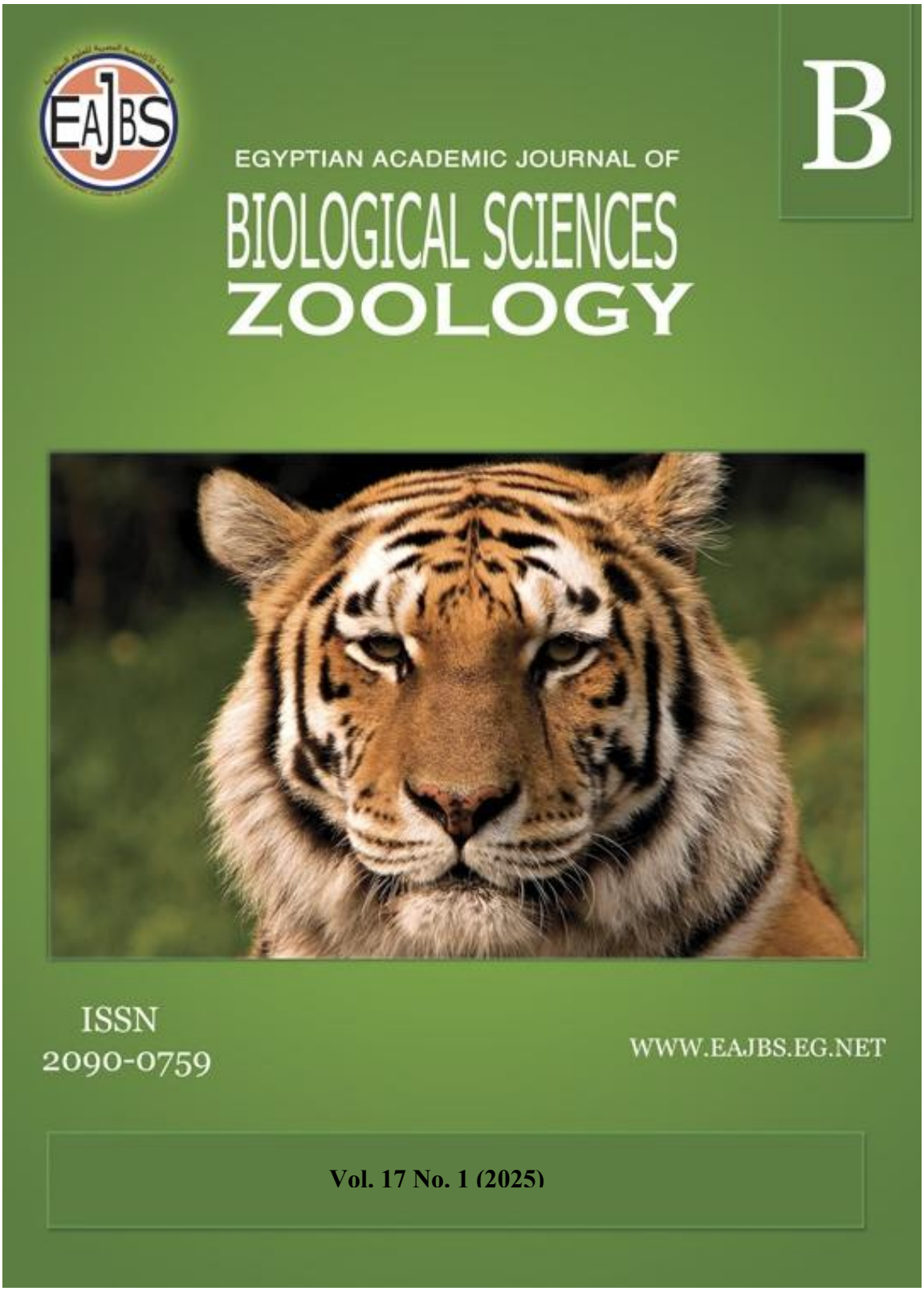

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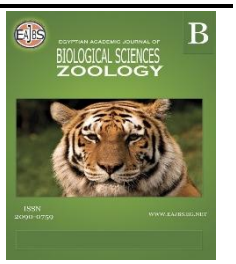

Elucidation of Hsa-miR-98's Role in Oncogenicity and Oxidative Stress in Breast Cancer Cell Lines

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ABSTRACT

The role of miR -98 is controversial in the literature which we aimed to clarify in breast cancer cell lines. To elucidate the role of miR-98, mimic and inhibitor were transfected into MCF-7and MDA-MB-231 cell lines. Q-RT-PCR was used to analyze Wnt pathway gene expression. Function analysis including wound healing, and determining oxidative stress parameters were conducted. miR-98 was up-regulated in most breast cancer tissue databases. Bioinformatics analysis target prediction and enrichment analysis clarified that miR-98 is incorporated in oxidative stress pathway. It targets several genes from which HIF-1A and VEGFA which were selected for real time analysis. Q-RT- PCR results revealed that HIF-1A and VEGFA were non-significantly increased upon inhibition of miR-98 in MCF-7 while HIF-1A and VEGFA were significantly decreased upon inhibition of miR-98 in MDA-MB-231. On the other hand, the level of catalase which is ROS tracker enzyme in cell lysate of MCF-7 was significantly increased upon inhibition of miR-98. miR-98 triggered the cells of MCF-7 and MDA-MB-231 to acquire great proliferative and migrative power assisting it to heal the manufactured scratch in both cell lines as compared to control. Overall, our result point out that miR-98 acts as oncomiR in breast cancer cell lines.

INTRODUCTION

Breast cancer is one of the most pervasive malignant tumors in females, accounting for 7-10% of all malignancies (Bray *et al.*, 2018; Xu *et al.*, 2023). Breast cancer comprises different cell categories from which MCF-7 and MDA-MB-231. MCF-7 cells depend on estrogen to proliferate, additional, it expresses estrogen receptor (ER^+), progesterone receptor (PR^+), and human epidermal growth factor receptor-2 ($HER2^+$) (Baguley and Leung, 2011; Martin *et al.,* 2012). The ER+ of breast cancer could be retained by the presence of small amounts of estrogen in the fetal bovine serum. In turn, sustaining raising with estrogen deficiency would select variants to get rid of them as HER2 and other cell receptor (Leung *et al.,* 2014). Interestingly, MDA-MB-231 (ER^-, PR^- and $HER2^-$ the triple-negative) sub-lines seem to have the origin in the ER-positive MCF-7 cell line (Klopp *et al.,* 2010).

The tumor microenvironment encompasses cancer cells, macrophages, fibroblasts, and extracellular matrix (ECM), cellular receptor of tumor cell itself and factors secreted by the cells such as cytokines, ATPs, etc. The sedimentation of ECM molecules in various solid cancers in companion with cellular receptor of tumor cell itself, including breast cancer,

causes bundling of the tumor mass during cancer progression (Anderson *et al.,* 2020).

