IJMCM
International Journal of Molecular and Cellular Medicine
p-ISSN: 2251-9637 o-ISSN: 2251-9645
Babol University of Medical Sciences
Cellular and Molecular Biology Research Center

Metformin as a Potential Therapeutic Agent in Breast Cancer: Targeting miR-125a Methylation and Epigenetic Regulation

Fatemeh Ahmadpour[1#], Somayeh Igder[2#], Ali Reza Eftekhari Moghadam[3], Bahman Moradipoodeh[4], Asma Sepahdar[5], Pooneh Mokarram[6], Jafar Fallahi[7], Ghorban Mohammadzadeh[8*]

1. Department of Clinical Laboratory Sciences, School of Allied Medicine, Lorestan University of Medical Sciences. Khorramabad. Iran.

2. Hyperlipidemia Research Center, Department of Clinical Biochemistry, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

3. Department of Anatomical Science, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran.

4. Department of Laboratory Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran.

5. Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences. Khorramabad. Iran.

6. Department of Biochemistry, School of Medicine, Autophagy Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

7. Department of Molecular Medicine, School of Advanced Medical Sciences and Technologies, Shiraz University of Medical Sciences, Shiraz, Iran.

8. Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

Article type: **Original Article**

Received: 2024/06/28
Revised: 2024/08/6
Accepted: 2024/08/26

ABSTRACT

Breast cancer, characterized by genetic diversity and molecular subtypes, presents significant treatment challenges, especially in human epidermal growth factor receptor type 2 (HER2)-positive cases, which are associated with poor prognosis. Metformin, widely known for its antidiabetic effects, has emerged as a promising candidate for cancer therapy. This study investigates the effect of metformin on miR-125a promoter methylation and its subsequent impact on the HER2 signaling pathway in HER2-positive breast cancer cells (SK-BR3). **SK**-BR3 cells were cultured and treated with various concentrations of metformin to assess its effects on cell viability, DNA methylation, HER2, and DNA Methyltransferase 1 (DNMT1) expression. Molecular analyses focus on the miR-125a signaling pathway modulation, DNA methylation, mRNA expression of DNMT1, and protein level of HER2. Research showed a dose-dependent reduction in cell viability, with IC50 values from 65 mM at 48 hours to 35 mM at 72 hours. Metformin treatment led to demethylation of the miR-125a promoter, which increased miR-125a expression and subsequently reduced HER2 levels. This suggests that metformin exerts its anticancer effects partly by regulation of the miR-125a-HER2 axis. Additionally, metformin inhibited vimentin expression, indicating its potential to interfere with epithelial-mesenchymal transition (EMT) processes. Metformin may serve as a targeted therapeutic agent in HER2-positive breast cancer by modulating the miR-125a-HER2 axis and influencing on the epigenetic and EMT regulation. Further research is warranted to elucidate the therapeutic potential of metformin through these mechanisms.

Keywords: Metformin, Breast cancer, miR-125a, methylation, HER2, DNMT1, epigenetics

Cite this article: Ahmadpour.F, *et al.* Metformin as a Potential Therapeutic Agent in Breast Cancer: Targeting miR-125a Methylation and Epigenetic Regulation. *International Journal of Molecular and Cellular Medicine.* 2024; 13(3):272-285.

# The first two authors contributed equally to this work.
*Corresponding: Ghorban Mohammadzadeh
Address: Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
E-mail: mohammadzadeh@ajums.ac.ir

[DOI: 10.22088/IJMCM.BUMS.13.3.272]

Introduction

Breast cancer, one of the most widespread malignancies, has shown a rapid increase in recent years. It is categorized into molecular subtypes based on its genetic profile, such as estrogen receptor (ER) positive, progesterone receptor (PR) positive, and HER2 positive. In patients with HER2 overexpression, low rates of disease-free survival, high rates of metastasis, and high mortality are observed (1). Several types of tyrosine kinase inhibitors (TKIs) have been approved for the treatment of breast cancer patients with HER2 positive (2). The patent medication has drawn much interest worldwide in the last 10 years due to its repositioning as a potential anti-cancer treatment (3). Studies show that the incidence of cancer and cancer-related mortality in diabetic patients treated with metformin is lower than in non-diabetic individuals (4, 5). Metformin (1,1-dimethylbiguanide hydrochloride) is an oral hypoglycemic medication frequently administered (6). Prospective, randomized phase III clinical studies are currently investigating the potential of this intervention to enhance overall survival and disease-free survival among patients diagnosed with breast cancer (7). There is uncertainty about the exact biochemical mechanisms underlying the therapeutic benefits of metformin. Increasing data suggests that metformin may inhibit cancer by altering microRNAs (miRNA) expression (8). Due to metformin's completely modulating several signaling pathways, we hypothesized that the miRNA regulation plays a role in this drug's effects.

Mature miRNAs are single-stranded, containing 18–26 nt, and post-transcriptionally represses gene expression by binding to the 3'-untranslated regions (3'-UTR) of different target mRNAs (9). Depending on the type of cancer, the effects of metformin on the miRNAs may occur by epigenetic changes. Recent research has shown that DNA methylation is responsible for tumor suppressor gene silencing, cancer chemoresistance, and metastasis, as well as altering miRNA, and mRNA expression (10, 11). DNA methylation is done by methyltransferase enzymes (DNMT1, DNMT3a, and DNMT3b) that catalyze transfer of methyl group from S-adenosyl-L-methionine (SAM) to the carbon 5 position of cytosine, yielding 5-methylcytosine. Cancer cells are characterized by the reversible suppression of tumor suppressor genes by locus-specific DNA hypermethylation in CpG promoter islands (12). In several malignancies, miRNAs are accurate biomarkers for predicting response to therapy. In breast cancer, they can be blocked by drugs or added to liposomal transporters for targeted drug delivery (13). The miR-125a-5p gene is located on chromosome 19q13. It is a tumor suppressor miRNA and plays a crucial role in acquiring characteristics such as growth inhibition, and tumor inhibition by blocking the HER-2 expression, and the autophagy pathway (14). Banerjee SM et al. reported that inhibiting DNA methyltransferases with 5-Aza-dC can suppress DNA methylation (15). The precise molecular mechanisms by which metformin influences the methylation of the miR-125a promoter in breast cancer remain unknown. Regarding the miR-125a signaling pathway, the objective of the current study was to examine a previously unexplored mechanism by which metformin may be utilized to treat breast cancer.

