and no impedance was obtained approximately from the 40th hour. ET-C and METDG-C administrated group produced a cytotoxic effect soon after the administration. The cells were reduced to a basal level at approximately 30th hour and cell viability completely disappeared (Figure 1).

RT-PCR

The MET doses, which were determined for RTCA (0.31, 2.5, 10 mM of MET, and the combination of these doses with 2-DG) were administrated into the MCF-7 cells that became confluent 50-55% in the flask of 25 cm^2. The gene expression levels of cDNA, obtained in 48 hours, were determined by RT-PCR. According to the results: except for the MET-A administrated group, in all of the groups the expression levels of HMOX-1 mRNA were increased significantly with the dose compared to the control group. In the MET-B administrated group, the expression levels of HMOX-1 mRNA were increased by 5.3 times the control group (p(H1) = 0.023) whereas in the METDG-B administrated group the increase was 12.6 times with a synergistic effect (p(H1) = 0.013). In the MET-C administrated group the increase was 19,8

Table II. Sequences of polymerase chain reaction primers.

Genes	Forward primer	Reverse primer	Probes
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC	tggggaag (probe 60, Roche)
CHOP	CAGAGCTGGAACCTGAGGAG	TGGATCAGTCTGGAAAAGCA	catcacca (probe 9, Roche)
HMOX-1	GGGTGATAGAAGAGGCCAAGA	AGCTCCTGCAACTCCTCAAA	catccagc (probe 42, Roche)
SGK-1	GACAGGACTGTGGACTGGTG	TTTCAGCTGTGTTTCGGCTA	gggagctg (probe 24, Roche)
PAR-4	GCAGATCGAGAAGAGGAAGCCAT	CATCTTCGTACTCATCTAAGCA	gggagaag (probe 7, Roche)
CAV-1	ACAGCCCAGGGAAACCTC	GATGGGAACGGTGTAGAGATG	catccagc (probe 42,Roche)

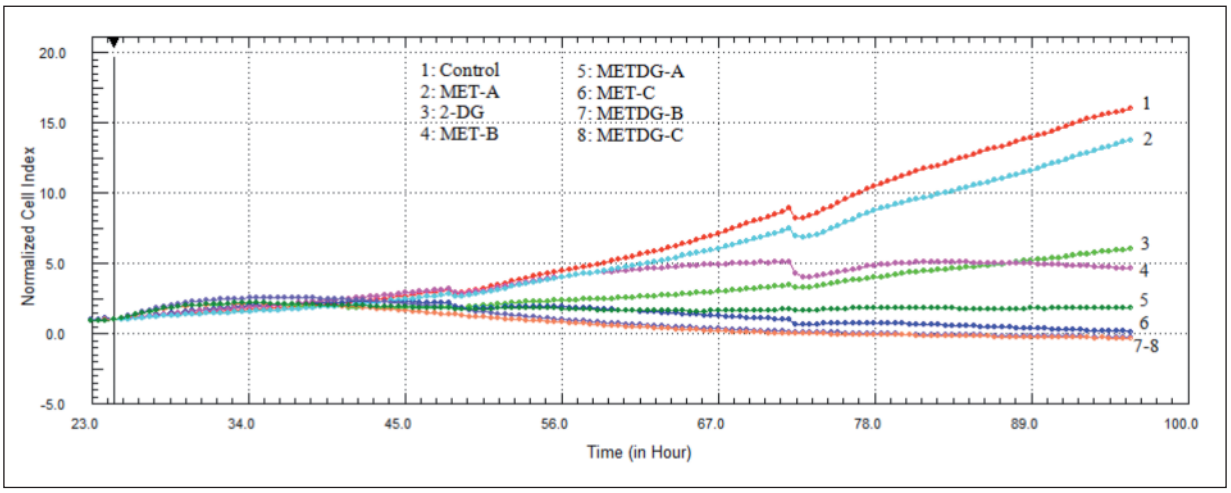

Figure 1. The graph for a 48-hour proliferation follow-up following administration of 0.31, 2.5 ve 10 mM of metformin and the combination of the same doses with 3mM of 2-DG into the MCF-7 cells.

times (p(H1) = 0.019); in the METDG-C administrated group it was 10 times (p(H1) = 0.005). In the last group, 2-DG appeared to have partly suppressed the increasing effect of metformin on HMOX-1 mRNA expression (Figure 2 A).

