



Role of actin binding protein Twinfilin 1 in breast cancer

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Abstract: Background: The cytoskeleton is a central factor providing numerous hallmarks of cancer. Actin regulatory proteins are involved in breast malignancy, and their dysregulation was shown to predict poor clinical prognosis. Twinfilin1, an actin dynamic regulator, has been identified as an oncoprotein in breast cancer, as it promotes the proliferation of MCF-7 cells (luminal A cell line). Twinfilin1 role in Triple-negative breast cancer is not fully comprehended. Aim: This study aimed to assess the role of actin dynamic regulators Twinfilin1, destrin and the effect of their knockdown by mir-320a mimic in Triple-negative breast cancer cell lines. Materials and methods: Triple-negative breast cancer TNBC cells were transfected with either a Negative Control or a miR-320a mimic. Cell proliferation was evaluated using a colony-forming assay. Reverse-transcription quantitative PCR was used to determine the expression levels of Twinfilin1 and DSTN mRNA. Results: Cell proliferation in Triple-negative breast cancer cells decreased after transfection with miR-320a. DSTN expression was significantly impaired upon transfection with miR-320a in Triple-negative breast cancer cells, whereas the level of Twinfilin1 expression did not change. Conclusion: These observations suggested that miR-320a may inhibit DSTN expression, which, in turn, could contribute to tumorigenesis. Meanwhile, the Twinfilin1 expression level indicated that the interactions between miRNAs and their targets aren't always one-to-one, and the functions of miRNAs are interactive and complex.

Keywords: Breast cancer; Actin regulatory proteins; Twinfilin1; Destrin; microRNA; miR-320.

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1. INTRODUCTION

Breast cancer (BC) is the most frequently diagnosed cancer in the world¹, and is the fifth leading cause of cancer death, with 2.3 million women newly diagnosed as BC cases in 2020, accounting for about 1 in every 4 cancer cases among women². In Egypt, it accounts for 33% of female cancer cases, with over 22,000 new cases are diagnosed each year³. Despite significant progress in surgical techniques and drug development, metastasis still happens regularly, representing the leading cause of death in advanced BC patients⁴. Consequently, a deeper understanding of BC molecular mechanisms, especially in patients with poor prognosis, is essential for developing more conventional treatment strategies and realizing effective therapeutic protocols against BC.

MicroRNAs (miRNAs) play critical roles in numerous physiological and pathological processes at the cellular level. Also, they are involved in almost all aspects of cancer biology, such as invasion, metastasis, apoptosis, cell proliferation, and cell cycle regulation^{5,6}. The differential expression of miRNA has been extensively discussed in BC and suggested that specific miRNAs may function as oncogenes or tumor suppressors⁷. Thus, miRNAs are supposed to be significant in biomarkers development as well as therapy and diagnosis of BC patients⁸.

Twinfilin-1 (TWF1) is a conserved actin-binding protein with two actin-depolymerizing factor homology (ADF-H) domains⁹. Destrin (DSTN) is an actin-depolymerizing protein with low MW ~18 kDa; DSTN can bind to actin monomers (G-actin) and actin filaments (F-actin). TWF1 and DSTN are related to a specific family called ADF/cofilin

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proteins¹⁰. They play a role in cell motility, migration, invasion, epithelial-mesenchymal transition (EMT), and F actin formation^{11,12}. TWF1 has been linked to the development of human BC chemotherapy resistance as well as the invasion, migration, and proliferation of MCF-7 cells (luminal A cell line)¹³. DSTN was reported to promote malignancy in lung adenocarcinoma¹¹. These studies suggest that TWF1 and DSTN were playing a crucial role in cancer. However, studies investigating the functional role of TWF1 and DSTN in Triple-negative breast cancer TNBC cell lines are limited. Also, the exact mechanism of their overexpression is not yet completely understood.

miRNA target prediction algorithms were used to look for miRNAs that regulate TWF1 expression. TWF1's 3' untranslated region (UTR) contains a binding site for members of the miR-320 family. Current researches have revealed that miR-320 was downregulated significantly in many cancers, such as cervical cancer¹⁴, colon cancer¹⁵, oral cancer¹⁶, and BC¹³. However, the patterns of biological functions and expression of miR-320 and its target gene TWF1 in BC are not completely explained. The purpose of this study was to evaluate the role of actin dynamic regulators TWF1, DSTN and the effect of their knockdown by mir-320a mimic in TNBC cell lines.

