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MicroRNA-496 inhibits triple negative breast cancer cell proliferation by targeting Del-1

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Abstract

Del-1 has been linked to the pathogenesis of various cancers, including breast cancer. However, the regulation of Del-1 expression remains unclear. We previously reported the interaction between *microRNA-137* (*miR-137*) and the *Del-1* gene. In this study, we investigated *miR-496* and *miR-137* as regulators of *Del-1* expression in triple negative breast cancer (TNBC). *Del-1* mRNA and *miR-496* were measured by quantitative PCR in breast cancer cells (MDA-MB-231, MCF7, SK-BR3, and T-47D) and tissues from 30 patients with TNBC. The effects of *miR-496* on cell proliferation, migration, and invasion were determined with MTT, wound healing, and Matrigel transwell assays, respectively. In MDA-MB-231 cells, *miR-496* levels were remarkably low and *Del-1* mRNA levels were higher than in other breast cancer cell lines. Luciferase reporter assays revealed that *miR-496* binds the 3′-UTR of *Del-1* and *Del-1* expression is downregulated by *miR-496* mimics. Furthermore, *miR-496* inhibited the proliferation, migration, and invasion of MDA-MB-231 cells. The effects of *miR-496* on cell proliferation were additive with those of *miR-137*, another miRNA that regulates *Del-1* expression. Moreover, in the 30 TNBC specimens, *miR-496* was downregulated ($P < .005$) and the levels of Del-1 in the plasma were significantly elevated as compared with in normal controls ($P = .0142$). The Cancer Genome Atlas (TCGA) data showed the correlation of *miR-496* expression with better overall survival in patients with early TNBC. In in silico and in vitro analyses, we showed that *Del-1* is a target of *miR-496* in TNBC and thereby affects cancer progression. Our findings suggest that *miR-496* and *miR-137* additively target *Del-1* and act as modulating factors in TNBC. They are potentially new biomarkers for patients with TNBC.

Abbreviations: DEL-1 = developmental endothelial locus-1, miRNAs = MicroRNAs, TCGA = The Cancer Genome Atlas, TNBC = triple negative breast cancer, WT = wild type.

Keywords: breast cancer, cancer biomarker, microRNA

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1. Introduction

Triple negative breast cancer (TNBC) accounts for only 15% to 20% of all breast cancer cases, but it accounts for the majority of breast cancer-related deaths.[1] Therefore, it is important to identify novel biomarkers for TNBC for the purpose of accurately classifying and hopefully treating TNBC.

Developmental endothelial locus-1 (Del-1; also called EGF Like Repeats and Discoidin Domains 3 or Edil-3) is widely expressed and Del-1 levels are high in circulating extracellular vesicles in the plasma of patients with breast cancer. After curative surgery, plasma Del-1 levels are decreased, suggesting it is a new diagnostic marker for early breast cancer.[2,3] Notably, *Del-1* expression is significantly associated with unfavorable histology, high Ki67 expression, and the TNBC subtype, indicating its potential as a prognostic marker for TNBC.[4] However, it is not known how *Del-1* expression is regulated in normal or tumor cells.

MicroRNAs (miRNAs) suppress gene expression through sequence-specific base paring with the 3′ untranslated region (3′UTR) of their target mRNAs, resulting in their translational repression or degradation. Evidence has suggested that miRNAs regulate gene expression by controlling diverse cellular and metabolic pathways in cancer cells as either tumor suppressors or oncogenes and, therefore, could emerge as promising biomarkers for a variety of cancers.[5–8] We hypothesized that specific miRNA(s) may affect *Del-1* expression in TNBC and, if so, such miRNA(s) could be novel therapeutic target(s) together with Del-1. Recently, we identified the functional role of *miR-137* and

the interaction between *miR-137* and the *Del-1* gene.[9] Since *miR-496* has also been predicted to bind to the *Del-1* gene, we investigated its interaction with *miR-137* and role in Del-1 expression in TNBC.

2. Material and methods

2.1. Selection of miR-496 as a candidate

Since miRNAs negatively regulate gene expression, any miRNA upregulated in cancer cells can be a candidate to downregulate mRNAs of target genes. The miRNA candidates possibly affecting *Del-1* expression were selected from a list created using 3 web-based algorithms: miRanda (http://www.microrna.org/microrna/home.do), Target Scan (http://www.targetscan.org/vert_71/), and miRDB (http://mirdb.org/miRDB/).

2.2. Clinical specimens to measure miR-496 and Del-1 expression

All clinical breast cancers and paired adjacent normal breast tissues were acquired from 30 patients with early TNBC (Stage I through IIIA). Total RNA was extracted using an RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. *Del-1* mRNA and miRNA expressions were measured and analyzed considering both clinical and pathological characteristics, such as age, tumor size, lymph node involvement, histological grade, lymphovascular invasion, and BRCA 1/2 mutation status. All procedures were performed under a protocol approved by the institutional review board at Kyungpook National University Chilgok Hospital (#2013-09-009-001). At the time of recruitment, patients were given an information leaflet and a consent form for storage and collection of biological materials, including blood and tissue samples, as well as future use of their samples for research purposes.

2.3. Breast cancer cell lines

A human breast epithelial cell line (MCF10A) and breast cancer cell lines (MDA-MB-231, MCF7, and SK-BR3) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). MCF10A cells were maintained in Dulbecco Modified Eagle medium (DMEM)/F-12 (1:1) medium (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 10 ng/ml epidermal growth factor (Sigma-Aldrich Co., St. Louis, MO), 0.5 μg/ml hydrocortisone (Sigma–Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), and 10 μg/ml insulin (Sigma–Aldrich). MDA-MB-231, MCF7, and SK-BR3 cells were maintained in DMEM (Gibco) supplemented with 10% FBS.

