

ORIGINAL RESEARCH ARTICLE

Regulation of the DDAH/ADMA/NO axis by miR-193a and miR-939-5p in triple-negative breast cancer

Malak Ibrahim¹, Heba Nafea^{1,2} , Reham M. Abdel-Kader^{3,4}, Rana A. Youness^{5*} , and Mohamed Z. Gad^{1*} 

¹Biochemistry Department, Faculty of Pharmacy and Biotechnology, German University in Cairo, Cairo, Egypt

²Biochemistry Division, Faculty of Dentistry, German University in Cairo, Cairo, Egypt

³Pharmacology and Toxicology Department, Faculty of Pharmacy and Biotechnology, German University in Cairo, Cairo, Egypt

⁴University of London at European Universities in Egypt, Cairo, Egypt

⁵Molecular Genetics and Biochemistry Department, Faculty of Biotechnology, German International University, Cairo, Egypt

Abstract

Introduction: Dysregulated nitric oxide (NO) signaling plays a role in the viciousness of triple-negative breast cancer (TNBC). Dimethylarginine dimethylaminohydrolase 1 (DDAH1), which hydrolyzes asymmetric dimethylarginine (ADMA), an endogenously produced inhibitor of nitric oxide synthase (NOS), enhances NO production and promotes tumor vascularization, linking it to tumor progression. However, the roles of DDAH1 and its isoform DDAH2, and their regulation in the DDAH/ADMA/NO axis, remain poorly defined. Given their emerging role in gene regulation, microRNAs (miRNAs) may offer novel approaches for modulating this pathway in TNBC.

Objective: To investigate the regulatory effects of miR-939-5p and miR-193a on the DDAH/ADMA/NO axis in TNBC.

Methods: RNA was extracted from breast cancer (BC) tissues, and MDA-MB-231 TNBC cells were subjected to miRNA mimics. Gene expression was analyzed by quantitative reverse transcription-polymerase chain reaction, ADMA levels were quantified using a competitive enzyme-linked immunosorbent assay, and NO levels were measured with the Griess assay.

Results: *DDAH1*, but not *DDAH2*, expression was elevated in BC samples. Overexpression of either miR-939-5p or miR-193a suppressed *DDAH1* and *DDAH2* in TNBC cells, elevating ADMA levels and reducing NO bioavailability. In addition, overexpression of miR-193a directly influenced *NOS2* and *NOS3* transcripts, further suppressing NO synthesis. These dual effects highlight the capacity of miRNAs to regulate NO signaling at multiple levels.

Conclusion: This study highlighted the overexpression of *DDAH1* in 20 Egyptian BC patients, a previously underexplored area. Moreover, it provided the first evidence that miR-939-5p and miR-193a modulate the ADMA/NO pathway in TNBC. This was achieved by simultaneously suppressing *DDAH1/DDAH2* and *NOS* enzymes; the miRNAs shifted the balance toward ADMA accumulation and reduced NO signaling. These findings identify miR-939-5p and miR-193a as novel regulators of the DDAH/

*Corresponding authors:

Mohamed Z. Gad
(mohamed.gad@guc.edu.eg);
Rana A. Youness
(rana.youness@giu-uni.de)

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ADMA/NO axis in TNBC and provide new insights into the molecular mechanisms governing this pathway.

Keywords: Triple-negative breast cancer; Nitric oxide synthases; Nitric oxide; Dimethylarginine dimethylaminohydrolase; Asymmetric dimethylarginine; MicroRNAs; miR-193a; miR-939-5p

1. Introduction

Triple-negative breast cancer (TNBC) accounts for 15%–20% of all breast cancer (BC) cases and is considered one of the most aggressive BC subtypes. It is defined by the lack of expression of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2).¹ Owing to the lack of therapeutic intervention on these targets, TNBC patients have limited treatment options and are primarily managed with chemotherapy, often resulting in higher rates of recurrence, early metastatic dissemination, and poorer overall survival compared with other BC subtypes.^{1–4} TNBC is a highly heterogeneous disease at both the molecular and clinical levels, displaying enhanced proliferative capacity, genomic instability, and increased invasive and metastatic potential.

Among the pathways implicated in TNBC biology, nitric oxide (NO) has emerged as a context-dependent regulator of tumor progression. Elevated NO levels have been associated with angiogenesis, vasculogenic mimicry, tumor invasion, therapy resistance, and poor clinical outcomes, particularly in ER-negative BCs and TNBC.^{5–8} In breast tumors, NO is primarily generated by inducible nitric oxide synthase (iNOS/NOS2) and endothelial nitric oxide synthase (eNOS/NOS3), whose overexpression has been linked to larger tumor size, increased tumor aggressiveness, reduced survival, and activation of oncogenic signaling pathways, including epidermal growth factor receptor (EGFR)-mediated signaling.^{9–12}

The MDA-MB-231 cell line has been employed as an *in vitro* model to explore the molecular mechanisms governing NO-related signaling pathways in TNBC. MDA-MB-231 cells are among the most extensively characterized TNBC cell lines and exhibit a mesenchymal-like phenotype with high migratory and invasive capacities.¹³

