MicroRNA-874 inhibits cell proliferation and induces apoptosis in human breast cancer by targeting CDK9

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**ABSTRACT**

It has been demonstrated that miR-874 plays important roles in many types of cancers. Nevertheless, its biological function in breast cancer remains largely unknown. In this study, we found that the expression level of miR-874 is down-regulated in breast cancer in comparison with the adjacent normal tissues. The overexpression of miR-874 is able to inhibit cell proliferation and induce cell apoptosis and cell cycle arrest in MCF7 and MDA-MB-231 cells. Using a bioinformatics method, we further show that CDK9 is a direct target of miR-874 and that its protein level is negatively regulated by miR-874. Therefore, the data reported in this manuscript demonstrate that miR-874 is an important regulator in breast cancer and imply that the miR-874/CDK9 axis has potential as a therapeutic target for breast cancer.

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1. Introduction

Breast cancer is the most common cancer in women around the world, which can be influenced by a number of environmental factors and is characterized by molecular heterogeneity [1]. Despite various existing screening programs and new therapeutic strategies have been implemented to treat breast cancer and significantly reduced mortality rates, the molecular mechanisms underlying breast cancer pathogenesis are only partially understood [2]. MicroRNAs (miRNA) are 19–25 nucleotide non-coding RNAs and have critical role in cancer progression through either protein translation repression or mRNA degradation to regulate their target mRNAs [3]. It has been shown that miRNAs serve as oncogenes or tumor suppressor genes to control cell proliferation, invasion, migration, differentiation and apoptosis [4–6]. With deregulated expression in several cancers including breast cancer, miRNAs are evolving as potential diagnostic and therapeutic markers [7]. Recently, the tumor suppressive role of miR-874 by inhibition of cell proliferation and invasion was reported in gastric cancer [8]. It has been demonstrated that miR-874 can inhibit NSCLC-initiating cells invasion and migration via directly regulating the protein expression of MMP-2 and uPA in vitro and in vivo [9]. Nohata et al. [10] found that comparing with adjacent normal epithelia, the expression levels of miR-874 were significantly downregulated in HNSCC (head and neck squamous cell carcinoma) tissues. Furthermore, the overexpression of miR-874 in HNSCC cell lines (SAS and FaDu) revealed that miR-874 can directly target HDAC1 (histone deacetylase 1) to inhibit cell proliferation and induce cell apoptosis. In another study conducted in maxillary sinus squamous cell carcinoma (MSSCC), miR-874 was found downregulated in MSSCC specimens and PPP1CA (protein phosphatase 1, catalytic subunit, a isoform) was identified as a direct target of miR-874 [11]. Cyclin-dependent kinase 9 (CDK9), a member of the cdcl2-like serine/threonine kinase family, interacts with specific types of cyclins to exert its biological activity [12,13]. This interaction forms a heterodimer, in which the Cdk serves as catalytic domain, and the cyclin partner, T cyclins (T1, T2a and T2b) and cyclin K, functions as the regulatory subunit [14,15]. It has been described that a number of human malignancies occur deregulations of the CDK9-related pathway, such as lymphomas [16], neuroblastoma [17], primary neuroectodermal tumor [18], rhabdomyosarcoma [19], and prostate cancer [20].

In the present study, the role of miR-874 and CDK9 in breast cancer tissues and cells was investigated. Our findings verified that

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the expression of miR-874 was downregulated, while CDK9 was upregulated in the breast cancer tissues compared with adjacent normal tissues. Overexpression of miR-874 can lead to suppressing of breast cancer cells growth, colony formation, cell cycle and increasing of cell apoptosis. Furthermore, we experimentally validated that miR-874 can directly target the 3’UTR of CDK9 and suppress its expression. Finally, we determined that knockdown of CDK9 can promote apoptosis and induced cell cycle arrest in breast cancer cells. Therefore, our results demonstrated that miR-874 can mediate its tumor suppressor function, at least in part, by suppressing the expression of CDK9. Altogether, our study characterized a novel microRNA-mediated mechanism of CDK9 regulation and suggests tumor inhibiting actions of miR-874 in breast cancer cells.

2. Materials and methods

2.1. Patient samples

Breast cancer specimens and adjacent normal tissues were collected in the First Affiliated Hospital of Nanjing Medical University (Nanjing, China) from January 2011 to December 2013. All the patients recruited into the present study did not receive radiotherapy, chemotherapy or any other treatment before and after operation. Surgical specimens of the tumor resection were collected, and lumps of tumors as well as adjacent normal tissues, which were at least 2 cm distal to tumor margins, were snap-frozen in liquid nitrogen for miR-874 and CDK9 assays. Written informed consent was obtained from all the study participants. The use of tissue samples were approved by the ethical committees of the ethical committees of the First Affiliated Hospital of Nanjing Medical University. The detailed patient information is shown in Table 1.