2.4. RNA extraction and quantitative PCR (qPCR)

Total RNA from cells was isolated using RNAiso Plus (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. The Super Script III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) were used to reverse transcribe the mRNAs and miRNAs, respectively. qPCR was performed to assess the relative expressions of *Del-1* using the Power SYBR Green PCR Master Mix (Applied Biosystems). Additionally, the abundance of miRNA was measured using TaqMan Universal Master Mix II (Applied Biosystems). The relative amount of each miRNA was normalized to *U6* snRNA, whereas mRNA expression was normalized to *β-actin*. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The TaqMan probe sets for *miR-496* and U6 were purchased from Applied Biosystems. The primers used for qPCR were as follows: *Del-1* (5′-TCGAAGACATTGCACTTTGC-3′ and 5′-ACCCAGAGGCTCAGAACAAC-3′) and *β-actin* (5′-TTGCCGACAGGATGCAGAA-3′ and 5′-GCCGATCCACACG GAGTACT-3′).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Del-1 levels in culture medium were detected by ELISA since Del-1 is known to be secreted from the cells as previously described.[3]

2.6. miRNA transfection

miR-496 mimics and the negative control were purchased from Sigma–Aldrich Co., LLC (St. Louis, MO, USA). *miR-496* inhibitors were purchased from Ambion by Life Technologies Inc. (Carlsbad, CA). Cells were plated in 6-well plates (2×10^5 cells/well) for wound healing assays or 96-well plates (5×10^3 cells/well) for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. After culturing overnight, the cells were transfected using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions.

2.7. Plasmid vector construction for luciferase report assays and mutagenesis

To construct a reporter plasmid containing the wild type (WT) *Del-1* 3′-UTR, a 533-bp fragment of the *Del-1* 3′-UTR was amplified by PCR using MDA-MB-231 cDNA as a template and cloned into the pTOP Blunt V2 vector (Enzynomics Co. Ltd, Daejeon, South Korea). Thereafter, the *Del-1* 3′-UTR was cloned in the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI). To construct the plasmids with a mutant (Mut) *Del-1* 3′-UTR, the putative *miR-496* target sequence was mutated using a Muta-Direct Site-Directed Mutagenesis Kit (iNtRON Biotechnology, Gyeonggi-do, South Korea). All the products were confirmed by sequencing.

2.8. Dual luciferase reporter assays

MDA-MB-231 cells were plated into 24-well plates (4×10^4 cells/well). After 24 hours, the cells were co-transfected with 100 ng of the pmirGLO Dual-Luciferase expression construct containing the 3′-UTR of *Del-1* (WT or Mut), and 10 pmol of *miR-496* mimics or its negative control using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after transfection, the cell lysates were collected and set aside, and the luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) and normalized to *Renilla* luciferase activity.

2.9. MTT assays to assess cell proliferation

Cell proliferation was estimated using an MTT assay (Sigma-Aldrich). Briefly, cells were seeded in 96-well plates. After

transfection with 1 pmol of miRNA mimics, miRNA inhibitors, or the negative control, cells were incubated for 1, 2, or 3 days without changing the medium after transfection. At each time point, 50 μL of MTT (2 mg/mL) were added to the wells, and the cells were cultured at 37°C for 4 hours. After removing the media, 150 μL of dimethyl sulfoxide (DMSO) were added and the solution was mixed for 10 minutes. The optical density was determined at 570 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT).

2.10. Wound scratch assays for cell migration

To examine the effects of *miR-496* on the migration of MDA-MB-231 cells, the cells were seeded in 6-well plates. After culturing overnight, a line was scratched into the cell monolayer using a sterile pipette tip and the medium was changed. Subsequently, the cells were transfected with 25 pmol of negative controls, *miR-496* mimics or *miR-496* inhibitors and further incubated for 48 hours. Images were then captured with the aid of a microscope and camera system (Olympus Co., Tokyo, Japan) after 0, 24, 48, or 72 hours of incubation.

2.11. Transwell assays for cell invasion

For the invasion assays, transwell chambers with 8 μm pores were coated with Matrigel (Corning Inc., Tewksbury, MA) and incubated at 37°C for 2 hours. After transfection for 24 hours with 25 pmol of the negative control, *miR-496* mimics, or *miR-496* inhibitors, the cells were resuspended in DMEM containing 1% FBS and 1×10^4 cells were plated in the upper chamber. The lower chamber contained the complete medium supplemented with 10% FBS. After incubation for 48 hours, the cells on the upper surface of the chamber were swabbed using a cotton swab, and those at the bottom were fixed with 2% paraformaldehyde, stained with 0.5% crystal violet, and then rinsed with phosphate-buffered saline. Finally, 4 random views were chosen for each culture well under a light microscope (200×; Olympus Co., Tokyo, Japan) and the number of invading cells in each view was counted.

2.12. Survival according to miR-496 levels

To validate whether the levels of *miR-496* were related to the survival of patients, we used a The Cancer Genome Atlas (TCGA) dataset (n = 1,077) relative to patients with TNBC and divided it into subgroups based on the levels of *miR-496* and *Del-1*. The prognostic value of *miR-496* was estimated using the Kaplan–Meier Plotter (http://kmplot.com/analysis/).[10]

2.13. Statistical analysis

All quantitative experiments were performed at least in triplicate. The data are expressed as the mean ± standard deviation and were analyzed using Student *t* test and Mann–Whitney *U* test with GraphPad Prism version 7 (GraphPad Software, San Diego, CA). *P* value of ≤.05 was considered statistically significant.

3. Results

3.1. miR-496 expression in breast cancer cell lines and tumor tissues

Based on a bioinformatics search using 3 prediction algorithms, *miR-496* was selected as a potential miRNA targeting *Del-1*. Next, we corroborated this interaction. We measured *miR-496* levels in various breast cancer cell lines and found that *miR-496* was significantly downregulated in a triple negative breast cell line (MDA-MB-231) compared with in MCF10A, and it was similarly expressed in the other cancer lines ($P < .005$; Fig. 1A). Furthermore, the expression of *miR-496* in breast tumor tissues of 30 patients with TNBC showed the level was significantly lower than that in the paired normal breast tissues, irrespective of clinical stage, pathologic characteristics, and BRCA mutation status ($P = .0142$; Fig. 1B, Supplementary Fig. 1, http://links.lww.com/MD2/A33).

