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Investigation the Role of miR-506 in Metformin-Induced Cell Death Mechanism in MCF-7 and MDA-MB-231 Breast Cancer Cells

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Abstract

Micro RNAs (miRNA) play a role in basic cellular processes such as cell growth, development, cell cycle and apoptosis by affecting gene expression. Abnormal regulation or changes in expression of miRNAs can be observed in many diseases, especially cancer. Therefore, miRNAs are also being investigated as potential therapeutic targets. It has been suggested that miR-506 may be expressed at low levels in various types of cancer and this may contribute to cancer development. In this study, the effect of miR-506 on the sensitivity of breast cancer cells to metformin was evaluated in terms of its effect on cell survival and apoptotic mechanism. Metformin caused a dose-dependent decrease in cell viability and induced loss of mitochondrial membrane potential in MCF-7 cells while MDA-MB-231 cells were more resistant. The colony formation and migration potential of both cell lines with increased miR-506 expression were significantly suppressed after metformin treatment. Additionally, apoptotic cell death triggered by metformin was induced in both cell lines when miR-506 expression was increased. In conclusion, miR-506 acts as a tumor suppressor in MCF-7 and MDA-MB-231 cells and increases their sensitivity to metformin, indicating the potential of miR-506 to be an important therapeutic target in future studies.

Keywords: Apoptotic cell death, Breast cancer, Metformin, miR-506

1. Introduction

MicroRNAs (miRNAs) are non-translated RNAs of almost 22 nucleotides in length which are found in various organisms, including plants, animals, and some viruses [1]. MicroRNAs mostly bind to the 3' untranslated region (3' UTR) of the mRNA and in a minority can bind to the 5'-UTR, ORF (open reading frame) or promoter regions to carry out their transcription regulation functions [2]. Irregularities in miRNA expressions are related with various types of cancer initiation, progression and metastasis. Abnormally low expression of tumor suppressor miRNAs in cancer cells causes such abnormalities in cellular processes like as decrease in cell death mechanism, increased cell growth and metastasis. However, suppressing the functions of tumor suppressor miRNAs causes a decrease in sensitivity to treatment [3]. OncomiRs generally target genes involved in normal cell regulation, causing these genes to support uncontrolled cell growth, metastasis (spread of tumor cells to other regions) and other cancer features [4]. Studies have shown that miR-506 expressed at significantly low levels in various types of cancer and has tumor suppressor role by targeting oncogenes such as N-Ras, PIM3 and ROCK1 [5]. Many studies have investigated the role of miR-506 in breast cancer cases, which have the second highest incidence worldwide. Accordingly, it has been shown that increased miR-506 expression reduces c-Myc and E2F protein levels in breast cancer cells, resulting in suppression of cell viability and migration capacity [6]. The molecular mechanism of the tumor suppressor role of miR-506, which is found at low levels in breast cancer, continues to be investigated, and its role in combined treatments, which is one of the aims of our project, is a matter of curiosity.

Metformin, used in type 2 diabetes therapies, has an anti-proliferative effect on various cancer cells [7]. In fact, a meta-analysis conducted in 2014 showed that individuals using metformin had a lower incidence of breast cancer [8]. Metformin activates AMP-activated protein kinase (AMPK) by suppressing the mammalian target of rapamycin (mTOR), thus inhibiting cell survival [9]. The

effect of metformin on glucose metabolism in breast cancer cells are related to the duration of administration and has been shown to cause different effects on the uptake of 3H-deoxy-D-glucose (3H-DG) into the cell [10]. In studies conducted on different cancer cells such as pancreatic, gastric or osteosarcoma, metformin has been shown to induce apoptosis, which is programmed cell death [11-13]. Extrinsic and intrinsic molecular mechanisms can be triggered in apoptosis, which is determined by blebbing on the cell membrane, chromatin condensation and the formation of apoptotic bodies. The mitochondrial signaling mechanism, which is activated in the intrinsic pathway, is activated by increasing Bax/Bak protein expressions, which cause polarization in the mitochondrial membrane, and especially by triggering the loss of mitochondrial membrane potential (MMP). At this stage, cytochrome c, which is translocated to the cytosol, forms an apoptosome complex with APAF-1 in the cytosol, triggering caspase activation that takes part in the apoptotic process. Members of the caspase protease family are involved in the initiation and progression of apoptosis. The caspases have the ability to activate each other proteolytically. Caspase 9 known as the initiator caspase in the intrinsic pathway, stimulates PARP cleavage by activating caspase 3 and caspase 7 in the downstream signaling pathway [12]. There are studies showing that the effect of metformin on the intrinsic pathway is due to an increase in reactive oxygen species [7]. However, the effect of metformin on cellular death mechanisms may vary depending on both the application time and the cancer cell. For this reason, studies are continuing to increase the cell viability inhibitory effect of metformin with combined agents.