Materials and methods

High-glucose Dulbecco Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin/ streptomycin solution were Gibco Brand (Grand Island, NY, United States). MTT powder and sodium bisulfite were procured from Sigma-Aldrich (St. Louis, MO, United States), in addition to metformin

[DOI: 10.22088/IJMCM.BUMS.13.3.272]

(Glucophage, a product of Merck Pharmaceutical). Paraformaldehyde was obtained from Merck Pharmaceutical Company (Darmstadt, Germany). 100 X trypsin/EDTA solution and bovine serum albumin (BSA) were prepared by Invitrogen (Thermo Fisher Scientific, United States). DNA extraction and total RNA extraction were purchased from Favorgen Company (Taiwan). cDNA synthesis kits were obtained by Yekta Tajhiz and Zist Pooyesh (Tehran, Iran). Hot-Start Taq Master Mix and SYBR Green Real-Time PCR Master Mix were ordered from Ampliqon (Denmark). Antibodies were such as a primary antibody (Anti-HER2/neu rabbit IgG RMPD 008-Diagnostic Biosystems, Pleasanton, CA, USA), anti- vimentin antibody (rabbit antihuman; Bio Legend), and HRP conjugated antibody (goat anti-rabbit IgG- HRP: sc-2030, Santa Cruz, CA, USA).

Cell culture and cytotoxicity assay

The human cell line SK-BR3 (cells strongly expressing HER2) was provided by the Institute Pasteur Cell Bank (Tehran, Iran). Cells were cultured at 37°C in high-glucose DMEM, 10% FBS, and 0.5% Pen-Strep. To determine the optimal concentration for preconditioning metformin and examine the cytotoxic effect of metformin on cells, viability was determined using the MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl 2H-tetrazolium bromide) assay. SK-BR3 cells (4000 cells/well) were seeded in 96-well microtiter plates and incubated with metformin ranging from 5-80 mM for 48-72 hours, according to previous studies (16). Then, the medium was removed, and 20 µL of MTT reagent (0.6 mg/mL) were administered to the cells. The MTT reagent was removed after 4 hours of incubation and replaced with 100 µL of DMSO for 20 minutes at 37°C. The absorbance plate was subsequently scanned using a Biotek EIX800 microplate reader (Winooski, USA) at a wavelength of 570 nm. For the computation of half-maximal inhibitory concentration (IC_{50}) values, drug dose-response curves were utilized in conjunction with GraphPad Prism (GraphPad Software, San Diego, CA). The Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.REC.1398.888) approved this project.

After the detection of IC_{50} concentration, cells were divided into three groups: cells without treatment, metformin (20 µM), and metformin (40 µM), which received the drug for 48 hours to determine its impact on gene expression, methylation, and protein levels.

DNA extraction and methylation-specific PCR (MSP)

The DNA Extraction Kit was used to separate genomic DNA from SK-BR3 breast cancer cells. The methylation level of the miR-125a promoter was chemically evaluated with sodium bisulfite and subsequent MSP. Using a modification of bisulfite, this method converts unmethylated cytosine into uracil. This distinction is used by the MSP to amplify methylated or unmethylated DNA. Using a T1000 thermocycler (Bio-Rad, Hercules, CA, USA) and primers designed on the Meth Primer 2 website, MSP sodium bisulfite-treated DNA was amplified. The primer sequences for methylation were miR-125a-MF (5'-GTATAGAGCGAGGAGGAGGAGGC-3') and miR-125a-MR (5'-CCGAAAAACGACGAAACCG-3'), and the length of the amplicon was 161 bp. Primers miR-125a-UF (5'-GTATAGAGTGAGGGAGGAGGT-3') and miR-125a-UR (5'-CCAAAAACAACAACAACCAACAACA-3') were used in the demethylation reaction. The length of the amplicon was 164 bp. The final products were imaged by gel electrophoresis.

RNA extraction and real‑ time PCR

[DOI: 10.22088/IJMCM.BUMS.13.3.272]

Total RNA was isolated from cells using an RNA isolation kit. Accurate gene expression data was obtained using high-quality purified RNA evaluated spectrophotometrically at 260 nm. According to the kit instructions, cDNA synthesis processes were amplified using M-MLV and produced by priming with oligo-dT or a gene-specific stem-loop-based miRNA primer. A real-time instrument (Applied Biosystems Step One, ABI) was used to analyze mRNA expression. Primer pairs used in the study are presented in Table 1. Amplification started with an initial 15-second incubation at 94°C and was completed with 40 cycles of the extension step (94°C for 30 seconds, 60°C for 30 seconds). The comparative threshold cycle (Ct) method was employed to compute the results, with the ratio of miRNAs to SNORD-47 miRNA and mRNA to GAPDH mRNA serving as internal controls.

Immunocytochemical staining for HER2

Table 1. Primer Sequences.

Gene	Forward primer	Reverse primer	Product size
GAPDH	GGTCGGAGTCAACGGATTTGG	TGATGACAAGCTTCCCGTTCT	194
ERBB2	CGGAGAGCTTTGATGGGGAC	CCGGCCATGCTGAGATGTAT	117
DNMT1	CCAAACCCCTTTCCAAACCTC	CCTGGTGCTTTTCCTTGTAATCC	154

To perform an immunocytochemical (ICC) staining assay to detect the abundant presence of HER2 protein, 1×10^4 SK-BR-3 cells were cultured in four-well plates and subjected to a two-day treatment at 37°C. The cells were then fixed in 4% paraformaldehyde at ambient temperature for approximately 10 minutes. 15 minutes after three PBS treatments, 3% H_2O_2 inhibited the activity of endogenous peroxidase. For fifteen minutes, cells were obstructed with 2% BSA. Following that, the sample was incubated overnight at 4°C in a humid environment, during which the primary antibody (1:200) was added. Following a PBS wash, the primary antibody was incubated at room temperature for 2 hours with HRP-conjugated antibody (1:500). It was subsequently rinsed three times with PBS. At ambient temperature, 20 µL of substrate reagent solution was added to each slide. Following this, the specimen was stained with hematoxylin. HER2 results were determined based on the frequency and severity of stained cells and staining severity as follows: 0= 10%, 1= 10% to 25%, 2= 25% to 50%, 3= 50% to 75%, and 4 = 75%.