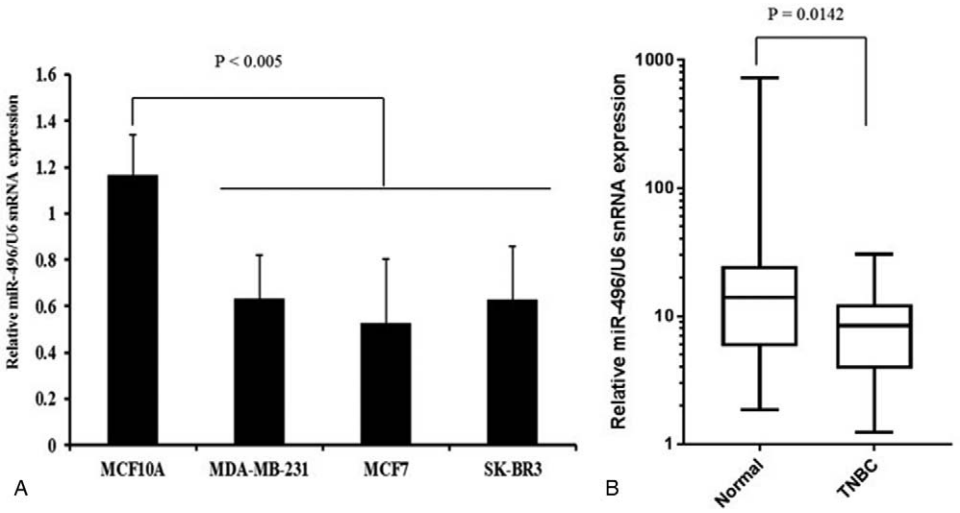

Figure 1. Expression of *miR-496* in breast cancer cell lines and tissues. (A) The expression of *miR-496* was downregulated in all breast cancer cells including MDA-MB-231 cells compared with MCF10A breast epithelial cells, (B) Expression of *miR-496* in patients with TNBC: the breast tumor tissues of patients with TNBC showed a significant downregulation of *miR-496* expression irrespective of the patients' clinical and pathological characteristics (n = 30 for each group). TNBC = triple negative breast cancer.

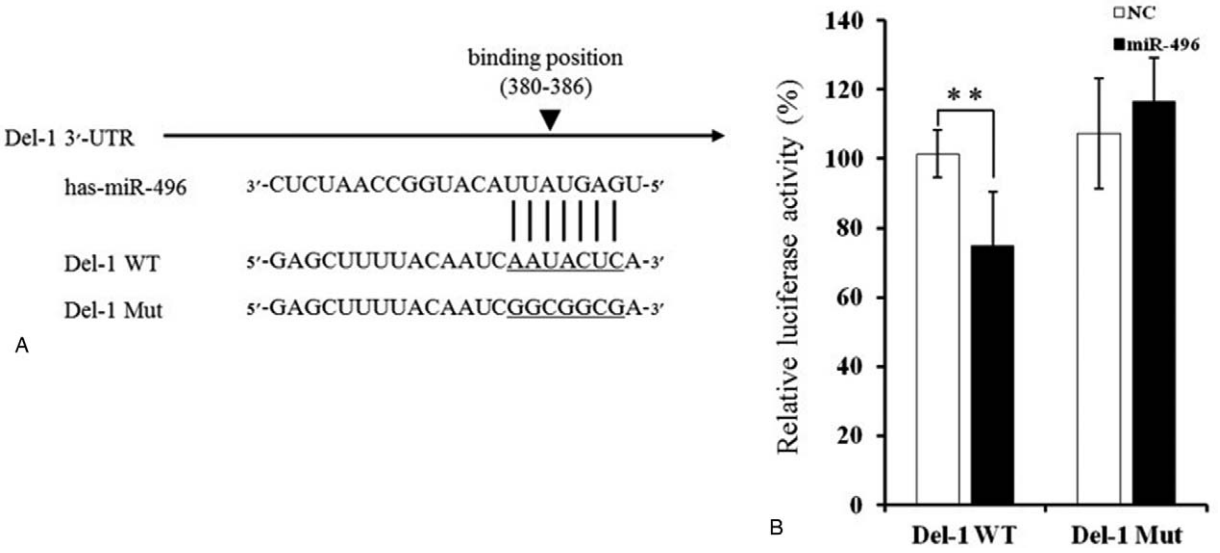

Figure 2. Identification of *miR-496* target site. (A) The putative target site of *miR-496* was predicted to be located within 3'-UTR of *Del-1* at nt 380 to 386. (B) Luciferase reporter assay to evaluate the interaction between *miR-496* and *Del-1* 3'-UTR: MDA-MB-231 cells were transfected with luciferase constructs containing the wild-type (*Del-1* WT) or mutated (*Del-1* Mut) 3'-UTR of *Del-1* and *miR-496* mimics or NC oligos. Luciferase activity was determined 24 hours after transfection. Relative luciferase expression values are the ratio of the luciferase activity in *miR-496*-treated cells and that in NC-treated cells. Data are representative of 3 independent experiments. Error bars: SD, **: $P < .01$. Del-1 = developmental endothelial locus-1, NC = negative control.

3.2. miR-496 directly targets the 3′-UTR of Del-1

Bioinformatic analyses predicted the 380 to 386 regions in the 3′-UTR of *Del-1* mRNA as a putative target of miR-*496* (Fig. 2A). To determine whether the site was functional, a constructed mutant of the Del-1 plasmid was transfected in MDA-MB-231 cells and tested using luciferase assays with miR-496 mimics. As shown in Figure 2B, *miR-496* mimics caused significant downregulation of the luciferase activity of the plasmid with WT *Del-1* 3′-UTR ($P < .01$). There was no effect on the plasmid with Mut *Del-1* 3′-UTR. These findings suggested that the 3′-UTR of *Del-1* is a functional target of *miR-496*.

