INDIAN JOURNAL OF SCIENCE AND TECHNOLOGY

RESEARCH ARTICLE

Knockdown of microRNA-375 suppresses cell proliferation and promotes apoptosis in human breast cancer cells

Rajesh Kannan Moorthy[1], Chandhru Srinivasan[1], Sridharan Jayamohan[1], Mahesh Kumar Kannan, Siva Sankari Thirugnanam[1], Janaki Sankar Ganesh[2], Antony Joseph Velanganni Arockiam[1]*

1 Molecular Oncology Lab, Department of Biochemistry, School of Life Sciences, Bharathidasan University, Tiruchirappalli-620 024, Tamilnadu, India
2 Department of Biomedical Sciences, School of Life Sciences, Bharathidasan University, Tiruchirappalli620 024, Tamilnadu, India

OPEN ACCESS

Received: 17.09.2021
Accepted: 25.11.2021
Published: 14.12.2021

Citation: Moorthy RK, Srinivasan C, Jayamohan S, Kannan MK, Thirugnanam SS, Ganesh JS, Arockiam AJV (2021) Knockdown of microRNA-375 suppresses cell proliferation and promotes apoptosis in human breast cancer cells. Indian Journal of Science and Technology 14(43): 3199-3209. https://doi.org/10.17485/IJST/v14i43.1719

* **Corresponding author**.

ajvelanganni@gmail.com

Funding: University Grant Commission (UGC), New Delhi (F.No.41-1274/2012/(SR) dt:26.7.2012). DST for providing funds for the improvement of the infrastructure facility (FIST) to the Department of Biochemistry, Bharathidasan University

Competing Interests: None

Published By Indian Society for Education and Environment (iSee)

ISSN
Print: 0974-6846
Electronic: 0974-5645

Abstract

Background: The purpose of the present investigation is to unravel the influence of the miR-375 inhibitor on cell survival descent and apoptosis stimulation, mediating PI3K/Akt/mTOR signaling network in human breast cancer cells. **Methods**: MCF-7 cells were transfected with miR-375 inhibitor for 72 h. Then, cell survival assay was measured by MTT. Cells were stained with EtBr/AO, DAPI, and DCFH-DA to assess the effect of the miR-375 inhibitor on cell death. A scratch experiment was performed to observe the cell migration ability. The expression of anti-apoptotic and apoptotic genes such as BCL-2, BAX, and PI3K/Akt/mTOR and miR-375 were evaluated in miR-375 inhibitor transfected cells by qRT-PCR. **Findings**: MiR-375 inhibitor sensitized tumor cells and influenced significant loss in the breast cancer cell proliferation with obvious cell death elevation. MiR-375 inhibitor effectively augmented ROS generation. Also, miR-375 inhibition hampered migratory ability. Furthermore, our qRT-PCR analysis showed that inhibition of the miR-375 was able to significantly reduce the constitutive expression of PI3K/Akt/mTOR mRNAs. Additionally, miR-375 suppression decreased the anti-apoptotic gene, Bcl-2 expression and enhanced pro-apoptotic gene, Bax expression along with potentially decreasing miR-375 level compared to control. **Novelty and applications**: Inhibition of the miR-375 has considerably attenuated cell proliferation and stimulated apoptotic cell death in the breast cancer cells. Thus, miR-375 represent a potential therapeutic target for the breast cancer.

Keywords: Breast Cancer; Proliferation; Apoptosis; Migration; miR-375

1 Introduction

Cancer is one of the leading causes of death worldwide, accounting for 1,92,92,789 (19.3 million) new cases and 99,58,133 (9.9 million) deaths in 2020 alone. Breast cancer is the most common cancer that prevalent all over the world including India. Breast cancer cases and fatalities were significantly increased in 2020, with an estimated 22,61,419 (11.7%) and 6,84,996 (6.9%) globally[1]. Breast Cancer is currently the most common malignancy among women in India with 13,92,179 cases in 2020, creating a serious public health issue[2]. The inherent ability of the cancer cells is to multiply indefinitely and overcome apoptosis, so that it invades into normal tissues and develop the disease[3]. Cancer cell migration is the result of a variety of biological processes with distinct features. Metastasis is the final stage of cancer progression, in which cancer cells separate from the initial tumour, move into blood vessels, diffuse throughout the body, and eventually seed in distant organs, results in the formation of a new tumour. The biochemical mechanism underlying aberrant proliferation and death of the breast cancer cell has yet to be fully understood. As a result, getting a detailed knowledge of the mechanisms that underpin the breast cancer cell proliferation and apoptosis pave the way to enhance prognosis, diagnosis, and prevention.

MicroRNAs (miRNAs) are small, non-coding endogenous RNAs (18–21 nt) that alter target gene expression post-transcriptionally by mismatch binding to the 3′-untranslated region (3′-UTR) of protein-coding mRNA transcripts, results in mRNA cleavage via 3' uridylation and argonaute mediation[4,5] or translational repression through effector proteins (GYF-1 & IFE-4)[6]. According to the evidence, microRNAs govern major biological functions such as lipid and glucose metabolism[7], apoptosis, angiogenesis, migration, invasion and cell proliferation[8,9], and oncogenesis[10–14] by directly influencing signaling pathway. Based on the cellular context and tissue specificity, miRNAs act as oncogenic promotors and/or tumor suppressors[8,9]. Diverse regulation of miRNA disrupts the molecular mechanism of normal biological characteristics; so that miRNAs evade apoptosis and promote angiogenesis. Ultimately, all these dysregulated functions contribute to carcinogenesis.

In recent times, miR-375 has been proved as a tumor-induced regulator in different cancers by activating important regulatory networks. For example, Xin et al demonstrated that overexpression of the miR-375 mediates phosphorylation and methylation of STAT3; also inhibits FOXO1 and p53 axis thereby stimulating the breast cancer progression[15]. Overexpression of circ-0072088 or miR-375 inhibitors promoted hepatocellular carcinoma proliferation, migration, invasion and reduced the cell death by activating JAK2/STAT3 network whereas circ-0072088 downregulation or miR-375 mimics explored opposite function[16] and also miR-375 activated JAK2/STAT3 signaling pathway to inhibit stemness of the breast cancer[17]. Moreover, in cervical cancer cells, miR-375 ectopic expression significantly suppressed cancer proliferation process through altering astrocyte elevated gene-1 (AEG-1)[8]. Alam et al found that MiR-375 regulates cell growth, proliferation, cell migration, and apoptosis by modulating the CTGF-EGFR-PIK3CA-AKT signaling axis in human colorectal cancer cells[18]. Furthermore, several investigations were demonstrated that constitutive expression of miR-375 promotes oncogenicity in the breast[19–23], prostate[24–27], and lung cancer[28]. In addition, few pieces of literature reported that miR-375 functions as a tumor suppressor[16,29–31]. MiR-375 suppresses cervical cancer development by regulating epithelial-mesenchymal transition by targeting Yes-associated protein 1[31]. The exact role and function of the miR-375 in the breast cancer development remains uncertain. Thus, finding new therapeutic approaches is necessary for treating cancer. So, describing biological consequences of aberrant miRNA expression and identifying miRNA targets are necessary for a thorough understanding of pathways underlying miRNA molecular mechanisms.