In the current study, the expression of miR-506, which has a tumor suppressor role, was increased in estrogen receptor positive (ER+) MCF-7 and triple negative breast cancer (ER-, PR-, HER2-) MDA-MB-231 breast cancer cells. Then, the effect of metformin on colony formation potential, migration and effect on cell death mechanism were examined in these cells. In the results obtained, it was observed that miR-506 expression was at a lower level in MDA-MB-231 cells compared to MCF-7 cells. Metformin treatment increased miR-506 expression in both cell lines. When miR-506 expression was increased in both cell lines by synthetic miRNA transfection, the effect of metformin in suppressing colony-forming potential was significantly increased. Additionally, in cells with increased miR-506 expression, metformin suppressed cell migration and triggered apoptotic cell death. As a result, our study revealed that miR-506 is an important target to increase the therapeutic effectiveness of metformin in both types of breast cancer cell lines.

2. Material and Methods

2.1. Cell Culture

MDA-MB-231 (HTB-26) and MCF-7 (HTB-22) cell lines were obtained from ATCC (American Type Culture Collection). Cells were incubated in DMEM (Dulbecco's modified Eagle serum) medium containing 10% FBS and 1% penicillin/streptomycin in an incubator at 5% CO_2 and 37 °C. Passaging was performed when the cells reached a certain density. For the passaging process, the upper media was removed and the cells were washed with 1 X PBS, an isotonic solution. Then, 0.25% trypsin-EDTA was added to cover the surface of the flask and the cells were allowed to lift off the surface for 3-5 minutes at 37 °C. The medium was added with trypsin at a ratio of 1:1, and after trypsin inactivation was achieved, the cells were centrifuged at 500 g for 4 minutes. After centrifugation, the cell pellet was homogenized with media and counting was performed using hemocytometry. miR-506 mimic (5 nM) miRCURY LNA miRNA Mimics, (Cat. No: 339173; Qiagen, Hilden, Germany) and mirVana™ miRNA Mimic Negative Control (Cat. No: 4464058; Thermo Scientific, Massachusetts, USA) were transfected by lipofectamine reagent at 1:3 (µg/ml) ratio for 24-48 hours [13].

2.2. Cell survival test

A cell survival test was performed using the 3-(4,5-Dimethylthiazol-2-yl (MTT) agent. In our study, cells were seeded in a 96-well/plate as 1 X10^4 cells/well and incubated overnight for adhesion. Following this, different concentrations of metformin (1.5-25 mM) were treated to the cells for 24 hours. Then, the supernatant was carefully removed from the cells to which 10 µL MTT agent was applied in an incubator of 5% CO_2 and 37 °C for 4 hours and then 100 µl DMSO was added. Absorbance measurement due to the color change was made in a plate reader at dual wavelengths (570 nm and 655 nm).

2.3. Fluorescence microscopy

DAPI staining: Cells were seeded in a 12-well/plate as 5x10^4 cells/well. After adhesion, 2.5 mM and 5 mM metformin was treated to the cells for 24 hours. After a 15-minute waiting period with the medium containing DAPI (5 mg/ml), it was observed that the nuclei were stained blue in the fluorescence microscope with at 350/470 nm (Ext./ Em.). DiOC6 staining: Cells were treated as mentioned above. Then, cells treated with 4mM DiOC6 for 15 minutes. The cells with intact mitochondrial membrane structure was observed in green color under a fluorescence microscope at 488/525 nm (Ext./Em). Propidium iodide (PI) staining: Drug-treated/un-treated cells were incubated with 5 mg/ml PI for 30 minutes. Under a fluorescence microscope, dead cells were observed in red color at 536/617 nm (Ext./Em).

2.4. miRNA analysis

miR-506 level was analyzed by qRT-PCR after miR-506 mimic transfection into cells. At the same time, miR-506 level in metformin-treated cells was also examined. Purification of miRNA from cells was carried out with the mirnEasy miRNA isolation Kit with the protocol prescribed by the commercial kit (Qiagen, Cat No: 217004). miScript II RT Kit was used for cDNA synthesis (Qiagen, Cat No: 218161). Following the conversion to cDNA, RT-PCR for miR-506 and endogenous control RNU6B primer was performed on the CFX Connect (BioRad), using miScript SYBR Green PCR Kit (Qiagen, Cat No: 218073) following the steps of 15 s denaturation at 94 °C, 30 s binding at 55°C and 30 s extension at 70°C, 40 cycles were continued. Data analysis was normalized by the ΔCT values of miRNAs of each sample against the internal control RNU6B and was performed in at least 2 replicates.

