Long Non-Coding RNA ANRIL Promotes Doxorubicin Resistance in Triple-Negative Breast Cancer via Suppressing Glycolysis Through the MicroRNA-125a/ENO Pathway

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Research Article

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Abstract

Background

Breast cancer is the main cause of death among women worldwide. More and more long non-coding RNAs (lncRNAs) have been identified as oncogenes or tumor suppressors during cancer development. However, whether ANRIL is involved in drug resistance in triple-negative breast cancer (TNBC) has not been investigated.

Methods

Luciferase reporter assay was conducted to verify the binding of miR-125a and ANRIL. RT-PCR and western blot were performed to detect the expression of miR-125a, ANRIL and ENO1. Gene silence and overexpression experiments as well as CCK-8 and colony formation assays on TNBC cell lines were performed to determine the regulation of molecular pathways. Glycolysis analysis was performed with Seahorse extracellular flux methodology.

Results

ANRIL expression in TNBC patients and TNBC cells was examined and we found that ANRIL expression was upregulated in both TNBC patients and TNBC cell lines. Knockdown of ANRIL increased the cytotoxic effect of ADR and inhibited HIF-1α-dependent glycolysis in TNBC cells. In addition, we found that ANRIL negatively regulated miR-125a expression in TNBC cell lines. Besides, a dual-luciferase reporter assay proved ANRIL functioned as a sponger of miR-125a. Further investigation revealed that ENO1 was a target of miR-125a and positively regulated by ANRIL in TNBC cells. Additionally, ANRIL upregulation reversed miR-125-mediated inhibition on HIF-1α-dependent glycolysis in TNBC cells. More notably, 2-deoxy-glucose (2-DG) attenuated ANRIL-induced increase of drug resistance in TNBC cells.

Conclusions

Taken together, our study was the first to identify that knockdown of ANRIL plays an active role in overcoming the drug resistance in TNBC by inhibiting glycolysis through the miR-125a/ENO1 pathway, which maybe serve useful for the development of novel therapeutic targets.

Introduction

Breast cancer is the main cause of death among women worldwide[1, 2]. Triple-negative breast cancer (TNBC) which lacks the expression of HER2, progesterone, and estrogen receptors, possesses an aggressive clinical phenotype[3-5]. Chemotherapy rather than a hormone or targeted immunotherapy is now used as standard treatment for TNBC patients[6-8]. Adriamycin/doxorubicin (ADR) is a cytotoxic drug (an anthracycline antibiotic) that is commonly used in chemotherapy in TNBC patients. Nevertheless, many TNBC patients develop resistance to ADR-therapy, which leads to relapse and patient death.

Dysregulation of lncRNAs is frequently observed during carcinogenesis and contributes to cancer development[9, 10]. Accumulating evidence suggests that lncRNAs are involved in the development of TNBC by regulating DNA damage repair, cell cycle, apoptosis and angiogenesis[11-13]. For example, lncRNA HEIH was shown to modulate cell proliferation and apoptosis through miR-4458/SOCS1 axis in TNBC[14]. LINC00096 increased cell proliferation by targeting miR-383-5p/RBM3 axis in TNBC[15]. LncRNA GAS5 was reported to promote apoptosis and inhibit proliferation of TNBC cells by targeting miR-196a-5p and miR-378a-5p/SUFU signalling[16]. LncRNA CCAT1 could promote TNBC cells migration and invasion by suppressing miR-218/ZFX signalling[17]. These studies evoke the potential of altering lncRNAs expression in future to represent a novel therapeutic approach to reverse anti-chemotherapy in TNBC patients. However, further studies and mechanistic investigations of the regulation mechanism of lncRNAs-mediated drug resistance in TNBC are needed.

LncRNA ANRIL is an antisense non-coding RNA in the INK4A locus and is widespread in many types of human cancers such as breast cancer[18]、ovarian cancer[19, 20], gastric cancer[21], melanoma[22, 23] and non-small cell lung carcinoma[24, 25]. In nasopharyngeal carcinoma cells, downregulation of lncRNA ANRIL inhibits proliferation, induces apoptosis, and enhances radiosensitivity in nasopharyngeal carcinoma cells through regulating miR-125a[26]. In TNBC tissue and cells, LncRNA ANRIL promoted carcinogenesis via sponging miR-199a in TNBC tumor pathological[27]. However, the role and the regulation mechanism of lncRNA ANRIL-mediated chemoresistance in TNBC remains unclear.

LncRNAs regulate gene expression via functioning as microRNA sponge[28, 29]. miRNAs are short, non-coding RNA molecules that bind to 3'UTR of mRNAs to regulate target gene expressions. Many miRNAs are closely related to TNBC tumor progression and chemoresistance[30, 31]. miR-125a has been reported to be abnormally expressed in prostate carcinoma[32]. Various studies have shown miR-125a to be closely related to tumor cell growth, differentiation, and metastasis[33-35]. Especially, miR-125a has been reported to control the proliferation, apoptosis, migration in MCF-7 breast cancer cells[36]. Nevertheless, whether and how miR-125a regulates drug resistance in TNBC is largely unknown.

In this study, we provide compelling evidence that ANRIL was upregulated in TNBC patient tumor tissues and TNBC cell lines. Mechanistically, we discovered an ANRIL/miR-125a/ENO1 axis in mediating chemotherapy sensitivity against adriamycin. Our findings may provide insights into conquering chemotherapy resistance of TNBC patients.

Methods

Cell culture

The TNBC cell line MCF10A, MCF7, T47D, MDA-MB-231, BT549, and HS578T cells were kindly provided by the Cell Bank / Stem the Cell Bank, Chinese Academy of Sciences. The cells were cultured in

Dulbecco's Modified Eagle's Medium (DMEM; ThermoFisher) containing 10% fetal bovine serum (FBS; ThermoFisher), 1% penicillin, and streptomycin, and cultured at 37 °C, 5% CO_2.

Total RNA isolation and qRT-PCR assays

RNA was extracted from the OS cellular samples using with TRIzol reagent (Thermo, USA). cDNA was generated by High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). qRT-PCR was performed using the VII7 system (Applied Biosystems) with the SYBR™ Green PCR Master Mix plus (ThermoFisher) for quantification analysis. RIP experiments were performed with the Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Co-precipitated RNAs were subjected to RT–qPCR analysis. U1 RNA was used as the housekeeping control. The primers used in this study are as follows: ANRIL-F: 5'-GCCTCATTCTGATTCAACA-3'; ANRIL-R: 5'-TAGAAAGCAGTACTGACTCGG-3'.