Hence, we aimed to figure out the functional correlation between miR-375 inhibition and apoptosis via the PI3K/Akt/mTOR pathway in the breast cancer cell line. Finally, our study shows that inhibition of the miR-375 suppressed MCF-7 breast cancer cell proliferation and induced apoptosis. Moreover, this study highlights miR-375 could be a potential new therapeutic target for the breast cancer.

2 Material and methods

2.1 Cell Line and Reagents

The Human MCF-7 breast cancer cell line was procured from National Center for Cell Science (NCCS), Pune, India. The cell line was immediately sub-cultured using Dulbecco's modified Eagle's medium (DMEM) containing high glucose with the supplementation of 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin antibiotics. Cells were incubated at 37°C under 5% CO_2 for 48 h.

2.2 Transfection of the miR-375 inhibitor

Breast cancer cells seeded in a 6-well culture plate (1×10^6). After 24 h cells were transiently transfectedwith miR-375 inhibitor (Sigma Aldrich, USA), after 48 h incubation, the medium was replaced with new medium devoid of antibiotics. The final concentration of 100nM miR-375 inhibitor was transfected using lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

2.3 MTT assay for cell survival

Breast cancer cells were seeded at 1×10^4 cells/well in 96 well plates. After overnight incubation, cells were transiently transfected and then incubated with miR-375 inhibitor for 72 h. After 72 h, the medium was removed and 200μl of fresh medium and 20μl freshly prepared MTT (5 mg/ml Phosphate Buffer Saline (PBS)) was added to each well and the plates were incubated in a dark at 37°C for 4 h. Then, the culture medium was removed and 200μl of Dimethyl Sulphoxide (DMSO) was added to each well to dissolve the crystal formation and the absorbance was read at 595 nm using a microplate reader (iMark Microplate Absorbance Reader, California, USA).[8,9]

2.4 Ethidium Bromide /Acridine Orange (EtBr/AO dual staining)

Cells were grown in 6 well plates at the density of 1×10^5 and incubated with miR-375 inhibitor for 72 h. The medium was removed and cells were washed with PBS and stained with 10μl of EtBr/AO (1 mg/ml) for 5 min. Morphological changes were visualized under a fluorescence microscope (a Floid Cell Imaging Staion, Life Technologies Corp, Carlsbad, CA, USA.)[8,9].

2.5 4',6-diamidino-2-phenylindol (DAPI Staining)

Cell's nuclear morphology was analyzed by DAPI staining. Cells were cultured at 1×10^5 cells/well in 6 well plates and incubated with miR-375 inhibitor for 72 h. The medium was removed and cells were washed with PBS and stained with 10μl of DAPI (100 μg/ml) and incubated for 30 min at 37°C. Finally, the stain-fixed cells were observed under a fluorescence microscope (a Floid Cell Imaging Station, 20x)[8,9].

2.6 Measurement of Reactive Oxygen Species (ROS) levels

Cells seeded in 6 well plates at the density of 1×10^5 cells/well. Then, the MCF-7 cells were transfected with miR-375 inhibitor and incubated for 72 h. After incubation, cells were washed with PBS once and incubated with 10 μM Dichlorodihydrofluorescein Diacetate (DCFH-DA) dye in a complete medium for 30 min at 37°C to assess the ROS production. The staining intensity was monitored under a fluorescence microscope (a Floid Cell Imaging Station, 20x)[8,9].

2.7 Wound healing assay

Breast Cancer cells were seeded at 1x104 density in 6 well plates. After the confluency, cells were treated with or without miR-375 inhibitor for 72 h. A wound was created by using a micropipette (0.2-2 μl) tip in the middle area of confluent cells. Cell migration was evaluated with time and images were taken Floid Cell Imaging Station at 20x magnifications in a phase contrast mode[8,9].

2.8 Quantitative Real-Time PCR analysis

Breast cancer cells were incubated with miR-375 inhibitor for 72 h. After incubation, total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). 2 μg of RNA was used to synthesize cDNA from each sample with a cDNA synthesis kit (Takara Biosystems, India). Real-Time PCR was carried out using KAPA SYBR FAST qPCR Master mix kit (Kapa Biosystems, Cape Town, South Africa) in 10μl by Step one plus RT-PCR (Applied Biosystems, USA): 40 cycles at 95°C for 20 s, 60°C for 30 s and 95°C for 15 s[8,9]. Fold changes were calculated by 2^ (-ΔΔCT) for gene expression. The β-actin and U6 were used for RNA template normalization.

2.9 Statistical analysis

Statistical significance was analyzed for three independent experiments (n = 3) by one-way ANOVA using GraphPad Prism software Version 6.0 (Graphpad software, Inc., La Jolla, CA, USA). Statistical significance was given as *P<0.05.

3 Results

3.1 MiR-375 inhibition affects MCF-7 breast cancer cell proliferation

MiR-375 inhibitor was transfected into MCF-7 cells and the cell proliferation rate was determined by MTT assay. As depicted in Fig. 1, miR-375 inhibitor remarkably suppressed MCF-7 cell proliferation after increasing its concentration at 72 h of exposure time, and the inhibition concentration was 100 nM/ml. In addition, cell shrinkage, apoptotic bodies, and cell breakage were observed (Fig. 2). This result proved that miR-375 inhibition affects the breast cancer survival ability.

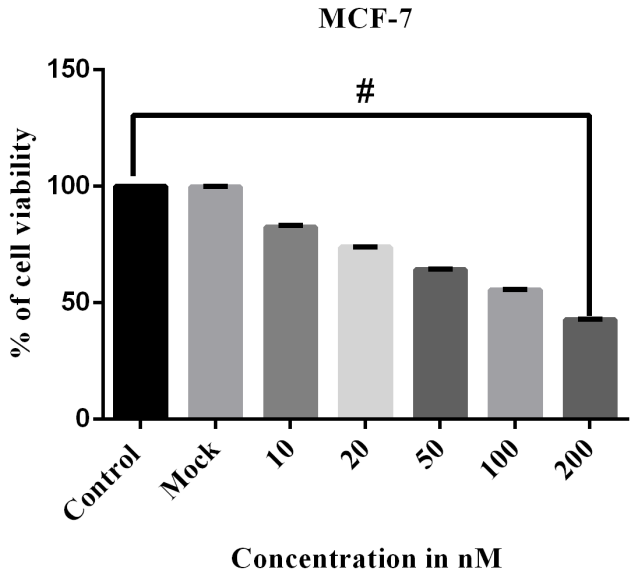

Fig 1. Cell viable effect of the miR-375 inhibitor in human breast cancer cells by MTT assay. Cells were treated with different concentrations (0,10,20,50, 100, 200 nM/ml) of the miR-375 inhibitor for 72 h and cell viability was assessed and the IC_{50} was calculated by Graphpad Prism 6 software (Graphpad software, Inc., La Jolla, CA, USA). The mean percentage and standard deviation estimates of living cells were plotted against miR-375 inhibitor concentration. The data is presented as the mean and standard error of the mean of 3 independent experiments. Statistical significance was measured by one-way ANOVA as compared to control and treated. *$p < 0.05$