The resilience of ECM enclosing cancer cells drives tumor to become more aggressive accompanying with massive cytoskeletal rearrangement and alterations in oncogenic signals. Moreover, tumor mass raised on a stiff matrix showed fierce cytoskeletal tension, causing cell–ECM adhesion enhancement, instability of cell junctions, and improving cell proliferation (Anderson *et al.,* 2020; Butcher *et al.,* 2009). It is clarified that increasing microtubule acetylation and reducing expression of ER stress markers in MDA-MB-231 cells raised on a stiff matrix prevents the progression of breast cancer, particularly migration, invasion, proliferation, and spheroid formation, as αTAT1 gene expression reduced (Ko *et al.*, 2021). In colon cancer rising microtubule acetylation by α-tubulin N-acetyltransferase 1 (αTAT1) enhance the invasiveness of tumor cells via Wnt/β-catenin signaling (Lee *et al.,* 2016).

The genetic and epigenetic triggered within the microenvironment of tumor cells; such as hypoxia, and metabolites, disrupts metabolic balance and tends to support pro-angiogenic agents. The released angiogenic agents by tumor cells, vascular endothelial growth factor (VEGF), fibroblastic growth factor (FGF), matrix metalloproteinase (MMP1), platelet derived growth factor (PDGF), angiobioten (ANGT1and 2), and hypoxia inducible factor alpha (HIF-1A), cause vasodilation and increase the vascular permeability of blood vessels in the vicinity of the tumor. That turns on disruption of the basement membrane, loosening of pericyte covering, plasma protein diffusion, and providing matrix for migration of endothelial cells (Eilken *et al.*, 2010).

Also, high ROS level in tumour microenviroment can lead to unbalance in redox hemostasis, which results in cell proliferation, activation of redox sensitive proteins (HIF) and pro-survival mechanisms in cancer cells. In which activation of HIF binding sites by HIF-1A induce activation of expression of genes incorporated in neovascularization, glycolysis, cellular proliferation and metastasis. Thus, the HIF-1A response is due for tumor proliferation by authorizing cancer cells to accommodate to depleted oxygen environment (Siragam *et al.,* 2014). Cancer is composite disease associated with disturbance of numerous genes. In addition, surplus cellular pathways brass the efficiency of mono-target drugs in cancer therapy (Kellman *et al.,* 2025).

MicroRNAs are a category of RNA therapeutics, which run on targeting several pathways via incorporated genes (Jones *et al.*, 2008; Saliminejad *et al.*, 2019). Herein, the role of miR-98 is going to be surveyed (mimic and inhibitor) in two folds of breast cancer cell lines (MCF-7, MDA-MB-231), which are ER+ and ER- cell lines respectively. The purpose miR-98 is thought to be alternated between oncomir and tumor suppressor associated with various cancers [Siegel *et al.,* 2018]. This controversy about the role of miR-98 in various cancers, is the inspiration to find its exact assignment in breast cancer. This study intended to investigate the role of miR-98 in Wnt angiogenesis and oxidative stress pathways. It was found that miR-98 has oncogenic role in MCF-7 and MDA-MB-231 cell lines through investigation of Wnt angiogenesis and oxidative stress pathway.

MATERIALS AND METHODS

In Silico Study:

Target Prediction of miR-98:

The MirWalk is a broad available comprehensive resource that accommodates the predicted as well as validated micro-RNA target interaction pairs and possible miRNA-binding site predictions within the complete sequence of all known genes of three genomes (human, mouse, and rat). The Mir Walk settings were to get the intersection between; Miranda, PICTAR, TARGET SCAN. There are three types of binding between mRNA and micro-RNA which are 3'UTR compensatory, 5'prime dominant, and canonical. The tools such as RNA22, RNA-hybrid, target scan, and intaRNA had been utilized to detect mode of binding of miRNA and mRNA.

Enrichment Analysis of miR-98-3p:

The database for annotation, visualization, and integrated discovery (DAVID), presents a comprehensive set of functional annotation tools to understand the biological meaning behind the submitted gene list. This is via introducing the incorporated pathway maps for genes (KEGG, Reactome) which links gene disease association. The uploaded gene

list undergoes annotation managements through which pathways (Reactome, KEGG), gene ontology terms (GO), Functional annotations (cellular component, molecular function, biological process) for the listed genes were determined with p-value<0.05.

Expression Level of miR-98-3p in Breast Cancer Tissue:

Using dbDEMC2 software (accessed on 18 October 2023), the expression status of miR-98-3p in normal and tumor tissues, including breast cancer, was determined. An integrated database called database of Differentially Expressed miRNAs in Human Cancers (dbDEMC) holds high throughput data on how miRNAs are expressed differentially in human cancers [Xu *et al.,* 2022]. Other miRNA disease association databases, HMDD [Huang *et al.*, 2018], miR2Disease [Jiang *et al.*, 2009], and miRcancer [Xie *et al.*, 2013], which are widely used in the literature were utilized to clarify the differential expression status of miR-98- 3p in breast cancer.

Survival Analysis:

Star Base V.20 (accessed on 18 October 2023) was used to perform the survival analyses for miR-98-5p sourced [Li *et al.*, 2013]. Encyclopedia of RNA interactomes (Star base v2.0) shows gene expression data of 32 types of cancer which have been utilized to determine protein analysis through evolutionary relationship database (pan-cancer) analysis on RNA-RNA and RBP-RNA interaction, and also provide platforms to perform survival and differential analysis of miRNA.

In vitro experimental and molecular methods for surveying the role of miR-98 in MCF-7 and MDA-MB-231 cell lines**:**

Statistical Analysis:

Excel, it is utilized in order to calculate mean, standard deviation, mean error, ttest and instruct bar chart graph. While Image J tool is utilized in order to adjust the resolution of image, measure image dimension and compare the treated image with control.

Preparation of Cell Lysate for HIF-1A Protein by ELISA and Oxidative Stress Parameters:

Following cell transfection cells were washed with ice-cold DPBS and lysed in RIPA lysis buffer (Pierce RIPA, Thermo Fisher). Samples were kept on ice for 20 minutes with regular shaking. Lysates were then centrifuged (14,000 rpm for 20 minutes at 4 °C), and protein extracts (supernatants) were collected and frozen at −80 °C (Yahya *et al*, 2016).

Cell Line and Cell Culture:

Wild breast cancer cell lines were acquired from American Type Culture Collection (ATCC). These cells were culture and propagated in 75 cm2 flasks. In Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Belgium); supplemented with 10% Fetal Bovine Serum (Biochrom, Berlin), 1% Penicillin–streptomycin (Lonza, Belgium) and 4 μM L-glutamine (Lonza, Belgium) at 37°C in a 5% CO2 incubator.

Cell Transfection with RNA Oligonucleotides:

Breast cancer cells were transfected with 100nM of miSCript miR-98 mimics and miSCript Inhibitor Negative Control (Qiagen, Valencia, CA, USA) using 1μL of HiperFect transfection reagent (Qiagen, Germany). After 24 h of transfection, transfected media were replaced by fresh media. At once, treated cells were gathered for microRNA isolation and detection, while protein extracts and total RNA were isolated after 48 h of transfection. Then these RNAs undergo cDNA (Complementary DNA) transformation for further investigation analysis.