Lentivirus vector construction and siRNA transfection

The knockdown and overexpression lentivirus vectors for ANRIL were produced by OBiO (Shanghai, China). The siRNAs against ANRIL (si-ANRIL) and negative control (NC) were synthesized by OBiO (Shanghai, China). TNBC cells were transfected with siRNAs with the Lipofectamine 3000 Reagent (Thermo, USA) according to the manufacturer's protocol.

Protein extraction and western blot analysis

Total proteins from the breast cancer tissues or the TNBC cells were extracted and analyzed as previously reported. The antibodies for the western blot analysis in this study were as follows: anti-HIF1a (Abcam, ab82832), anti-ENO1 (Abcam, ab85086), anti-β-actin (CST, #3700).

Cell viability assay and colony formation assay

Cell Counting Kit-8 (CCK8) assay (Abcam, ab228554) was used for the Cell viability assessment according to the manufacturer's protocol. For the colony formation assay, cells were seeded in 12-well plates and then treated with ADR. The cells were harvested using trypsin, collected, and reseeded at a density of 2000 cells/well in 12-well plates 2 hours later. Then, the colonies were fixed and stained with 0.1% crystal violet 2 weeks later.

Cell apoptosis assay

Cell apoptosis was measured using an Annexin-V-FITC/PI double staining apoptosis kit (Abcam) according to the manufacturer's instructions. Flow cytometry (Beckman Coulter, CA, USA) was used to analyze apoptosis. Annexin-V^+/PI^- cells were the apoptotic cells.

Xenograft tumor

All live mice manipulations were performed according to guidelines and protocol approved by the Institutional Animal Care and Use Committee at Harbin Medical University Cancer Hospital. For xenograft experiments, BT-549 cells transfected with shANRIL or negative control vector were harvested and suspended in a density of 1×10^7 cells/100 μL in saline and injected subcutaneously into the right flank of each mouse. The volume of tumor was measured every 5 days and determined according to the equation: $0.5 \times \text{length} \times \text{width}^2$. At 4-week post injection, the mice were killed by cervical dislocation, and the tumors were excised and photographed.

Immunohistochemistry

The tumor tissues were embedded with OCT and quickly frozen in the liquid nitrogen after weighting. 5-μm-thick sections were made in a Cryostat (Leica, Germany). Slides were blocked with 5% BSA at RT for 1 hour. The first antibody against Ki67 was incubated at RT for 2 hours a 37 ℃. The second antibody was incubated for 1 hour at RT, and then uses diaminobenzidine (DAB) as chromogen.

Statistical analysis

The results are shown as the mean ± SEM. The differences between the 2 groups were analyzed by a two-tailed unpaired Student's t-test. One-way ANOVA followed by the Tukey multiple-comparisons test was applied for paired comparisons. Values of $p<0.05$ were considered statistically significant.

Results

ANRIL overexpression augmented Adriamycin (ADR)-induced cytotoxic effect in TNBC cells. To observe the expression change of ANRIL in TNBC, qRT-PCR was performed to detect its expression levels in 21 TNBC tissues and 10 normal breast tissues. We found a significantly higher level of ANRIL in TNBC tumor tissues (**Figure 1A**). Consistently, the expression level of ANRIL is also significantly higher in TNBC cell lines (MDA-MB-231, BT549, and HS578T) in comparison with ER + breast cancer cell lines (MCF7 and T47D) and normal breast cell line (MCF10 A) (**Figure 1B**). This analysis of ANRIL expression suggested that ANRIL might involve in TNBC progression. Due to the higher expression level of ANRIL in MDA-MB-231 and BT549 cells, these 2 cell lines were transfected with si-ANRIL or si-NC and Vector or pcDNA-ANRIL to further investigate the role of ANRIL during drug resistance of TNBC cells in vitro. ANRIL expression was strikingly decreased in MDA-MB-231 and BT549 cells after transfection with si-ANRIL (**Figure 1C**). CCK8 assay implicated that cell viability was inhibited in MDA-MB-231 and BT549 cells treated with ADR, while si-ANRIL remarkably increased ADR-induced inhibition on cell viability (**Figure 1D, E**). Meanwhile, ANRIL overexpressed cells showed an obvious increase in ANRIL expression compared to vector-introduced cells (**Figure 1F**). Reversely, the results in si-ANRIL- introduced MDA-MB-231 and BT549 cells, CCK8 assay showed that ANRIL overexpression led to a marked increase in cell viability in ADR-treated MDA-MB-231 and BT549/ADR cells with respect to vector group (**Figure 1G, H)**. Together, the above data revealed that ANRIL was related to the ADR resistance of TNBC cells.

ANRIL knockdown inhibited HIF-1α-dependent glycolysis in TNBC and TNBC/ADR cells. More evidence demonstrated that increased glycolytic contributes to the development of drug resistance in different kinds of tumors[37, 38]. Therefore, we investigated whether siANRIL negatively regulated aerobic glycolysis or not. We used the Seahorse Extracellular Flux Analyzer for glycolysis and mitochondrial respiration measurements, and ECAR to reflect aerobic glycolysis and OCR to indicate mitochondrial respiration. ANRIL knockdown in TNBC/ADR cells decreased glycolysis and glycolytic capacity, which was reflected in ECAR measurement (**Figure 2A and 2B**). Mitochondrial respiration was impaired in the process of aerobic glycolysis, resulting in a reduction of oxygen consumption that was reflected by OCR measurement. We observed that ANRIL knockdown enhanced OCR, indicating the positive roles of ANRIL in mitochondrial respiration (**Figure 2C and 2D**). Transcription factor hypoxia-inducible factor 1α (HIF-1α) controls the expression of a large number of gene products involved in energy metabolism and glycolysis. Therefore, we next investigated whether ANRIL knockdown mediated regulation of glycolysis was HIF-1a dependent. WB analysis indicated that knockdown of ANRIL effectively decreased HIF-1a in TNBC cells (**Figure 2E and 2F**). Therefore, these results demonstrated that ANRIL downregulation suppressed HIF-1α-dependent glycolysis.