2. 2. MATERIALS AND METHODS

2.1. Cell line

The human BC cell line MDA-MB-231 (ATCC®) (Manassas, VA) was cultured as a monolayer at 37 °C in complete RPMI medium (Gibco Life Technologies, Carlsbad, USA), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 g/ml streptomycin sulphate, and 3 mM L-glutamine. Cells in culture were divided into three groups, Control group (cells were incubated with RPMI medium only), miR-320a mimic transfection group (transfected with miR-320a mimic) and Negative Control (NC) group (cells were transfected with short interfering (si)RNA non-specific sequence Control).

2.2. Cell transfection

AllStars Negative Control siRNA, HiPerFect transfection reagent, and miScript miRNA mimic of miR-320a were purchased from Qiagen (Valencia, CA). The procedure for transfection was performed according to the instructions of manufacturer. In brief, cells were seeded and transfected with a transfection complex of 100 nM miR-320a mimic, or 100 nM NC and HiPerFect transfection reagent

diluted with serum-free medium and incubated until downstream analysis.

The prediction algorithms TargetScan (version 7.2¹⁷), co-expression meta-analysis of miRNA target (CoMeTa)¹⁸ and miRTargetLink Human¹⁹ were used to predict for TWF1 gene that can be targeted by the miR-320 family.

2.3. Colony formation assay

Cells were seeded in triplicate at a density (500-1000/well) in 6-well plates at and were transfected with 100 nM miR-320a mimic and respective NC. The cells were cultured for 10 days in a 37 °C incubator containing 5% CO₂ and the medium was changed every 2 days. At day 12, the cells were washed twice with PBS after removing the medium. The colonies were fixed for 10 minutes in 25% cold methanol, dried, and stained for 30 minutes in 0.5 percent crystal violet solution. The stained plates were photographed, and cell colonies with more than 50 cells were counted.

2.4. Gene expression analysis (qRT-PCR):

Total RNA was extracted from the transfected cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purity and the concentration of extracted RNA samples were measured by DS-11 spectrophotometer/Fluorometer (DeNovix). For expression analysis, samples with an A260/A280 ratio of 1.85–2 were considered. QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA) was used for reverse transcription of extracted mRNA into single-stranded complementary DNA (cDNA). For quantitative real time PCR of TWF1 and DSTN, amplification mixtures were prepared using QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA). Primers were designed using the Primer-BLAST algorithm, revised with the IDT PrimerQuest™ tool, and purchased from sigma scientific services co. (willow fort, UK). GAPDH was used as an internal housekeeping reference gene. The 2- $\Delta\Delta C_t$ method²⁰, was used to express the results as a ratio of reference to the target gene. The primers are shown in Table 1.

2.5. Statistical analysis

Graph Pad Prism version 8 (GraphPad Software, CA, USA) was used to analyses the data, which was expressed as mean \pm standard deviation. All statistics were carried out using one-way analysis of variance (ANOVA), then Tukey's multiple comparison test. A statistically significant difference was defined as P<0.05.

Table 1. Primers sequences for quantitative reverse transcription polymerase chain reaction.

Target	Primer sequence
TWF1	F: 5'-AAGAGAGCGGATGCTGTATTC-3'
	R: 5'-CCCATTGTCTATCTCGATCTTTCT-3'
DSTN	F: 5'-GGCACTACGAGAATGGAGTTTAG-3'
	R: 5'-GCGACATACTTCATCAGCTACTT-3'
GAPDH	F: 5'-GGTGTGAACCATGAGAAGTATGA-3'
	R: 5'-GAGTCCTCCACGATACCAAAG-3'