QRT- PCR Analysis for the Expression Level miR-98 and mRNAs Gene Targets:

For microRNA, miSCript miRNA PCR system (miRneasy mini kit for miRNA extraction, miSCript RT II for miRNA reverse transcription, 10X miR-98a-1 miSCript Primer Assay and miSCript SYBR Green PCR kit for PCR amplification) (Qiagen, Valencia, CA, USA) was used to analyze the expression level of miR-98 after 24h transfection of miRNA according to the manufacturer and instruction of protocol. The Cycling conditions for Q T PCR were set: 95 °C for 15 min, 94 °C for 15 s, 55 °C for 30 s, and then 70 °C for 30 s, the number of cycles was 40 cycles. Fluorescence measurements were performed with real-time PCR (MiniOpticon Real-Time PCR System, Bio-Rad, France). After 48 hours of transfection of miRNA, total RNA was extracted using QIAZOL (Qiagen, Valencia, CA, USA) following manufacturer's instructions, expression levels of HIF-1A and VEGFA were estimated using their corresponding primers (Table 1) by QRT-PCR according to manufactures' instructions, and these results were normalized to B-actin as housekeeping gene. The RT and subsequent

PCR cycling conditions were as follows: 42 °C for 60 min, 70°C for 5 min; followed by 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 sec, 60 °C for 60 sec, and the number of cycles was 40 cycles. Bio-Rad MiniOpticon™ real-time PCR cycler was used for quantitative estimation (Yahya et al., 2016).

Table 1 Represents the supposed genes that going to be elucidated by Q T PCR and their corresponding genes.

Genes	Primer	citation
B-actin	F CCTTCCTGGGCATGGAGTCCT	Yahya et al., 2017
	R GGAGCAATGATCTTGATCTTC	
(HIF-1A)	F GCAAGCCCTGAAAGCG	Yahya et al., 2017
	R GCAAGCCCTGAAAGCG	
VEGF	F TACCTCCACCATGCCAAGTG	Yahya et al., 2017
	R ATGATTCTGCCCTCCTCCTTC	

Wound Healing Assay (migration property):

The wound healing assay triggers migration and interactive properties of treated cells which are influenced by FGF1, MMP and VEGFA cytokines that are secreted by tumor and mutation in the ER alpha axis [Ball *et al.,* 2007; Ding *et al.,* 2010]. After several passage of MCF-7 and MDA-MB-231 cell lines verifying their viability using trypan blue dye, cells were seeded in 6 well plate, and treated by miR-98 mimic and inhibitor until be confluent. Then 100 µl pipette tips were applied to create a homogeneous scratch wound on the monolayer. After 24h the width of scratches was quantified by the mean of image J tool (https://imagej.net/) at three different positions (bottom, middle, and top), and the mean of width was calculated. Finally, the obtained underwent statistical analysis using Excel format.

Oxidative Stress:

Catalase:

The level of catalase an antioxidant enzyme is an indicator of oxidative dose that cells of MCF-7 and MDA-MB-231 suffered from. Catalase enzyme was measured by (catalase, Bio diagnostic, Egypt), in the cell lysate of transfected cells of both cell lines according to manufacture instructions.

Nitric Oxide:

The level of nitric oxide is an indicator of ROS that was found in the tested sample. Also, nitric oxide synthase (NOS) is predicted to be downregulated by miR-98 MIMIC. Nitric oxide, an indicator of nitric oxide synthase, was measured by (Nitric oxide, Bio diagnostic, Egypt) in the cell lysate of transfected cell lines according to manufacture instructions.

Enzyme-Linked Immune sorbent Assay (ELISA) for Detection of HIF-1A:

HIF-1A protein level was gauged in cell lysate of MCF-7 and MDA-MB-231 cell lines by mean of (HIF-1A Human ELISA kit, Thermo Fisher, Germany), according to manufacture instructions.

Toxicity Assay:

Cell viability was measured by neutral red assay (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride). It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. Additionally, it depends upon the ability of the viable cells to maintain a PH gradient in which the dye present net charge close to zero which could penetrate the cell membrane and retain in lysosome as the proton gradient less than that in the cytoplasm [Repetto *et al.*, 2008]. Cells were seeded in 96 well plate and transfected as mentioned previously. After 48hr of transfection, cells were treated with neutral red. A positive control Doxorubicin (Mr=543.525) was used as cytotoxic natural agent giving 100% inhibition. Dimethyl sulfoxide (DMSO) was the vehicle used for dissolution of tested compound and its final concentration on the cells was less than 0.2%. All tests and analyses were done in triplicate and the results were averaged.

RESULTS

Insilco Studies Results:

MirWalk V.20 after undergoing analysis on miR-98-3p suggested 28 target genes that are engaged in different pathways. After the comprehensive analysis of the mode of binding between miR-98 and its target genes HIF-1A and VEGFA were selected as to be a potential canonical target and tumor oncogenes associated with malignant progression in breast carcinomas for experiment as declared by RNA22, RNA-hybrid, target scan, and intaRNA.The majority of database utilized to detect the expression status of miR-98 in breast cancer declare that miR-98 is upregulated. The survival analysis was obtained from the Star Base V.20 database and the Kaplan Meier analysis curves indicated that there is a significant difference statistically between the population survival curves for the low and high expression of miR-98 (Fig.1a) with ($p = 0.032$). Also, the differential expression level of miR-98 of Star Base v2.0 in breast cancer and normal samples reveals that miR-98 is significantly increased in breast cancer sample (Fig.1b). The enrichment analysis that was carried out on miR-98 targets by the DAVID server revealed that these genes are incorporated in oxidative pathways and their related cascades as demonstrated by KEGG and Reactome platforms. Also, functional annotations of the DAVID clarified that the target genes of miR-98-3p were engaged in cellular components, molecular function and biological processes that contributed oxidative process. Moreover, DAVID server declared that the target genes of miR-98-3p were in collaborative in gene ontology terms of endothelial cell migration regulation, epithelial cell proliferation and response to hypoxia (p value≤ 0.05) (Fig.2a, b, c).