ANRIL directly recognizes miR-125a in TNBC/ADR cells. The subcellular location of ANRIL determined by qRT-PCR showed that ANRIL was mainly inside the cytoplasm, indicating that ANRIL could contact with miRNAs in the cytoplasm (**Figure 3A and 3B**). We used microRNA.org, and miRBase to analyze the potential targets of ANRIL. The results provided the potential binding sequence of miR-125a to ANRIL, as shown in **Figure 3C**. Luciferase reporter assay further confirmed that miR-125a mimics but not miR-125a inhibitors reduced the luciferase activities of ANRIL-wt reporter vector in TNBC cells, but had no obvious inhibitory effect on ANRIL-mut reporter vector (**Figure 3D and 3E**). Anti-Ago2 RIP assay and luciferase reporter assay was conducted to further investigate whether ANRIL function as a sponger of miR-125a. The results showed that ANRIL and miR-125a have highly existed in Ago2-containing beads in TNBC cells relative to control IgG immunoprecipitates (**Figure 3F**). These results demonstrated that ANRIL is a sponge of miR-125a in TNBC cells.

ENO1 is a target of miR-125a and is positively regulated by ANRIL in TNBC cells. Bioinformatics analysis using targeting algorithms (TargetScan and microRNA.org) were used to further investigate the target protein of miR-125a. We found that 3’-UTR of ENO1 mRNA is a binding site of miR-125a (**Figure 4A**). Further luciferase reporter assay demonstrated that miR-125a upregulation significantly decreased the luciferase activity of ENO1-wt but not ENO1-mut in TNBC cells (**Figure 4B**). We next conducted qRT-PCR and western blot to see whether ANRIL knockdown or miR-125a overexpression could reduce ENO1 expression in TNBC cells. We found that both ANRIL silencing and miR-125a overexpression decreased the ENO1 expression both in mRNA level and protein level (**Figure 4C and 4D**). These results revealed that ANRIL increased ENO1 expression by functioning as a sponge of miR-125a in TNBC cells.

ANRIL increases TNBC cell chemoresistance via ENO1 in vitro. Except boosting tumorigenesis, the aerobic glycolysis of cancer cells provides an environment in which drug resistance is often increased. We used siRNA of ENO1 and 2-DG to see whether ENO1 was important for ANRIL during drug resistance

in TNBC cells. The effect of ANRIL on TNBC cell drug resistance against ADR was determined by clone formation assay. We found that ANRIL overexpression significantly increased the clone formation ability of TNBC cells under ADR treatment (**Figure 5A**). Additionally, cell viability assay indicated that ANRIL promoted ADR resistance of TNBC cells (**Figure 5B**). Flow cytometry analysis also demonstrated that ANRIL-overexpression decreased apoptosis of TNBC cells treated with ADR. ENO1knockdown by siRNA and 2-DG was used to treat ANRIL-overexpressing TNBC cells to investigate whether the TNBC cell resistance to ADR by ANRIL upregulation happens through increased glucose metabolism. The results revealed that drug resistance of ANRIL-upregulated TNBC cells to ADR was reversed by ENO1 siRNA or 2-DG (**Figure 5**).

ANRIL silencing inhibited tumor growth in vivo. To study the effect of ANRIL on TNBC ADR resistance in vivo, NOD-SCID mice were used for the xenograft tumor model. shANRIL cells were subcutaneously injected into the mammary fat pad of mice. To elucidate the role of ANRIL in chemoresistance in vivo model, ADR was administered locally in shNC- and shANRIL-treated mice. Macroscopic observation of the tumor tissue revealed that tumor volume was smaller in shANRIL mice than in the shNC mice under ADR treatment or not. Further measurement on tumor volume and weight results showed that both the tumor volume and weight in the shANRIL and ADR groups are smaller than the control group, while shANRIL+ADR group has smaller tumor volume and weight than the shANRIL-treated group, indicating that ANRIL suppression enhanced ADR sensitivity in TNBC (**Figure 6A and 6B**). We further evaluated whether ANRIL knockdown influences the apoptosis and proliferation ability of TNBC cells in chemoresistance in vivo model. We found that both shANRIL and ADR stimulation promoted the ratio of TUNEL positive cells and shANRIL+ADR has more TUNEL positive cells. Ki67 positive cells decreased in shANRIL and ADR groups and shANRIL+ADR has lesser Ki67 positive cells (**Figure 6C and 6D**). Taken together, our results suggested that ANRIL contributed to tumorigenesis of TNBC through regulating chemoresistance (**Figure 7**).

Discussion

In this study, we found that lncRNA ANRIL expression was upregulated in TNBC patients. ANRIL knockdown promoted the toxic effects of ADR and decreased HIF-1α-dependent glycolysis in TNBC cells. In TNBC cells, we discovered an ANRIL/miR-125a/ENO1 pathway in mediating chemotherapy sensitivity against ADR. Furthermore, 2-deoxy-glucose (2-DG) suppressed HIF-1α-dependent glycolysis and attenuated ANRIL-induced increase in ADR resistance in TNBC cells. We come to a conclusion that ANRIL plays a positive role in ADR resistance in TNBCby inhibiting glycolysis through the miR-125a/ENO1 pathway, contributing to a better understanding of the molecular mechanisms underlying TNBC chemoresistance.

Adriamycin has been widely used as a chemotherapy drug for the treatment of TNBC. However, TNBC patients often develop drug tolerance during chemotherapeutic treatment[39, 40]. Recent studies have shown that long non-coding RNA (lncRNA) may play an important regulatory role in tumor cell behavioral activities including invasion, proliferation, and drug resistance[41, 42]. Jiang et al. found a novel lncRNA

ARA plays an important role during adriamycin resistance in breast cancer. Our current findings have firstly found a relationship between lncRNA ANRIL and ADR resistance in TNBC. It was proved by experiments that down-regulation of ANRIL could promote ADR toxicity, while overexpression of ANRIL could partially promote ADR resistance in TNBC[43]. In vivo experiments confirmed that knockdown of ANRIL reversed ADR resistance in TNBC cells xenograft in vivo. These results provided more evidence on the importance of lncRNA against drug-resistant TNBC.

Aerobic glycolysis has been raised been related to the development of drug-resistance[37, 44, 45]. The faster glucose metabolism is well recognized as one of the major hallmarks of malignancy and is related to tumor oncogenesis, progression, and metastasis. Besides, more and more evidence suggested that higher glycolysis may contribute to the development of drug resistance in multiple tumors, including TNBC. HIF-1α plays a critical role in the regulation of glycolysis of cancer cells, has been demonstrated to be related to chemoresistance in many kinds of cancer cells[46, 47]. We demonstrated in this study that downregulation of ANRIL decreased HIF-1α-dependent glycolysis. Except that, 2-DG treatment suppressed HIF-1α-dependent glycolysis and attenuated ANRIL-induced increase of drug-resistance in TNBC cells. Taken together, we conclude that ANRIL knockdown inhibited ADR-resistance by blocking HIF-1α-dependent glycolysis in TNBC cells.