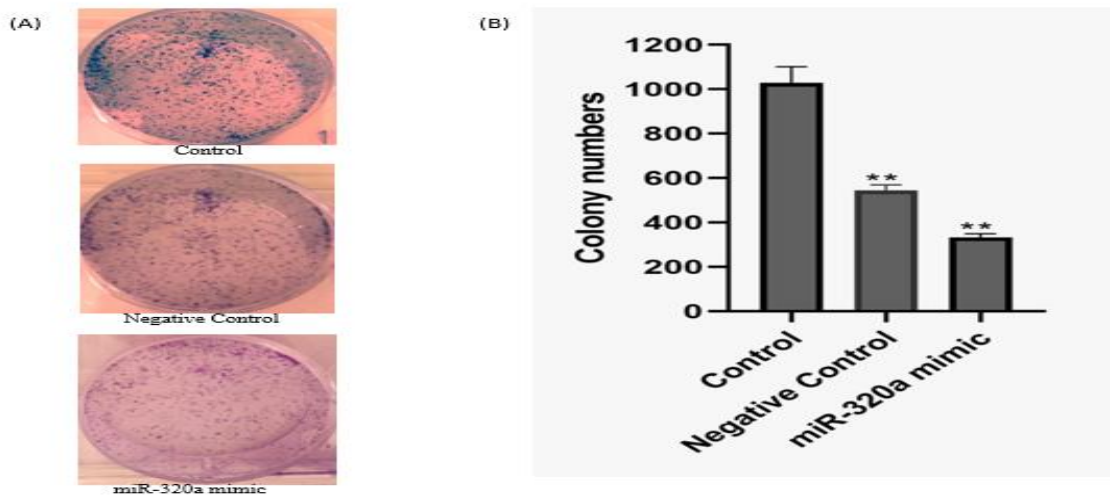


Figure 1. miR-320a inhibits the BC proliferation. (A) Colony formation in BC cell line (MDA-MB-231) was evaluated at 12 days after transfection with miR-320a mimic or Negative Control by colony formation assays. (B) The graph shows the mean colony numbers \pm SD for MDA-MB-231 cells that were transfected with miR-320a mimic, or Negative Control compared to Control, $p < 0.05$.

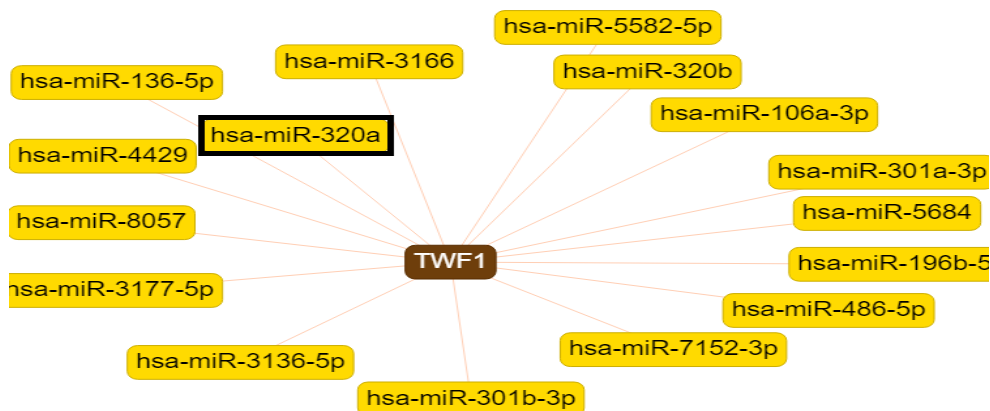


Figure 2. Different microRNAs that are predicted to target TWF1 in miRTargetLink Human Networks.

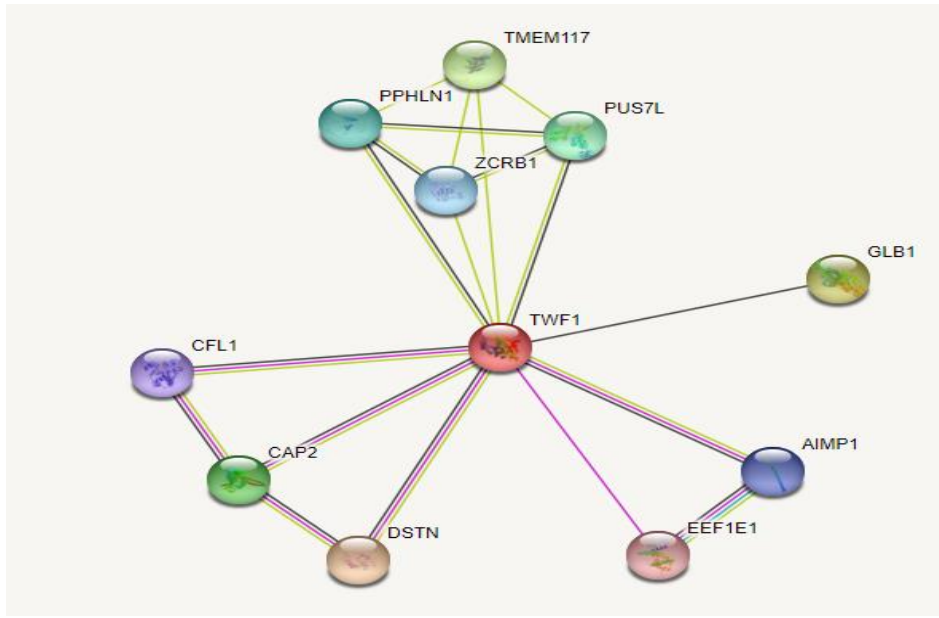


Figure 3. TWF1 and DSTN were predicted as functional partners in STRING Protein Interaction Networks.