Immunofluorescence staining for vimentin

To detect SK-BR3 mesenchymal protein (vimentin), cells were cultured in four-well plates, washed with PBS, and fixed with ice-cold 4% paraformaldehyde for 10-15 minutes at room temperature. Following three consecutive PBS washing, cells were permeated with 0.1% Triton X-100 for 10 minutes at room temperature. Next, block the cells with 1 % BSA for 40 minutes, and then incubate the cells with an anti-vimentin antibody (1:200) overnight at 4°C. Cells were washed three times with PBS before being exposed to a 1:500 dilution of a goat anti-rabbit antibody conjugated to fluorescein isothiocyanate for an hour at room temperature. Next, wash with PBS three times. The nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI). A fluorescence microscope was used for visualization (Leica M205 FA; Leica Microsystems).

In silico studies on miRNA targets

We investigated the signaling pathways that could be affected by miRNA expression and then evaluated them using the KEGG database (http://www.genome.jp/kegg/pathway_facility). The study identified

[DOI: 10.22088/IJMCM.BUMS.13.3.272]

three miR-regulated genes, miR-125a-5p, associated with tumor pathways. Targets were verified using the miRTarBase database (http://mirtarbase.mbc.nctu.edu.tw/php/download.php).

We used the RNA-hybrid program (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/) to link the biological function of miR-125a-5p and target-related signaling to better understand the tumor suppressor biology of this miRNA expressed in SK-BR3 cells. Only first-level interactions between the target mRNA (DNA Methyltransferase 1 (DNMT1) and HER2) and has-miR-125a-5p (UCCCUGAGACCCUUUAA CCUGUG A) were noticed.

Statistical analysis

The study used SPSS for statistical analysis, using the Kolmogorov-Smirnov test, independent t-tests, ANOVA, Tukey's post hoc analysis, Mann-Whitney U and Kruskal-Wallis H tests, Spearman's method, and chi-squared test to assess correlations among quantitative data and qualitative categorical relationships. Triplicates were conducted with a significance level of $p < 0.05$.

Results

Metformin arrests the growth of SK-BR3 cells

Initially, the impact of metformin on cellular viability was evaluated by employing the MTT viability assay at five distinct concentrations (0, 10, 20, 40, and 80 mM) after treatment for 48 and 72 hours. To examine the impact of metformin on SK-BR3 cell proliferation, *in vitro* cultures were prepared using metformin concentrations of 20, and 40 mM. The cells were incubated for durations of 48 and 72 hours. The optical density (OD) of cells was quantified using the MTT method as the concentration of metformin increased. As shown in Figure 1, the average OD value decreased gradually, whereas the rate of inhibition of cell proliferation increased markedly. The experiments demonstrated the sensitivity of SK-BR3 to metformin. Using the MTT assay, the IC_{50} values (65 mM for 48 hours and 35 mM for 72 hours) were determined, as illustrated in Figures 1A and B. Metformin reduced cell viability at 48 hours at concentrations of 20 mM or higher by 19%, 37%, and 65%, respectively (Figure 1). To examine the impact of metformin on SK-BR3, in vitro cultures were prepared using metformin concentrations from 20, to 40 mM for 48 hours.

Metformin induces hypomethylation of miR‑125a promoter

We assessed the CpG dinucleotides associated with the signature promoter of miR-125 using the MethPrimer2 online utility software. To validate our hypotheses, we designed specific primers for methylation-specific PCR (MSP) (Figure 2A). The methylation quality of the expected CpG sites was analyzed using MSP in control, 20 mM, and 40 mM treated with metformin for 48 hours (Figure 2B). The findings indicated that control cells exhibited positive hypermethylation, whereas cells subjected to metformin treatment displayed only partial methylation of the miR-125a promoter. In general, research indicates a robust correlation between aberrant methylation and diminished miR-125a expression. Following treatment with 20 mM, and 40 mM metformin, the expression of miRNA in SK-BR3 cells increases by approximately 1.7 and 2-fold, respectively (Figure 2C, $p<0.05$).

Metformin decreases the expression of DNMT1 and HER2

The mRNAs of DNMT1 and HER2 were examined in this investigation; DNMT1 and HER2 are targets of has-miR-125a-5p (Figure 3). Metformin induces a dose-dependent decrease in DNMT1 and HER2 mRNAs. They were identified in SK-BR3 cells via real-time PCR (Figure 4A, B).

[DOI: 10.22088/IJMCM.BUMS.13.3.272]