3.3. Del-1 expression is negatively regulated by miR-496

To investigate whether *miR-496* negatively regulates Del-1 protein expression, we transfected *miR-496* into MDA-MB-231

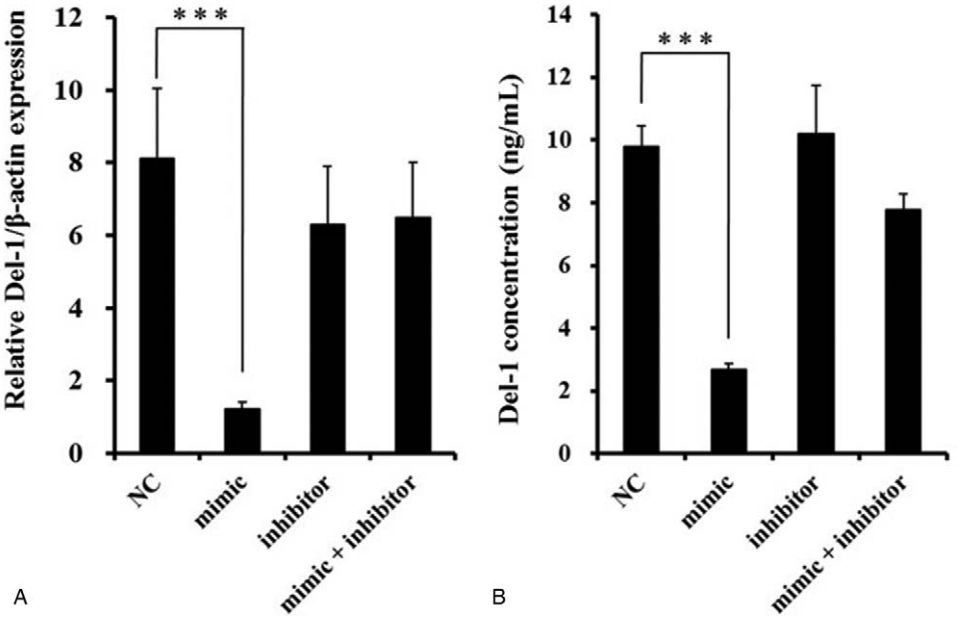

Figure 3. *Del-1* expression upon *miR-496* overexpression or knockdown. (A) Relative *Del-1* expressions were evaluated by qPCR 48 hours after transfection with mimics, inhibitors, or mimics plus inhibitors of *miR-496*. (B) The concentration of Del-1 in the culture medium was measured 48 h after transfection with mimics, inhibitors, or mimics plus inhibitors of *miR-496* in MDA-MB-231 cells with an ELISA. Bars represent the mean ± SD. ***$P < .001$. Del-1 = developmental endothelial locus-1, SD = standard deviation.

cells and measured *Del-1* mRNA and protein levels using qPCR and ELISA, respectively. Del-1 expression was significantly downregulated (85%) in MDA-MB-231 transfected with *miR-496* mimics, whereas *miR-496* inhibitors reversed this effect ($P < .001$; Fig. 3A). Additionally, Del-1 protein levels were significantly decreased (72.5%) upon *miR-496* mimics transfection and, again, *miR-496* inhibitors reversed this effect ($P < .001$; Fig. 3B). These findings suggested that *miR-496* may suppress the transcription of Del-1 or induce its degradation.

3.4. miR-496 inhibits the proliferation, migration, and invasion of MDA-MB-231 cells

Del-1 has been shown to promote cancer progression.[3] Because *miR-496* negatively regulates *Del-1*, it might reverse the oncogenic effect of *Del-1* in TNBC. To investigate this hypothesis, we assessed the effect of *miR-496* on the proliferation, migration, and invasion of MDA-MB-231. MTT assays showed that the proliferation of MDA-MB-231 cells were significantly decreased upon transfection of *miR-496* mimics ($P < .0001$; Fig. 4A), whereas *miR-496* inhibitors had little effect on cell proliferation compared with the negative control miRNAs. The migration and invasion of MDA-MB-231 cells were significantly inhibited by *miR-496* mimics, whereas the effect of miR-496 inhibitors was similar to that of the negative control miRNAs (Figure 4B and C). In addition, we evaluated the functional effects of *miR-496* in another TNBC cell line, Hs578T, to exclude cell-line dependent effects Transfection of miR-496 mimics significantly inhibited proliferation, migration, and invasion of Hs578T cells, which showed consistent results with MDA-MB-231 cells (Supplementary Fig. 2, http://links.lww.com/MD2/A33). Collectively, these results showed that *miR-496* had a tumor suppressive role during the progression of TNBC via *Del-1* downregulation.

3.5. Effects of miR-496 and miR-137 on MDA-MB-231 cells

Among several miRNAs predicted to target *Del-1*, our previous work revealed *miR-137* also targets *Del-1*.[9] We therefore tested whether *miR-496* and *miR-137* act additively or synergistically in MDA-MB-231 cells. As shown in Figure 5, co-transfection of the 2 miRNAs additively reduced *Del-1* mRNA and protein expression ($P < .001$ and $P < .01$, respectively). Additionally, we showed their additive effect on the proliferation of MDA-MB-231, but not on the invasion and migration of these cells.

3.6. High miR-496 expression predicts a better prognosis

We then used the Kaplan–Meier Plotter database to evaluate the relation between *miR-496* levels and the prognosis of 97 patients with TNBC in whom *miR-496* levels were available in the TCGA database. We set a cutoff value of *miR-496* levels of 2 to separate the high and low expression groups. Although high *miR-496* expression was slightly associated with better survival (HR = 0.43; 95% CI = 0.14–1.37; $P = .14$; Fig. 6A), when node-negative TNBC cases (n = 53) were selected to minimize the impact of the clinical characteristics, a significant association was observed between *miR-496* and survival (HR and 95% CI were not computed due to no death event in the high *miR-496* group; $P = .022$; Fig. 6B). Meanwhile, *Del-1* expression was estimated as an unfavorable prognostic factor (Fig. 7). These results provide additional evidence that *miR-496* or Del-1 may play a role in TNBC progression.