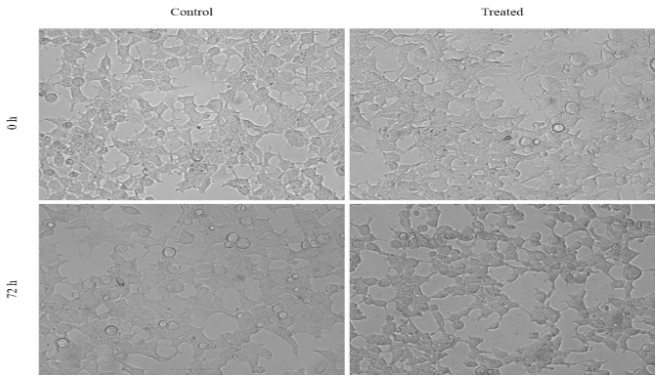

Fig 2. Analysis of morphological changes in miR-375 inhibitor-treated breast cancer cells after 72 h. Cells were exposed to IC_{50} concentration of the miR-375 inhibitor and morphological changes were observed in phase contrast mode following 72 h of treatment. Images were captured using Floid cell imaging station microscope (magnification, 20x). Control cells showed no alteration structurally and miR-375 inhibitor-treated cells showed cell shrinkages and round morphologically

3.2 MiR-375 inhibition causes cell death in MCF-7 breast cancer cell line

After 72 h transfection of the miR-375 inhibitor, cells stained with EtBr/AO stain. Then, apoptotic nuclear morphology was observed under fluorescence microscopy in MCF-7 cells. As illustrated in Fig. 3. Under EtBr/AO staining, live cells in the control group showed uniform bright green color with intact nuclei. However, the nuclei were dense in the miR-375 inhibitor transfected MCF-7 cells, and also apoptotic bodies were observed in several dead cells (bright red color). Also, bright blue color fluorescence from DAPI staining representing fragmented apoptotic nuclei and DNA damage were observed in miR-375 inhibitor-treated cells (Fig. 4). These experimental results revealed that survival of cancer cells was suppressed by triggering apoptotic cell death under miR-375 inhibition in the breast cancer cell line.

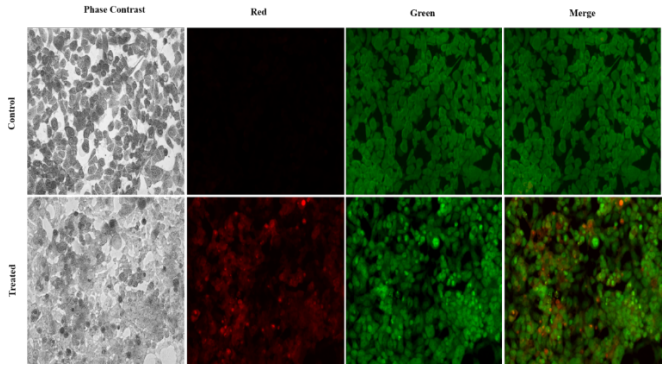

Fig 3. Morphological observation following EtBr/AO dual staining (magnification, 20x). MCF-7 breast cancer cells were treated with or without miR-375 inhibitor at IC_{50} concentration for 72 h. Treated cells showed more cell death compared to control

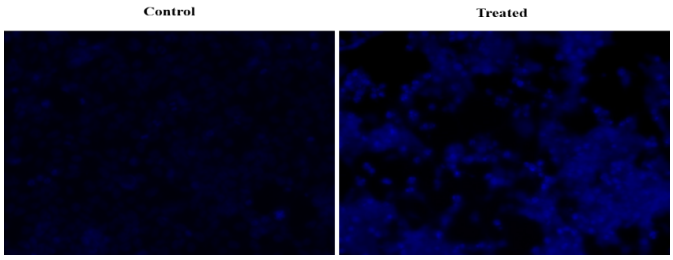

Fig 4. Identification of chromatin condensation and nuclear fragmentation in breast cancer cells. Cells were treated with 100nM miR-375 inhibitor for 72 h. After treatment, cells were stained with DAPI and morphological changes of breast cancer cells were visualized under fluorescence microscopy. The control cells showed healthy nuclei and miR-375 inhibitor-treated cells showed nuclear fragmentation and chromatin condensation

3.3 Inhibition of the miR-375 enhances intracellular ROS production in the breast cancer cells

Abnormal concentration of free radicles, specifically ROS generated by oxidative stress, has been associated with tumour growth and can behave as a signaling molecule to mediate apoptosis. Recent studies have shown that ROS regulates miRNA biogenesis and controls miRNA expression, thereby facilitating cancer cell growth. On the contrary, miRNAs have been demonstrated to regulate the expression of proteins involved in ROS homeostasis [(32)]. Dysregulation of the miR-375 in turn promotes cancer via aberrant ROS regulation [(33)]. So, we further evaluated intracellular ROS condition by DCFH-DA staining. MCF-7 cells were transfected with miR-375 inhibitor. Then green color fluorescence DCHF-DA stain was pre-stained into miR-375 inhibitor transfected MCF-7 cells, and the fluorescence intensity was analyzed in a Floid Cell Imaging Station (Life Technologies Corp, Carlsbad, CA, USA). As depicted in Fig.5, knockdown of the miR-375 markedly increased intracellular ROS generation. So, these findings indicates that the miR-375 inhibition induced cell death via ROS generation in the breast cancer cells.