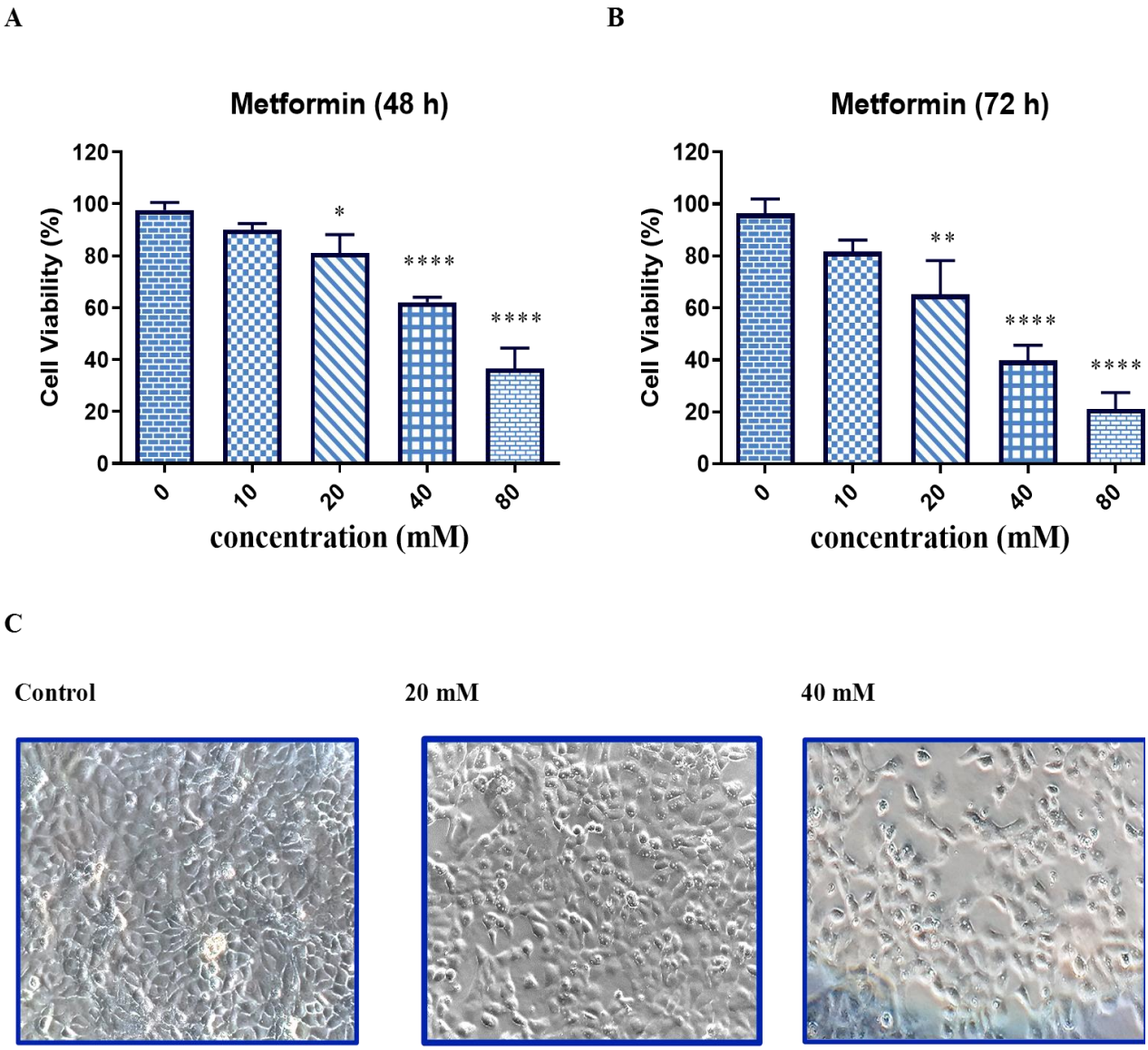


Fig. 1. Effects of Metformin on Viability and Morphology of the SK-BR3 Cells. **A** MTT viability test with five different concentrations (0, 10, 20, 40, and 80 mM) at 48 and 72 h of Metformin treatment. **B** Morphology of the SK-BR3 treated at concentrations of 20 mM and 40 mM with Metformin.* P<0.05, ** P<0.01, *** P < 0.001; ****P<0.0001, * symbol indicates the comparison of treated groups and non-treated controls. Results are presented as means ± standard deviation (SD) and are representative of three independent experiments.

Metformin represses the protein level of HER2 and Vimentin

Metformin therapy with 20 mM metformin decreased HER2 protein expression by 25% after 48 hours and by 50% at 40 mM. The dose-dependent potency of metformin was confirmed through the utilization of immunocytochemistry techniques (Figure 5). By employing a vimentin-targeting antibody, the cells' expression of an EMT marker was assessed. Using immunofluorescence staining (Figure 6), vimentin expression was confirmed. After subjecting SK-BR3 cells to metformin at different concentrations, it was

[DOI: 10.22088/IJMCM.BUMS.13.3.272]

observed that the intensity of vimentin fluorescence light was diminished in cells that received the highest concentration of metformin.

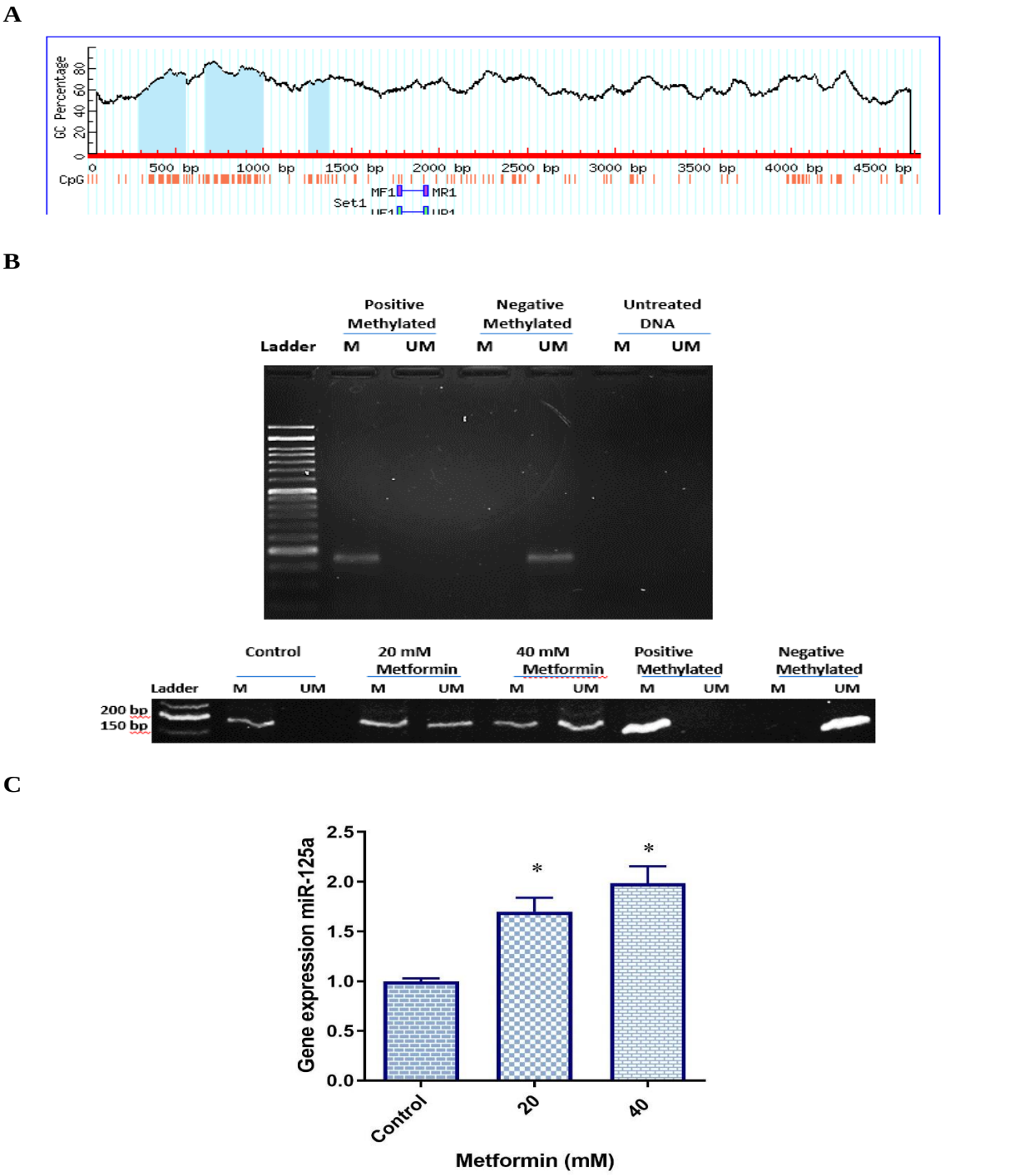


Fig. 2. **A** location of three CpG islands 3,500 bp upstream of miR-125a-5p. **B** MSP was used to analyze the methylation status of the first CpG island from the miR-125a gene in SK-BR3 cells treated at concentrations of 20 mM and 40 mM with Metformin. Arrows indicate the unmethylated (UM) or methylated (M) products. **C** miR-125a expression was upregulated after treated with 20 and 40 mM of Metformin for 48 h in the SK-BR3 cell line.