Endogenous methylarginines tightly constrain NO production. Asymmetric dimethylarginine (ADMA) and N^G-monomethyl-L-arginine (L-NMMA) competitively inhibit NOS at the L-arginine site, while symmetric dimethylarginine (SDMA) limits L-arginine transport without directly inhibiting NOS.^{14–17} Dimethylarginine dimethylaminohydrolase 1 (DDAH1) is broadly expressed

and considered the principal ADMA-metabolizing enzyme.¹⁸ Across malignancies, upregulated DDAH expression has been linked to reduced ADMA, enhanced NO signaling, pro-angiogenic remodeling, and aggressive behavior.^{19–21} However, the expression levels of DDAH isoforms in BC have not been clearly investigated. In TNBC models, pharmacologic DDAH1 inhibition (e.g., ZST316, ZST152) elevates ADMA, reduces L-citrulline/NO output, and suppresses endothelial-like network formation *in vitro*, supporting the therapeutic rationale for targeting this axis; consistent effects are seen with the endogenous NOS inhibitor L-NMMA, while SDMA is inactive in this context.^{22–23}

Post-transcriptional control further tunes this pathway. MicroRNAs (miRNAs) regulate NOS isoforms and upstream activators and can directly target DDAH: miR-193b represses DDAH1, and other non-coding RNAs modulate NO signaling through phosphoinositide 3-kinase (PI3K)/Akt/eNOS and related cascades, adding a layer of context-dependent regulation in cancer cells and the tumor microenvironment.²⁰ In TNBC, additional miRNAs, such as miR-939-5p, intersect with gasotransmitter metabolism (e.g., cystathionine β -synthase [CBS]/cystathionine γ -lyase [CSE]), underscoring broader crosstalk between NO control and related pathways.^{24,25}

Together, these observations suggest that dysregulation of the DDAH1/DDAH2/ADMA/NO axis is a critical, yet underexplored, determinant of NO-driven aggressiveness in TNBC. This study unravels the expression of DDAH isoforms in Egyptian BC patients. Moreover, it investigates the regulation of this axis in TNBC, aiming to (i) define expression and functional relationships among DDAH isoforms, ADMA, and NOS activity; (ii) test how genetic perturbation of DDAH alters NO signaling and pro-tumorigenic behaviors; and (iii) explore miRNA-mediated regulation as a potential control point.

2. Materials and methods

2.1. Subjects

Breast cancer tissue specimens were collected from 20 Egyptian female patients diagnosed with BC who

underwent either breast-conserving surgery or lumpectomy at the National Cancer Institute (IORG0003381). For each patient, paired tumor and adjacent histologically normal safety-margin tissues, located 5–7 cm from the tumor boundary, were obtained. Fresh tissue specimens were rapidly frozen in liquid nitrogen upon collection and maintained at -80°C pending downstream analyses. The expression status of ER, PR, and HER2 was established using standard histopathological evaluation. Ethical approval for the study was obtained from the Institutional Review Board of the National Cancer Institute, Cairo University (IRB approval no. PA2407-106-067) and the Ethics Committee of the Faculty of Pharmacy and Biotechnology at the German University in Cairo. All study procedures complied with the ethical standards outlined in the Declaration of Helsinki.

2.2. Materials

The key equipment, reagents, kits, and supplies used in this research are listed in [Table A1](#).

2.2.1. Equipment and supplies

Standard cell-culture and molecular biology equipment were used (CO_2 incubator, Class II biosafety cabinet, centrifuges, micropipettes, plate reader, inverted microscope, thermal cycler, real-time polymerase chain reaction [PCR] system). Consumables were sterile and DNase/RNase-free.

2.2.2. Chemicals and reagents

The chemicals and reagents included Dulbecco's modified Eagle's medium (DMEM; 4.5 g/L glucose) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin; trypsin–ethylenediaminetetraacetic acid (EDTA); HiPerFect[®] transfection reagent; TaqMan[®] Gene Expression and MicroRNA assays *DDAH1* (Assay ID: Hs00201707_m1), *DDAH2* (Assay ID: Hs00967863_g1); hsa-miR-939-5p (Assay ID: MSY0004982); hsa-miR-193a-3p (Assay ID: MIMAT0000459), corresponding reverse transcription (RT) kits; QIAzol for RNA extraction; Griess Reagent System for NO (nitrite) quantification; and competitive ELISA kit for ADMA.

2.2.3. Solutions

Full culture medium and freeze mix were prepared as described in Section 2.3.2.

2.2.4. Cell lineage

Experiments were conducted using the MDA-MB-231 cell line, a well-established model of human TNBC.

2.3. Methods

2.3.1. Bioinformatics analysis

In silico analysis was performed to identify novel miRNAs potentially targeting *DDAH1* and *DDAH2* transcripts using four prediction tools: microRNA.org, miRDB, DIANA Lab, mirWalk, and TargetScan. Candidate miRNAs were evaluated based on binding complementarity, scores, hybridization energy, and the number of 3' untranslated region (UTR) binding sites, with more negative energy values indicating stronger interactions.