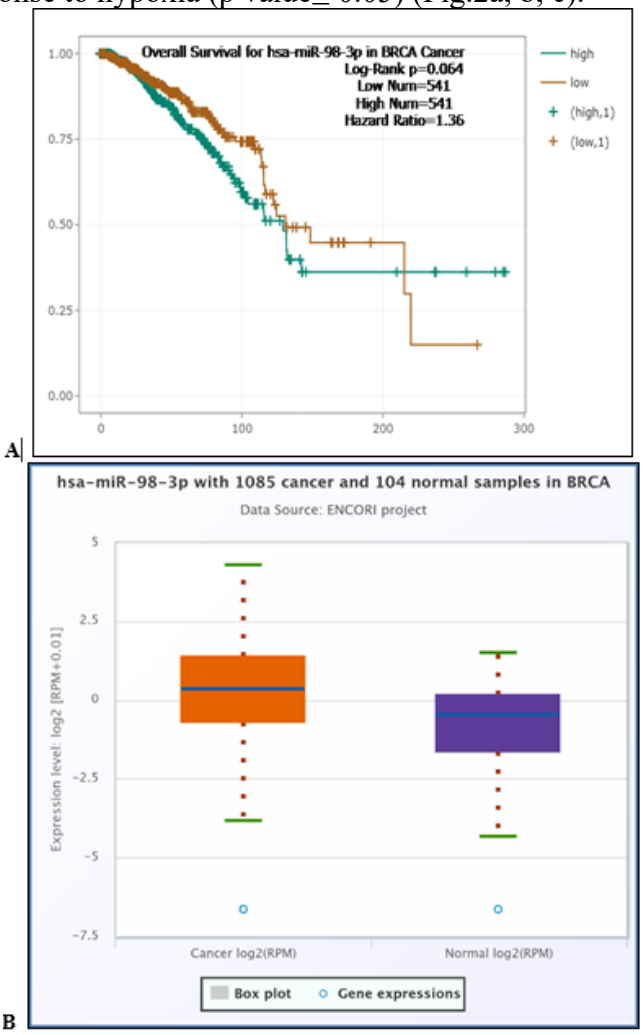

Fig. 1 Represents survival analysis of miR-98. (a) pan cancer survival analysis Kaplan–Meier curves for miR-98-3p with survival time of breast cancer patients from star base v.2. (b) Pan-Cancer Differential Expression Analysis across invasive breast cancer plot for expersion level of miR-98-3p in normal and breast cancer patient.

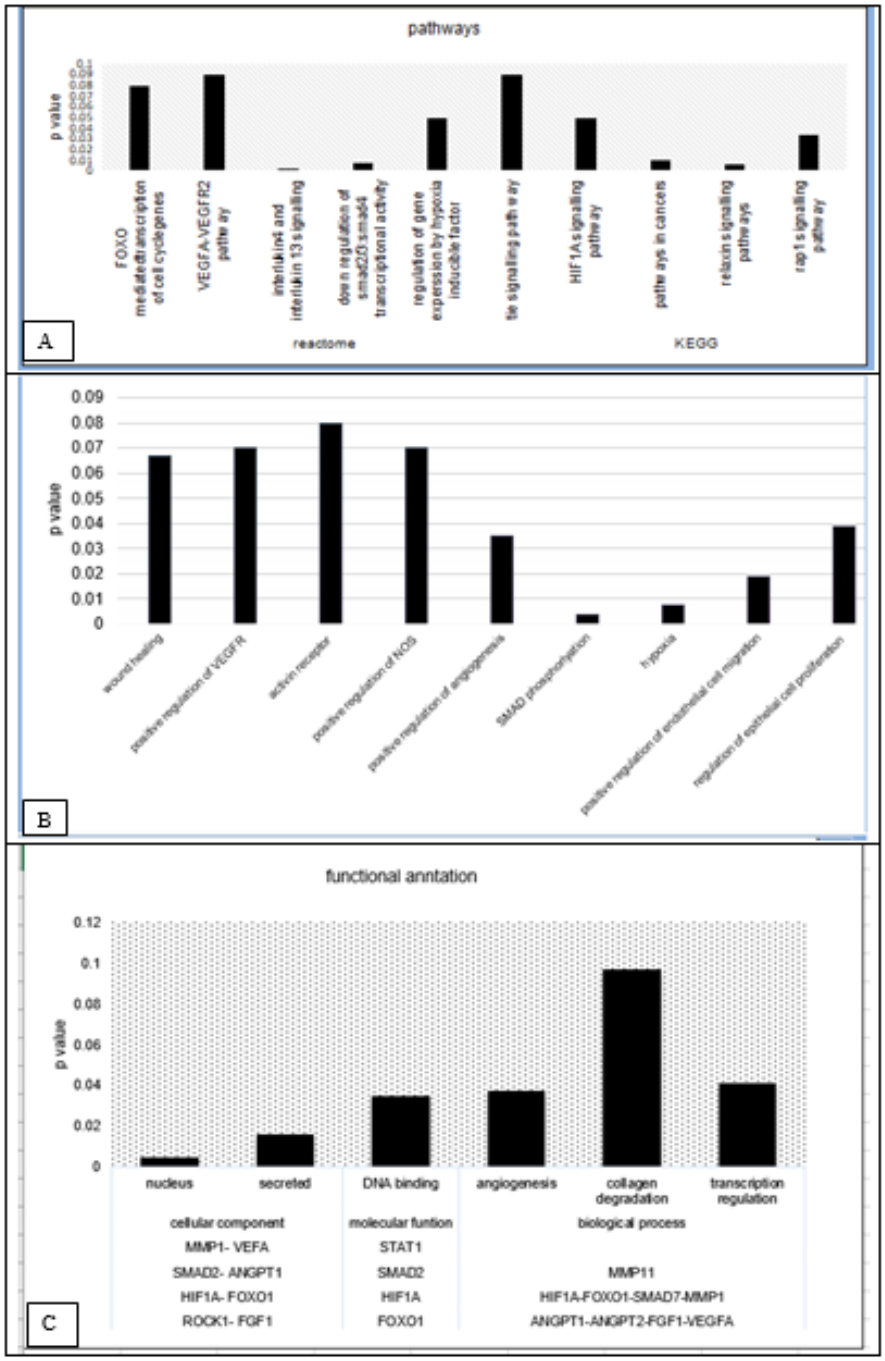

Fig 2. Represents bar chart of the enrichment analysis data of miR-98 target genes by DAVID server. (a) pathway acording to determined significance threshold($p \leq 0.05$), and pathway analysis of target genes of reactome and KEGG.(b) threshold significance of gene ontology terms of miR-98 target genes. (c) threshold significant of functional annotation of miR-98 target genes.

Effect of miR-98-3p Inhibition On Gene Expression Levels in MCF-7 and MDA-MB-231 Cell Lines:

The expression level of miR-98-3p in MCF-7 and MDA-MB-231 cell lines revealed that miR-98 mimic, and miR-98 inhibitor were successfully transfected as compared to control (NC) (Fig 3 a, b). Such that MCF-7 Cells transfected with miR-98 mimic showed a dramatic increase in miR-98 expression level as compared to negative control treatment (6723.3%), while cells transfected with miR-98 inhibitor showed a significant reduction in miR-98 expression level as compared to negative control treatment (-89%). Similarly, MDA-MB-231 Cells transfected with miR-98 mimic showed a dramatic increase in miR-98

expression level as compared to negative control treatment (703.8%), while cells transfected with miR-98 inhibitor showed a significant reduction in miR-98 expression level as compared to negative control treatment (-92.6%). The reduction of miR-98 in the MCF-7 cell line it induced an insignificant increase in HIF-1A and VEGFA gene levels. On the other side, down regulation of miR-98-3p in MDA-MB-231 cell lines induced a significant reduction in HIF-1A, VEGFA gene expression level (Fig 3 c, d).