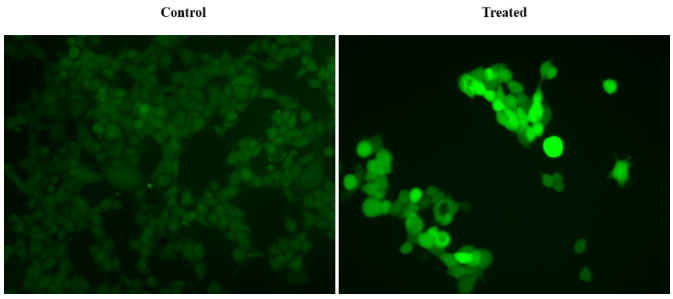

Fig 5. MiR-375 inhibitor increased the ROS production in breast cancer cell line. Breast cancer cells were treated with IC_{50} concentration of the miR-375 inhibitor for 72 h. The medium was discarded and stained with DCFH-DA. Intracellular ROS production was examined and visualized under Floid Cell Imaging Station fluorescence microscope (magnification, 20x). MiR-375 inhibitor-treated cells showed an increased ROS generation levels when compared to control

3.4 Inhibition of the miR-375 suppressed the migratory ability of MCF-7 breast cancer cell line

To examine the potential metastatic function of the miR-375 in MCF-7 breast cancer cell line, we performed scratch wound closure assay. Control cells showed maximum wound closure at 72 h and in miR-375 inhibitor transfected MCF-7 cells, cell migration evidently reduced and closure was minimal (Fig. 6). This result suggests that the miR-375 inhibitor greatly inhibited MCF-7 cell migration.

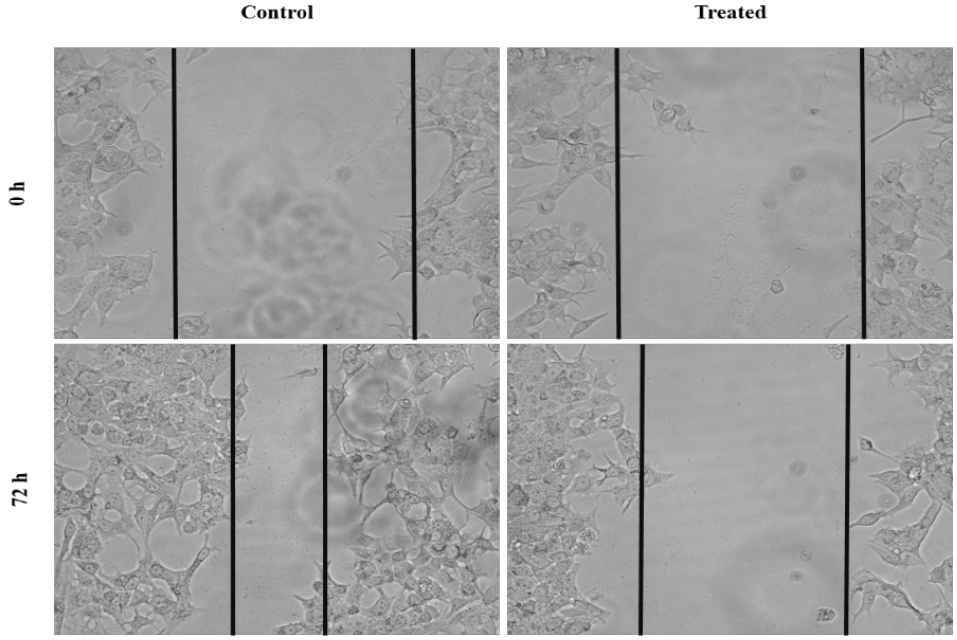

Fig 6. The effect of the miR-375 inhibitor on the migration of MCF-7 by wound healing assay. Breast cancer cells treated with or without 100nM of the miR-375 inhibitor. Scratch was created in the adherent cells and images were taken in a Floid cell imaging station microscope (magnification, 20x) after 72 h of the miR-375 treatment respectively. MiR-375 inhibitor suppressed the migration capability of MCF-7 human breast cancer cells.

3.5 Inhibition of the miR-375 promotes apoptosis via regulating PI3K/Akt/mTOR in the breast cancer cell line

To examine the molecular mechanism involved in the knockdown of the miR-375 inhibition on apoptosis, we mainly focused on the expression of the PI3K/ Akt mTOR signaling pathway that participates in the intracellular signaling required for cell survival. As shown in Fig. 7, the expression of the miR-375 remarkably decreased in miR-375 inhibitor transfected cells. The level of PI3K/Akt pathway components PI3K/Akt and mTOR, were dramatically down-regulated in the presence of the miR-375 inhibitor cells. Additionally, anti-apoptotic mRNA, Bcl-2 drastically decreased and pro-apoptotic mRNA, Bax significantly increased compared to control cells. Interestingly, the results indicated that the inhibition of the miR-375 in the breast cancer MCF-7 cells triggered apoptosis by inhibiting the PI3K/Akt/mTOR signaling network in the breast cancer cells.