In conclusion, our study shows for the first time that overexpression of ANRIL may lead to ADR resistance in TNBC, and downregulation of ANRIL partially reverses drug resistance. Moreover, down-regulation of ANRIL attenuated ADR resistance of TNBC by downregulating ENO1 expression. In vivo experiments confirmed that downregulation of ANRIL reversed ADR resistance in the TNBC xenograft mice model. Our results provided useful clues for exploring the mechanism of ADR resistance in TNBC, with certain application value and practical significance.

Declarations

Availability of data and materials

We hereby undertake that all data and materials are available.

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Authors' Contributions

Conceptualization: JM and WZ; Data curation: DT; Formal analysis: HZ and ZC Funding acquisition: DT; Investigation: JM and DT; Methodology: HZ, ZC, HL and XF, Project administration: JM and WZ. Supervision: DT; Roles/Writing—original draft: DT; Writing-review and editing: All authors. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Harbin Medical University Cancer Hospital.

Consent for publication

All listed authors have participated in the study and approved the submitted manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

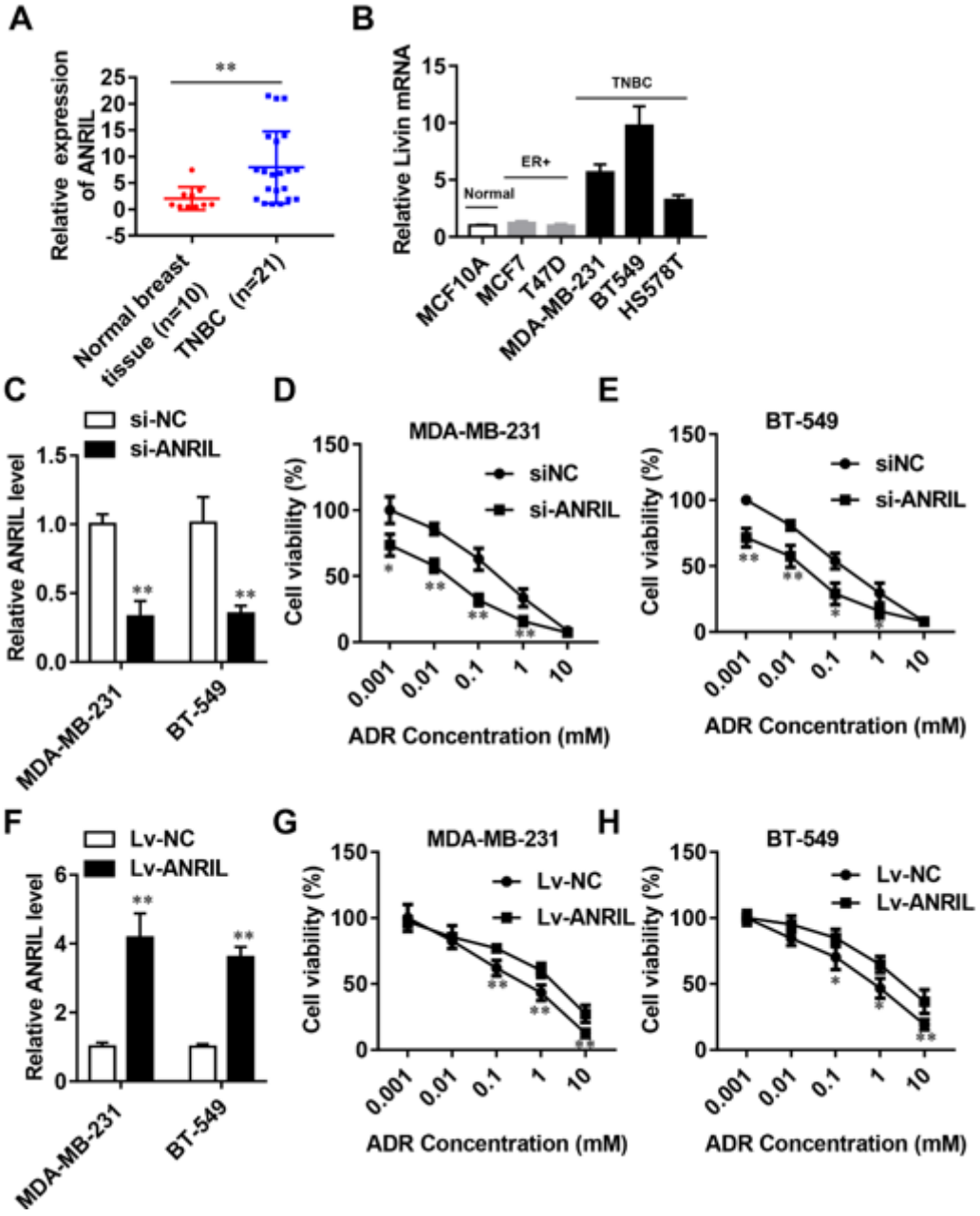

Figure 1

ANRIL overexpression augmented adriamycin-induced cytotoxic effect in TNBC cells. (A) The QRT-PCR analysis of ANRIL expression in TNBC tissues and normal breast tissue from different clinical samples. (B) The expression level of ANRIL in a panel of normal breast epithelial cell lines and breast cancer cell lines. (C) QRT-PCR analysis of ANRIL in primary TNBC cells transfected with ANRIL siRNA. (D, E) The cell viability of MDA-MB-231 and BT549 cells transfected with ANRIL siRNA were evaluated by CCK8 assay. (F) QRT-PCR analysis of ANRIL in primary TNBC cells infected with lentivirus carrying ANRIL sequence (Lv-ANRIL). (G, H) The cell viability of MDA-MB-231 and BT549 cells infected with Lv-ANRIL were evaluated by CCK8 assay. *p< 0.05, **p<0.01.