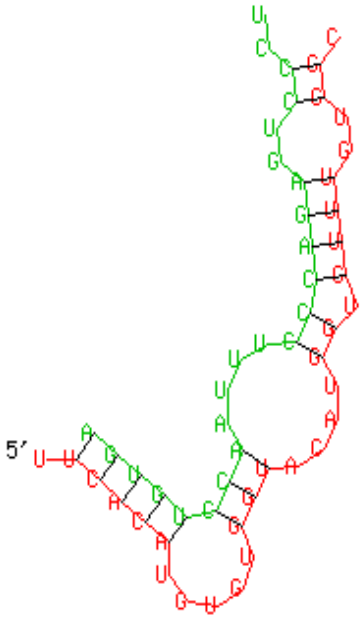
[DOI: 10.22088/IJMCM.BUMS.13.3.272]

```
dataset: 1
Target: DNMT1
length: 320
MiRNA: hsa-miR-125a-5p
length: 24

mfe: -21.3 kcal/mol
p-value: 1.000000e+00

Position: 86
target 5' U     UGUGU   ACAU  U    GU  C  3'
           UCACA     GGU    GG GUUU  GG
           AGUGU     CCA    CC CAGA  CC
miRNA  3'               AUUU       GU  CU 5'

plot as png,jpeg or ps (in a new window)
```



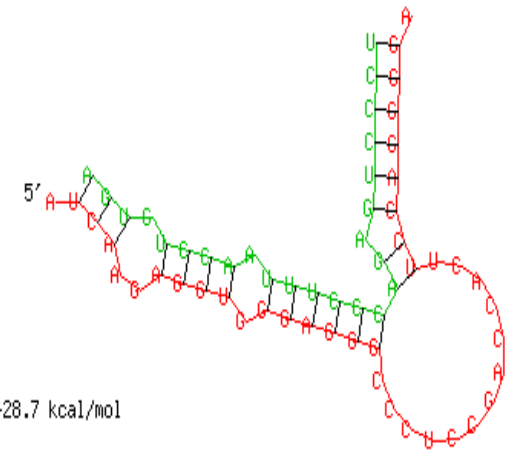
mfe: -21.3 kcal/mol

```
dataset: 1
Target: HER2
length: 614
MiRNA: hsa-miR-125a-5p
length: 24

mfe: -28.7 kcal/mol
p-value: 1.000000e+00

Position: 71
target 5' A   AG    G      CCCUCCGACCACU         A 3'
          UCA  AGGU GGAGGG             UC CAGGGG
          AGU  UCCA UUUCCC             AG GUCCCU
miRNA  3'     G     A                    A       5'

plot as png,jpeg or ps (in a new window)
```



mfe: -28.7 kcal/mol

Fig. 3. Prediction of post-transcriptional interaction between DNMT1 (**A**) and HER2 (**B**) mRNA 3'UTR with miR-125a-5p assessed by RNA-Hybrid program. The interaction produced a significant minimum free energy (−21 mfe for DNMT1 and – 28 mfe for HER2) forming a stable hybrid structure.

[DOI: 10.22088/IJMCM.BUMS.13.3.272]

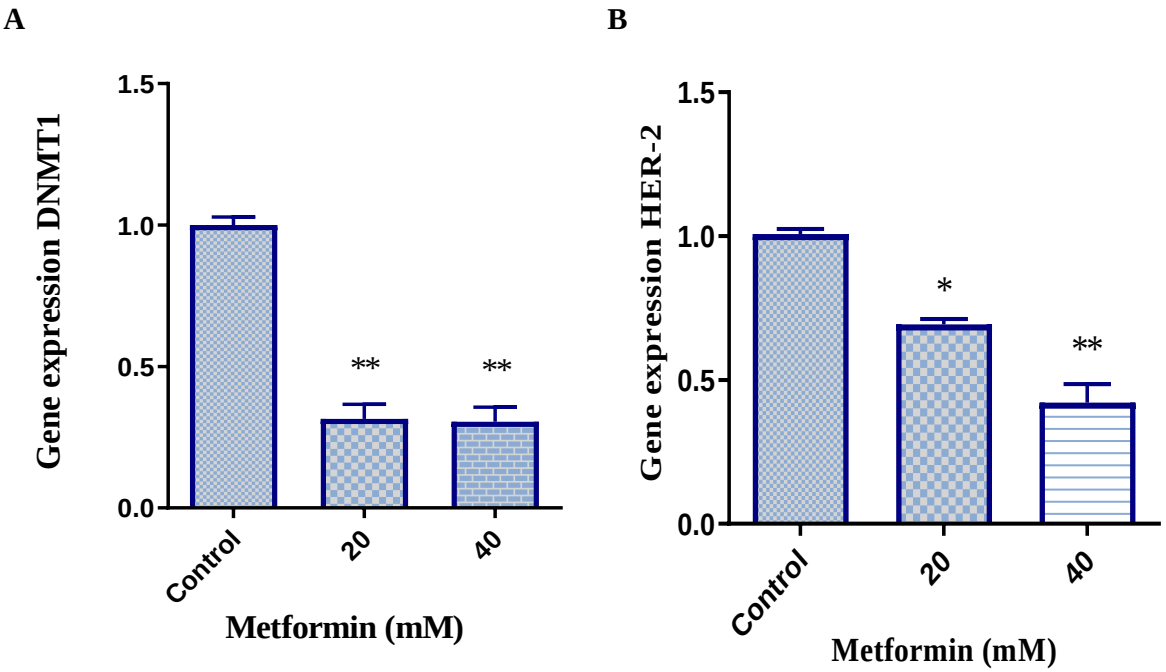


Fig. 4. Expression of DNMT1 and HER2 in SK-BR3 cells after treated with 20 and 40 mM of Metformin for 48 h. **A** DNMT1 expression was downregulated after treatment with Metformin for 48 h in the SK-BR3 cell line. **B** HER-2 expression was downregulated treatment with Metformin for 48 h in the SK-BR3 cell line. * P<0.05, * symbol indicates the comparison of treated groups and non-treated controls. Results are presented as means ± standard deviation (SD) and are representative of three independent experiments.