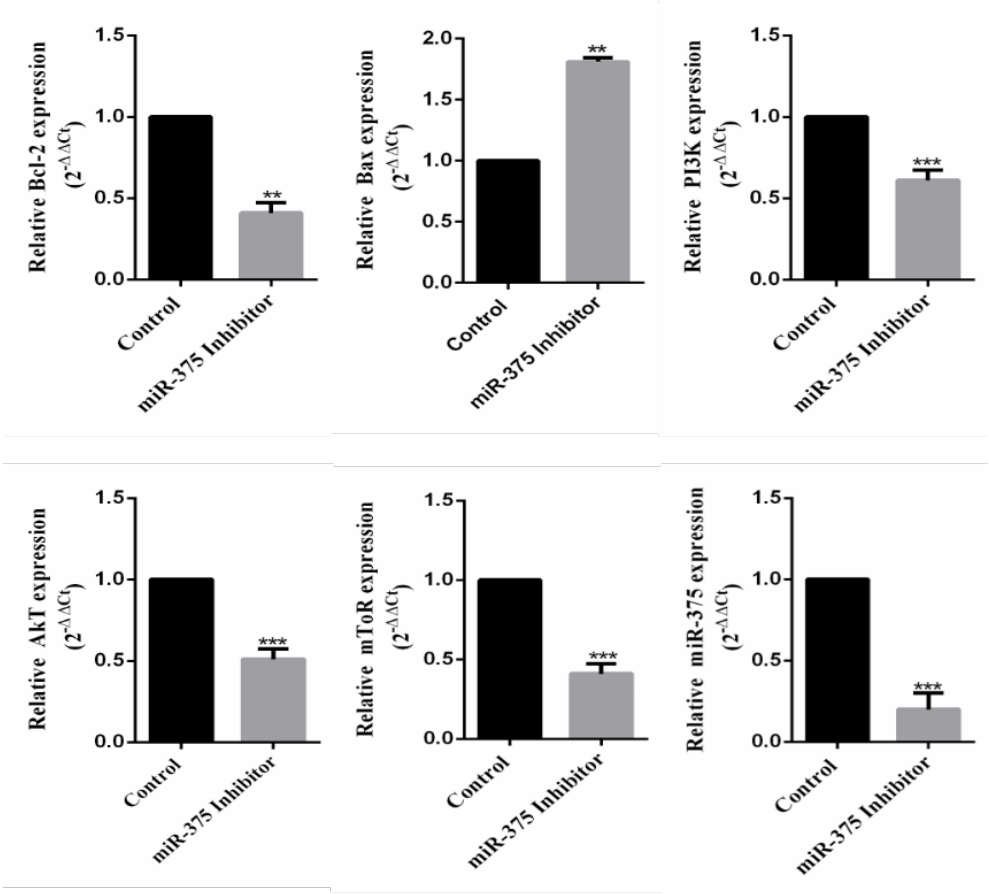

Fig 7. The apoptotic gene mRNA expression in breast cancer cell lines by qRT-PCR. MCF-7 cell lysate treated with miR-375 inhibitor for 72 h. RNA was isolated from breast cancer cell line. Real-Time PCR was performed. MiR-375 regulates Bcl-2, Bax, PI3K, Akt, mToR mRNA, miR-375 and β-actin, GAPDH and U6 were served as an internal control to normalize the gene expression. Each experiment was repeated for a minimum of 3 times and plotted as bar graphs with error bars. One-way ANOVA was conducted and the p-value was calculated between control and miR-375 inhibitor-treated groups, where, *p-value < 0.05

4 Discussion

MiRNAs are crucial negative regulators of gene expression in a wide range of malignancies and hold promise for enhancing the novel cancer therapeutic approaches through RNAi Interference. According to the findings, single miRNAs can stimulate or repress particular biological function by controlling their target mRNAs. Abnormalities in miRNA expression have been correlated with various diseases, including cancer. Increasing shreds of evidence proposed that miRNAs can act as an oncogenes or tumour suppressor genes in cancer conditions [34–36]. Recent findings were demonstrated the pathological significance of the miR-375 in a variety of human cancers. In breast cancer, miR-375 was found to accelerate tumour growth, so that, miR-375

was reported as an oncogene [20,23,37]. Overexpression of miRNAs has also been demonstrated to promote carcinogenesis by stimulating cancer-related pathways such as the PI3K/Akt axis [35]. The regulatory function of miRNAs in the activation of the PI3K signaling pathway results in the breast cancer regression, suggests the therapeutic potency of miRNAs for the breast cancer therapy. Hence, in this study, we investigated whether miR-375 inhibitor affects cell proliferation suppression and promotes apoptosis through PI3K/Akt/mTOR signaling pathway in the breast cancer cells.

Previously Simonini et al [20] found that blockage of the miR-375 reduced cell proliferation. In contrast, miR-375 inhibition increases cervical cancer cell growth. [8] So, we sought to monitor the potential anti-proliferative activity of synthetic anti-miR-375 inhibitors in MCF-7 breast cancer cells. Therefore, we blocked oncomiR-375 with a miR-375 inhibitor in the breast cancer cells. Compared to control cells, miR-375 inhibitor remarkably decreased the cell proliferation rate. Cell structure rapture, swelling, shrinkage, plasma membrane blebbing, chromosomal DNA fragmentation, nuclear condensation, and apoptotic body formation are some known cancer morphological hallmarks [38]. In this study, we found the cell shrinkage, cell damage, and chromosomal DNA fragmentation events in anti-miR-375 transfected MCF-7 cells compared to control.

Incomplete oxygen species are generally termed ROS. Cells produce ROS such as peroxides, singlet oxygen, and superoxides under a hypoxic environment. Increased level intracellular ROS generation has been found in most cancer types. They play a vital role in tumourigenesis. Oxidative stress accelerate apoptosis during the greater accumulation of ROS. Besides, over synthesis of ROS beyond the normal condition can causes oxidative stress and finally lead to cell death [39]. Moreover, ROS production is critical in inducing apoptosis by anticancer medicines. So, increased ROS generation can result in $\Delta\Psi m$ loss, which leads to apoptosis. This finding has been confirmed in a study that a high quantity of reactive oxygen species (ROS) production triggers apoptosis in cervical cancer cells [40]. Furthermore, there is some crosstalk between miRNAs and ROS in cancer development. Few research studies have exerted that ROS participated in the regulation of miRNA expression. Reversibly, some miRNAs regulate oxidative stress-inducing processes such as redox sensor expression and anti-oxidant factors alteration [41]. ROS controls ERBB2/3 via miR-125b and miR-199a expression through elevating promotor methylation by DNA methyltransferase 1 (DNMT1) gene reduction in ovarian cancer [42]. Overexpression of miR-142 through acetyltransferase Ep300 attenuation accumulates ROS thus inhibiting pexophagy [43]. Moreover, dysregulation of the miR-375 promotes cancer cell death via aberrant reactive oxygen species regulation through targeting HIGD1 in porcine Sertoli cells [33]. So, we wanted to check whether miR-375 inhibitor generates ROS in MCF-7 breast cancer cells. Compared to control cells, antagonizing miR-375 stimulating ROS generation in miR-375 inhibitor transfected breast cancer cells. These findings revealed that miR-375 inhibitor reduced MCF-7 breast cancer cell line growth through ROS regulation.

Wound healing process is a multifaceted function comprising various stages such as hemostasis, proliferation, remodeling, and inflammation [44]. Generally, the cancer cell break away from the site where they initially formed and grow newly in other site of the body where it attached. Also, metastasis causes more cancer cell death because of tumour cell dissemination from the primary tumour during early phase cancer growth and the cancer cells adapt for evading immune surveillance and developing metastatic stage to transform as a secondary tumour. So this transition signal can trigger a migration process that is important for wound healing [45]. Increasing evidence suggests that miRNAs also contributed with the development of cancer [44]. MiR-375 ectopic expression significantly inhibited invasive and migratory function in gastric cancer through targeting on JAK2 [45]. Similarly, miR-375 expression suppressed cervical cancer cell migration through AEG-1 [8]. Inhibition of miR-941 predominantly prevents the cell multiplication and migration of the breast cancer cells by altering cell cycle protein expression [46]. In our study, we found that miR-375 inhibition notably reduced wound closure movement. Thus, our data suggest that miR-375 inhibition prevents the migratory ability in aggressive metastatic breast cancer cells.

Apoptosis is a kind of programmed cell death and evasion of apoptosis favors cancer cells to grow uncontrollably due to the impairment of tumour suppressor genes and elevation of anti-apoptotic genes. Interestingly, avoiding apoptosis is a critical process for transformed cancerous cells [47]. Control of cancer-related oncogenic miRNA expressions through synthetic miR-375 inhibitor is anticipated to play a role in developing new therapeutic intervention in cancer treatment [48]. Generally, cells commit to die due to their own pro-anti apoptotic proteins in apoptosis there by, it is generally referred to as a cell suicidal process and the balance between oncoproteins and tumour suppressor proteins are more responsible to determine when the cells die. So, this apoptotic mechanism is accomplished by both intrinsic (mitochondrial) and extrinsic (death receptor) pathway signaling protein molecules activation [49,50].