2.3.2. Cell culture

MDA-MB-231 cells were cultured in DMEM containing 4.5 g/L glucose, *L*-glutamine, 10% FBS, and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2 , using appropriate plate formats for each experiment. Cells were subcultured at 70%–80% confluence to maintain logarithmic growth. For cryopreservation, cell suspensions were mixed 1:1 with freeze mix (final 10% DMSO, 20% FBS) and stored at -80°C ; rapid thawing was followed by medium exchange and recovery.

2.3.3. Transfection (HiPerFect[®])

The principle of transfection is as follows: cationic lipid complexes facilitate the cellular uptake of miRNA oligonucleotides (lipofection) via endocytosis, enabling transient gene silencing. Cells were seeded to 60%–80% confluence and transfected with miR-193a-3p or miR-939-5p according to the manufacturer's guidelines (optimized oligo and reagent amounts per plate format). Mock wells received reagent only. Knockdown was assessed 48 h post-transfection.

2.3.4. RNA isolation and complementary DNA preparation

RNA extraction was carried out from both tissue samples and cultured cells using QIAzol reagent following the supplier's recommended protocol. The resulting RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water and preserved at -80°C until analysis. For downstream gene expression studies, RT was performed to produce complementary DNA (cDNA). A high-capacity RT kit was employed for messenger RNA (mRNA) targets, whereas miRNA-specific cDNA was generated using the TaqMan[®] MicroRNA RT Kit.

2.3.5. Quantitative PCR-based expression analysis

TaqMan[®] assays use a hydrolysis probe (5' reporter/3' nonfluorescent quencher–minor groove binder [NFQ–MGB]). Probe cleavage during extension releases

fluorescence proportional to amplicon quantity. *DDAH1* and *DDAH2* were quantified using gene-specific assays; 18S ribosomal RNA and β -actin served as endogenous controls (in a multiplex format). MiRNAs were quantified with specific TaqMan® miRNA assays and appropriate small-RNA controls. Data were analyzed using the comparative Ct method (Δ Ct, $\Delta\Delta$ Ct; relative quantification [RQ] = $2^{-\Delta\Delta$ Ct}).

2.3.6. Nitric oxide measurement (Griess)

Nitric oxide production was assessed indirectly by measuring nitrite (NO_2^-), a stable oxidation product of NO, using the Griess assay. Cell culture supernatants were incubated with the Griess reagents, resulting in the formation of a colored azo compound that was quantified spectrophotometrically at 540–550 nm. A sodium nitrite standard curve was used to calculate nitrite concentrations, and the data were subsequently expressed relative to controls and reported as percentage values.

2.3.7. ADMA measurement (competitive ELISA)

Sample ADMA competes with plate-bound ADMA for binding to a biotinylated anti-ADMA antibody; signal (horseradish peroxidase [HRP]/3,3',5,5'-tetramethylbenzidine [TMB]) is inversely proportional to ADMA concentration. Standards and samples were incubated per kit protocol (E-EL-0042), washed, developed,

stopped, and read at 450 nm; concentrations were derived from a four-parameter logistic standard curve. Results are expressed as percentages of ADMA in mock cells.

2.4. Statistical analysis

Prior to analysis, data were evaluated for normality. Differences between two groups were assessed using the unpaired Student's *t*-test, whereas comparisons involving more than two groups were examined using one-way ANOVA with Dunnett's multiple comparisons post hoc tests. Gene expression data obtained from qPCR assays were reported as RQ values and presented as mean $\pm$ SEM. Statistical significance was assigned at $p < 0.05$, with significance levels denoted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; non-significant results are indicated as ns. Statistical analyses were performed using GraphPad Prism v8.0 (United States of America).

3. Results

3.1. Expression of *DDAH1* and *DDAH2* in Egyptian breast cancer patients

Screening of *DDAH1* and *DDAH2* was performed in 20 female BC patients, and the expression patterns in BC and normal counterparts from the same patients were compared. The transcripts of *DDAH1* (Figure 1A) were significantly higher than those of their normal counterparts. Meanwhile, *DDAH2* (Figure 1B) was not significantly altered.