2.5. Colony formation assay

It was conducted to determine the effect of metformin and miR-506 on colony formation potential of MDA-MB-231 and MCF-7 breast cancer cells. 2000 cells/well were seeded into a 6-well plate. Cells were transfected with synthetic miR-506 miRNA mimic for 24 hours. Then, 5mM metformin was treated for 24 hours. Then, fresh medium was replaced with fresh medium. For approximately two weeks, when the control group reached 80% colony density, the cells were fixed with methanol: acetic acid (3:1 ratio) for 10 minutes at room temperature and observed by staining with 0.5% crystal violet. Colony numbers were counted by ImageJ image processing program.

2.6. Wound healing assay

Wound healing test, also referred to as cell migration test, helps to analyze the effect of metformin and miR-506 on migration capacity of breast cancer cells. The cells were transfected with miR-506 mimic after seeding them in a 6-well petri dish at $6x10^5$ cells/well. At the end of 24 hours, a wound was created by scratching the petri dish with a 10 µl sterile pipette tip. After washing with 1 X PBS, fresh media was added to control groups and 5 mM metformin was treated for drug-treated groups. The change in wound width was measured with the help of a light microscope for 24, 48 and 72 hours. At the ends of the 72 hours, cells were stained with DiOC6 and observed with fluorescence microscope.

2.7. Flow cytometry-PI staining

$6x10^5$ (cells/well) cells were seeed and miR-506 transfection protocol was proceeded. Then, metformin was applied and after 24 hours, the cells were Trypsinized and fixed with ice-cold 70% ethanol at +4 °C for 2 days. Following fixation,cells were stained with 50µg/mL PI dye and 100mg/mL RNase. Finally analyzed by cell flow cytometry (BD AccuriTM C6 Flow Cytometer).

2.8. Immunoblotting

Following transfection and the treatment procedures, cells were harvested using 1 X PBS. Total protein was obtained with the cell extraction buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, NP-40 0.5%, (v/v), 1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, protease inhibitor cocktail (Complete EDTA -free, Roche) and then centrifuged at 16000 g at 4 °C. Protein concentrations were determined by Bradford analysis. 30 µg total protein lysate were loaded and proceeded to 10-12% SDS-PAGE gel electrophoresis method. After being separated according to protein weight, the transfer process was carried out to transfer them to the PVDF membrane. AMPK, Bak, Puma, Bax, Caspase 9, Caspase 7 and PARP (CST; Danvers, MA) primer antibodies were incubated for 24 h in TBS-T containing %5 non-fat milk. Then, membranes were incubated with HRP-conjugated-anti-rabbit secondary antibody (CST; Danvers, MA) for 24 h at 4°C at 1:3000 ratio. β-actin was used as a loading control. After membranes were exposed to chemiluminescence reagent, results were detected by Chemidoc (Bio-Rad, California, USA).

2.9. Statistical analysis

All results obtained regarding the effect mechanism of metformin on cell viability and cell migration were examined with the Tukey's multiple comparisons test with Two-way ANOVA statistical method compatible with the number of data using the Graphpad program and presented as a graph/table.

3. Results and Discussion

3.1. Metformin treatment reduced cell viability in MCF-7 and MDA- MB-231 breast cancer cells

MTT analysis was performed in order to determine the effect of metformin on cell viability in MCF-7 and MDA-MB-231 breast cancer cells depending on the concentration (1.5, 2.5, 5, 10, 15, 20, 25 mM). The viability of each breast cancer cell line was decreased after metformin treatment for 24 hours depending on the increasing dose (Figure 1a-b). Treatment of 2.5 mM and 5 mM metformin to MCF-7 cells decreased cell viability by 20% and 40%, respectively. The same concentrations suppressed cell survival by 15% and 30%, respectively in MDA-MB-231 cells. Metformin exerted more potent cytotoxic effect against MCF-7 (IC_{50}, 10 mM) compared with MDA-MB-231 cells (IC_{50}, 25 mM).

The effect of metformin on cell death and MMP loss was detected by fluorescence staining (Figure 1c). Dead cells take PI into the cell with the deterioration of membrane

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permeability. It was observed that the number of PI stained cells were increased after 2.5 mM and 5 mM metformin treatment. DiOC6 is a fluorescent dye that visualizes living cells as green at certain wavelengths, depending on the MMP maintained in living cells.