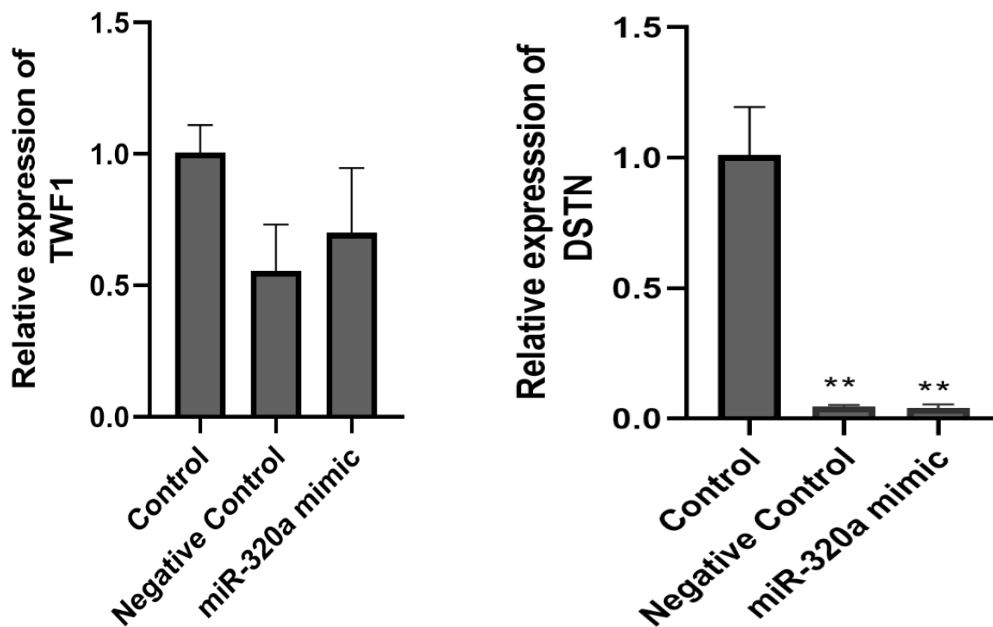


Figure 4. The effect of transfection with miR-320a on the expression level of (A) DSTN and (B) TWF1 genes. The changes expression levels of DSTN and TWF1 were assessed by qRT-PCR, after transfection with miR-320a of MDA-MB-231 (TNBC cells), calculated using the $\Delta\Delta$ CT method and expressed as fold change, $p < 0.05$.

3. RESULTS

3.1. miR-320a decreases colony formation of BC cells

The data revealed that transfection of MDA-MB-231 cells with miR-320a mimic resulted in a reduction in the formation of cell colony formation compared with Control (Figure 1).

3.2. Relation between TWF1, DSTN, and miR-320a

TargetScan Algorithm, CoMeTa database and miRTargetLink Human interactions Network showed that miR-320a is one of the miRNAs that regulate TWF1 (Figure.2). We revealed protein-protein interaction by STRING platform and the ARCHS4 database, Possible interaction of TWF1 with DSTN was found as shown in (Figure. 3), the combined score in STRING platform is (0.857)²¹, while the Pearson correlation score in ARCHS4 database is (0.5545) and DSTN rank 27²².

3.3. Effect of miR-320 modulation on the expression of the TWF1 and DSTN

The RT-PCR data demonstrated that transfection with miR-320a mimic resulted in a significant decrease of DSTN expression at the mRNA level. However, there was no change in the expression of TWF1, Compared with Control (Figure. 4).

4. DISCUSSION

Breast cancer is the most common malignancy in women around the world, posing a significant threat to women's health worldwide²³. Previous research has shown that TWF1 is Participates in cell migration, tumor growth, and metastatic potential of tumor^{24,25}. Its overexpression also promotes breast cancer cells EMT (Epithelial-mesenchymal transition) by activating transcription factors associated with mesenchymal lineages, like MKL1 and SRF and increasing cytoskeletal dynamics⁹. It has also been reported to increase cyclin D1 expression in breast epithelial cells and luminal A/B breast cancer cells^{26,27}. A recent pan-cancer analysis study has reported that TWF1 protein expression was significantly higher in breast cancer tissues compared to normal tissues. Also, there is a link between the high expression level of TWF1 and poor RFS (Relapse-free survival) and PPS (Post-progression survival) for breast cancer, implying that TWF1 is a potential biomarker for predicting breast tumor prognosis. Moreover, Focal adhesion and vesicle transportation were among the top hits in the GO|KEGG pathway analysis, implying that these pathways are involved