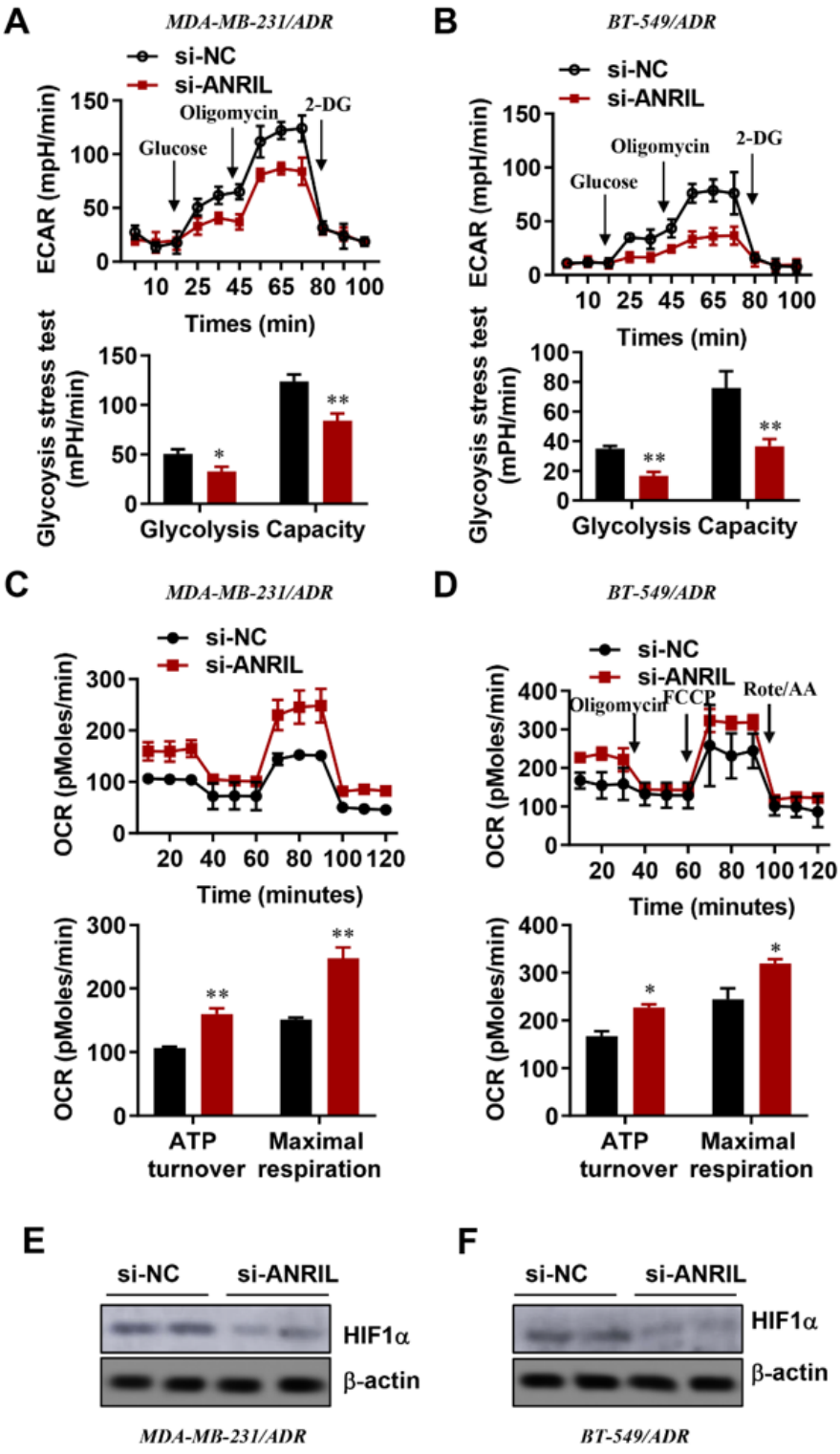

Figure 2

ANRIL knockdown inhibited HIF-1α-dependent glycolysis in TNBC and TNBC/ADR cells. (A, B) Diagram and quantitative of ECAR results obtained by Seahorse extracellular flux analyzer to determine the impact of ANRIL knockdown on aerobic glycolysis in MDA-MB-231 and BT549 cells. (C, D) Diagram and quantitative of OCR measurement with Seahorse analyzer to confirm the role of ANRIL knockdown in

mitochondrial respiration. (E, F) WB analysis showed that knockdown of ANRIL effectively lowered HIF-1α level in MDA-MB-231 and BT549 cells treated by ADR. *p<0.05, **p< 0.01.