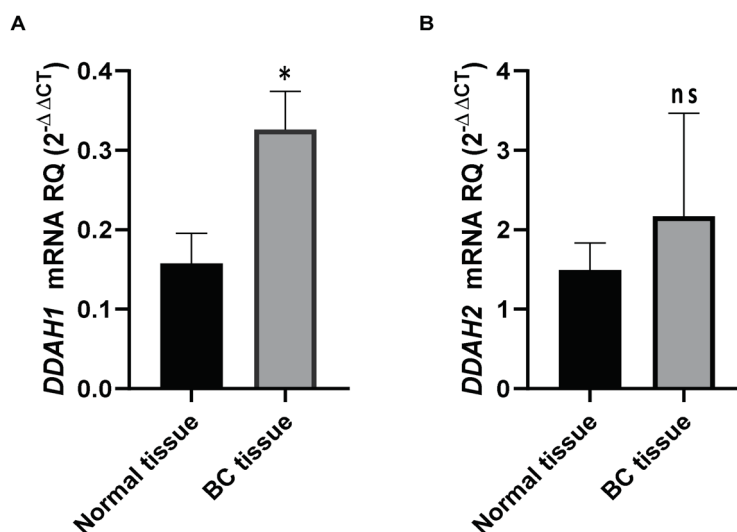


Figure 1. Quantitative reverse transcription polymerase chain reaction analysis of *DDAH1* and *DDAH2* mRNA expression in breast cancer (BC) tissues and matched adjacent normal tissues. (A) *DDAH1* expression was significantly increased in BC tissues compared with the corresponding normal controls. (B) No significant difference was observed in *DDAH2* expression between BC and normal tissues.

Notes: Statistical comparisons were conducted using an unpaired Student's *t*-test. Data are presented as mean $\pm$ SEM. * $p < 0.05$; ns: Non-significant. Abbreviations: mRNA: Messenger RNA; RQ: Relative quantification.

3.2. Effects of miR-939-5p on DDAH1/DDAH2/ADMA axis in MDA-MB-231 cells

In an earlier study by our group, NOS2 and NOS3 were shown to be upregulated in BC tissues, accompanied by elevated NO production. We further identified miR-939-5p as a key regulator of this pathway. Transfection of BC cells with miR-939-5p suppressed NOS2 and NOS3 expression, thereby reducing NO levels.²⁵ Building on these findings, we explored whether miR-939-5p could also regulate *DDAH1* and *DDAH2*.

To evaluate the effect of miR-939-5p, MDA-MB-231 cells were transfected with miR-939-5p and scrambled (Scr)-miRNA oligonucleotides. The expression levels of *DDAH1* and *DDAH2* in the transfected cells were evaluated via quantitative RT-PCR. ADMA was analyzed by ELISA, and NO levels were measured by the Griess assay. Transfection with miR-939-5p resulted in a significant reduction in the expression of both *DDAH1* and *DDAH2* in MDA-MB-231 cells compared with that in control cells (Figure 2A–2B). Figure 2C–2D show a significant increase in ADMA levels and a decrease in NO levels, respectively, post-transfection with miR-939-5p, which coincides with a significant decrease in *DDAH1/DDAH2*. These observations highlight the regulatory influence of miR-939-5p on *DDAH1/DDAH2* and its subsequent role in ADMA metabolism in MDA-MB-231 cells.

3.3. Bioinformatics analysis

Recent advances have highlighted effective tools for regulating gene expression, including the use of silencing enzymes involved in cancer and tumor progression. Building on this foundation, miRNAs were used to target *DDAH1* and *DDAH2* in TNBC. A computational algorithm is the most common technique for predicting miRNA–target transcript interactions. The search for tumor suppressor miRNAs, which simultaneously target *DDAH1* and *DDAH2*, required extensive sorting of data from five different online algorithms: miRDB (www.mirdb.org/mirdb/), DIANA Lab (www.diana.cslab.ece.ntua.gr/), TargetScan (www.targetscan.org/), mirWalk (<http://mirwalk.umm.uni-heidelberg.de/>), and microRNA.org (www.microrna.org/). The final search identified the candidate miRNA hsa-miR-193a-3p as a potential target for *DDAH1* and *DDAH2* in miRBase (<https://mirbase.org/>). The accession number and the mature sequence were obtained (Table 1).

3.4. Effects of miR-193a on DDAH1/DDAH2/ADMA axis in MDA-MB-231 cells

To investigate the effect of miR-193a on the *DDAH1/DDAH2/ADMA* pathway, MDA-MB-231 cells were

transfected with either miR-193a or Scr-miRNA oligonucleotides. Overexpression of miR-193a resulted in a significant reduction in both *DDAH1* and *DDAH2* expression levels compared with the mock group (Figure 3A–3B). Consistent with the observed decrease in *DDAH1* expression, a significant increase in ADMA levels was detected following miR-193a transfection (Figure 3C). These findings suggest that miR-193a modulates the *DDAH1/DDAH2/ADMA* pathway in MDA-MB-231 cells.

3.5. Effects of miR-193a on NOS2/NOS3 expression and NO levels in MDA-MB-231 cells

After demonstrating the direct effect of miR-193a on the *DDAH/ADMA* axis, it was worth investigating whether it also has an indirect role in NOS enzymes. Overexpression of miR-193a in MDA-MB-231 cells resulted in significant suppression of *NOS2* and *NOS3* transcript levels compared with those in mock cells (Figure 4A–4B). Moreover, a reduction in NO levels was observed (Figure 4C).