in TWF1's effect on cancer pathogenesis²⁸. Regulation of TWF1 inhibits tumor invasion and resistance to chemotherapy of human breast tumor^{13,29}. Those studies indicating that TWF1 may perform an essential role in the BC pathogenesis¹³. Findings indicate that miRNAs can act as tumor suppressors or oncogenes in many types of cancers by targeting mRNA degradation or regulating translational inhibition³⁰. However, the miRNA that regulates TWF1 in BC patients is not fully understood.

Previous research has revealed that some miRNAs such as miR-720³¹, miR-493³², and miR-125b-1³³ can affect the development of BC. regarding the miR-320 role in BC, increased expression of miR-320, according to Bai et al.⁸, inhibits breast cancer colony formation through upregulation of SRY-box transcription factor 4. Furthermore, miR320 inhibits the migration, invasion, and proliferation of BC by regulating aquaporin1(AQP1)³⁰. These previous studies are consistent with our study, we reported that cell proliferation was significantly decreased after transfecting BC cells with miR-320 as shown by colony formation assay (Figure.1), implying that miR-320 may act as a tumor suppressor.

Previous studies have shown that a single miRNA can control several target genes, or multiple miRNAs can control a common target gene in cancers³⁴. Many genes have been identified as miR-320 targets in various cancers. For example, miR-320 regulates Forkhead box protein M1, which influences colorectal cancer progression³⁵. Notably, TWF1 has been shown in some studies to be a target gene for specific miRNAs in other cancers and to function as an oncogene. TWF1 is upregulated, according to Sun et al.²⁵, and acts as a target of miR-30c to regulate the pancreatic cancer progression. DSTN works as an oncogene and develops lung cancer malignancy through facilitating β -catenin nuclear translocation and encouraging EMT¹¹.

In the present study, the connection between the expression of TWF1, DSTN and miR-320a was examined by qRT-PCR to evaluate the effect of miR-320a on mRNA expression of actin regulatory proteins. The results indicated that DSTN may be a target of miR-320a in TNBC (Figure.3). As far as we know, the current study is the first to show that miR-320a regulates DSTN in BC cells, this suggests a possible explanation for miR-320a's role in breast cancer progression. TWF1 mRNA expression did not change in miR-320a mimics transfected cells (Figure.3). This result suggests that miRNA regulatory effects may be less obvious than gene silencing by siRNA. The effect of using miRNA mimics and inhibitors to change the endogenous level of a single miRNA can be offset by other

miRNAs. Furthermore, miRNAs often have less than 50% repression of their target genes^{36,37}. Gene expression may also be regulated by multiple miRNAs and an effect may not be observed when using a single mimic or inhibitor^{34,38}.

5. CONCLUSION

This study suggested that miR-320a could be a tumor suppressor and a negative regulator of DSTN, which may play an important role in the occurrence and progress of breast cancer. Individual transfection with miR-320a cannot alter TWF1 expression. A series of additional alterations in the expression levels of RNA binding proteins or/and other miRNAs may be required in parallel to alter TWF1 gene expression. More studies are recommended to explain the role and mechanism of actin regulator proteins in the pathogenesis of BC.

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Conflicts of Interest: The authors declare no conflicts of interest.

Ethical Statement: The protocol followed in this study was approved by Al-Azhar Faculty of Pharmacy (Girls) scientific research ethical committee (no. 97, 2016).

Author contribution: This work was carried out in collaboration between all authors. Iman Hassan Ibrahim and Mohammed Mostafa Nooh designed the study. Hagar Salah Mahmoud performed the experiments and wrote the manuscript. Iman Hassan Ibrahim, Mohammed Mostafa Nooh and Hagar Salah Mahmoud analyzed the data and revised the manuscript.

List of abbreviations: TWF1: Twinifilin1; BC: Breast cancer; TNBC: Triple negative Breast Cancer; DSTN: Destrin; NC: Negative Control; miRNAs: MicroRNAs; EMT: Epithelial-mesenchymal transition; (si)RNA: short interfering RNA; UTR: untranslated region; cDNA: Complementary DNA; AQP1: Aquaporin 1; ATCC®: American Type Culture Collection

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