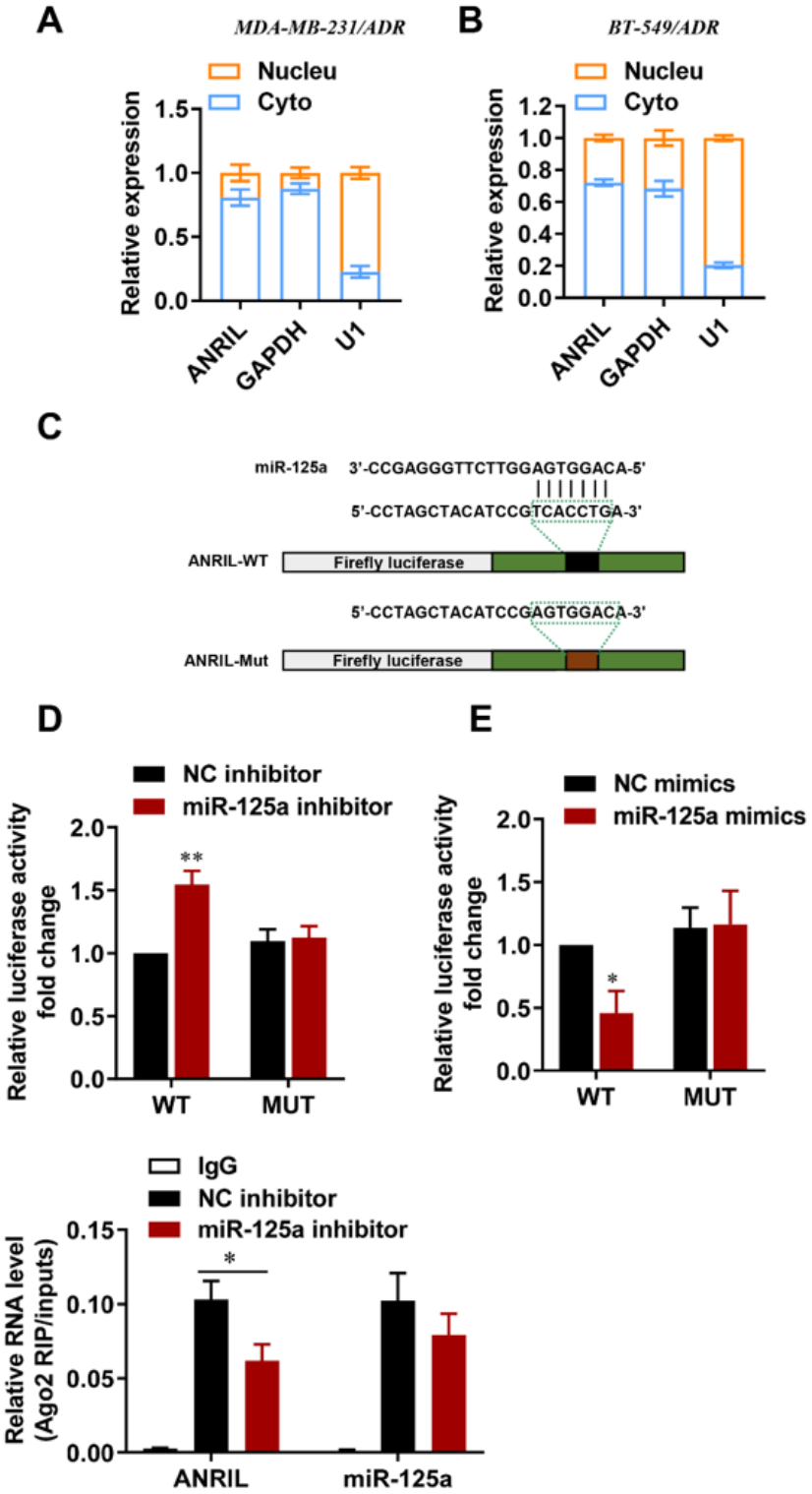

Figure 3

ANRIL directly binding to miR-125a in TNBC/ADR cells. (A) The subcellular location of ANRIL was determined by quantitative real-time PCR. (B) Sequence complementarity of ANRIL and miR-125a. Short vertical lines indicated complementary nucleotides. (C) The dual-luciferase reporter gene assay was

utilized in TNBC cells to verify the target relationship between ANRIL and miR-125a-mimic or miR-125a-inhibitor (D). (E) The amount of ANRIL and miR-125a bound to Ago2 or IgG was measured by RT–qPCR in the presence of miR-125a inhibitor or negative control. Data represent the mean ± SEM of three independent experiments. *p<0.05, **p < 0.01.

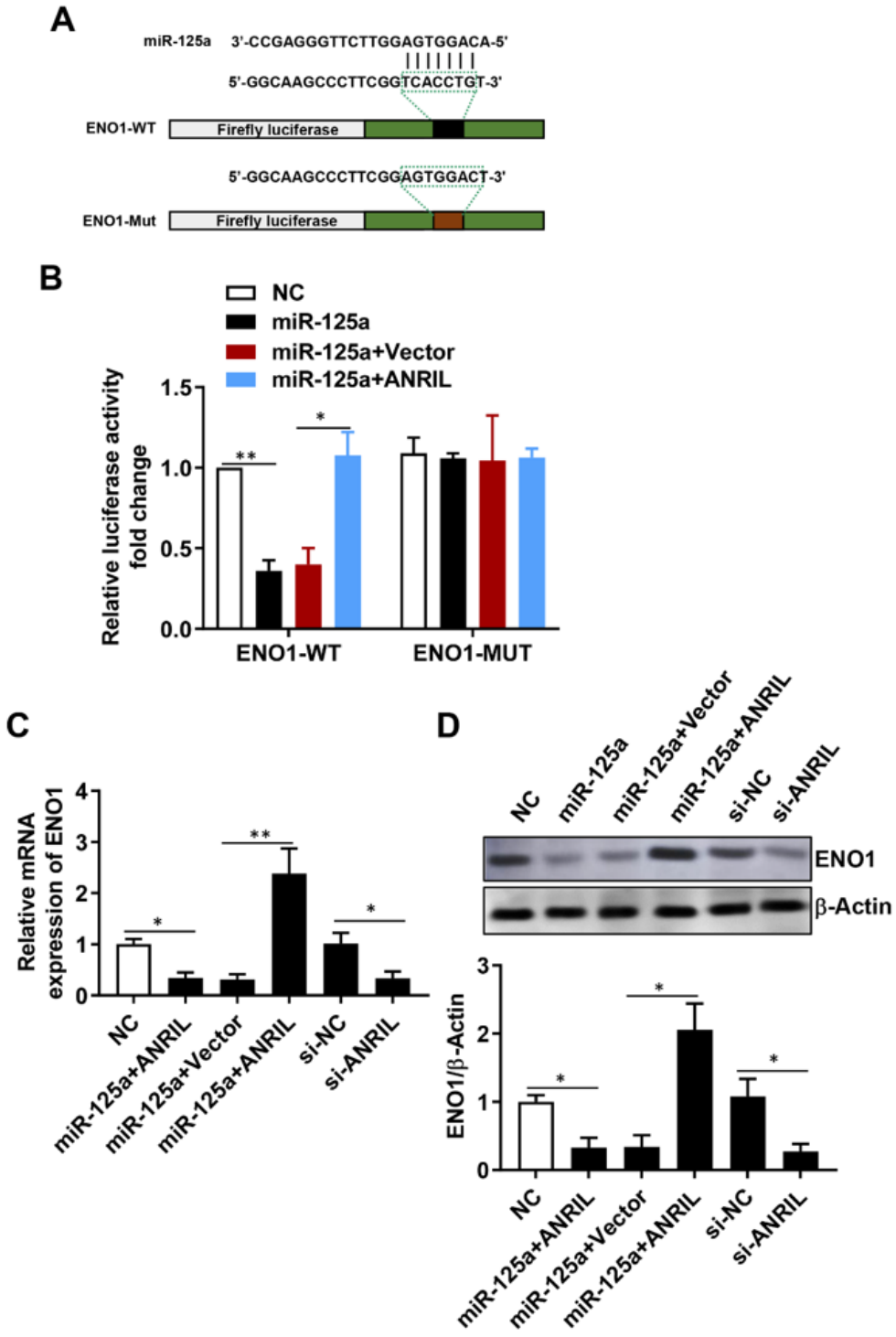

Figure 4

ENO1 is a target of miR-125a and is positively regulated by ANRIL in TNBC cells. (A) Bioinformatics analysis using targeting algorithms showing that miR-125a had candidate binding sites in the 3'-UTR of ENO1 mRNA. (B) Luciferase reporter assay implied that miR-125a overexpression remarkably inhibited the luciferase activities of ENO1-wt but not that of ENO1-mut in TNBC cells. (C) qRT-PCR result of ANRIL silencing and miR-125a overexpression reduced mRNA of ENO1 in TNBC cells. (D) Western blot analysis of ANRIL silencing and miR-125a overexpression reduced protein level of ENO1 in TNBC cells. *p<0.05, **p < 0.01.