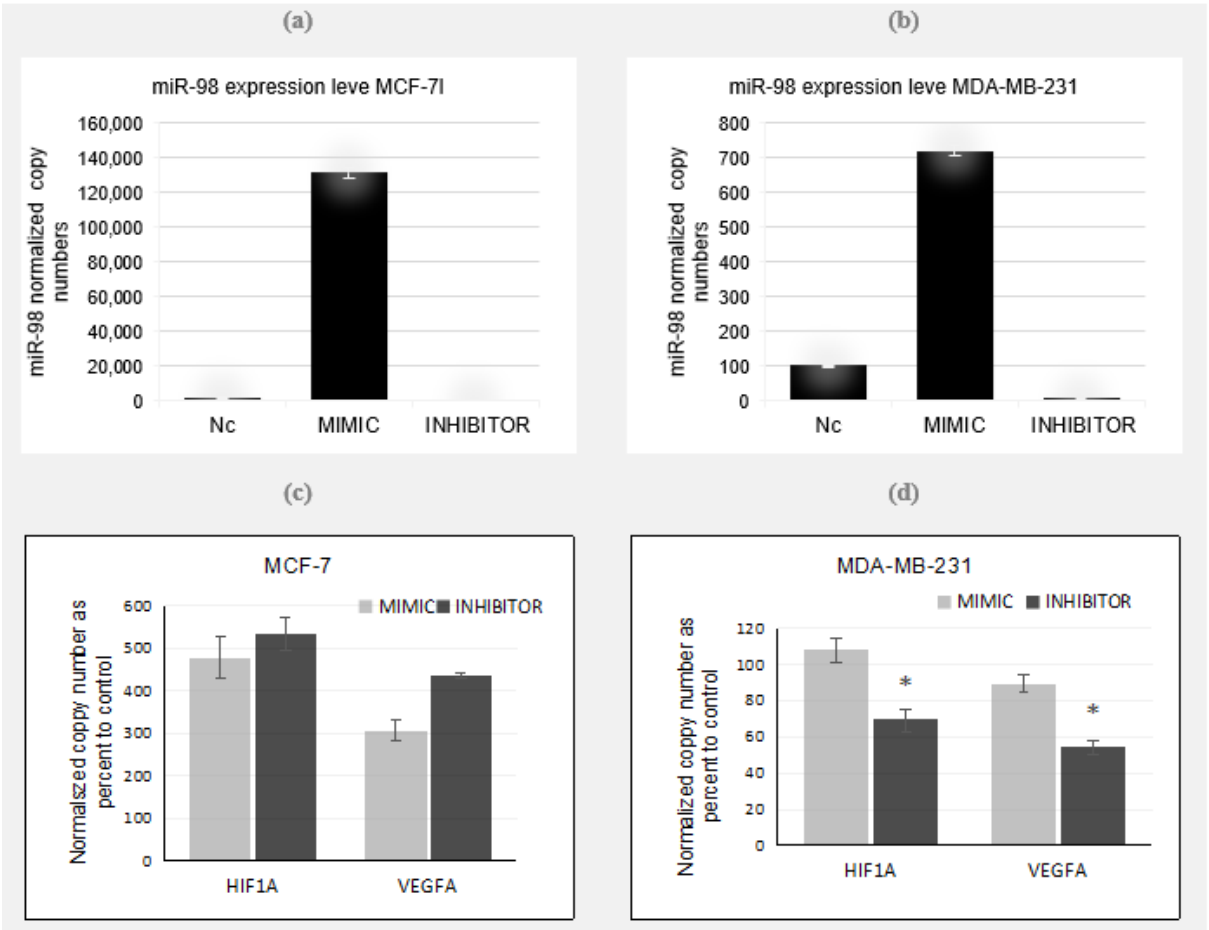

Fig .3 Graphical representation of expression level of RNA in MCF-7 and MDA-MB-231 cell lines after being treated by NC, miR-98-3p mimic, miR-98-3p inhibitor. (a) Expression level of miR-98-3p in MCF-7 cell line, (b) expression level of miR-98-3p in MDA-MB-231 cell line. (c) Gene expression level of HIF-1A, VEGFA in MCF-7 cell line. (d) Gene expression level of HIF-1A, VEGFA in MDA-MB-231 cell line. * p value ≤ 0.05.

The Inhibition of miR-98-3p Counters the Migration Property of MCF-7 and MDA-MB-231 Cell Lines:

The angiogenesis process is tightly engaged with the migration and invasive function of tumor mass cells under the influence of its secreted cytokines [Pons *et al*., 2008; Kidacki *et al*., 2017]. The wound healing survey of MCF-7 and MDA-MB-231 cell lines clarifies that miR-98-3p reduction decreases the migration property of cells. This can be inferred from microscopic observation of cells that reveal the width of scratch has widened down as the cells lose their migration power upon miR-98 reduction by inhibitor (Fig.4). This can be endorsed by statistical analysis of scratch by image J tool that showed the width of scratch was significantly increased with miR-98 reduction. However, this scratch width was dramatically reduced upon miR-98-3p up-regulation (Fig.5 a, b). Also scratch percent was significantly increased upon miR-98-3p inhibition while it was significantly reduced with miR-98-3p up-regulation (Fig 5 c, d)

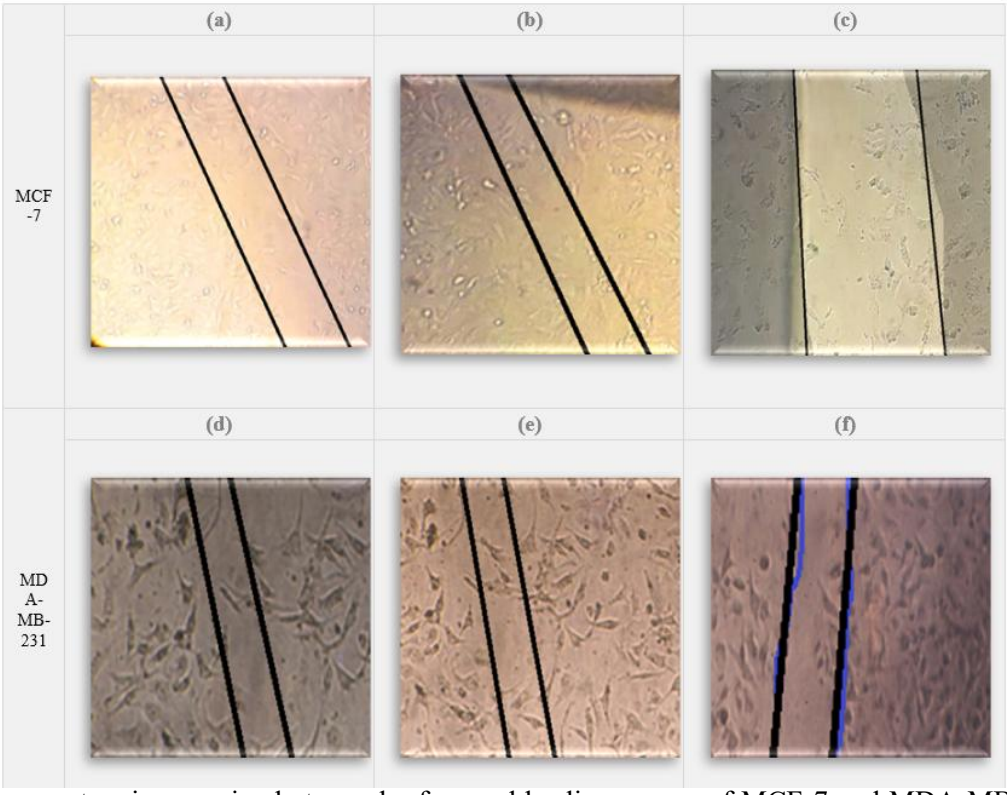

Fig.4 Represents microscopic photograph of wound healing survey of MCF-7 and MDA-MB-231cell lines. (a) MCF-7 cells treated by control (NC). (b) MCF-7 cells treated by miR-98-3p mimic. (c) MCF-7 cells treated by miR-98-3p inhibitor. (d) MDA-MB-231 cells treated control (NC). (e) MDA-MB-231 treated by miR-98-3p mimic. (f) MDA-MB-231 cells treated by miR-98-3p inhibitor.