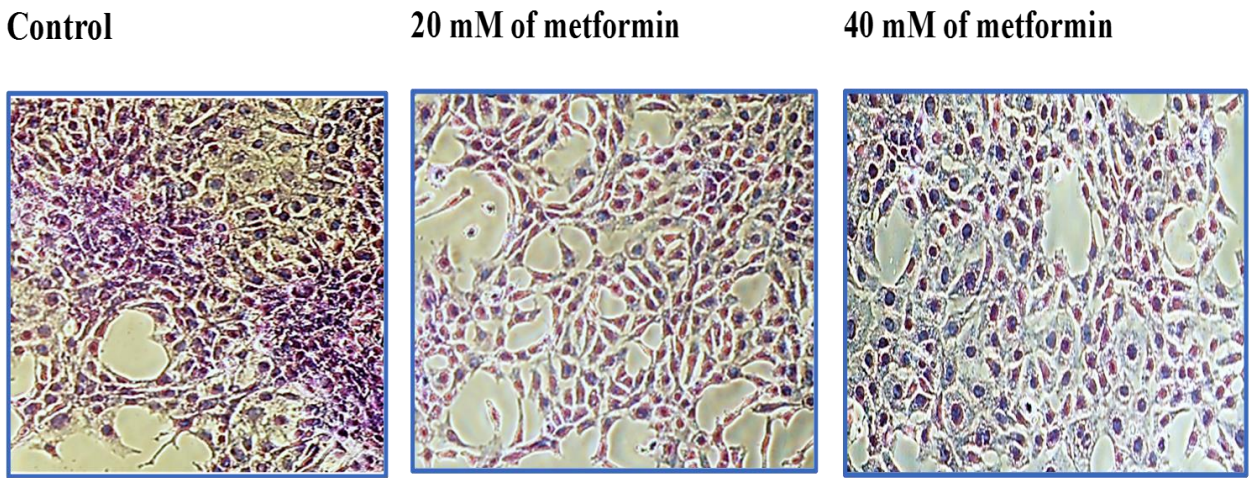


Fig. 5. Representative image of HRP staining of HER-2 expression in the SK-BR3 cell line. Brown dots was associated with HER-2 positive staining and blue was related with differential staining of the nuclei by hematoxylin. HER-2 protein level was significant after Metformin treatment for 48 h. (Control:4 +, 20 mM of metformin: 3+, 40 mM of metformin:2+). Immunofluorescence test of cells at 48 h: proliferating cells were immunostained (brown) for HER2 HRP, the nucleus of all cells was counterstained with hematoxylin. Mean percentage of proliferating cells compared with total counted cells.

[DOI: 10.22088/IJMCM.BUMS.13.3.272]

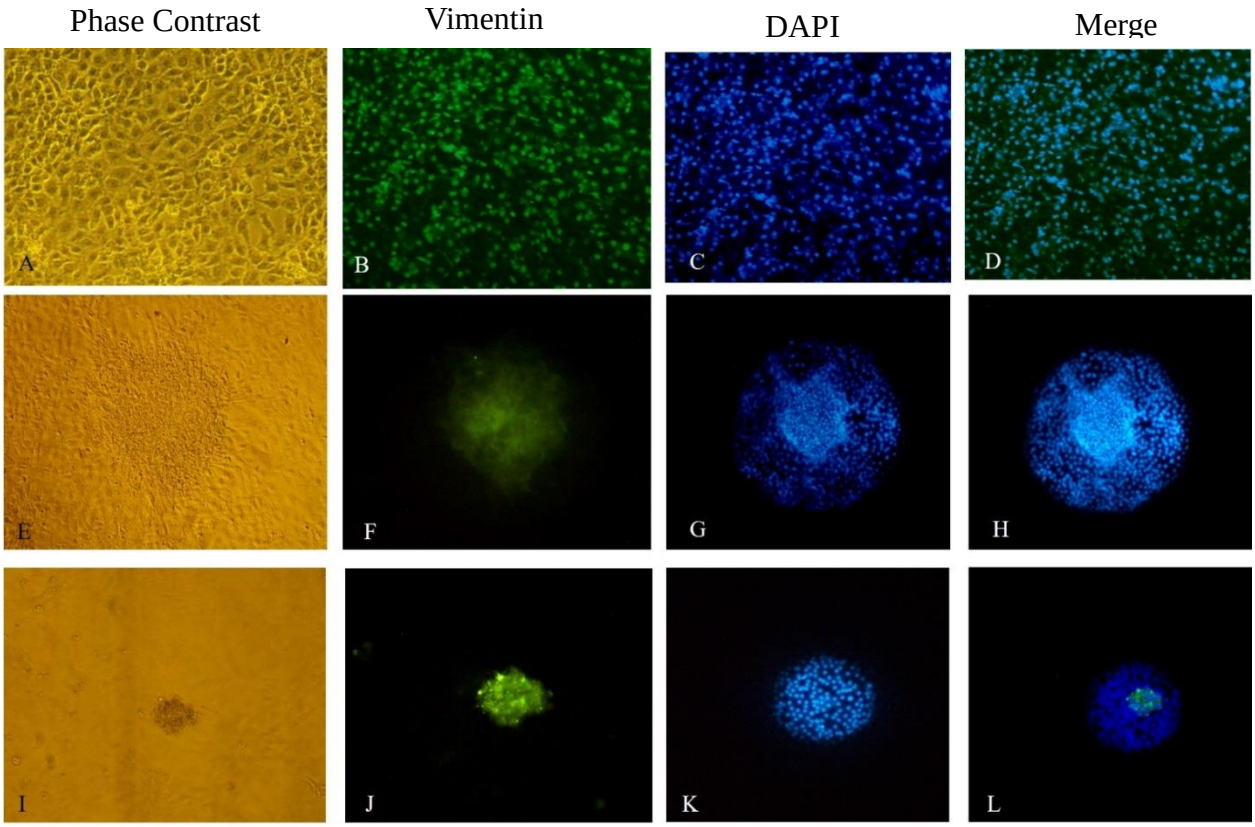


Fig. 6. SK-BR3 cell line cultured in vitro for 24 and 48 h (A–D). All cells exhibit cytoplasmic immunostaining for Vimentin. Nuclear DNA was stained with DAPI (C, blue fluorescence). (E-L) Vimentin immunostaining of SK-BR3 adherent cells in 20 and 40-mM metformin supplemented medium after 48 h, respectively. Fluorescent light intensity was interpreted by ImageJ software. DAPI, 4′,6- diamidino- 2- phenylindole**.**