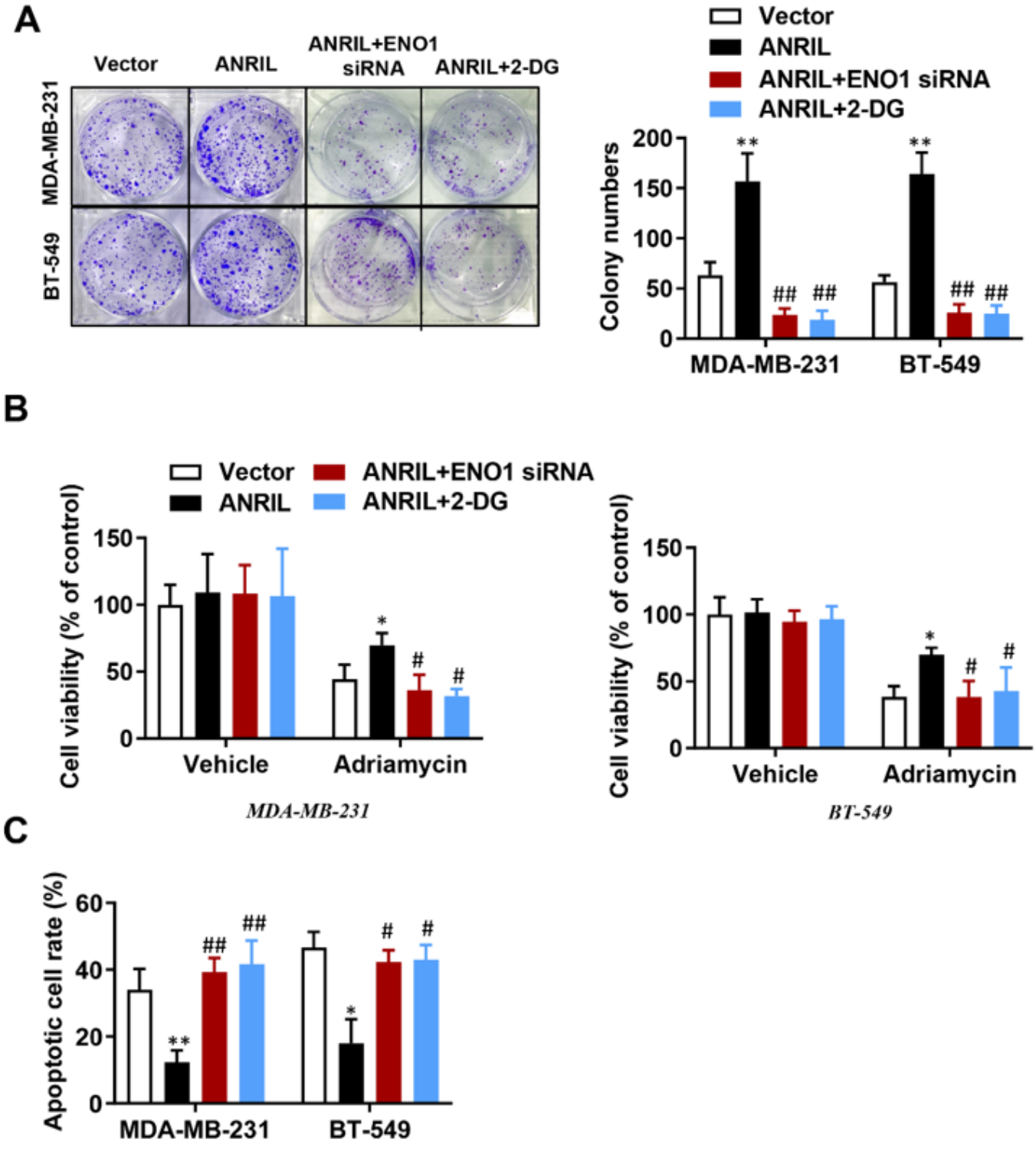

Figure 5

ANRIL increases TNBC cell chemoresistance via ENO1 in vitro. (A) ANRIL overexpression significantly enhanced the clonogenic potential of TNBC cells upon exposure to ADR. (B) Cell viability analysis showed that ANRIL enhanced ADR resistance of TNBC cells. (C) Flow cytometry analysis showed less apoptosis in ANRIL-overexpressing TNBC cells than in control cells treated with ADR.