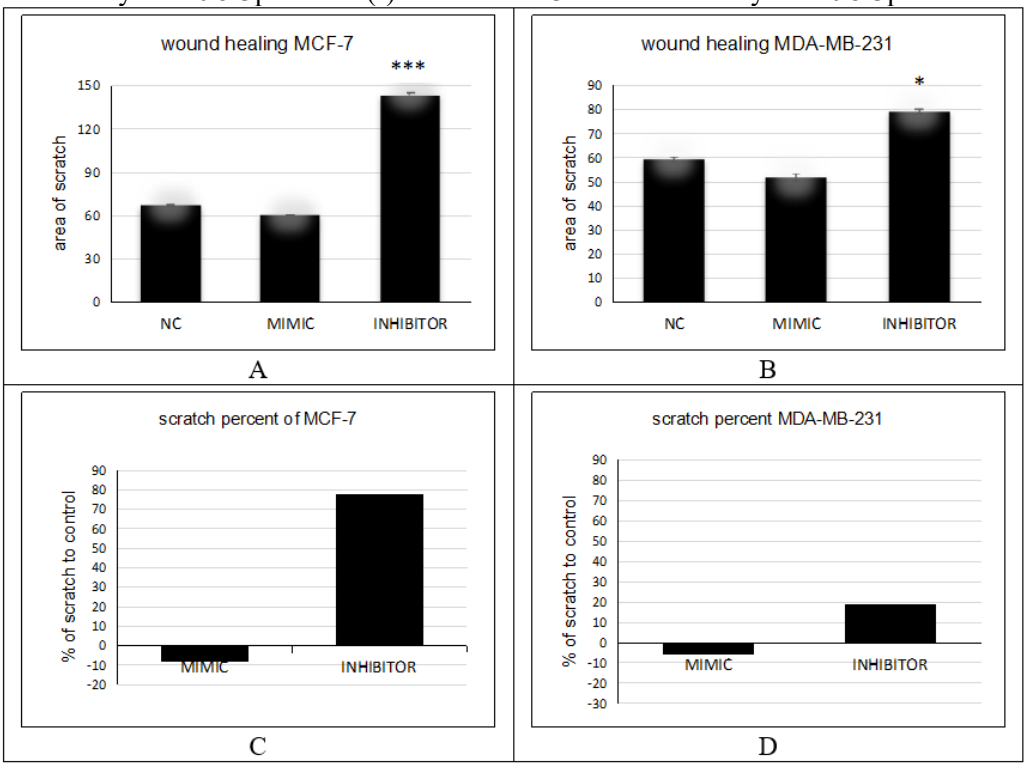

Fig. 5 Represents statistical analysis chart graph for wound healing assay. (a) Wound healing survey of MCF-7 after being transfected by NC, miR-98 mimic, miR-98 inhibitor. (b) Wound healing survey of MDA-MB-231 after being transfected by NC, miR-98 mimic, miR-98 inhibitor, (c) chart bar of scratch percent of MCF-7 cell line formed after being transfected by miR-98 mimic and inhibitor to control, (d) chart bar of scratch percent of MDA-MB-231 cell line formed after being transfected by miR-98 mimic and inhibitor to control, * stated for p value ≤ 0.05, ** stated for p value ≤ 0.01

Oxidative Stress (HIF-1A, NO and catalase):

The degree of oxidative stress that MCF-7 and MDA-MB-231 suffered from was surveyed by HIF-1A protein level, nitric oxide, and catalase enzyme. The survey of HIF-1A protein level by ELISA revealed that the HIF-1A level upon up- regulation of miR-98-3p wassignificantly decreased in MCF-7 and significantly increased in MDA-MB-231 (Fig. 6a, b). Additionally, the inhibition of miR-98-3p increased the HIF-1A, which was significant only in the case of MCF-7 cells. Meanwhile, the concentration of catalase enzyme was significantly increased in MCF-7 and decreased in MDA-MB-231 upon inhibition of miR-98-3p (Fig 6c, d). Finally, the concentration level of nitric oxide was significantly increased as a percent to control upon up- regulation of miR-98-3p in MCF-7 and MDA-MB-231 (Fig.6 e, f).