4. Discussion

Given the lack of validated molecular targets and the poor outcomes, a greater understanding of TNBC at all levels is critical.[1] This study found that *miR-496* as well as *miR-137* is significantly downregulated in TNBC and *Del-1* is a direct target of these miRNAs. Furthermore, we found that the overexpression of *miR-496* inhibits TNBC cell proliferation, invasion, and migration by decreasing *Del-1* expression, and these effects were reversed by *miR-496* inhibitors. Since *miR-496* can downregulate both *Del-1* mRNA and protein, this miRNA might affect both *Del-1* mRNA degradation and translation. In summary, this study identified novel mRNA/miRNA interactions that contribute to TNBC via Del-1 and therefore represent a potential target for the development of new therapeutic strategies for TNBC. Notably, it corroborated the findings of previous studies investigating Del-1 as a diagnostic and prognostic marker of breast cancer.[2–4,9]

Del-1, initially identified as an extracellular matrix protein with epidermal growth factor-like domains, is over-expressed in several cancers and has been shown to induce tumorigenesis or cancer progression via direct mechanisms such as apoptosis and epithelial-mesenchymal transition[11–15] or indirect mechanisms such as angiogenesis and immune tolerance against tumor cells.[16–19] In breast cancer, Del-1 seems to enhance cancer progression via angiogenesis [20] and a different study showed the direct impact of Del-1 on breast cancer invasion and metastasis through the integrin-FAK signaling pathway.[3] The diagnostic and prognostic value of Del-1 has been first suggested in a proteomic study in 2 separate cohorts (n = 320 and 242, respectively) showing that Del-1 was upregulated as in the plasma of patients with breast cancer as well as in the media of MDA-MB-231 cells compared with controls.[2] Additionally, in a survival analysis of a Kyungpook National university Hospital cohort, Del-1 expression in tumor tissues has been found to be independently associated with worse survival in patients with TNBC.[4] Similarly, in this study, a survival analysis of a TCGA dataset showed that high *Del-1* expression was correlated with worse survival in TNBC early breast cancer. (Supplementary Table 1, http://links.lww.com/MD2/A36).

Despite evidence showing the role of Del-1 in tumor progression, how its expression is regulated in cancer cells is not clear. Since miRNAs act as post-transcriptional regulators of a variety of target genes, functioning as either tumor suppressors or oncogenes,[21,22] the identification of one or more miRNAs regulating *Del-1* expression might suggest their targeting ability to regulate *Del-1*. In this study, bioinformatic analyses pointed to *miR-496* as a potential regulator of *Del-1* expression. *miR-496* was significantly downregulated in TNBC cell lines and was found to suppress *Del-1* expression by directly binding to its mRNA. Additionally, functional studies demonstrated the inhibitory effect of *miR-496* on the proliferation, migration, and invasion of TNBC cells.

Interestingly, the examination of a public dataset revealed that high levels of *miR-496* correlate with the survival of patients in early-stages TNBC. Similarly, a survival analysis from a TCGA dataset relative to patients with pancreatic cancer has demonstrated the association of *miR-496* with survival.[23]

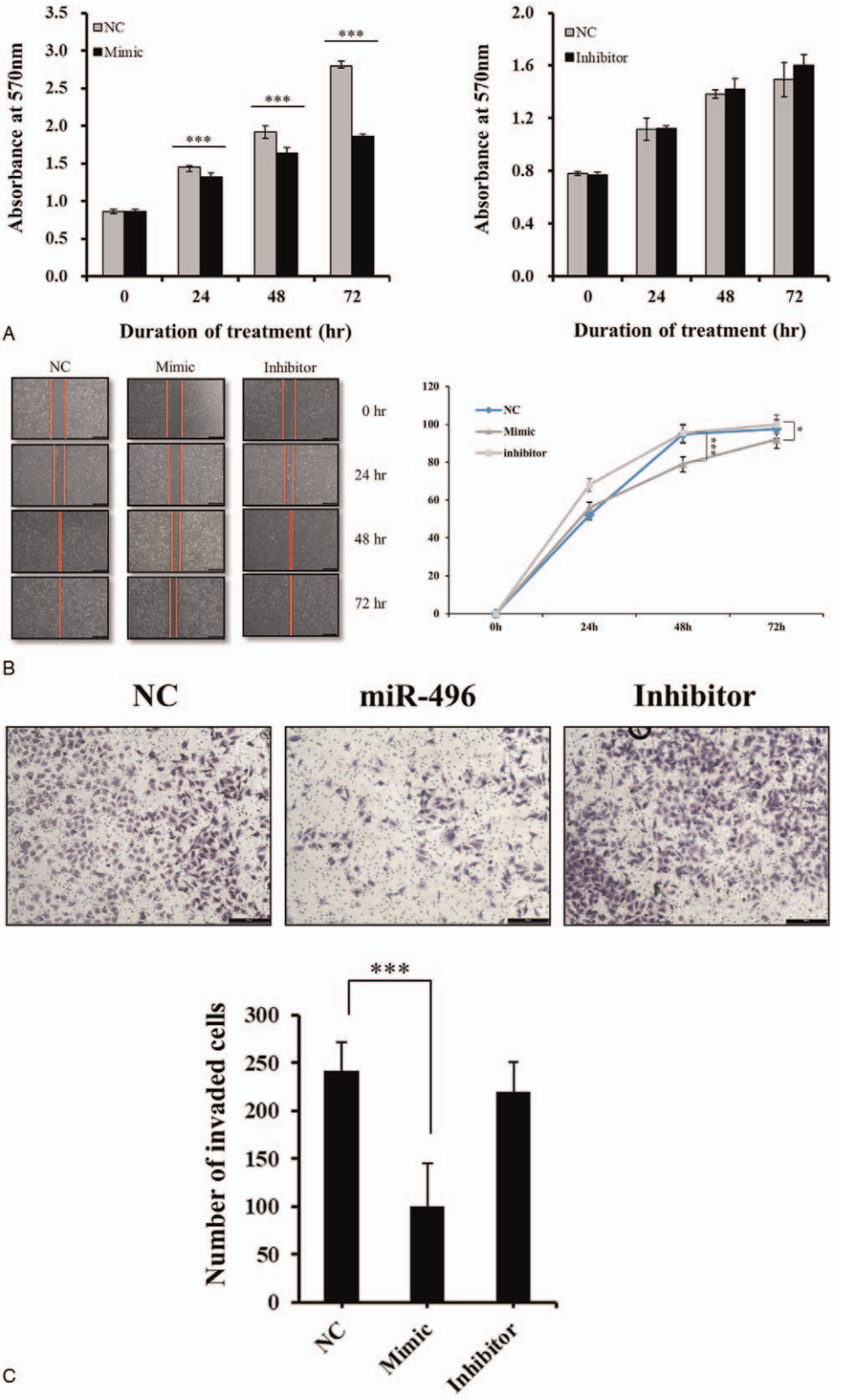

Figure 4. Proliferation, migration, and invasion of MDA-MB-231 cells following the overexpression and knockdown of *miR-496*: (A) MTT assays, (B) Wound scratch assays, and (C) Matrigel transwell assays. *: $P < .05$, ***: $P < .001$.