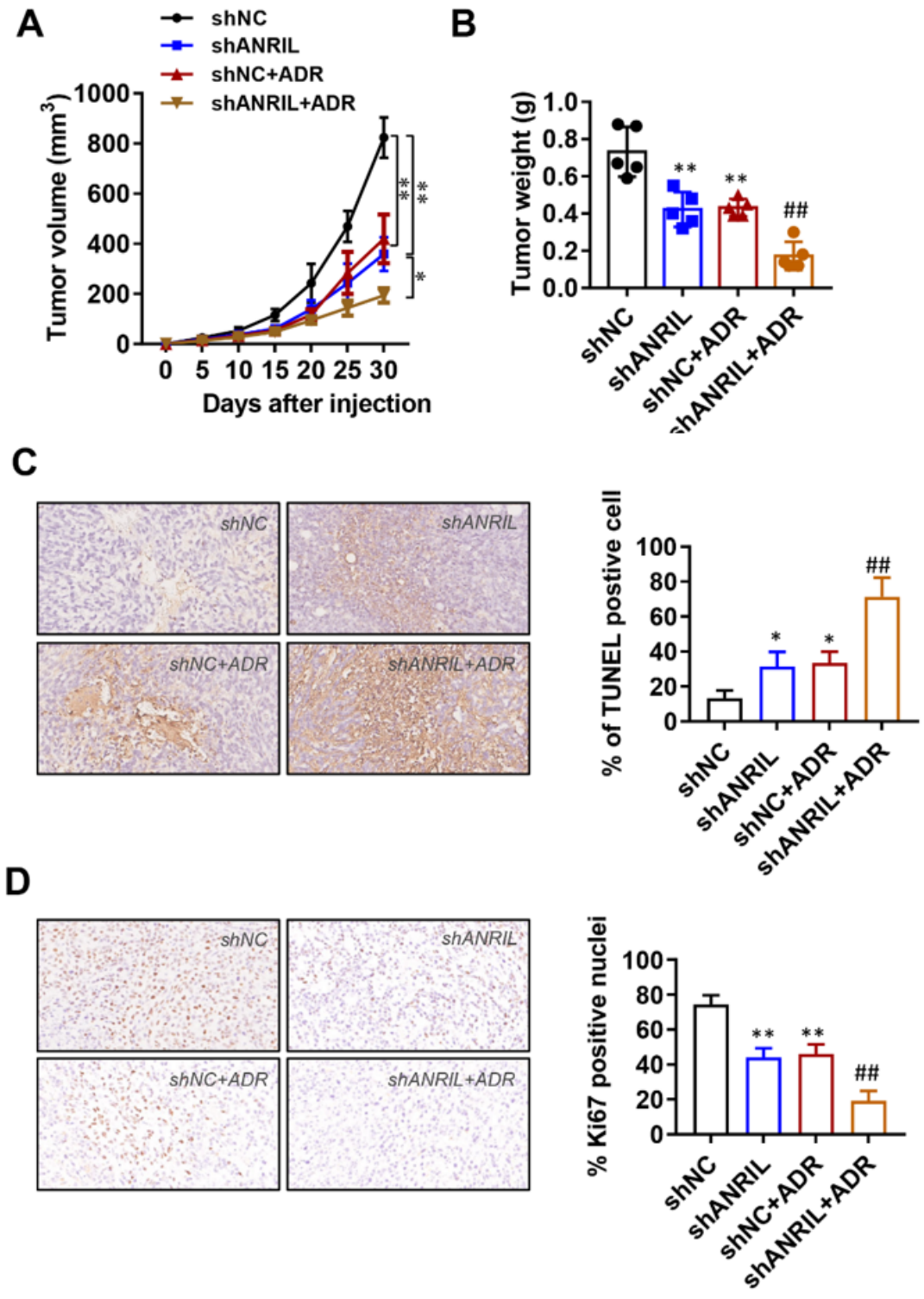

Figure 6

ANRIL silencing inhibited tumor growth in vivo. (A) Quantitative analysis of the NB tumor volume at the indicated time. (B) Quantitative analysis of the NB tumor weight. (C, D) TUNEL assay (C) and immunohistochemistry of Ki-67 protein (D) in the TNBC tissues. TUNEL and Ki-67-positive cells indicate apoptotic and proliferative cells, respectively. *p<0.05, **p< 0.01.

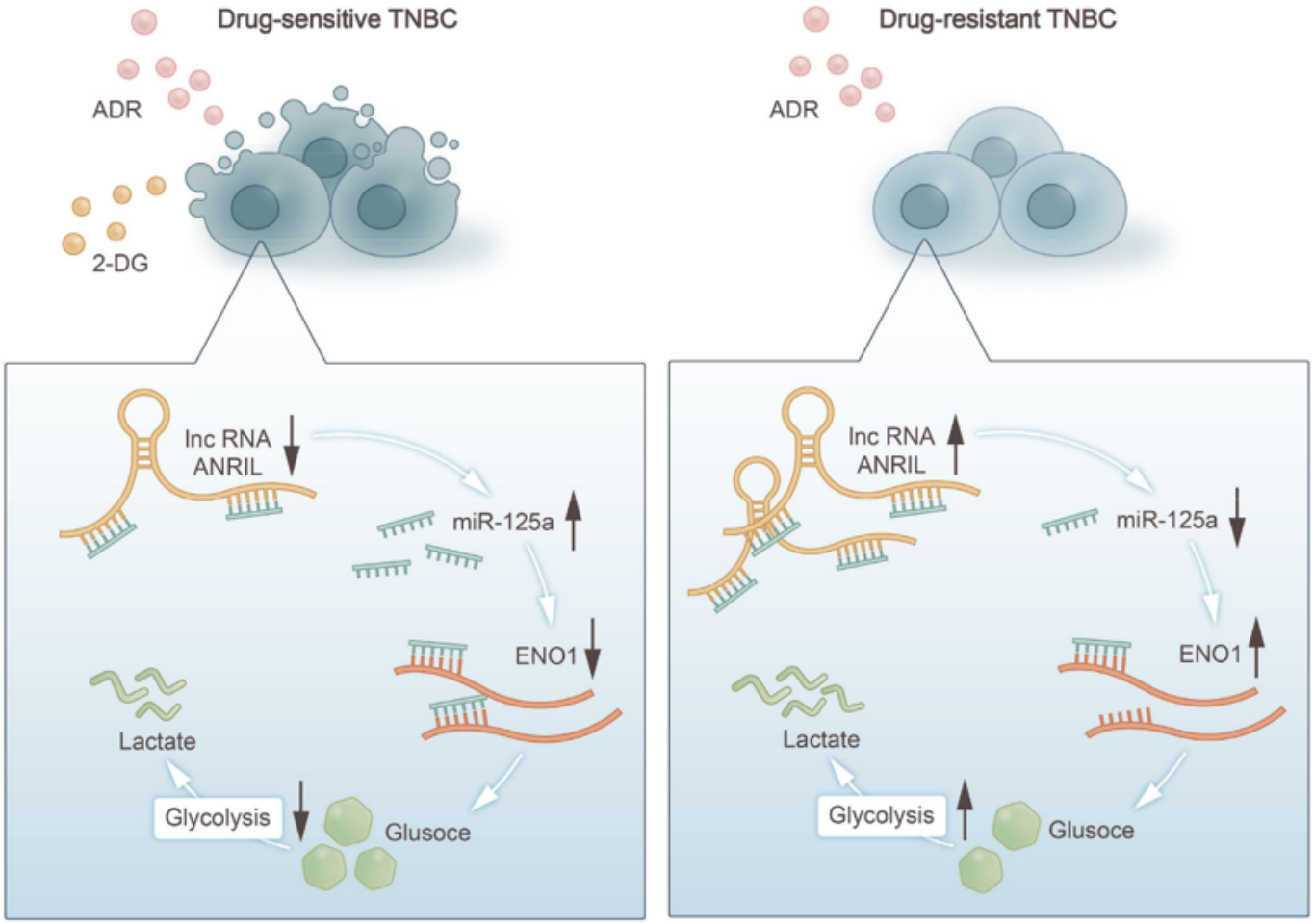

Figure 7

Schematic diagram of lncRNA-ANRIL-based regulatory mechanism in ADR resistance of TNBC cells.