In all groups with metformin administrated alone or in combination with 2-DG, CHOP mRNA expression levels were increased. In the MET-B administrated group, the increase was 17.1 times (p(H1) = 0), and in the MET-C administrated group it was 42.7 times (p(H1) = 0.008). In the METDG-B administrated group, the increase was 18,1 times (p(H1) = 0.004) whereas it was 39 times (p(H1) = 0.003) in the METDG-C administrated group (Figure 2 B). The significant increase was particularly observed in the MET-C and METDG-C administrated groups.

In the groups with metformin administrated alone or in combination with 2-DG, the dose-dependent increase was observed in the Cav1 mRNA expression levels. No significant change was present in the MET-A administrated group whereas the increase was 1.9 times (p(H1) = 0.019) in the MET-B administrated group, and 1.8 times (p(H1) = 0.017) in the MET-C administrated group. The dose-dependent increase was observed in the combined groups. The increase was 1.8 times (p(H1) = 0.012) in the METDG-A administrated group, 2.8 times (p(H1) = 0.005) in the METDG-B administrated group, and 3.8 times (p(H1) = 0.008) in the METDG-C administrated group (Figure 2 C).

Except for the MET-A administrated group, in all of the groups a dose-dependent increase was seen in the Par-4 mRNA expression levels in comparison to the control group. No significant change was present in the MET-A administrated group but the increase was 1.6 times (p(H1) = 0.036) in the METDG-A administrated group. The increase was 3.4 times (p(H1) = 0.000) in the MET-B administrated group, and 7.2 times (p(H1) = 0.009) in the METDG-B administrated group with a synergistic effect. The increase was 5.1 times (p(H1) = 0.017) in the MET-C administrated group, and 8.4 times (p(H1) = 0.001) in the METDG-C administrated group with a synergistic effect (Figure 2 D).

In all of the groups with metforminin administrated alone or in combination, a decrease was observed in the SGK-1 mRNA expression levels. However, not all dose groups had a significant decrease. The decrease was significant and 0.5 times (p(H1) = 0.008) in the MET-C administrated group. In particular, both the METDG-B administrated group had a significant decrease which was 0.6 times (p(H1) = 0.008), and the METDG-B administrated group had a significant decrease which was 0.6 times (p(H1) = 0.005) (Figure 2 E).

Discussion

The recent studies have demonstrated that use of metformin in patients with Type 2 diabetes is associated with lower risk of cancer[10]. It is considered that metformin shows the anticancer effect by affecting the cell proliferation and apop-