2.2. Cell culture and transfection

Two widely used cell lines, a more aggressive breast cancer cell line (MCF-7) and a less aggressive breast cancer cell line (MDA-MB-231) were used in this study, which were obtained from ATCC and maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% antibiotics (Invitrogen, USA). Transfection of the cells with miR-874 mimics or miR-Control (GenePharma, China) was performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions. The transfected amount of miR-874 mimics and miR-Control was 10 pmol per 1 × 10⁵ cells.

2.3. Detection of cell phenotypes

The effect of miR-874 on proliferation of breast cancer cells was evaluated by the MTT assay. MCF-7 and MDA-MB-231 cells were plated in 96-well culture plates (3 × 10³ per well). After 24 h incubation, the cells were transfected with miR-874 mimics (30 pmol) or miR-Control (30 pmol) for 12, 24 and 48 h. Then the MTT (0.5 mg/ml; Sigma–Aldrich, USA) was added to each well (20 µl/well). After 4 h of additional incubation, MTT solution was discarded and 200 µl of DMSO (Sigma, USA) was added, and the plates were shaken gently. The absorbance was measured on an ELISA reader at a wavelength of 570 nm. For the colony formation assay, cells were counted and seeded in 12-well plates (in triplicate) at 100 cells per well. Fresh culture medium was replaced every three days. The number of viable cell colonies was determined after 14 days, and the colonies were fixed with methanol, stained with crystal violet, photographed and counted. Each experiment was performed in triplicate.

2.4. Cell cycle and apoptosis assays

The transfected MCF-7 and MDA-MB-231 cells were seeded into six-well plates for 24 h in complete medium. Then, the cells were deprived of serum for 48 h followed by returning complete medium for an additional 24 h. After that, the cells were collected by centrifugation, fixed in 95% ethanol, incubated at −20 °C overnight and washed with phosphate buffered saline (PBS). The cells were then resuspended in 1 ml of FACs solution (PBS, 0.1% TritonX-100, 60 µg/ml propidium iodide (PI), 0.1 mg/ml DNase free RNase, and 0.1% trisodium citrate) with final incubation on ice for 30 min. The cells were analyzed using a FACScalibur flow cytometer (Beckman Coulter). A total of 10000 events were counted for each sample. For the Annexin V assay, the MCF-7 and MDA-MB-231 cells were transfected. After 48 h, the DNA content was determined by propidium iodide staining as described by Hwang et al. [21], and Annexin V staining was performed with the Vybrant Apoptosis Assay Kit (Invitrogen).

2.5. Western blotting

Western blotting was performed to determine protein expression of CDK9. The total protein was extracted by TRIzol reagent (Invitrogen, USA) and the protein concentration in the supernatants were determined using Bradford protein dye reagent (Bio-Rad, Hercules, CA), and the volumes of the supernatant were then adjusted for equal protein concentration in all of the samples. Immunoblotting was performed as described previously [22]. Antibodies specific to CDK9 and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.6. RT-PCR

The RNAs were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed using gene-specific reverse primers and reverse transcriptase (Takara, Japan), and the resulting cDNAs were PCR-amplified on an ABI 7500 thermocycler (Applied Biosystems). For the detection of miR-874, the following primers were used: miR-874, qRT-PCR stem-loop primer, 5’-GTCGTATCCAG TGCAGGGTCCAGGTATCGCACTGGATACGACTCTTAGG-3’, Qpcr

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Table 1
Breast cancer patient clinical information.

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forward primer, 5′-TGCGGCGGCCACGCACCAG-3', Qpcr reverse primer, 5′-CCAGTGCAGGGTCCGAGGT-3'; U6, Qrt-pcr stem-loop primer, 5′-GTGCGTATCCAGTCCAGGGTCCGAGGTCACTGGATACGAAAAATATGG-3′, qPCR forward primer, 5′-TGCGGTGTGCTCGCTTGTGCAGCAACTGGAATACGA CAAAATATGG-3′, qPCR reverse primer, 5′-CCAGTGCAGGGTCCGAGGT-3′. U6 was used to standardize the amounts of miRNAs in each samples.