The PI3K/Akt/mTOR signaling cascade is the overexpressed intracellular signaling axis mediating cell proliferation, growth, and survival. So, it is well known that uncontrolled regulation of the PI3K pathway is central in human cancer advancement including breast cancer [51]. Generally, microRNAs activates PI3K/Akt pathway to regulate malignant properties and represent as a novel biomarker for CRC patients [35]. Several studies have found that abnormal expression of PI3K/Akt signaling pathway activated by miRNA leads to uncontrolled cell proliferation in the breast carcinogenesis [51–54]. In addition, Mahesh et al. found that inhibition of miR-221 causes apoptosis through targeting PTEN/PI3K/Akt [9]. Also, our previous report indicated that miR-

375 inhibition reverses the regulatory mechanism to cause apoptosis evasion [8]. Increasingly, miR-375 stimulated ferroptosis in gastric cancer [55]. In our study, miR-375 inhibitor elicits apoptosis via modulating PI3K/Akt/ mTOR pathway. Also, apoptosis is always implicated with the synergistic activation of pro and anti-apoptotic proteins. It is well-known that Bcl-2 is the cancer cell growth enhancer and Bax is the apoptotic promotor [56]. So, we found that increased Bax expression and decreased Bcl-2 expression in miR-375 inhibitor transfected MCF-7 cells MCF-7 cells.

Ultimately, miR-375 inhibitor promotes apoptosis in cancer cells nonetheless the mechanism by which miR-375 triggers cell death in the breast cancer cells is still not well understood. Hence, the efficacy of the miR-375 inhibitor on apoptosis via modulation of PI3K/Akt/mTOR in the breast cancer was demonstrated in this study.

5 Conclusion

Finally, we have demonstrated that miR-375 inhibitor significantly inhibits MCF-7 breast cancer cell proliferation and triggered intracellular ROS generation, hindered cell migration and induced apoptotic cell death. The miR-375 inhibitor also caused dramatic mRNA changes and eventually triggered apoptosis via activating PI3K/Akt/mTOR network. To the best of our knowledge, for the first time, our findings endorsed that miR-375 inhibitor suppresses cancer cell proliferation and promoted apoptosis by modulating the expression of PI3K/Akt/mTOR signaling. Besides, the association between miR-375 and PI3K/Akt/mTOR requires further investigations.

6 Acknowledgement

We sincerely thank the University Grant Commission (UGC), New Delhi, India for providing financial assistance for this project (F.No.41-1274/2012/(SR) dt:26.7.2012) and DST for providing funds for the improvement of the infrastructure facility (FIST) to the Department of Biochemistry, Bharathidasan University, Tiruchirappalli, Tamilnadu, India.

References

1) Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians*. 2021;71(3):209–249. Available from: https://dx.doi.org/10.3322/caac.21660.
2) Mathur P, Sathishkumar K, Chaturvedi M, Das P, Sudarshan KL, Santhappan S, et al. Cancer Statistics, 2020: Report From National Cancer Registry Programme, India. *JCO Global Oncology*. 2020;6(6):1063–1075. Available from: https://dx.doi.org/10.1200/go.20.00122.
3) CHEN CP, SUN ZL, LU X, WU WX, GUO WL, LU JJ, et al. miR-340 suppresses cell migration and invasion by targeting MYO10 in breast cancer. *Oncology Reports*. 2016;35(2):709–716. Available from: https://dx.doi.org/10.3892/or.2015.4411.
4) Xu K, Lin J, Zandi R, Roth JA, Ji L. MicroRNA-mediated target mRNA cleavage and 3′-uridylation in human cells. *Scientific Reports*. 2016;6(1):1–4. Available from: https://dx.doi.org/10.1038/srep30242.
5) Nowak I, Sarshad AA. Argonaute Proteins Take Center Stage in Cancers. *Cancers*. 2021;13(4):788–788. Available from: https://dx.doi.org/10.3390/cancers13040788.
6) Mayya VK, Flamand MN, Lambert AM, Jafarnejad SM, Wohlschlegel JA, Sonenberg N, et al. microRNA-mediated translation repression through GYF-1 and IFE-4 in C. elegans development. *Nucleic Acids Research*. 2021;49(9):4803–4815. Available from: https://dx.doi.org/10.1093/nar/gkab162.
7) Agbu P, Carthew RW. MicroRNA-mediated regulation of glucose and lipid metabolism. *Nature Reviews Molecular Cell Biology*. 2021;22(6):425–438. Available from: https://dx.doi.org/10.1038/s41580-021-00354-w.
8) Jayamohan S, Kannan M, Moorthy RK, Rajasekaran N, Jung HS, Shin YK, et al. Dysregulation of miR-375/AEG-1 Axis by Human Papillomavirus 16/18-E6/E7 Promotes Cellular Proliferation, Migration, and Invasion in Cervical Cancer. *Frontiers in Oncology*. 2019;9:847–847. Available from: https://dx.doi.org/10.3389/fonc.2019.00847.
9) Kannan M, Jayamohan S, Moorthy RK, Chabattula SC, Ganeshan M, Arockiam AJV. AEG-1/miR-221 Axis Cooperatively Regulates the Progression of Hepatocellular Carcinoma by Targeting PTEN/PI3K/AKT Signaling Pathway. *International Journal of Molecular Sciences*. 2019;20(22):5526–5526. Available from: https://dx.doi.org/10.3390/ijms20225526.
10) Long X, Shi Y, Ye P, Guo J, Zhou Q, Tang Y. MicroRNA-99a Suppresses Breast Cancer Progression by Targeting FGFR3. *Frontiers in Oncology*. 2020;9:1473–1473. Available from: https://dx.doi.org/10.3389/fonc.2019.01473.
11) Surapaneni SK, Bhat ZR, Tikoo K. MicroRNA-941 regulates the proliferation of breast cancer cells by altering histone H3 Ser 10 phosphorylation. *Scientific Reports*. 2020;10(1):1–7. Available from: https://dx.doi.org/10.1038/s41598-020-74847-7.
12) HU J, SHAN Z, HU K, REN F, ZHANG WEI, HAN M, et al. miRNA-223 inhibits epithelial-mesenchymal transition in gastric carcinoma cells via Sp1. *International Journal of Oncology*. 2016;49(1):325–335. Available from: https://dx.doi.org/10.3892/ijo.2016.3533.
13) Ma L. MicroRNA and metastasis. *Advances in cancer research*. 2016;132:165–207. Available from: https://doi.org/10.1016/bs.acr.2016.07.004.
14) Braicu C, Raduly L, Morar-Bolba G, Cojocneanu R, Jurj A, Pop LA, et al. Aberrant miRNAs expressed in HER-2 negative breast cancers patient. *Journal of Experimental & Clinical Cancer Research*. 2018;37(1):1–6. Available from: https://dx.doi.org/10.1186/s13046-018-0920-2.
15) Guan X, Shi A, Zou Y, Sun M, Zhan Y, Dong Y, et al. EZH2-Mediated microRNA-375 Upregulation Promotes Progression of Breast Cancer via the Inhibition of FOXO1 and the p53 Signaling Pathway. *Frontiers in Genetics*. 2021;12:382–382. Available from: https://dx.doi.org/10.3389/fgene.2021.633756.
16) Li L, Xiao C, He K, Xiang G. Circ_0072088 promotes progression of hepatocellular carcinoma by activating JAK2 / STAT3 signaling pathway via miR -375. *IUBMB Life*. 2021;73(9):1153–1165. Available from: https://doi.org/10.1002/iub.2520.