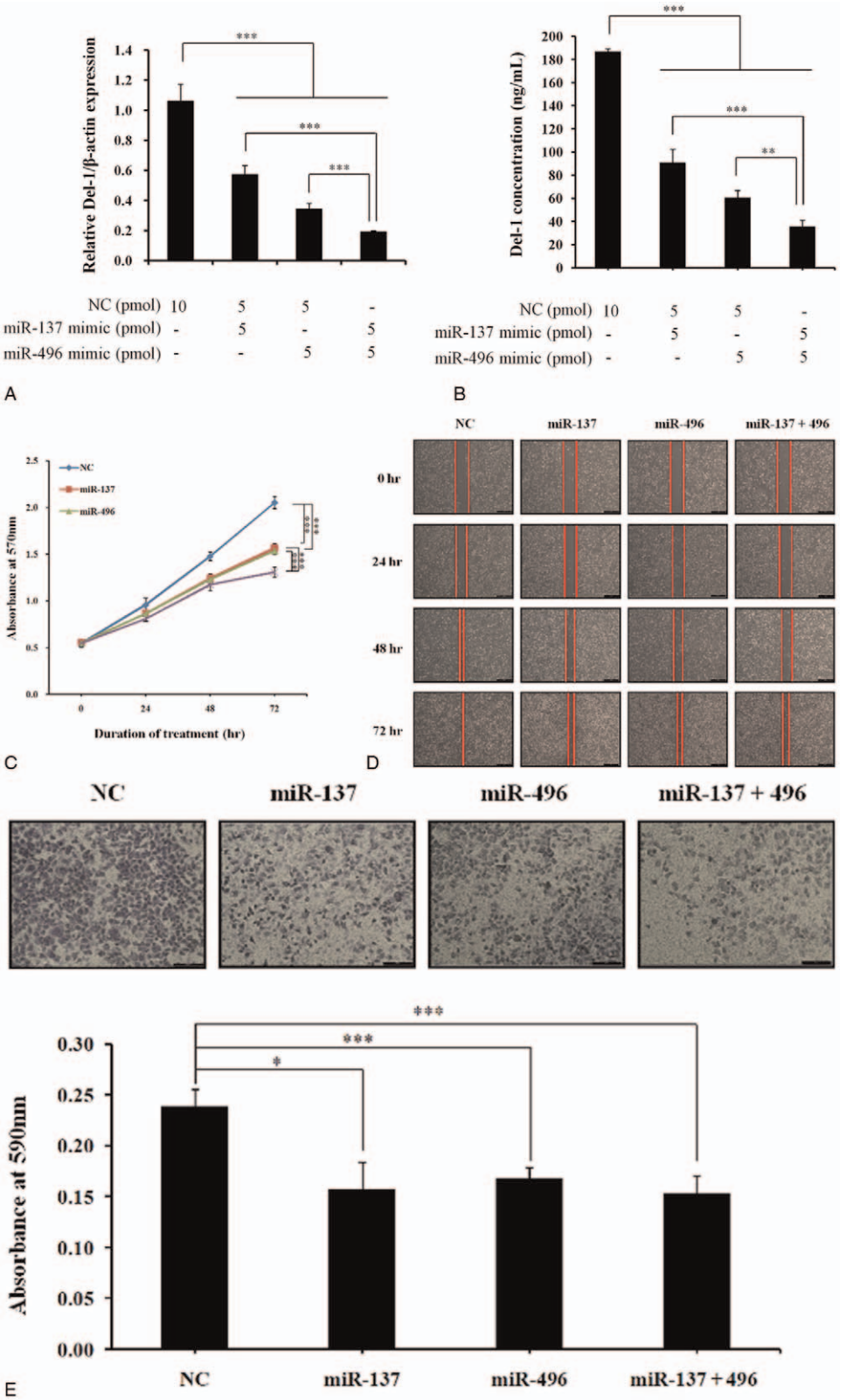

Figure 5. Additive inhibitory effect of both *miR-137* and *-496* in *Del-1* expression. (A) qRT-PCR analysis for *Del-1* mRNA expression level: upregulation of both *miR-137* and *-496* in MDA-MB-231 cells by transfecting *miR-137* and *-496* mimics resulted in additive reduction in *Del-1* mRNA transcription. (B) Western blot analysis for Del-1 protein levels in the cell lysate and soup from MDA-MB-231. (C) Proliferation of MDA-MB-231 cells was additively impaired by both miRNAs transfection in a MTT assay. (D) For cell migration using a wound scratch assay, both miRNAs respectively suppressed breast cancer cell migration compared with in normal controls, but there was an additive effect with both miRNAs. (E) Similarly, a transwell assay for cell invasion revealed that there was on additive effect on tumor cell migration and invasion although the significant decrease was observed with both miRNAs, separately under a light microscope (200 ×) (E upper) and confirmed by microplate reader at 590 nm (E bottom). *: $P < .05$, **: $P < 0.01$, ***: $P < .001$. Del-1 = developmental endothelial locus-1.