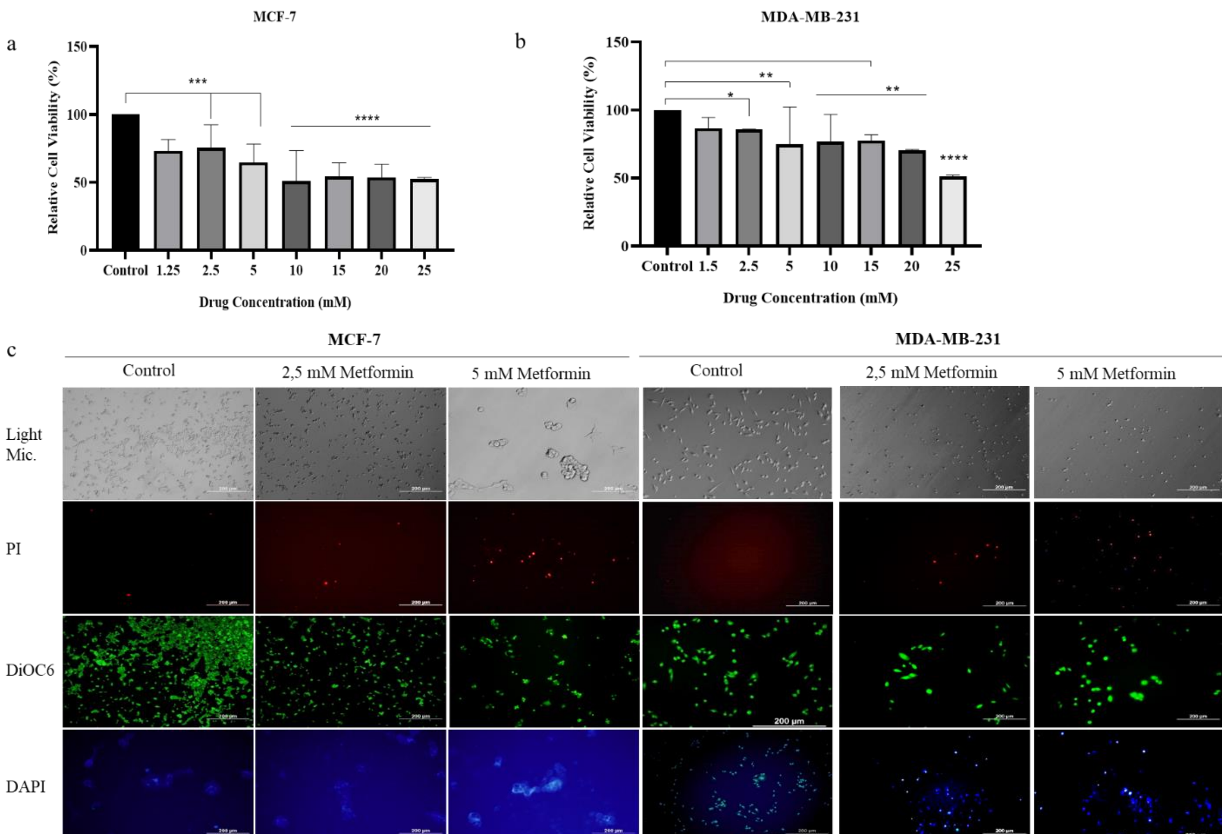

Figure 1. Metformin reduced cell viability in MCF-7 and MDA-MB-231 cells in a dose dependent manner. (a-b) MTT cell viability test was proceeded after metformin treatment in various concentration (1.5-25 mM) for 24 hours in MCF-7 and MDA-MB-231 cells.* $p<0.05$, ** $p<0.01$ *** $p<0.001$, **** $p<0.0001$ (c) Cells were treated by metformin (2.5 and 5 mM) for 24 hours and then the ratio of cell death was examined by PI staining, the MMP loss was observed by DiOC6 staining and the DNA breaks was analyzed by DAPI staining with fluorescence microscopy.

Metformin treatment to each breast cancer cells caused a noticeable decrease in viable cells. DNA breaks in cells caused by metformin were analyzed by DAPI staining. Metformin caused an increase in DNA breaks in each breast cancer cells compared to the control groups. Similar to our study, a decrease in cell survival was shown with the treatment of metformin (2.5 and 5mM) for 24-hours [14]. The indicated concentrations induced MMP loss in both breast cancer cells and caused a decrease in cell survival.