Table 1. Accession number and mature sequence of miR-193a-3p

ID	miR-193a-3p
Accession number	MIMAT0000459
Mature sequence	5'AACUGGCCUACAAAGUCCAGU-3'

4. Discussion

Breast cancer remains the second-most common malignancy and the leading cause of cancer-related mortality among women worldwide.²⁶ Among its subtypes, TNBC is the most aggressive, accounting for 10%–20% of all BC cases. TNBC is typically associated with high histological grade, early systemic relapse, elevated recurrence rates, and enhanced metastatic potential, all of which contribute to poor clinical outcomes and low survival rates. A defining feature of TNBC is the absence of ER, PR, and HER2 expression, significantly restricting therapeutic options compared with other BC subtypes. Consequently, extensive research efforts have focused on developing molecularly targeted strategies by identifying key genes and pathways implicated in TNBC initiation and progression.²⁷

Nitric oxide is a small, diffusible free radical that functions as a signaling molecule in diverse physiological

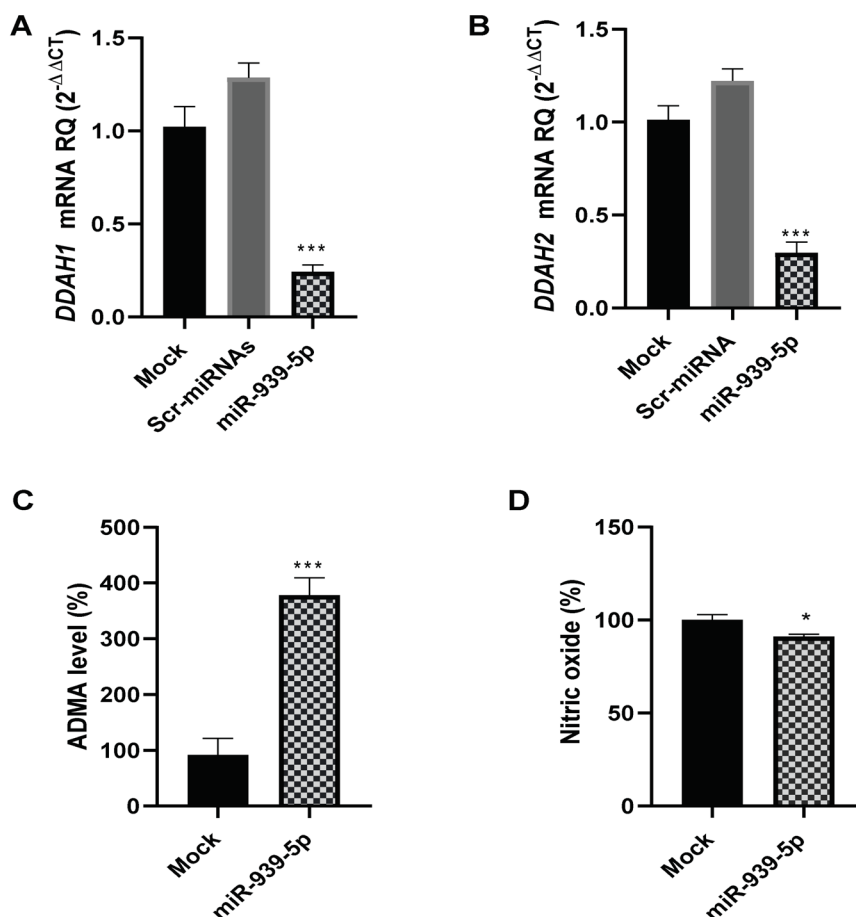


Figure 2. Effects of miR-939-5p transfections on the DDAH1/DDAH2/ADMA/NO axis in MDA-MB-231 cells. (A) *DDAH1* mRNA level. (B) *DDAH2* mRNA level. (C) ADMA level. (D) Nitric oxide level.

Notes: Data presented as mean $\pm$ SEM from three independent experiments. Statistical analyses were performed using one-way ANOVA and Student's *t*-test, as appropriate. * $p < 0.05$; *** $p < 0.001$ versus the mock group.

Abbreviations: ADMA: Asymmetric dimethylarginine; DDAH: Dimethylarginine dimethylaminohydrolase; miRNA: MicroRNA; mRNA: Messenger RNA; NO: Nitric oxide; RQ: Relative quantification.

processes, including vascular tone regulation, angiogenesis, and immune responses. Beyond these physiological roles, NO has been implicated in pathological conditions such as cancer, where its effects are complex and context-dependent, ranging from tumor-promoting to tumor-suppressive activities.²⁸⁻³⁰ NO is generated by NOS enzymes, of which three isoforms exist: NOS2, NOS3, and NOS1. Among these, NOS2 and NOS3 are most frequently linked to tumorigenesis and metastasis. Elevated NOS2 expression correlates with poor prognosis, including reduced overall survival in patients with stage III melanoma,³¹ and has also been reported in subsets of

BC.³² Conversely, the absence of NOS2 expression in stage III ovarian cancer has been associated with improved responses to first-line chemotherapy.³³ Furthermore, in oral squamous cell carcinoma, iNOS overexpression has been linked to tumorigenesis and disease prognosis, indicating its potential prognostic value.³⁴