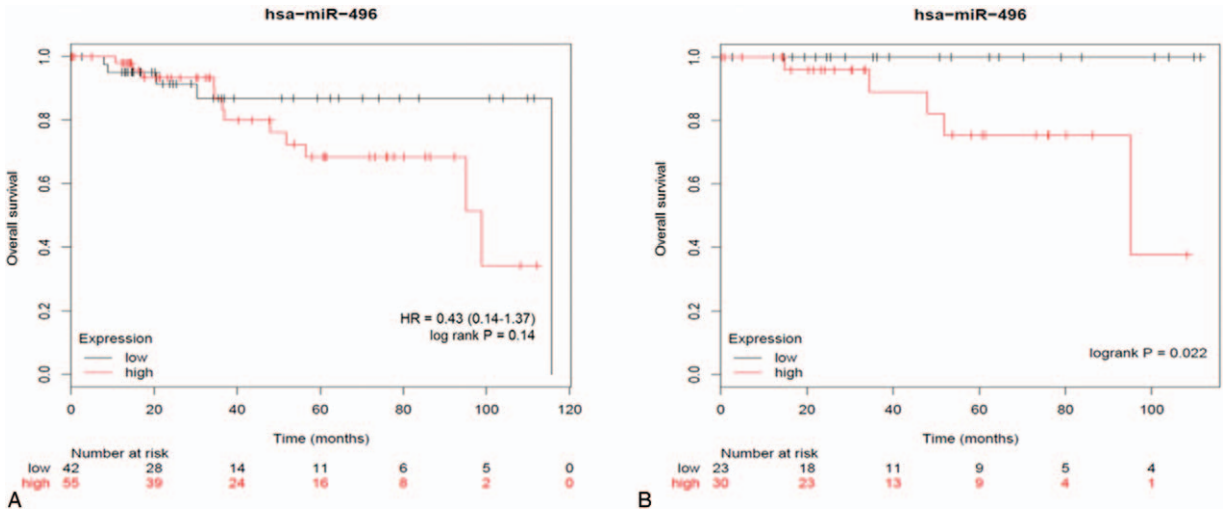

Figure 6. Survival analysis with the TCGA dataset (n = 1,077) using Kaplan–Meier Plotter (http://kmplot.com/analysis/) according to miR-496 expression among 97 TNBC patients for whom miRNA data were available (A) and 53 node-negative TNBC patients (B). TCGA = The Cancer Genome Atlas, TNBC = triple negative breast cancer.

To our knowledge, this is the first study describing the tumor suppressor role of *miR-496*, with the exception of a recent study showing that *miR-496* reduces mTOR expression, a key molecule of the PI3K/Akt/mTOR signaling pathway.[24] Thus, when considering the cross-talk between p53 and mTOR in cell growth, proliferation, and death, it is conceivable to hypothesize that *miR-496* affects the Del-1/p53/mTOR axis.

Notably, the correlations of *miR-496* with breast cancer have not been fully elucidated in a large cohort or prospective studies and, moreover, the underlying mechanisms are still unclear. In this study, we investigated the effect of *miR-496* with *miR-137* on *Del-1* expression, and found they have an additive effect on cancer cell proliferation and invasion, suggesting their independent action on *Del-1*.

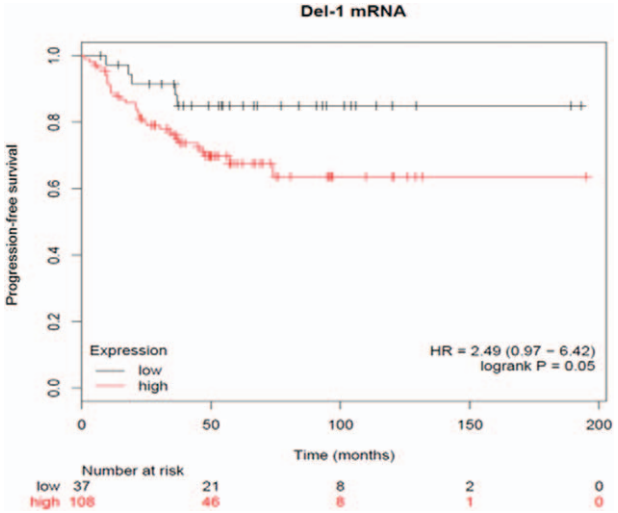

Figure 7. Survival analysis with web-based datasets (n = 3,951) using Kaplan–Meier Plotter (http://kmplot.com/analysis/) according to *Del-1* mRNA among 145 node-negative TNBC patients. Del-1 = developmental endothelial locus-1, TNBC = triple negative breast cancer.

Breast cancer cell lines have been proven to be useful in laboratory and preclinical investigations to predict the outcome of endocrine or anti-HER2 therapeutics in clinical trials for hormone responsive or HER2-overexpressing breast cancer subtypes, respectively. TNBC, however, is a heterogeneous disease both pathologically and molecularly and comprises several subtypes based on gene expression.[25] Therefore, there are several limitations in the investigation of TNBC with cell lines. Currently, there are ≥27 human breast cancer cell lines classified as TNBC but with different characteristics.[26] MDA-MB-231 cells, for example, which were used in this study, are the most commonly studied cell line for TNBC, but they were established in the 1970s, before routine evaluation of the expression of hormone receptors and HER2. The development of molecular science has subsequently revealed that MDA-MB-231 is a basal cell line with features of high-grade histology,[27,28] mutations in *P53*[29,30] or *KRas*,[29] and wild type *BRCA*[31,32] and genes in the PI3K pathway. [29] As such, the preclinical success of targeted agents using TNBC/basal cell lines does not translate into the success of clinical trials.[33,34] Given that TNBC is heterogeneous and the role of specific miRNAs are ultimately exclusive to different types of cells or tissues, [23] further studies with different types of TNBC cell lines are necessary to validate the exact role of miRNAs in breast cancer. We have shown that *miR-496* regulates Del-1 expression by interacting *miR-137* and affects the proliferation, migration, and invasion of MDA-MB-231 cells.

In summary, *miR-496* was found to be involved in *Del-1* regulation with *miR-137* via binding with the *Del-1* gene, affecting cancer progression by altering Del-1 expression, which suggests that *miR-137* and *miR-496* exert a tumor suppressive role by targeting *Del-1* in TNBC. Thus, we provide new insights into the potential mechanisms of *Del-1*-related oncogenesis and reveal the potential role of *miR-496* as a new biomarker for TNBC. However, when considering that *Del-1* is not the only target gene of *miR-137* and *miR-496*, and it was not down-regulated in all the TNBC tissues, further studies are warranted to investigate the exact mechanisms of *Del-1* regulation based on the heterogeneity of TNBC.