3.2. Examination of miR-506 level in MCF-7 and MDA-MB-231 breast cancer cells

The expression of miR-506, a tumor suppressor, in both cell lines was examined by real-time PCR following miRNA isolation. Normalization were accomplished according to the RNU6 expression, an internal control. miR-506 expression was observed to be higher in MCF-7 cells. A concentration of 5 mM metformin was chosen for subsequent experiments to determine the molecular mechanism triggered in relation to metformin and miR-506. Treatment of 5 mM metformin for 24 hours increased miR-506 expression by 2.4 in MCF-7 and 2 fold MDA-MB-231 cells (Figure 2a-b). Synthetic miR-506 was transfected into both cell lines and then expression of miR-506 was examined by qRT-PCR. After transfection, miR-506 expression increased 4.3-fold in MCF-7 cells and 6-fold in MDA-MB-231 cells. These transfection rates were continued to be used in subsequent experiments (Figure 2a-b). It has been shown that the effect of metformin on cancer cells is due to its inhibition of mTOR by activating AMPK [7]. A significant increase in AMPK level was observed in both cell lines treated with metformin. When miR-506 expression was increased in both cell lines, AMPK levels increased. However, a significant increase in AMPK level was observed in metformin-treated miR-506+ cells compared to the metformin-only group (Figure 2c). Studies have shown that activation of AMPK, one of the

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main targets of metformin treatment, triggers apoptosis by using the p53/p21 axis in the cell [17]. However, the exact molecular mechanisms of metformin are needed to be clarification in breast cancer. For this reason, in the current study, the effect of metformin on cell survival, migration and cell death was investigated through miR-506 expression.

3.3. High expression of miR-506 increased the effect of metformin on colony formation in MCF-7 and MDA-MB-231 breast cancer cells

After increasing miR-506 expression, the long-term effect of metformin treatment was investigated by colony formation assay (Figure 3a).

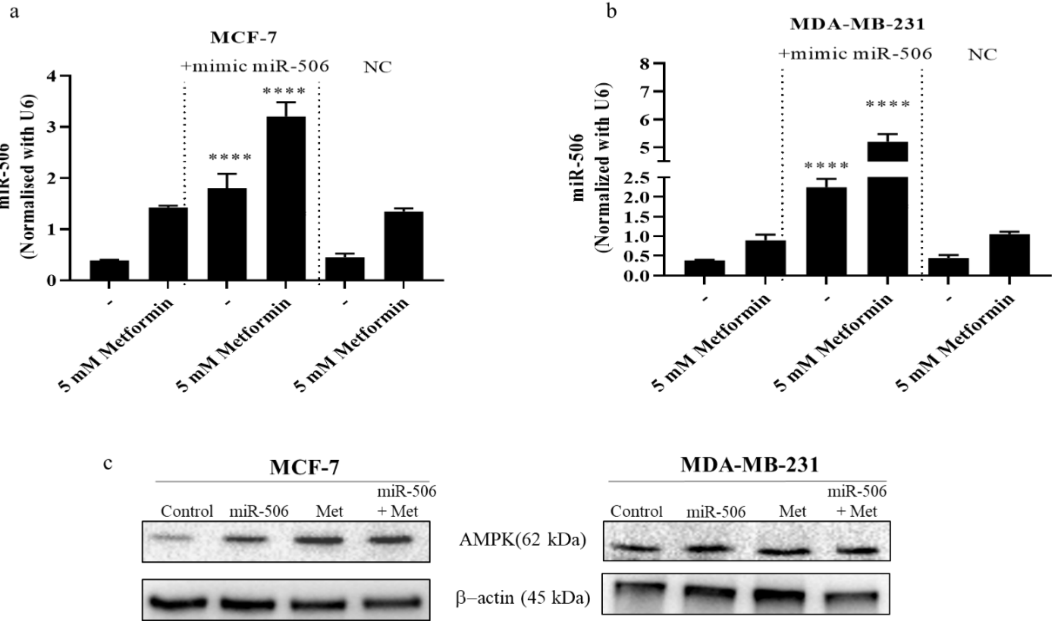

Figure 2. Metformin treatment increased the miR-506 levels in each cell line. (a) MCF-7 and (b) MDA-MB-231 cells were transfected with synthetic miR-506 and negative control (NC) miRNA for 48 hours then treated with 5 mM metformin for 24 hours. qRT-PCR was performed in non-transfected and transfected cells after metformin treatment. Results were normalized with RNU6 internal miRNA control. The results obtained are the average/st. deviation values of two replicate data of three different experiments **** $p<0.00001$. (c) After transfection of miR-506 and 5 mM metformin treatments total protein isolation was proceeded. AMPK levels were determined in each cell line by immunoblotting. β-actin was used as a loading control.