17) Zhao Q, Liu Y, Wang T, Yang Y, Ni H, Liu H, et al. MiR-375 inhibits the stemness of breast cancer cells by blocking the JAK2/STAT3 signaling. *European Journal of Pharmacology*. 2020;884:173359–173359. Available from: https://dx.doi.org/10.1016/j.ejphar.2020.173359.
18) Alam KJ, Mo JS, Han SH, Park WC, Kim HS, Yun KJ, et al. MicroRNA 375 regulates proliferation and migration of colon cancer cells by suppressing the CTGF-EGFR signaling pathway. *International Journal of Cancer*. 2017;141(8):1614–1629. Available from: https://dx.doi.org/10.1002/ijc.30861.
19) Tang W, Li GS, Li JD, Pan WY, Shi Q, Xiong DD, et al. The role of upregulated miR-375 expression in breast cancer: An in vitro and in silico study. *Pathology - Research and Practice*. 2020;216(1):152754–152754. Available from: https://dx.doi.org/10.1016/j.prp.2019.152754.
20) de Souza Rocha Simonini P, Breiling A, Gupta N, Malekpour M, Youns M, Omranipour R, et al. Epigenetically Deregulated microRNA-375 Is Involved in a Positive Feedback Loop with Estrogen Receptor α in Breast Cancer Cells. *Cancer Research*. 2010;70(22):9175–9184. Available from: https://dx.doi.org/10.1158/0008-5472.can-10-1318.
21) Guan X, Shi A, Zou Y, Sun M, Zhan Y, Dong Y, et al. EZH2-Mediated microRNA-375 Upregulation Promotes Progression of Breast Cancer via the Inhibition of FOXO1 and the p53 Signaling Pathway. *Frontiers in Genetics*. 2021;12:382–382. Available from: https://dx.doi.org/10.3389/fgene.2021.633756.
22) Nurzadeh M, Naemi M, Hasani SS. A comprehensive review on oncogenic miRNAs in breast cancer. *Journal of Genetics*. 2021;100(1):1–21. Available from: https://dx.doi.org/10.1007/s12041-021-01265-7.
23) Frank AC, Ebersberger S, Fink AF, Lampe S, Weigert A, Schmid T, et al. Apoptotic tumor cell-derived microRNA-375 uses CD36 to alter the tumor-associated macrophage phenotype. *Nature Communications*. 2019;10(1):1–8. Available from: https://dx.doi.org/10.1038/s41467-019-08989-2.
24) Costa-Pinheiro P, Ramalho-Carvalho J, Vieira FQ, Torres-Ferreira J, Oliveira J, Gonçalves CS, et al. MicroRNA-375 plays a dual role in prostate carcinogenesis. *Clinical Epigenetics*. 2015;7(1):1–4. Available from: https://dx.doi.org/10.1186/s13148-015-0076-2.
25) Wang Y, Lieberman R, Pan J, Zhang Q, Du M, You M, et al. Abstract 4948: miR-375 induces docetaxel resistance in prostate cancer by targeting SEC23A and YAP1. *Clinical Research (Excluding Clinical Trials)*. 2016;15(1):1–1. doi:10.1186/s12943-016-0556-9.
26) Zedan AH, Osther PJS, Assenholt J, Madsen JS, Hansen TF. Circulating miR-141 and miR-375 are associated with treatment outcome in metastatic castration resistant prostate cancer. *Scientific Reports*. 2020;10(1):1–9. Available from: https://dx.doi.org/10.1038/s41598-019-57101-7.
27) Abramovic I, Vrhovec B, Skara L, Vrtaric A, Gabaj NN, Kulis T, et al. MiR-182-5p and miR-375-3p Have Higher Performance Than PSA in Discriminating Prostate Cancer from Benign Prostate Hyperplasia. *Cancers*. 2021;13(9):2068–2068. Available from: https://dx.doi.org/10.3390/cancers13092068.
28) Jin Y, Liu Y, Zhang J, Huang W, Jiang H, Hou Y, et al. The Expression of miR-375 Is Associated with Carcinogenesis in Three Subtypes of Lung Cancer. *PLOS ONE*. 2015;10(12):e0144187–e0144187. Available from: https://dx.doi.org/10.1371/journal.pone.0144187.
29) Zou Q, Yi W, Huang J, Fu F, Chen G, Zhong D. MicroRNA-375 targets PAX6 and inhibits the viability, migration and invasion of human breast cancer MCF-7 cells. *Experimental and Therapeutic Medicine*. 2017;14(2):1198–1204. Available from: https://dx.doi.org/10.3892/etm.2017.4593.
30) Liu J, Wang P, Zhang P, Zhang X, Du H, Liu Q, et al. An integrative bioinformatics analysis identified miR-375 as a candidate key regulator of malignant breast cancer. *Journal of Applied Genetics*. 2019;60(3-4):335–346. Available from: https://dx.doi.org/10.1007/s13353-019-00507-w.
31) Hu Y, Ma Y, Luo G, Liao W, Zhang S, Li G. Effect of MiR-375 Regulates YAP1 on the Invasion, Apoptosis, and Epithelial-Mesenchymal Transition of Cervical Cancer HeLa Cells. *Evidence-Based Complementary and Alternative Medicine*. 2021;2021:1–8. Available from: https://dx.doi.org/10.1155/2021/3088723.
32) Babu KR, Tay Y. The Yin-Yang Regulation of Reactive Oxygen Species and MicroRNAs in Cancer. *International Journal of Molecular Sciences*. 2019;20(21):5335–5335. Available from: https://dx.doi.org/10.3390/ijms20215335.
33) Guo J, Yang C, Zhang S, Liang M, Qi J, Wang Z, et al. MiR-375 induces ROS and apoptosis in ST cells by targeting the HIGD1A gene. *Gene*. 2019;685:136–142. Available from: https://dx.doi.org/10.1016/j.gene.2018.10.086.
34) Rahmani F, Avan A, Hashemy SI, Hassanian SM. Role of Wnt/β-catenin signaling regulatory microRNAs in the pathogenesis of colorectal cancer. *Journal of Cellular Physiology*. 2018;233(2):811–817. Available from: https://dx.doi.org/10.1002/jcp.25897.
35) Soleimani A, Rahmani F, Ferns GA, Ryzhikov M, Avan A, Hassanian SM. Role of Regulatory Oncogenic or Tumor Suppressor miRNAs of PI3K/AKT Signaling Axis in the Pathogenesis of Colorectal Cancer. *Current Pharmaceutical Design*. 2019;24(39):4605–4610. Available from: https://dx.doi.org/10.2174/1381612825666190110151957.
36) Niu T, Zhang W, Xiao W. MicroRNA regulation of cancer stem cells in the pathogenesis of breast cancer. *Cancer Cell International*. 2021;21(1):31–31. Available from: https://dx.doi.org/10.1186/s12935-020-01716-8.
37) Pronina IV, Loginov VI, Burdennyy AM, Fridman MV, Senchenko VN, Kazubskaya TP, et al. DNA methylation contributes to deregulation of 12 cancer-associated microRNAs and breast cancer progression. *Gene*. 2017;604:1–8. Available from: https://dx.doi.org/10.1016/j.gene.2016.12.018.
38) Bertheloot D, Latz E, Franklin BS. Necroptosis, pyroptosis and apoptosis: an intricate game of cell death. *Cellular & Molecular Immunology*. 2021;18(5):1106–1121. Available from: https://dx.doi.org/10.1038/s41423-020-00630-3.
39) Gao HE, Sun Y, Ding YH, Long J, Liu XL, Yang M, et al. Antineoplastic effects of CPPTL via the ROS/JNK pathway in acute myeloid leukemia. *Oncotarget*. 2017;8(24):38990–39000. Available from: https://dx.doi.org/10.18632/oncotarget.17166.
40) Carbonell T, Gomes AV. MicroRNAs in the regulation of cellular redox status and its implications in myocardial ischemia-reperfusion injury. *Redox Biology*. 2020;36:101607–101607. Available from: https://dx.doi.org/10.1016/j.redox.2020.101607.
41) He J, Xu Q, Jing Y, Agani F, Qian X, Carpenter R, et al. Reactive oxygen species regulate ERBB2 and ERBB3 expression via miR-199a/125b and DNA methylation. *EMBO reports*. 2012;13(12):1116–1122. Available from: https://dx.doi.org/10.1038/embor.2012.162.
42) Houri K, Mori T, Onodera Y, Tsujimoto T, Takehara T, Nakao S, et al. miR-142 induces accumulation of reactive oxygen species (ROS) by inhibiting pexophagy in aged bone marrow mesenchymal stem cells. *Scientific Reports*. 2020;10(1):1–3. Available from: https://dx.doi.org/10.1038/s41598-020-60346-2.
43) Soliman AM, Das S, Ghafar NA, Teoh SL. Role of MicroRNA in Proliferation Phase of Wound Healing. *Frontiers in Genetics*. 2018;9:38–38. Available from: https://dx.doi.org/10.3389/fgene.2018.00038.
44) Deyell M, Garris CS, Laughney AM. Cancer metastasis as a non-healing wound. *British Journal of Cancer*. 2021;124(9):1491–1502. Available from: https://dx.doi.org/10.1038/s41416-021-01309-w.
45) Xu Y, Jin J, Liu Y, Huang Z, Deng Y, You T, et al. Snail-Regulated MiR-375 Inhibits Migration and Invasion of Gastric Cancer Cells by Targeting JAK2. *PLoS ONE*. 2014;9(7):e99516–e99516. Available from: https://dx.doi.org/10.1371/journal.pone.0099516.
46) Tavazoie SF, Alarcón C, Oskarsson T, Padua D, Wang Q, Bos PD, et al. Endogenous human microRNAs that suppress breast cancer metastasis. *Nature*. 2008;451(7175):147–152. Available from: https://dx.doi.org/10.1038/nature06487.
47) Surapaneni SK, Bhat ZR, Tikoo K. MicroRNA-941 regulates the proliferation of breast cancer cells by altering histone H3 Ser 10 phosphorylation. *Scientific Reports*. 2020;10(1):1–7. Available from: https://dx.doi.org/10.1038/s41598-020-74847-7.