2.7. Construction of 3′UTR reporter plasmid and luciferase assay

The human CDK9 3′UTR harboring miR-874 target sequence as well as the seed-sequence-mutated version (miR-874–3′UTR-mut) were synthesized by GenPharm (Shanghai, China). The CDK9 3′UTR reporter was generated by inserting the entire 3′UTR or 3′UTR-mut of human CDK9 mRNA into XhoI/NotI sites of psiCHECK-2 vector (Promega) downstream of the Renilla luciferase gene. For the luciferase assay, 1 × 10^5 cells were transfected with the CDK9 3′UTR reporter and the miR-874 mimics in a 24-well plate using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. After 24 h, the firefly and Renilla luciferase activities were measured consecutively using Dual Luciferase Assay (Promega).

2.8. Knockdown of CDK9 by siRNA

The transient transfection of CDK9 siRNA was performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions. After 48–72 h, the cells were analyzed to determine the effects of the knockdown. The silencing efficiency was confirmed by western blot. The siRNA

![Graph A](image1.png)  
**Fig. 1.** Expression of miR-874 in breast cancer tissues. (A) The relative expression of miR-874 (normalized to U6) was detected using qRT-PCR in breast cancer tissues and matched adjacent normal tissues; (B) Relative expression of miR-874 in well- and moderately differentiation tissues as well as tissues with poor or no differentiation; (C) Relative expression of miR-874 in tumor size less than 5 cm and tumor sizes greater than 5 cm.
targeting CDK9 was purchased from Santa Cruz Biotechnology (sc-29268 company,货号).

2.9. Tumorigenicity in vivo

The lentiviral vector that over-expresses pre-miR-874 and the control lentiviral packaging plasmid was purchased from Genechem (Genechem, Shanghai, China). A total of sixteen nude mice (BALB/c nude mice, Vital-river, Nanjing, China; 4 weeks old) were randomly divided into two groups. MCF-7 cells stably transfected with pre-miR-874 mimics and pre-miR-Control were inoculated bilaterally and subcutaneously into the flanks of nude mice. Bidi-dimensional tumor measurements were taken with vernier calipers every seven days, and the mice were euthanized after four weeks. The volume of the implanted tumor was calculated using the formula: volume = (width^2 × length)/2.

2.10. Statistical analysis

A Student’s test was performed to analyze the significance of differences between the sample means obtained from three independent experiments. Differences were considered statistically significant at P < 0.05.

3. Results

3.1. Relative expression of miR-874 in breast cancer tissue

In this study, the expression levels of miR-874 were measured with qRT-PCR in 26 pairs of breast cancer tissues and adjacent normal tissues, which showed that miR-874 was downregulated in the breast cancer tissues (Fig. 1A). In addition, we showed that miR-874 expression was associated with pathological differentiation and tumor size in breast cancer tissues. As shown in Fig. 1B, the expression of miR-874 were down-regulated in breast cancer tissues with poor or no differentiation compared with well and moderate differentiation tissues. Furthermore, lower expression of miR-874 in breast cancer tissues with tumor size greater than 5 cm compared with tissues with tumor size less than 5 cm (Fig. 1C).

3.2. miR-874 suppresses breast cancer proliferation in vitro and in vivo

To investigate the function of miR-874 in the breast cancer cells lines, MCF-7 and MDA-MB-231 were transfected with miR-874 mimics. As shown in Fig. 2A, miR-874 mimics increased the expression of miR-874 by 17-fold and 16-fold in MCF-7 and MDA-MB-231 cells, respectively. Growth curve and MTT assays were performed to detect the effects of miR-874 on cell growth in vitro. Our data demonstrated that the relative cell growth was significantly inhibited in the miR-874 mimics transfected cells (Fig. 2B–D). As shown in Fig. 2E, our data showed that the cell apoptosis was induced obviously in miR-874 mimics transfected MCF-7 and MDA-MB-231 cells. To assess the effect of miR-874 on tumorigenicity in vivo, we packaged recombinant lentivirus producing the miR-874 precursor and infected the MCF-7 cells. The transfected cells were injected into a flank of nude mice to form ectopic tumors. Cells transfected with negative lentiviral vector was used as negative control and inoculated into the opposite flank of the same mice. Notably, miR-874 over-expression significantly suppressed tumorigenicity in a time dependent manner in vivo, where the tumor size was dramatically decreased compared with that of the controls (Fig. 2F).
3.3. miR-874 induces apoptosis and inhibits cell cycle in breast cancer cell lines

To address the mechanism underlying miR-874-modulated cell growth, TUNEL-TMR staining was used to detect the effect of miR-874 on cell apoptosis. The proportion of apoptotic MCF-7 cells transfected with miR-874 was significantly higher than the miR-control group (11.5% vs 21.6%). In the MDA-MB-231 cells, we also detected an increased percentage of apoptotic cells compared with that of miR-control group (17.8% vs 23.3%). Moreover, cell cycle arrest was observed in miR-874 transfected MCF-7 and MDA-MB-231 cells via increasing the percentage of cells in G0/G1 phase and reducing the cell population in S phase (Fig. 3C and D).