According to the results obtained, no significant difference was observed in the number of colonies of each cell with increased miR-506 expression compared to the untreated group. MCF-7 cells were observed to be more sensitive to metformin. When miR-506 expression was increased, both cell lines became more sensitive to metformin, and the number of colonies was significantly reduced compared to cells treated with metformin alone (Figure 3a). Similar to our study, overexpression of miR-506 has been reported as suppressor on the progression of various cancer types such as ovarian and cervical cancer [5]. However, Streicher and colleagues reported that high expression of miR-506 acts as an oncogene in melanomas [18]. Therefore, it can be concluded that the role of miR-506 might be vary in specific cell types. It has been uncovered that miR-506 suppressed cell proliferation and invasion of breast cancer cells through the ERK pathway by suppressing the expression of IQGAP1 [19]. However, the molecular mechanism of process of miR-506 on different breast cancer cells is still the focus of research.

Following metformin treatment to normal and miR-506+ breast cancer cells, the effect of cell migration was examined (Figure 3b-c). It was observed that increasing miR-506 expression, which had no effect on colony formation, suppressed cell migration in each cell. When metformin was treated to breast cancer cells with high miR-506 expression, it was observed that migration was significantly reduced in both cell lines. In addition, miR-506 led to an increase of the effect of metformin on cell migration compared to the metformin-only group. In the study on hepatocellular carcinoma cells (HCC), overexpression of miR-506 was shown to significantly inhibit the migration and invasiveness of HCC cells *in vitro* and *in vivo* [20]. In the same study, it was showed that the ectopic expression of miR-506 led to significant decrease in the expression of vimentin and an increase of E-cadherin level in HCC cells, suggesting that miR-506 inhibits HCC metastasis by suppressing EMT. In another study in MDA-MB-231 cells that examined the effects of miR-506 on invasion and migration, miR-506 played a suppressor role in the EMT mechanism by directly targeting CD151, VIM and SNAI2 [21]. In this context,

it has been shown in our study that the migration inhibitory effect of metformin is increased in miR-506 + breast cancer cells.