Discussion

Breast cancers, characterized by HER2 gene overexpression, affect 20% of patients, leading to severe forms and lower disease-free survival (17). Chemotherapy is often considered, but drug resistance and cardiomyopathy from HER2 receptor blockers limit its effectiveness (2). A unique therapy is needed to address these challenges .Metformin, an antidiabetic, is safe due to its ability to modulate S-adenosylhomocysteine activity, affecting genomic DNA methylation and epigenetic modifying enzymes, primarily through AMP-activated protein kinase (AMPK) activation , which can inhibit DNMTs (18). This study investigates the uncertain impact of metformin on the epigenome in breast cancer, focusing on its effects on miR-125a promoter methylation, DNA methylation, and its molecular mechanism. miRNAs play a crucial role in cancer cell functions like differentiation, proliferation, apoptosis, and metastasis (19). Our previous study showed that aberrant DNA methylation suppresses miR-125a production in breast cancer cells. We found that miR-125a significantly reduced and reversed the autophagy process. Utilizing miRNA mimics, HER2 protein expression was reduced in the SK-BR3 cancer cell line (20). In this study, we investigated whether metformin could change the methylation status and expression of miR-125a by

[DOI: 10.22088/IJMCM.BUMS.13.3.272]

inhibiting the DNMT1 expression and then we looked at miR-125a-related target genes, namely HER2 and vimentin in SK-BR3 cell line.

This study investigated how metformin affects the development and division of SK-BR3 cells and found IC_{50} at concentrations from 65 mM to 35 mM for 48 and 72 hours, respectively. Cell viability decreased with increasing dose of metformin; the inhibitory effect was time-and dose-dependent manner. Metformin also showed time-dependent cytotoxic activity toward MCF-7 cells (21). Fu YL et al. conducted a study in which they observed that an ovarian SKOV3 cancer cell line treated with metformin for 48 hours yielded an IC_{50} of 20 mM (22). Moreover, according to the findings of Yong P Hwang and Hye G Jeong, metformin at concentrations lower than 5 mM had no statistically significant impact on the viability of cancer cells after 24 hours, metformin at 25 mM showed about 28% decrease in cell viability in fibrosarcoma cells (23). Metformin can inhibit the expression of DNMTs in human cancer cells, according to two separate research groups. To illustrate, research suggests that the concurrent administration of metformin and berberine effectively suppressed the expression of DNMT1 in NSCLC cells via a synergistic mechanism involving the downregulation of transcription factor 3, phosphoinositide-dependent protein kinase 1(PDPK1), and SP1 (24). Additionally, they documented that metformin decreased DNMT1 expression at both the post-transcriptional and transcriptional levels in A549 lung cancer cells in a dose-dependent manner. In A549 cells treated for 72 hours with 10 mM metformin, the activity of DNMTs was diminished by approximately 2.6-fold (25). For the first time, however, the inhibitory effect of metformin on DNMT1 expression in SK-BR-3 cells was investigated in this study. Expression of DNMT1 decreased in a dose-dependent manner after metformin treatment (Figures 4A-B). A 75% decrease in expression was observed at 20 mM and 40 mM concentrations of metformin. Our results showed after 48 hours treatment with metformin the inhibitory effect of it was observed on the DNMT1 expression in SKBR3 cells.

We further clarified the probable downstream effectors to analyze the mechanism and understand the biological significance of the interaction between DNMT1, miR-125a, and HER2 (Figure 3). In this study, we elucidated the expression of DNMT1 and HER2, where HER2 is the target of miR-125a. Our findings indicate that the mechanism by which metformin inhibits HER2 via miR-125a augmentation and decreases DNMT1 mRNAs may be elucidated by hypomethylation of the miR-125a promoter. Investigations have demonstrated that DNMT1 inhibition can trigger cellular apoptosis by decreasing the expression of HER2 (26). The ICC staining results obtained in our study indicate that the induction of apoptosis by metformin was dose-dependent (Figure 5). Metformin concentrations from 20 mM to 40 mM induced apoptosis in SK-BR-3 cells about 25% and 50%, respectively, after 48 hours. Our study provides additional evidence by specifically linking metformin's action to the miR-125a-HER2 axis, something not extensively explored in earlier research. This novel insight contributes to the understanding of metformin's role in targeting HER2-positive breast cancer.

Moreover, the significance of vimentin in cancer cell migration and invasion has been well-documented over the past decade, a study showed that vimentin is a key marker of epithelial-mesenchymal transition (EMT) and a predictor of poor prognosis in breast cancer patients (27). Thompson et al. demonstrated that breast cancer patients' bone marrows may contain circulating vimentin-positive cells (28). Inhibiting vimentin could thus be a viable molecular target for combating the invasion and localization of tumor cells

[DOI: 10.22088/IJMCM.BUMS.13.3.272]

in other tissues. We documented elevated levels of vimentin expression in the SK-BR3 cell line in this investigation. Vimentin expression was also compared to the suppressive effects of the minor drug metformin. This molecule inhibited vimentin with considerable potency, according to our findings. Metformin is capable of selectively targeting mitochondria and disrupting certain signaling pathways and redox systems, such as the AMPK and EMT signaling pathways. Metformin inhibits the expression of transcription factors that are responsible for EMT signaling (29). Our study is unique in showing that metformin not only inhibits HER2 and DNMT1 but also significantly reduces vimentin levels, suggesting a broader impact on EMT and metastasis. These findings highlight metformin's potential as a multi-target therapeutic agent, which is in line with the results of Thompson et al., who showed that targeting vimentin could reduce metastasis in breast cancer (28). While our study shares similarities with previous research in demonstrating metformin's effects on miRNA expression and EMT, it also differs by providing a more detailed mechanistic explanation of how miR-125a modulates HER2 and vimentin expression through DNMT1 inhibition. This multifaceted mechanism suggests that metformin's anti-cancer effects extend beyond simple cytotoxicity and involve complex epigenetic and signaling pathway regulations.

We have concluded that metformin primarily modulates the expression of vimentin and HER2 in breast cancer cells by inhibiting the DNMT1-mediated methylation of miR-125a. The proliferation and migration of breast cancer cells are affected via the miR-125a/HER2/Vimentin axis by an epigenetic mechanism. The results of this study shed light on the mechanism by which metformin inhibits breast cancer proliferation and metastasis and contribute to the development of novel therapeutic approaches that target the miR-125a/HER2/Vimentin axis. Such an approach holds great promise for the treatment of breast cancer and other human malignancies according to these mechanisms.