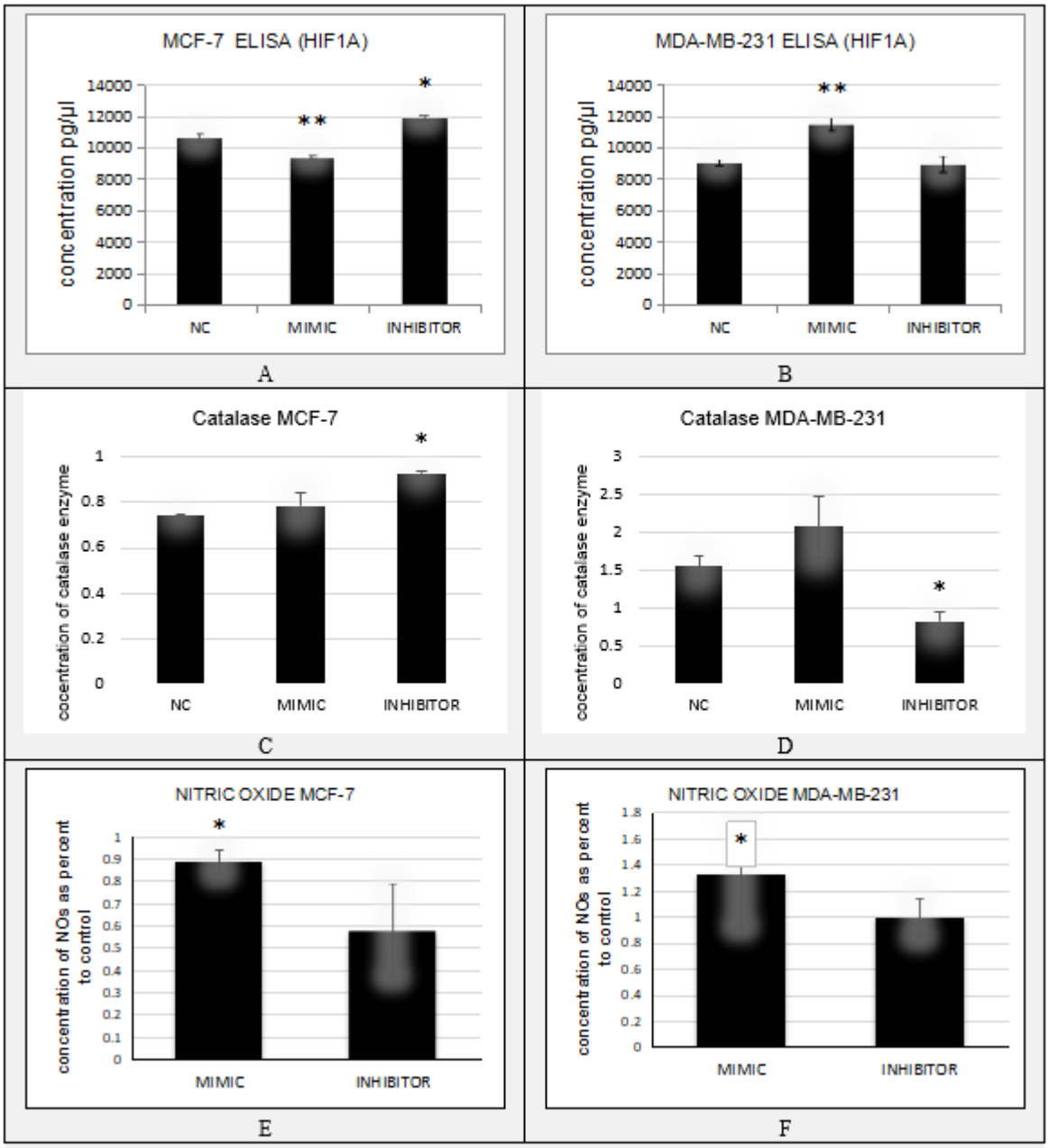

Fig.6 Represents the oxidative stress graphs for MCF-7 and MDA-MB-231. a, b ELISA chart repsent the ELISA bar chart result of MCF-7, MDA-MB-231 after transfection of NC, miR-98-3p mimic and inhibitor; c, d Catalase chart represet the concentration of catalase bar chart in both cell lines after NC, miR-98-3p mimic and inhibitor transfection; e, f Nitric oxide chart represent the concentration as percent to control of nitric oxide in both cell lines after transfection of NC, miR-98-3p mimic and inhibitor; * pvalue ≤ 0.05, ** pvalue ≤ 0.01.

Cytotoxic Assay:

The toxicity assay shows that the utilized concentration dose of miR-98 inhibitor was significantly lethal in comparison with control in MCF-7 cell line. While the viability of cells increased significantly as compared to control upon up- regulation of miR-98 by mimic MDA-MB-231 cell line (Fig. 7).

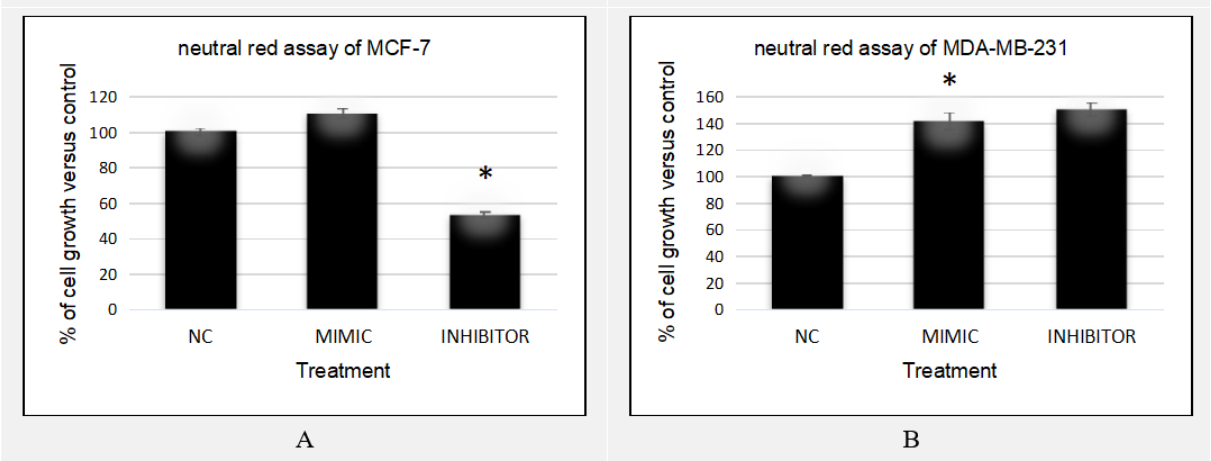

Fig. 7: Represents viability bar chart of MCF-7 (a) and MDA-MB-231 (b) cell line transfected by negative control (NC), miR-98-3p mimic and inhibitor, * state for significance pvalue ≤ 0.05

DISCUSSION

The expression of several genes involved in different stages of angiogenesis, including VEGF, ANGPT-2, and (FGF), is increased by hypoxia (Pugh *et al.*, 2023). Cancer proliferation, invasion, and migration are complicated multifactorial pathways regulated by many molecules, of which miRNAs are included. The miRNAs have been recognized as either tumor suppressors or oncogenic factors based on the established functions of their target mRNAs (Kenneth *et al.,* 2023). microRNA alterations in cancer are very important as many cancer cells show genetic alterations that are microRNA –dependent mechanisms. These mechanisms include altered target binding, processing, and post-transcriptional editing. A common feature in cancer cell is the variation in binding site in the 3' UTR of the target mRNA which has been widely reported (Ziebarth *et al.,* 2012). In cancer cells, mRNAs become insensitive to microRNA regulation through mutations, single nucleotide polymorphisms (SNPs), and deletions of 3'UTRs during the mRNA splicing process (Sun *et al.*, 2009). It appears that miRNAs miRNAs play a crucial role in orchestrating cancer proliferation and metastasis. Consequently, miRNAs have been introduced as vital therapeutic molecules and diagnostic markers for cancer treatment. Further understanding of the importance and molecular pathways of miRNAs in cancer progression is needed. miR-98, a member of the let-7 family, still has many controversies regarding its role in breast cancer proliferation and metastasis.

Recently, microRNA miR-98 was reported to inhibit tumor proliferation, invasion and migration by targeting and binding to Wnt signaling pathway-related genes and oxidative stress in many cancer types; including gastric cancer (Zhan *et al.*, 2021), ovarian cancer (Wang *et al.*, 2021), glioblastoma (Xu *et al.*, 2017), pancreatic ductal adenocarcinoma [Wang *et al.*, 2021], non-small cell lung cancer [Fu *et al.*, 2018], and breast cancer [Shi *et al.*, 2019]. Moreover, Kenneth (Kenneth *et al.*, 2023) reported that miR-98-5p may suppress an important Wnt protein receptor expression level (FZD3), leading to suppressed proliferation and metastasis of colorectal tumor. Additionally, Siragam *et al.* (2012) reported that miR-98 mimic suppresses ALK4 and MMP11 expression resulting in diminished angiogenesis and tumor invasion. Similarly, Shi *et al* (2019) reported that the up-regulation of miR-98-5p noticeably reduced Gab2 expression. However, other studies, as per our current findings reported an oncogenic activity of miR-98 (Deng *et al.,* 2014; Hamed *et al.,* 2018). Where Deng *et al.* (2014) stated that miR-98 was up-regulated in FFPE tissues of breast cancer. Meanwhile, Hamed *et al.* (2018) found that ABCC5 and ABCC10, important ABC transporters affecting the drug efflux process negatively, were down regulated upon miR-98

inhibition in HepG2 hepatocellular carcinoma cells.