3.4. miR-874 directly targets and inhibits CDK9 protein expression

To explore the potential target of miR-874, five computational algorithms, namely DIANA, miRDB, TargetScan, miRwalk and miranda, were used and a set of different target genes were predicted. Among these candidate targets, CDK9 attracted our attention immediately which was predicted by all five algorithms (Fig. 4A). Moreover, we found out that the binding of miR-874 with CDK9 seed sequence had a minimum free energy (mfe) of $-25.7$ kcal/mol, suggesting that the binding of miR-874 with CDK9 may form a stable structure. To investigate whether miR-874 can directly target CDK9, the 3'UTR luciferase reporter assay was performed, which showed the miR-874 had an obvious inhibitory effect on the luciferase intensity of wild-type 3'UTR luciferase reporter. However, the inhibitory effect of miR-874 was reduced in the presence of mutant 3'UTR luciferase reporter (Fig. 4C). We then tested whether miR-874 can affect the expression of endogenous CDK9. The restoration of the miR-874 expression resulted in a reduction of endogenous CDK9 protein expression in MCF-7 and MDA-MB-231 cells, while miR-874 had no effect on the CDK9 RNA level (Fig. 4D). These results provide evidence that miR-874 can directly target the 3'UTR of CDK9 mRNA, resulting CDK9 translation inhibition and suppressing its expression.

3.5. The expression of CDK9 in breast cancer tissues

Since we have demonstrated the expression levels of CDK9 in breast cancer cell lines and tissues, qRT-PCR was used to measure the mRNA expression levels of CDK9 in the 20 pairs breast cancer tissues and adjacent normal tissues (ANT). Compared with the ANT, the expression of CDK9 were markedly increased in breast cancer tissues (Fig. 5A). Furthermore, the protein expression of CDK9 in three delegated breast cancer tissues and ANT are showed in Fig. 5B, which demonstrated a significantly higher protein expression of CDK9 in breast cancer tissues compared with ANT.

3.6. Knockdown of CDK9 inhibits cell proliferation

To investigate the function of CDK9 in breast cancer cells lines, CDK9 siRNA was transfected into MCF-7 and MDA-MB-231 cells. As shown in Fig. 6A, CDK9 siRNA decreased 70% and 80% of the expression of CDK9 in MCF-7 and MDA-MB-231 cells, respectively. Colony formation and MTT assays were performed to assess the effects of CDK9 on cell growth in vitro. Our data showed that relative cell growth was significantly inhibited in CDK9 siRNA transfected cells (Fig. 6B–D). As shown in Fig. 6E and F, our data

Fig. 3. miR-874 induces cell apoptosis and cell cycle arrest. (A and B) TUNEL-TMR staining was used to detect DNA fragmentation during programmed cell death in MCF-7 and MDA-MB-231 cells transfected with miR-874 mimics or miR-control; (C and D) FACS analysis was used to detect the effect of miR-874 on the cell cycle of MCF-7 and MDA-MB-231 cells.
Fig. 4. miR-874 targets CDK9 to repress its expression. (A) Bioinformatic analysis using DIANA, miRDB, Targetscan, miRwalk and miRanda predicted that miR-874 may target CDK9; (B) Putative miR-874 sites in the 3'UTR region of CDK9 as determined by RNAhybrid program; (C) MCF-7 cells were transfected with miR-874 mimics to analyze the effect on the luciferase intensity of pGL3/Luciferase–CDK9–3'UTR reporter; (D) Real-time PCR and western blot assays were used to analyze the effect of miR-874 on CDK9 mRNA in MCF-7 and MDA-MB-231 cells.

Fig. 5. CDK9 levels in breast cancer tissues. (A) qRT-PCR was used to detect the expression of CDK9 in breast cancer tissues and adjacent normal tissues (ANT); (B) Western blot was used to detect the expression of CDK9 in three pairs of breast cancer tissues and ANT.
demonstrated that cell apoptosis was induced obviously in CDK9 siRNA transfected MCF-7 and MDA-MB-231 cells (13.8% in miR-control group vs. 33.6% in CDK9 siRNA group). Furthermore, we demonstrated that knockdown of CDK9 by siRNA lead to the cell cycle arrest in MCF-7 and MDA-MB-231 cells by increasing the percentage of cells in G0/G1 phase and reducing the cell population in the S phase (Fig. 6G and H).