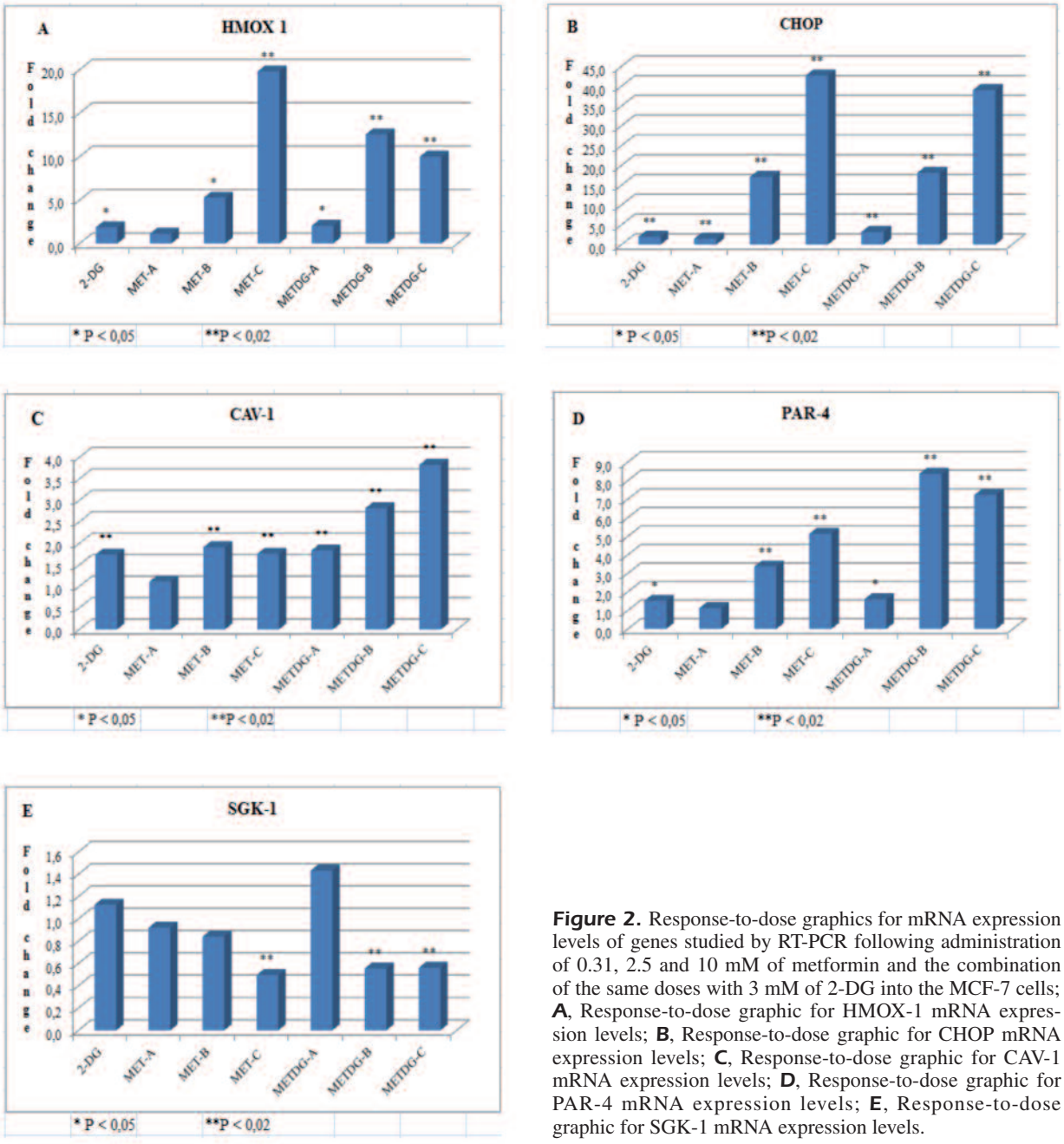

Figure 2. Response-to-dose graphics for mRNA expression levels of genes studied by RT-PCR following administration of 0.31, 2.5 and 10 mM of metformin and the combination of the same doses with 3 mM of 2-DG into the MCF-7 cells; **A**, Response-to-dose graphic for HMOX-1 mRNA expression levels; **B**, Response-to-dose graphic for CHOP mRNA expression levels; **C**, Response-to-dose graphic for CAV-1 mRNA expression levels; **D**, Response-to-dose graphic for PAR-4 mRNA expression levels; **E**, Response-to-dose graphic for SGK-1 mRNA expression levels.

tosis[11-12]. Activation of AMPK and inhibition of mammalian target of rapamycin (mTOR) are considered to be responsible for the basic antiproliferative effect of metformin[13]. Metformin activates the PERK-ATF4 pathway, resulting in endoplasmic reticulum stress signaling (ERSS), and strongly upregulates the CHOP mRNA level metformin, PERK-ATF4[14].

It is suggested that slightly expressed CHOP in most of the proliferated cells is upregulated in different tissues exposed to oxidative stress, DNA damage, and ER stress[24]. Increased CHOP mRNA expression has been shown to increase cell cycle arrest related to the decreased Bcl-2[25] as well as apoptosis related to the ER stress[26]. In the present study, dose-dependent significant increases in the CHOP mRNA expression levels were observed in all doses of metformin, either alone or combined (Figure 2 B). With the increased CHOP, the decrease in the cell index and

the cytotoxic effect were particularly conspicuous in the higher doses of metformin (Figure 1). The increasing effect of metformin on the CHOP in MCF-7 cells and associated increase in the apoptotic activity might be important in the treatment strategy of cancer.

HMOX-1 is a response protein to stress, which is increased by temperature, oxidants, heavy metals, and heme. In healthy cells, the HMOX-1 is reported to protect the cell against hydrogen peroxide, radiation, cisplatin, and inflammatory cytokines produced by cell damage[27]. On contrary, it is indicated that HMOX-1 induction reduces the risk of metastasis of lymph node in human colorectal and oral carcinomas[28], and inhibits the proliferation and increases the apoptosis in the breast cancer cells[29], and HMOX-1 downregulation is correlated with the increased malignant progression in the hepatocellular carcinomas[30]. In the present study, we demonstrated that the metformin, which is widely used in the treatment of diabetes mellitus Type 2, significantly increased the expression levels of HMOX-1 mRNA in the MCF-7 cells depending on the dose (Figure 2A). We also showed that it inhibited the proliferation of MCF-7 cells and produced a cytotoxic effect (Figure 1).