The role of NOS3 in cancer remains controversial, with conflicting findings across tumor models. For example, *NOS3*-deficient mice exposed to the carcinogen *N*-diethylnitrosamine developed significantly higher rates of liver tumors than their wild-type counterparts, while overexpression of *NOS3* in hepatic tumor cells reduced

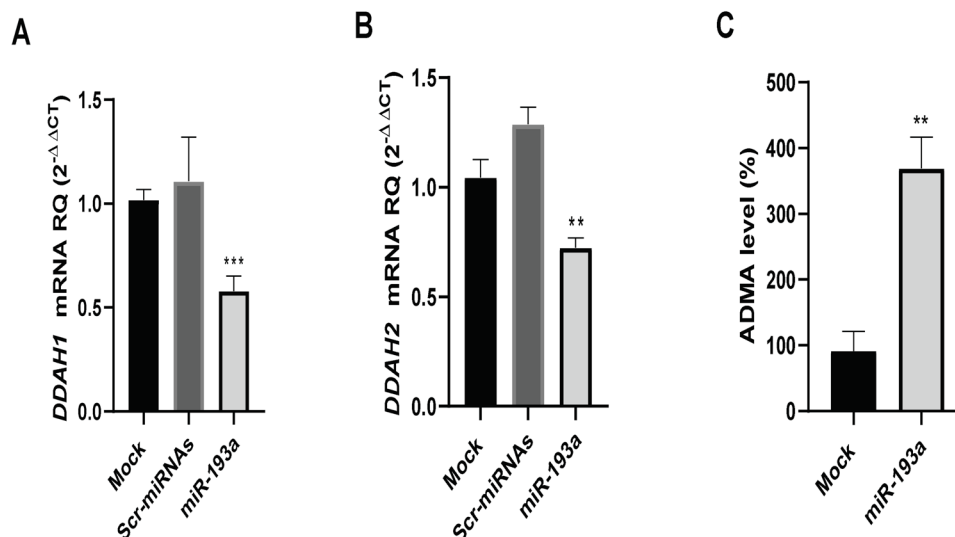


Figure 3. The impact of miR-193a overexpression on the DDAH1/DDAH2/ADMA/NO axis in MDA-MB-231 cells. (A) *DDAH1* mRNA level. (B) *DDAH2* mRNA level. (C) ADMA level.

Notes: Data presented as mean $\pm$ SEM from three independent experiments. Statistical analyses were performed using one-way ANOVA and Student's *t*-test, as appropriate. ***p* < 0.01; ****p* < 0.001 versus the mock group.

Abbreviations: ADMA: Asymmetric dimethylarginine; DDAH: Dimethylarginine dimethylaminohydrolase; miRNA: MicroRNA; mRNA: Messenger RNA; NO: Nitric oxide; RQ: Relative quantification.

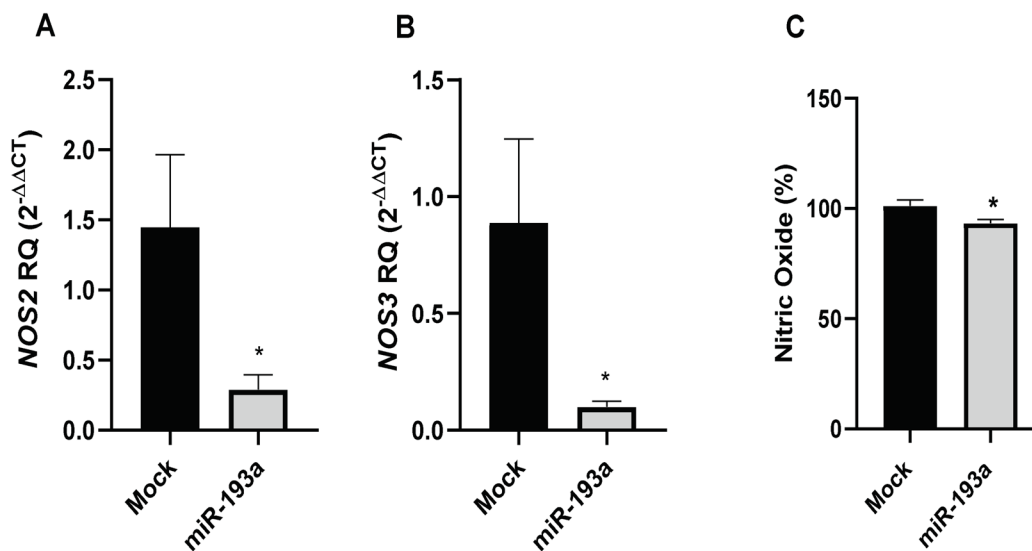


Figure 4. Effects of miR-193a overexpression on *NOS2*/*NOS3* expression and NO levels in MDA-MB-231 cells. (A) *NOS2*. (B) *NOS3*. (C) NO.

Notes: Data presented as mean $\pm$ SEM from three independent experiments. Statistical analyses were performed using Student's *t*-test. **p* < 0.05 versus the mock group.

