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

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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, the 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 expressions of 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 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, miR-375, Proliferation, Apoptosis, Migration.

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 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 regions (3'-UTR) of proteincoding 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 ^[29,30,16,31]. MiR-375 suppresses cervical cancer development by regulating epithelial-mesenchymal transition by targeting Yesassociated 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 the 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 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 μ g/mL streptomycin antibiotics. Cells were incubated at 37°C under 5% CO₂ 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 transfected with 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 incubation overnight, cells were 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 ^[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 Station, 20x) ^[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 transfection, cells were washed with PBS once and incubated with 10 μ M 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 1×10^4 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 using phase-contrast microscopy at 20x magnifications ^[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). 2µg 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 SYBER FAST qPCR master mix kit (Kappa Biosystems) in 20µl by Step one plus RT-PCR (Applied Biosystems): 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 $2^{\circ}(-\Delta\Delta CT)$ 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. 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.



Figure 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 IC₅₀ 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 concentrations. 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.



Figure 2. Analysis of morphological changes in miR-375 inhibitor-treated breast cancer cells after 72 h. Cells were exposed to IC₅₀ concentrations 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 alterations 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 labeled 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), and their control cells showed no apoptotic cell death. 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 lines.



Figure 3. Morphological observation following EtBr/AO (magnification, 20x). MCF-7 breast cancer cells were treated without and miR-375 inhibitor at IC₅₀ concentration for 72 h. Treated cells showed more cell death compared to control.



Figure 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-labeled into miR-375 inhibitor transfected MCF-7 cells, and fluorescence intensity was analyzed in a Floid Cell Imaging Station (Life Technologies). 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.



Figure 5. MiR-375 inhibitor increased the ROS production in breast cancer cell lines. Breast cancer cells were treated with IC₅₀ 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.



Figure 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 cell imaging station (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 transfected cells. The levels 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.





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 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 synthetic anti-miR-375 inhibitor generates ROS in MCF-7 breast cancer cells. Compared to control cells, antagonizing miR-375 stimulating ROS generation in miR-375 transfected breast cancer cells. These findings revealed that anti-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 through targeting on JAK2 ^[45]. Similarly, miR-375 expression suppressed cervical cancer cell migration through AEG-1^[8]. Inhibition of miRNA-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 anti-miR inhibitors 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 cancer 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, anti-miR-375 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 cancer cell growth enhancer and Bax is the apoptotic promotor ^[56]. So, we found that increased Bax expression and decreased Bcl-2 expression in anti-miR-375 inhibited 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.

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