Acknowledgments

This paper is issued from the research project and financial support was provided by the Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (grant no. CMRC-9816). The present study was designed and conducted with the permission of the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1398.888). We would like to thank Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Lorestan, Iran.

References

1. Feng Y, Spezia M, Huang S, et al. Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. Genes Dis 2018;5:77-106.

2. Singh DD, Lee H-J, Yadav DK. Clinical updates on tyrosine kinase inhibitors in HER2-positive breast cancer. Front Pharmacol 2022;13:1089066.

3. Liddicoat J, Liddell K, Darrow J, et al. Repositioning Generic Drugs: Empirical Findings and Policy Implications. Int Rev Intellect Prop Compet Law 2022;53:1287-322.

4. Orchard SG, Lockery JE, Broder JC, et al. Association of metformin, aspirin, and cancer incidence with mortality risk in adults with diabetes. JNCI Cancer Spectr 2023;7.

[DOI: 10.22088/IJMCM.BUMS.13.3.272]

5. Landman GW, Kleefstra N, van Hateren KJ, et al. Metformin associated with lower cancer mortality in type 2 diabetes: ZODIAC-16. Diabetes Care 2010;33:322-6.

6. Hernández-Velázquez ED, Alba-Betancourt C, Alonso-Castro ÁJ, et al. Metformin, a biological and synthetic overview. Bioorg Med Chem Lett 2023:129241.

7. Geng Y, Wang Z, Xu X, et al. Extensive therapeutic effects, underlying molecular mechanisms and disease treatment prediction of Metformin: a systematic review. Transl Res 2023;263:73-92.

8. Wang G, Lin F, Wan Q, et al. Mechanisms of action of metformin and its regulatory effect on microRNAs related to angiogenesis. Pharmacol Res 2021;164:105390.

9. Han J, Mendell JT. MicroRNA turnover: a tale of tailing, trimming, and targets. Trends Biochem Sci 2023;48:26-39.

10. Saviana M, Le P, Micalo L, et al. Crosstalk between miRNAs and DNA Methylation in Cancer. Genes 2023;14:1075.

11. Ghaznavi H, Kiani AA, Soltanpour MS. Association study between DNA methylation and genetic variation of APOE gene with the risk of coronary artery disease. Mol Biol Res Commun 2018;7:173-9.

12. Zhong F, Lin Y, Zhao L, et al. Reshaping the tumour immune microenvironment in solid tumours via tumour cell and immune cell DNA methylation: from mechanisms to therapeutics. Br J Cancer 2023;129:24-37.

13. Nel J, Elkhoury K, Velot E, et al. Functionalized liposomes for targeted breast cancer drug delivery. Bioact Mater 2023; 24:401-37.

14. Shinde SS, Ahmed S, Malik JA, et al. Therapeutic Delivery of Tumor Suppressor miRNAs for Breast Cancer Treatment. Biology (Basel) 2023;12.

15. Banerjee SM, Acedo P, El Sheikh S, et al. Combination of verteporfin-photodynamic therapy with 5-aza-2'-deoxycytidine enhances the anti-tumour immune response in triple negative breast cancer. Front Immunol 2023;14:1188087.

16. Moradipoodeh B, Mohammadzadeh G, Neamati D, et al. Synergistic Effects of Metformin-Lapatinib Combination on the Expression of Bax in SK-BR3 Cells. Jentashapir Journal of Cellular and Molecular Biology 2020;11.

17. Zhang H, Peng Y. Current Biological, Pathological and Clinical Landscape of HER2-Low Breast Cancer. Cancers (Basel) 2022;15.

18. Sharma A, Anand SK, Singh N, et al. AMP-activated protein kinase: An energy sensor and survival mechanism in the reinstatement of metabolic homeostasis. Exp Cell Res 2023;428:113614.

19. Darvish L, Bahreyni Toossi MT, Azimian H, et al. The role of microRNA-induced apoptosis in diverse radioresistant cancers. Cell Signal 2023;104:110580.

20. Ahmadpour F, Igder S, Babaahmadi-Rezaei H, et al. Methylation-mediated silencing of miR-125a-5p facilitates breast cancer progression by inducing autophagy. Mol Biol Rep 2022;49:6325-39.

21. Fatehi R, Rashedinia M, Akbarizadeh AR, et al. Metformin enhances anti-cancer properties of resveratrol in MCF-7 breast cancer cells via induction of apoptosis, autophagy and alteration in cell cycle distribution. Biochem Biophys Res Commun 2023;644: 130-9.

22. Fu YL, Zhang QH, Wang XW, et al. Antidiabetic drug metformin mitigates ovarian cancer SKOV3 cell growth by triggering G2/M cell cycle arrest and inhibition of m-TOR/PI3K/Akt signaling pathway. Eur Rev Med Pharmacol Sci 2017;21:1169-75.

23. Hwang YP, Jeong HG. Metformin blocks migration and invasion of tumour cells by inhibition of matrix metalloproteinase-9 activation through a calcium and protein kinase Calpha-dependent pathway: phorbol-12-myristate-13-acetate-induced/extracellular signal-regulated kinase/activator protein-1. Br J Pharmacol 2010;160:1195-211.

[DOI: 10.22088/IJMCM.BUMS.13.3.272]

24. Zheng F, Wu J, Tang Q, et al. The enhancement of combination of berberine and metformin in inhibition of DNMT1 gene expression through interplay of SP1 and PDPK1. J Cell Mol Med 2018;22:600-12.
25. Lee BB, Kim D, Kim Y, et al. Metformin regulates expression of DNA methyltransferases through the miR-148/-152 family in non-small lung cancer cells. Clin Epigenetics 2023;15:48.
26. Laranjeira ABA, Hollingshead MG, Nguyen D, et al. DNA damage, demethylation and anticancer activity of DNA methyltransferase (DNMT) inhibitors. Sci Rep 2023;13:5964.
27. Kidd ME, Shumaker DK, Ridge KM. The role of vimentin intermediate filaments in the progression of lung cancer. Am J Respir Cell Mol Biol 2014;50:1-6.
28. Thompson EW, Sung V, Lavigne M, et al. LCC15-MB: a vimentin-positive human breast cancer cell line from a femoral bone metastasis. Clin Exp Metastasis 1999;17:193-204.
29. Conza D, Mirra P, Fiory F, et al. Metformin: A New Inhibitor of the Wnt Signaling Pathway in Cancer. Cells 2023;12.

[DOI: 10.22088/IJMCM.BUMS.13.3.272]