Abbreviations: NO: Nitric oxide; RQ: Relative quantification.

micrometastatic burden.³⁵ In contrast, other studies have associated NOS3 overactivity with enhanced tumor growth, invasion, and angiogenesis, particularly in BC.³⁶ Evidence regarding NOS1 is limited and inconclusive; in astrocytoma, progressive increases in NOS1 expression were observed with advancing tumor grade,³⁷ but mechanistic insights into its role in oncogenesis remain lacking.

Endogenous inhibition of NO synthesis is primarily mediated by ADMA, a competitive inhibitor of NOS. Compared with *L*-NMMA, circulating ADMA exists at higher concentrations and is considered the predominant physiological NOS regulator.^{14,38} The enzyme DDAH metabolizes ADMA to *L*-citrulline and dimethylamine, thereby modulating NO bioavailability.³⁹ Dysregulation of the DDAH/ADMA/NO pathway enhances local NO levels and has been strongly implicated in tumor angiogenesis, growth, invasion, and metastasis.^{40,41} Elevated DDAH expression has been documented across various cancers; however, our study was one of its kind in highlighting *DDAH1* and *DDAH2* expression in BC patients.

Inhibition of DDAH activity has demonstrated therapeutic promise by suppressing tumor progression in both *in vitro* and *in vivo* models.^{21,42} Aberrant miRNA expression is a further driver of oncogenesis, arising through defects in biogenesis, genomic alterations, transcriptional dysregulation, or epigenetic modifications.⁴³ Functionally, miRNAs act as tumor suppressors or oncogenes, depending on their regulatory targets.⁴⁴ Moreover, miR-125b is considered a tumor suppressor in some tumor types and an oncogene in other types, although the mechanism remains the same across all tissues.⁴⁵ Another example is the miR-29 family, which has been reported to have dual roles. However, they can act as oncogenic miRNAs.^{46,47} Another miRNA that affects cluster expression in BC cell lines is miR-191/425, which is dependent on estrogen receptor α (ESR1) expression. ESR1-positive cells express high levels of the miR-191/425 cluster, leading to the suppression of the tumor suppressor gene early growth response 1 (EGR1). Moreover, ESR1-negative cells express low levels of the miR-191/425 cluster. When both miR-191 and miR-425 are overexpressed in ESR1-negative cells, tumor growth and metastasis are impaired. These examples illustrate that the same miRNA can act as an oncogene or a tumor suppressor, not only across different types of cancer, as with miR-125b and miR-29b, but also within the same type of cancer, as with the miR-191/425 cluster.⁴⁸

Our group has previously shown that NOS2 and NOS3 are upregulated in BC tissues, accompanied by elevated NO production.²⁵ We further identified miR-939-5p as a key regulator of this pathway: transfection of

BC cells with miR-939-5p suppressed NOS2 and NOS3 expression, thereby reducing NO levels. Building on these findings, we explored whether miR-939-5p could also regulate *DDAH1* and *DDAH2*. In MDA-MB-231 cells, miR-939-5p expression led to significant downregulation of both DDAH isoforms and an accumulation of ADMA, confirming a regulatory role in the DDAH/ADMA/NO axis. While miR-939-5p targeted both DDAH isoforms, the observed accumulation of ADMA can be attributed to the downregulation of *DDAH1*, as recent multicentric evidence confirms that *DDAH2* lacks the catalytic machinery to metabolize ADMA; consequently, the simultaneous reduction of *DDAH2* potentially exerted its functional effect through ADMA-independent pathways, such as the regulation of vascular endothelial growth factor (VEGF)-mediated angiogenesis or mitochondrial dynamics.¹⁸

Subsequently, we investigated miR-193a as another candidate regulator. *In silico* analysis and literature reports supported its tumor-suppressive role across several cancers, including prostate cancer,⁴⁹ colorectal cancer,⁵⁰ head and neck squamous cell carcinoma,⁵¹ and myeloid leukemia.⁵² Experimental validation in MDA-MB-231 cells demonstrated that miR-193a downregulated both *DDAH1* and *DDAH2*. Like the findings of miR-939-5p, the increased ADMA observed should be attributed to decreased *DDAH1*. Moreover, ectopic expression of miR-193a also suppressed NOS2/NOS3 levels and NO production, suggesting both direct (via DDAH/ADMA) and indirect (via NOS2/NOS3) regulatory effects on NO availability. These results highlight miR-193a as a versatile regulator of the DDAH/ADMA/NO pathway, consistent with the reported tumor-suppressive functions of its family member, miR-193b.²⁰

Taken together, our findings identify miR-939-5p and miR-193a as critical modulators of NO signaling in BC. By downregulating both DDAH enzymes and NOS isoforms, these miRNAs elevate ADMA levels and suppress NO bioavailability, thereby limiting tumor-promoting NO signaling. This dual regulatory mechanism underscores their potential as promising therapeutic candidates in the management of BC.