In the current study, gain- and loss-of-function experiments confirmed the oncogenic characteristics of miR-98-3p in both ER+ and ER- breast cancer cells. Additionally, miR-98 shows up-regulation in breast cancer tissue as indicated by miRNA disease-related databases such as; HMDD [Huang *et al.*, 2018], miR2Disease (Jiang *et al.*, 2009), and miRcancer (Xie *et al.*, 2013), which highlight it as a potential oncomir. The survival analysis result revealed a significant variation between low and high expression of miR-98-3p survival groups indicating the oncogenic function of this microRNA in breast cancer. Moreover, StarBase shows that miRNA expression in breast cancer is significantly higher than in normal tissue. Enrichment analysis suggested different pathways including angiogenesis and oxidative stress as target genes of miR-98. The restoration of miR-98-3p enhanced the spreading potential of both MCF-7 and MDA-MB-231 as demonstrated by wound healing. Conversely, miR-98 inhibition resulted in diminished spreading potential of both cell types as confirmed by the aforementioned assays. Additionally, the promotion of scratch healing by the miR-98 mimic suggests that miR-98 increases the proliferative and migratory capacity of cancerous cells in both cell lines. Based on these observations, it is concluded that the miR-98 mimic has an oncogenic effect. To elucidate the procedure beneath the effects of miR-98-3p on proliferation, migration, and invasion, putative targets of miR-98-3p were predidicted in both cell lines through bioinformatics analyses.

Amongst VEGF and HIF-1A were selected which were part of the Wnt pathway, especially the angiogenesis track. Also, the oxidative stress factors (Catalase and NO) have been gauged as they are considered regulators of angiogenesis process. In concomitant with functional assay results, gene expression levels of some previously mentioned angiogenic Wnt-related genes were inhibited upon miR-98-3p suppression. In MCf-7 cells, both VEGF and HIF-1A were non-significantly increased, while in MDA-MB-231, VEGF and HIF-1A were significantly diminished. In a paracrine manner, mesenchymal and stromal cells produce and secrete VEGF which is a strong mitogen and chemo-attractant factor for endothelial cells. The expression of VEGF is upregulated by Mutations in the Wnt signaling pathway. VEGF up-regulation results in the neovascularization of tumor cells. Hence, VEGF is considered an enhancer of endothelial survival, proliferation, and migration. Building on its previously mentioned roles, VEGF orchestrates physiological and pathological angiogenesis [Pugh *et al.*, 2003]. VEGFs and FGFs mediate their effects through acting as ligand for receptors that establish tyrosine kinase activity. Upon activation of the kinase receptor activity, several gene expression pathways start over that handle on cellular process as migration, proliferation, and differentiation of endothelial cells. Enhancement of FGF/ FGFR axes is a crucial step required to activate council of cross-linking pathways that up-regulate cell proliferation, survival, migration, or survival in many cell types (Yahya *et al.*, 2017). In colon carcinoma, suppression of FGF signaling resulted in anti-proliferative and anti-angiogenic effects (Bai *et al.*, 2015).

On the protein level, HIF-1A protein expression levels showed a significant reduction after miR-98-3p restoration in MCF-7 cells. On the contrary, miR-98-3p inhibition increases in HIF-1A protein expression significantly status. Meanwhile, miR-98-3p up-regulation increased HIF-1A protein levels significantly in MDA-MB-231 cells. HIF-1A protein is fellow of the bHLH-PAS family of proteins 12 and its structure contains $3O^2$-regulated α-subunits, HIF-1A, HIF-2A, and HIF-3A, and a constantly expressed β-subunit of the Aryl hydrocarbon nuclear translocator family, compromising, Arnt, Arnt2, and Arnt3. HIF-1A proteins were found to enhance a set of pro- and anti-angiogenic factors. The reported anti-angiogenic genes are Regulator of G-protein signaling 5, Thrombospondin-1, Angiostatin, DLL1-4, Carbonic anhydrase-9 (CA-9), Vasohibin-1, interferon, Endostatin, and Canstatin (Yahya *et al.*, 2017). The oxygen demand is met by transcription of hypoxia-inducible genes which meet the demand for oxygen needed for proteins that regulate the angiogenic switch to enhance VEGF5 production. Additionally, the new vasculature facilitates the removal of waste products and enhances metastasis (Kidacki *et al.*, 2017; Hua *et al.*, 2006). Taking into consideration the anti-angiogenic effects of HIF-1A, we may interpret the increase in HIF-α protein expression upon miR-98-3p inhibition. This, in turn, augments our postulation that miR-98-3p has oncogenic characteristics in breast cancer cells. Also, in cancer cells, HIF-1A was verified to have a crucial role in patience and adjustment to hypoxic stress. This action is mediated through contribution in production

angiogenic factors asVEGFA and promotion of glycolysis.

The increase in oxidative stress has a dual purpose, activating death channel receptors or inducing the angiogenesis process (Ryter *et al.*, 2007). Catalase activity was significantly increased upon miR-98-3p inhibition in MCF-7 cells; meanwhile, its activity was reduced in MDA-MB-231. These findings may indicate the anti-angiogenic effects of miR-98-3p inhibition hence confirming the angiogenic action of miR-98-3p in MCF-7 cells. Meanwhile, in MDA-MB-231 cells, the situation is reversed indicating activation of death channel receptors and consequently anti-oncogenic effects of miR-98-3p inhibition. Additionally, the restoration of miR-98-3p in both cell types increased nitric oxide levels, a finding which confirms its postulated oxidative and angiogenic effects.

Conclusion:

In summary, miRNA-98-3p has oncogenic properties against both ER+ and ER- cells. This was confirmed by following its effect on both gene protein and functional levels. This is proposed to be an alternative goal for chemotherapeutic regimens in breast malignancies in future. Collectively, the current research launched miRNA-98-3p as a negative regulator for some key players' angiogenic genes and spreading capabilities of breast cancer cells. Finally, miR-98-3p inhibitor could be utilized as a promising therapeutic molecule for inhibiting the angiogenic pathway and metastatic characteristics in breast cancer cells.

Declarations:

Ethical Approval: This study does not contain any studies with human participants or animals performed by any of the authors.

Competing interests: The authors declare that there is no conflict of interest.

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Availability of Data and Materials: All data sets are available in the manuscript and supplementary file.

The used databases and tools are as follows:

- MiRWALK: http://mirwalk.umm.uni-heidelberg.de.
- TargetScan: https://www.targetscan.org/vert_80.
- RNA22: https://cm.jefferson.edu/rna22/
- miRbase: https://www.mirbase.org/
- Ensembl genome browser: https://www.ensembl.org/index.html
- DbDEMC: http://www.picb.ac.cn/dbDEMC.
- HMDD: https://www.cuilab.cn/hmdd.
- miR2Disease http://www.mir2disease.org/.
- MiRcancer: https://mircancer.ecu.edu/.
- StarBase v2.0: http://starbase.sysu.edu.cn/

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