48) Thapa S, Rather RA, Singh SK, Bhagat M. Insights into the Role of Defective Apoptosis in Cancer Pathogenesis and Therapy. and others, editor;IntechOpen. 2021. doi:10.5772/intechopen.97536.
49) Inoue J, Inazawa J. Cancer-associated miRNAs and their therapeutic potential. *Journal of Human Genetics*. 2021;66(9):937–945. Available from: https://dx.doi.org/10.1038/s10038-021-00938-6.
50) Al-Aamri HM, Irving HR, Bradley C, Meehan-Andrews T. Intrinsic and extrinsic apoptosis responses in leukaemia cells following daunorubicin treatment. *BMC Cancer*. 2021;21(1):438. Available from: https://dx.doi.org/10.1186/s12885-021-08167-y.
51) Yu JSL, Cui W. Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. *Development*. 2016;143(17):3050–3060. Available from: https://dx.doi.org/10.1242/dev.137075.
52) Miricescu D, Totan A, Stanescu-Spinu II, Badoiu SC, Stefani C, Greabu M. PI3K/AKT/mTOR Signaling Pathway in Breast Cancer: From Molecular Landscape to Clinical Aspects. *International Journal of Molecular Sciences*. 2020;22(1):173–173. Available from: https://dx.doi.org/10.3390/ijms22010173.
53) Tokunaga E, Kimura Y, Mashino K, Oki E, Kataoka A, Ohno S, et al. Activation of PI3K/Akt signaling and hormone resistance in breast cancer. *Breast Cancer*. 2006;13(2):137–144. Available from: https://dx.doi.org/10.2325/jbcs.13.137.
54) Syeda ZA, Langden SSS, Munkhzul C, Lee M, Song SJ. Regulatory Mechanism of MicroRNA Expression in Cancer. *International Journal of Molecular Sciences*. 2020;21(5):1723–1723. Available from: https://dx.doi.org/10.3390/ijms21051723.
55) Ni H, Qin H, Sun C, Liu Y, Ruan G, Guo Q, et al. MiR-375 reduces the stemness of gastric cancer cells through triggering ferroptosis. *Stem Cell Research & Therapy*. 2021;12(1):1–7. Available from: https://dx.doi.org/10.1186/s13287-021-02394-7.
56) Portt L, Norman G, Clapp C, Greenwood M, Greenwood MT. Anti-apoptosis and cell survival: A review. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 2011;1813(1):238–259. Available from: https://dx.doi.org/10.1016/j.bbamcr.2010.10.010.