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References

[1] Anders CK, Carey LA. Biology, metastatic patterns, and treatment of patients with triple-negative breast cancer. Clin Breast Cancer 2009;9 (Suppl 2):S73–81.
[2] Moon PG, Lee JE, Cho YE, et al. Identification of developmental endothelial locus-1 on circulating extracellular vesicles as a Novel Biomarker for early breast cancer detection. Clin Cancer Res 2016;22:1757–66.
[3] Lee JE, Moon PG, Cho YE, et al. Identification of EDIL3 on extracellular vesicles involved in breast cancer cell invasion. J Proteomics 2016;131:17–28.
[4] Lee SJ, Lee J, Kim WW, et al. Del-1 expression as a potential biomarker in triple-negative early breast cancer. Oncology 2018;94:243–56.
[5] Lim LP, Lau NC, Garrett-Engele P, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 2005;433:769–73.
[6] Ambs S, Prueitt RL, Yi M, et al. Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. Cancer Res 2008;68:6162–70.
[7] Hobert O. Gene regulation by transcription factors and microRNAs. Science 2008;319:1785–6.
[8] Iorio MV, Croce CM. MicroRNAs in cancer: small molecules with a huge impact. J Clin Oncol 2009;27:5848–56.
[9] Lee SJ, Jeong JH, Kang SH, et al. MicroRNA-137 inhibits cancer progression by targeting Del-1 in triple-negative breast cancer cells. Int J Mol Sci 2019;20:
[10] Lanczky A, Nagy A, Bottai G, et al. miRpower: a web-tool to validate survival-associated miRNAs utilizing expression data from 2178 breast cancer patients. Breast Cancer Res Treat 2016;160:439–46.
[11] Goldhirsch A, Ingle JN, Gelber RD, et al. Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2009. Ann Oncol 2009;20:1319–29.
[12] Beckham CJ, Olsen J, Yin PN, et al. Bladder cancer exosomes contain EDIL-3/Del1 and facilitate cancer progression. J Urol 2014;192:583–92.
[13] Goldhirsch A. Personalized adjuvant therapies: lessons from the past: the opening address by the St. Gallen 2013 award recipient. Breast 2013;22 (Suppl 2):S3–7.
[14] Kim H, Lee SH, Lee MN, et al. Choi EY. p53 regulates the transcription of the anti-inflammatory molecule developmental endothelial locus-1 (Del-1). Oncotarget 2013;4:1976–85.
[15] Lee SH, Kim DY, Jing F, et al. Del-1 overexpression potentiates lung cancer cell proliferation and invasion. Biochem Biophys Res Commun 2015;468:92–8.
[16] Eskan MA, Jotwani R, Abe T, et al. The leukocyte integrin antagonist Del-1 inhibits IL-17-mediated inflammatory bone loss. Nat Immunol 2012;13:465–73.
[17] Choi EY, Chavakis E, Czabanka MA, et al. Del-1, an endogenous leukocyte-endothelial adhesion inhibitor, limits inflammatory cell recruitment. Science 2008;322:1101–4.
[18] Mitroulis I, Kang YY, Gahmberg CG, et al. Developmental endothelial locus-1 attenuates complement-dependent phagocytosis through inhibition of Mac-1-integrin. Thromb Haemost 2014;111:1004–6.
[19] Choi EY, Lim JH, Neuwirth A, et al. Developmental endothelial locus-1 is a homeostatic factor in the central nervous system limiting neuroinflammation and demyelination. Mol Psychiatry 2015;20:880–8.
[20] Aoka Y, Johnson FL, Penta K, et al. The embryonic angiogenic factor Del1 accelerates tumor growth by enhancing vascular formation. Microvasc Res 2002;64:148–61.
[21] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97.
[22] Meister G, Tuschl T. Mechanisms of gene silencing by double-stranded RNA. Nature 2004;431:343–9.
[23] Dou D, Yang S, Lin Y, et al. An eight-miRNA signature expression-based risk scoring system for prediction of survival in pancreatic adenocarcinoma. Cancer Biomark 2018.
[24] Rubie C, Kolsch K, Halajda B, et al. microRNA-496 - a new, potentially aging-relevant regulator of mTOR. Cell Cycle 2016;15:1108–16.
[25] Lehmann BD, Bauer JA, Chen X, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest 2011;121:2750–67.
[26] Chavez KJ, Garimella SV, Lipkowitz S. Triple negative breast cancer cell lines: one tool in the search for better treatment of triple negative breast cancer. Breast Dis 2010;32:35–48.
[27] Brinkley BR, Beall PT, Wible LJ, et al. Variations in cell form and cytoskeleton in human breast carcinoma cells in vitro. Cancer Res 1980;40:3118–29.
[28] Cailleau R, Young R, Olive M, et al. Breast tumor cell lines from pleural effusions. J Natl Cancer Inst 1974;53:661–74.
[29] Hollestelle A, Nagel JH, Smid M, et al. Distinct gene mutation profiles among luminal-type and basal-type breast cancer cell lines. Breast Cancer Res Treat 2010;121:53–64.
[30] O'Connor PM, Jackman J, Bae I, et al. Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. Cancer Res 1997;57:4285–300.
[31] Kao J, Salari K, Bocanegra M, et al. Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. PLoS One 2009;4:e6146.
[32] Elstrodt F, Hollestelle A, Nagel JH, et al. BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants. Cancer Res 2006;66:41–5.
[33] Burness ML, Grushko TA, Olopade OI. Epidermal growth factor receptor in triple-negative and basal-like breast cancer: promising clinical target or only a marker? Cancer J 2010;16:23–32.
[34] Finn RS, Bengala C, Ibrahim N, et al. Dasatinib as a single agent in triple-negative breast cancer: results of an open-label phase 2 study. Clin Cancer Res 2011;17:6905–13.