5. Conclusion

In conclusion, this study provides the first characterization of *DDAH1* expression in Egyptian female BC patients. Through *in silico* analysis, miR-193a was identified as a potential regulator of *DDAH1* and *DDAH2* and subsequently validated experimentally. In MDA-MB-231 cells, both miR-193a and miR-939-5p significantly reduced *DDAH1* and *DDAH2* expression. The reduction in *DDAH1* expression was associated with increased

ADMA levels, supporting DDAH1 as a key regulator of ADMA homeostasis in TNBC cells. Notably, this study demonstrated the regulation of the DDAH/ADMA pathway by miR-193a and miR-939-5p in TNBC. Collectively, these findings provide novel insights into the miRNA-mediated regulation of the DDAH/ADMA/NO pathway and contribute to a better understanding of the molecular mechanisms governing NO-related signaling in TNBC.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization: Heba Nafea, Rana A. Youness, Mohamed Z. Gad

Investigation: Malak Ibrahim, Heba Nafea, Rana A. Youness

Writing—original draft: Malak Ibrahim, Heba Nafea, Mohamed Z. Gad

Writing—review & editing: Heba Nafea, Reham M. Abdel-Kader, Rana A. Youness, Mohamed Z. Gad

Ethics approval and consent to participate

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Institutional Review Board (IRB) of the National Cancer Institute, Cairo University (Approval number: PA2407-106-067) and the Ethics Committee of the Faculty of Pharmacy and Biotechnology at the German University in Cairo. The approval covered all study procedures involving human participants and their biological specimens in accordance with institutional and national ethical guidelines. Informed consent was obtained from all participants prior to their enrollment.

Consent for publication

Informed consent was obtained from all individual participants included in the study to both participate and have their data published in a research article.

Availability of data

The study data are available upon reasonable request to the corresponding authors.

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Appendix

Table A1. Key equipment, reagents, kits, and supplies used in this study

Item	Description/use	Manufacturer (country)	Notes
Cell line	Human triple-negative breast cancer (TNBC) cell line, MDA-MB-231	Vacsera (Egypt)	–
Cell culture medium	DMEM, 4.5 g/L glucose + L-glutamine	Lonza (Germany)	–
Serum	Fetal bovine serum (FBS), heat-inactivated	Lonza (Germany)	–
Antibiotics	Penicillin/Streptomycin (100×)	Lonza (Germany)	–
Trypsin–EDTA	Cell detachment	Standard supplier	–
Cryopreservation	Cryovials, 1.8 mL	Apogent (Denmark)	–
Transfection reagent	HiPerFect® transfection reagent	Qiagen (Germany)	Cat. No. 301704
miRNA oligonucleotides	hsa-miR-939-5p (MSY0004982); hsa-miR-193a-3p (MIMAT0000459); scrambled control (MSY0000449)	Life Technologies/Applied Biosystems (USA)	IDs as listed
RNA extraction reagent	QIAzol/BIZOL reagent	Qiagen (Germany)	Cat. No. 79306
Reverse transcription kit (mRNA)	High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems (USA)	Cat. No. 4368814
Reverse transcription kit (miRNA)	TaqMan® MicroRNA RT Kit	Applied Biosystems (USA)	Cat. No. 4366596
qPCR master mix	TaqMan® Universal PCR Master Mix, 2× + ROX	Applied Biosystems/Thermo Fisher (USA)	Cat. No. 4304437
Gene expression assays	TaqMan® assays for DDAH1, DDAH2, 18S rRNA, β-actin	Thermo Fisher (USA)	IDs per assay
MicroRNA assays	TaqMan® assays for hsa-miR-939-5p, hsa-miR-193a-3p	Life Technologies (USA)	MSY0004982, MIMAT0000459
NO measurement kit	Griess Reagent System	Promega (USA)	Cat. No. G2930
ADMA ELISA kit	Competitive ELISA kit (human ADMA)	Elabscience® (China)	Cat. No. E-EL-0042
Spectrophotometer/plate reader	Wallac 1420 VICTOR2 multilabel counter	PerkinElmer (USA)	–
Real-time PCR system	StepOne™ Real-Time PCR	Applied Biosystems (USA)	–
CO ₂ incubator	Cell culture incubator	Medcenter Einrichtungen GmbH (Germany)	–
Laminar flow hood	Class II biosafety cabinet BH-EN2004	Faster (Germany)	–
Centrifuges	Benchtop (Eppendorf 5417C), refrigerated (Sigma 3K30), microcentrifuge (Hettich EBA20)	Eppendorf/Sigma-Aldrich/Hettich (Germany)	–
Microscope	Inverted microscope TCM400	Labomed (USA)	–
Freezer	–80 °C ultra-low	REVCO/Kendro (USA)	–
General reagents	DMSO, ethanol, isopropanol, chloroform, glutaraldehyde, quartz cuvettes	Sigma-Aldrich/Merck (Germany)	–
Consumables	Pipettes, sterile tips, tubes (DNase/RNase-free), tissue culture plates	Eppendorf (Germany), AHN Biotechnology (Germany), Greiner Bio-One (Austria)	–