4. Discussion

Numerous studies have suggested the potential role of microRNAs as oncogenic or tumor suppressive genes to cell cycle and apoptosis via the regulation of different target genes in cancers [23]. A set of miRNAs with differential expression genes in cancers [23]. A set of miRNAs with differential expression genes in cancers Fig. 6. Knockdown of CDK9 inhibits the cell proliferation and induces the cell apoptosis and cell cycle arrest in breast cancer cells. (A) Western blot was used to detect the transfection efficiency of siRNA CDK9 in MCF-7 and MDA-MB-231 cells; (B) The long-term cell growth was determined by colony formation assay; (C and D) Cell survival was determined by MTT assay; (E and F) The effect of siRNA CDK9 on cell apoptosis was detected using Annexin V-FITC/PI double staining method in MCF-7 and MDA-MB-231 cells by flow cytometry; (G and H) FACS was used to detect the effect of siRNA CDK9 on the cell cycle of MCF-7 and MDA-MB-231 cells.
including miR-145, miR-122a, miR-125a-5p, miR-126, miR-200c, miR-10b, and miR-96 [24]. Furthermore, multivariate analysis in breast cancer identified that downregulation of miR-155 and let-7 expression were significantly correlated with a shorter patient survival rate [25,26].

miR-874 is located on chromosome 5q31.2, a well-known frequent fragile site in the human genome which is often deleted in cancers and genetic disorders, and this site is also specifically correlated with chromosome rearrangements in cancer [9]. A previous investigation has identified a significant loss of miR-874 expression in NSCLC tissue samples. Here, we determined the miR-874 expression levels in breast cancer tissues and normal tissues by Real-time PCR analysis, which displayed remarkable down-regulation of miR-874 in the breast cancer tissues. Furthermore, down-regulation of miR-874 were also observed in poor- or no-differentiation tissues, and tissues with a tumor size greater than 5 cm. Thus, these results suggest the potential tumor suppressive role of miR-874. And this was further confirmed by the experiment in the breast cancer cell lines. Using breast cancer cell lines, MCF-7 and MDA-MB-231, we confirmed that overexpression of miR-874 decreased cell proliferation as determined by MTT and colony formation assays. Furthermore, miR-874 overexpression significantly suppressed tumor size in a time dependent manner in vivo.

Recently, an increasing number of studies have demonstrated that the disregulation of miRNAs can exert its functions in cell cycle progression and apoptosis in many human cancers. Researchers have identified that miR-15a can inhibit the cell cycle by targeting CCNE1 in breast cancer cells [27]. Functional assays revealed that MCF-7 cells growth can be suppressed by miR-497 through increasing the percentage of early apoptotic cells [28]. In our study, we identified that overexpression of miR-874 significantly increased the apoptosis of MCF-7 and MDA-MB-231 cells. Furthermore, we confirmed that miR-874 can induce cell cycle arrest.

In general, miRNAs exert its functions via binding to the 3’UTR of the target gene to repress its expression [29]. To identify the target genes of miR-874, bioinformatics analysis was performed via different computational algorithms and CDK9 was screened as our interest target gene. We then confirmed miR-874 had obvious inhibiting effect on the luciferase intensity of CDK9–3’UTR reporter, whereas it had no further inhibiting effect on the CDK9–3’UTR-mut luciferase reporter, in which the miR-874 seed sequence was mutated, which indicate that CDK9 is a direct target for miR-874. Furthermore, miR-874 significantly suppressed the protein level of CDK9 without affecting its mRNA level, indicating that miR-874 can directly target the 3’UTR of CDK9 mRNA, resulting CDK9 translation inhibition and suppressing its expression.

Recent studies have shown that the CDK9 is overexpressed in several tumors compared with normal tissues [16]. Furthermore, some of these studies have demonstrated that CDK9 can exhibit anti-apoptotic effects or sustaining cell growth on several cancer types [30]. In this study, our findings demonstrated that CDK9 expressed at higher levels in breast cancer tissues compared with normal tissues. To further confirm that miR-874 can directly target CDK9, the knockdown plasmid of CDK9 (siRNA CDK9) was used, which demonstrate that siRNA CDK9 can suppress MCF-7 and MDA-MB-231 cell lines proliferation. Furthermore, the knockdown plasmid promoted cell apoptosis and induced cell cycle arrest, which is consistent with the function of miR-874 overexpression.

In conclusion, the present study provides new insights into the specific function of miR-874 and its mechanism in breast cancer proliferation (Fig. 7), and suggests that targeting of miR-874 may provide a potential therapeutic strategy for blocking proliferation in breast cancer.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgement

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References


Fig. 7. A proposed model of the miR-874/CDK9 axis in breast cancer tumorigenesis.