Cav-1 plays a critical role in the regulation of AMPK[22] and biological function of insulin-like growth factor receptor (IGF-IR)[21]. Salani et al[31] found that metformin inhibited the IGF1-mediated proliferation in the non-small-cell lung cancer (NSCLC) cells and Cav1 played a role in this inhibition. How metformin affects the gene expression in MCF-7 cells and the association of this effect with the ctytotoxicity remain unclear. In this study, metformin increased the Cav-1 mRNA expression level depending on the dose. The increase in the Cav-1 was higher when the doses of metformin were combined with 2-DG (Figure 2C). It is particularly conspicuous that the doses of METDG-B and METDG-C, which had a cytotoxic effect on RTCA (Figure 1), were in parallel with the levels of gene expression. In our study, metformin increased the Cav-1 gene expression in the MCF-7 cells and showed a cytotoxic effect.

Par-4 has been studied as the potential molecular target in the treatment of cancer[32]. Par-4 downregulation is suggested to play a critical role in tumorigenesis[33]. It is reported[34] that overexpression of Par-4 induced apoptosis in most of the cancer cells without any additional stimulants. A correlation was detected between the high levels of Par-4 and the good prognosis in the pancreatic cancer in terms of lifetime[35]. Par-4 expression was found to increase the sensitivity to apoptosis in most of the cancer cells including breast cancer cells[36]. There are no studies on how metformin affects the Par-4 in MCF-7 cells. It is not clear how it affects the Par-4 gene expression and the association of this effect with the ctytotoxicity. In this study, the level of Par-4 mRNA expression was increased depending on the dose of the metformin (Figure 2). Some cytostatic and cytotoxic effect was observed in the RTCA in line with the significantly increased levels of gene expression particularly in doses of MET-C, METDG-B, and METDG-C (Figure 1). Considering the role of Par-4 in the apoptosis, it can be suggested that metformin increases the Par-4 in the MCF-7 cells, promoting the apoptotic activity.

SGK-1 enhances the amino acid, peptide, keratin, and glucose transport which are required for cell growth[37]. SGK1 was demonstrated to have been stimulated by proliferative signals and significantly upregulated in many tumors[23]. SGK1 upregulation was shown to inhibit the apoptosis in the breast cancer cells[38]. It was reported that in breast cancer cells increased SGK1 by glucocorticoids inhibited the apoptosis increased by the chemotherapeutic agents[39]. It was indicated that inhibition of SGK-1 mRNA expression increased the toxicity of chemotherapeutic drugs in the cancer cells with RNA silencing method[23]. Literature provides no satisfactory information on how metformin affects the level of SGK-1 mRNA expression. In this study, metformin significantly decreased the levels of SGK1 mRNA expression in the MCF-7 cells depending on the dose (Figure 2 E). There was a significant decrease in the MET-C with metformin administrated alone. There were also significant decreases in the METDG-B and METDG-C with combined doses. It can be suggested that metformin had a higher effect on the cells center of the tumor considering that an environment with 2-DG partly imitated the microenvironment of solid tumors. In the groups with significant decreases, there was a great decrease in the cell index in RTCA as well as cytostatic and cytotoxic effect (Figure 1). Considering that higher SGK-1 level develops resistance to chemotherapeutic agents in the cancer cells, it is quite important that metformin decreases the SGK-1 expression level. The level of SGK-1 expression can be reduced and/or the effect of chemotherapeutic agents can be enhanced by using metformin in the treatment of treatment-resistant cancer cells in a hypoglycemic environment.

The presence of resistance to chemotherapy is known in the cells forming the center of solid tumors. Reduced apoptotic activity is one of the primary causes of this resistance. In this study, metformin had an anti-proliferative and cytotoxic effect on the MCF-7 cells and caused transcriptional regulation of a large number of genes. Of these genes, the levels of CHOP, HMOX-1, CAV-1 and PAR-4 mRNA expression were increased. This increase probably caused increase in the apoptotic activity and decrease in the cell index. On contrary, the levels of SGK-1 mRNA expression were decreased, resulting in cytotoxic effect. Considering that SGK-1 is stimulated by proliferative signals[23] and the SGK-1 upregulation is increased by the chemotherapeutic agents and inhibited apoptosis[39] in the breast cancer cells, it can be a new treatment approach to breast cancers that metformin has an effect to reduce SGK-1 mRNA expression level and an associated decrease occurs in the cell index, and cytotoxic activity is increased.

Conclusions

Administration of metformin in combination with the chemotherapeutic agents can be an effective method for treatment of breast cancers in order to reduce the resistance to chemotherapy and increase the cytotoxic affect in the breast cancers.

Conflict of interest

The Authors declare that there are no conflicts of interest.

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