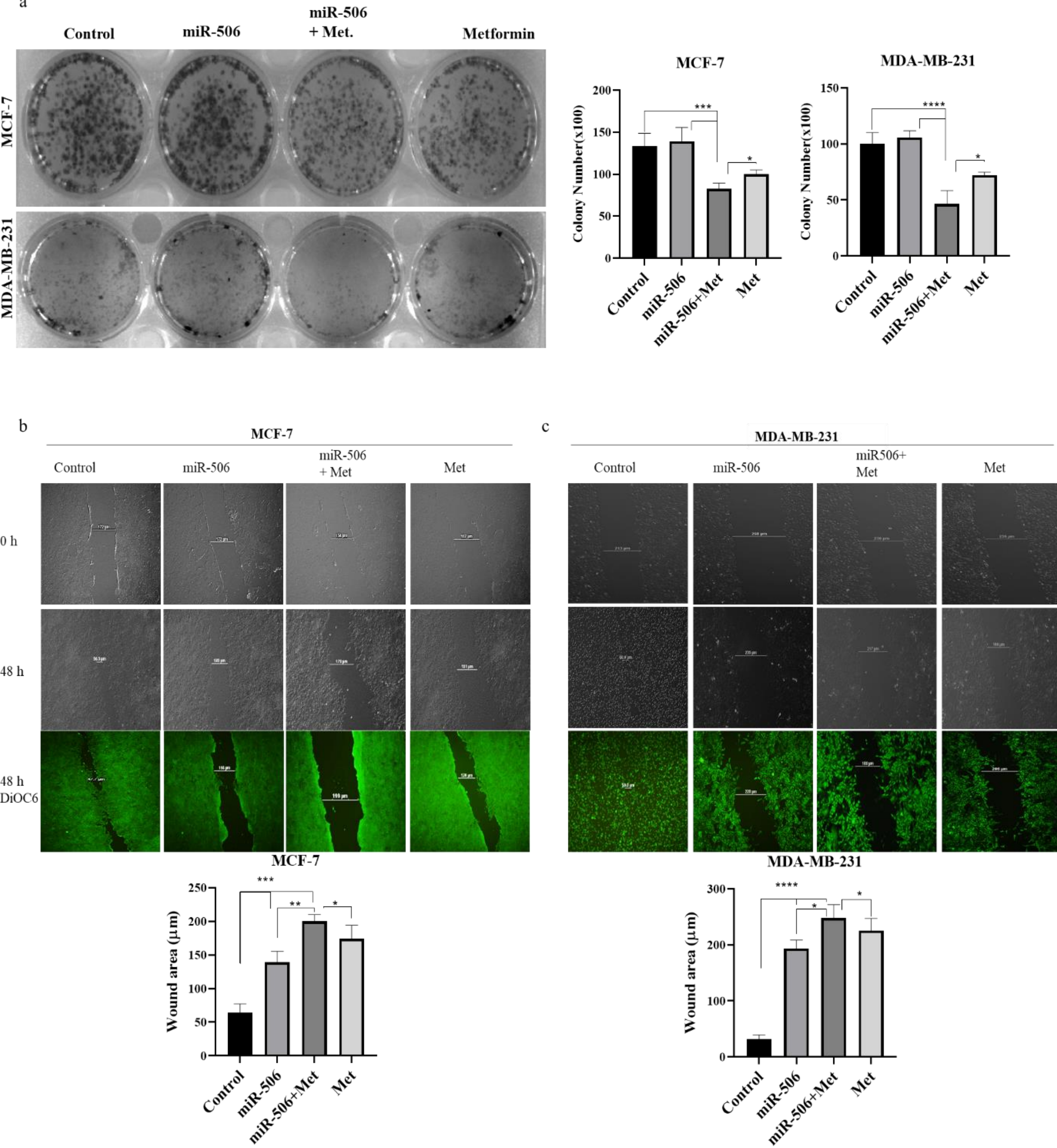

Figure 3. Metformin was more effective in the reduction of colony formation and cell migration capacity of the cell while miR-506 levels were increased in MCF-7 and MDA-MB-231 cells. (a) Colony formation test was performed in miR-506+ and normal breast cancer cells after 5 mM metformin treatment. (b-c) Cell migration assay was proceeded to observe the effect of increased level of miR-506 after metformin treatment in MCF-7 (b) and MDA-MB-231 (c) cells. Wound area was calculated and analyzed then presented as bar graphics for each cell line. * $p<0.05$, ** $p<0.02$, *** $p<0.001$, **** $p<0.0001$

3.4. Examining the effect of miR-506 and metformin treatment on cell cycle and apoptotic cell death

The effects of miR-506 and metformin treatments on cell cycle phases were examined by cell flow cytometry following PI staining (Figure 4a). Increasing miR-506 expression had different effects on cell cycle phases in both cell lines. When miR-506 expression was increased in MCF-7 cells, there was suppression in G1 phase.

Metformin alone treatment caused suppression of S phase in MCF-7 cells. When metformin was treated to MCF-7 miR-506+ cells, the arrest of S phase was observed compared to the control group and the metformin alone group. There were no similar results in MDA-MB-231 cells. Although the effect of miR-506 on protein expressions involved in the cell cycle is still a subject of research, studies have shown that miR-506-3p led to a significant decrease in E2F and cMyc levels, an increase in RB protein levels. Which is known that the potent proto-oncogene Myc is highly expressed in breast cancer [22]. There are studies showing that miR-506 suppresses breast cancer cell viability and migration capacity, but there are still many points of its molecular mechanism that need to be elucidated.

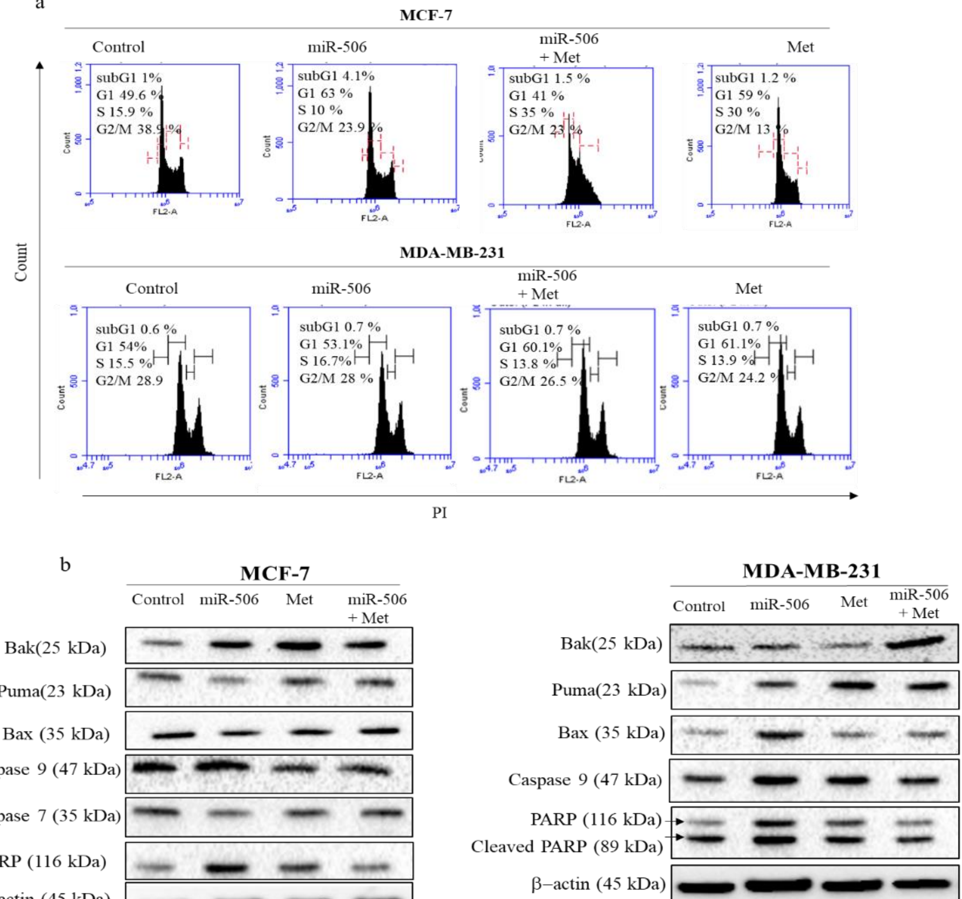

Figure 4. miR-506 and metformin combine treatment increased apoptotic cell death in MCF-7 and MDA-MB-231 cells. (a) The effect of miR-506 and metformin on cell cycle phases, the flow cytometry analysis was performed after PI staining. (b) The expression level of Bak, Puma, Bax, Caspase 9, Caspase 7 and PARP were determined by immunoblotting in MCF-7 and MDA-MB-231 cells. β-actin was used as a loading control.

To observe the effect of metformin on apoptotic cell death, total protein isolation was performed on normal cells and miR-506+ cells after 5 mM metformin treatment. Changes in the pro-apoptotic protein expressions (Bak, Bax, puma, caspase 9, PARP) were examined in each breast cancer cells by immunoblotting (Figure 4b).

When miR-506 expression was increased, an increase in Bak expression was observed in both cell lines. While metformin further increased Bak expression in MCF-7 cells, there was no similar result in MDA-MB-231 cells. However, metformin significantly increased Bak expression in miR-506+ MDA-MB-231 cells. Puma and Bax expression were higher in miR-506+ MDA-MB-231 cells compared to miR-506+ MCF-7 cells. Puma expression increased further in MDA-MB-231 cells when metformin was treated to these cells. However, Bax expression is higher in metformin-treated miR-506+ MCF-7 cells than in MDA-MB-231 cells. Studies was conducted that metformin increased the Bax expression in prostate cancer cells [23]. However, a study in breast cancer cells showed that metformin had no significant effect on Bax expression but, decreased the anti-apoptotic Bcl-2, c-Myc and Akt expression levels.

Therefore, metformin showed anti-tumorigenic effect on breast cancer cells [24].

Metformin treatment resulted in a higher reduction of the inactive caspase 9 form in miR-506+ cells. Metformin led to decrease of inactive caspase 7 expression in miR-506+ MCF-7 cells compared to the control group. The numerous DNA strand breaks that are likely to be present in the cell undergoing apoptosis lead to PARP activation which is a DNA repair protein. A decrease in full PARP (116 kDa), inactive PARP, expression was observed after metformin treatment in both cells. Moreover, increased miR-506 expression further increased the cleavage of PARP in both cell lines. Similar to the current study, miR-506 showed a pro-apoptotic effect by increasing cytochrome c, Bax and cleaved caspase 9 expression levels while by decreasing the anti-apoptotic Bcl-2 and Bcl-xL expression levels in lung cancer cells [25]. Moreover, overexpression of miR-506 has a significant potential to prevent pancreatic cancer progression and chemoresistance *via* Akt/NF-κB signaling [26]. Therefore, in the current study the tumorsuppressor role of miR-506 was used to increase the effect of anti-proliferative capacity of metformin in different breast cancer cells.

4. Conclusions

In conclusion, metformin treatment induced cell viability loss and induced MMP loss in MCF-7 and MDA-MB-231 cells. However, the MDA-MB-231 cells were more resistant to metformin treatment than MCF-7 cells. The expression level of miR-506 which has a tumor suppressor role, was lower in MDA-MB-231 cells. Therefore, elevated expression level of miR-506 increased the effect of metformin on the reduction of cell migration capacity and induction of cell death mechanism in each cell line. However, the signaling pathways through which metformin and miR-506 activate the cell death mechanism need to be further investigated. In our study, miR-506, which has the potential to be used as a combined therapy model in the future, has been shown for the first time to have a synergistic effect with metformin.

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Author's Contributions

Özge Rencüzoğulları: Conceptualization, Validation, Writing- original draft, Supervision, Writing- review and editing.
Suraya Qayoumi: Methodology, Data curation, Software, Visualization, Investigation.

Ethics

There are no ethical issues after the publication of this manuscript.

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