Research Article

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Negative regulation of CDC42 expression and cell cycle progression by miR-29a in breast cancer

Abstract: Objective: The inhibitory role of microRNA-29a (miR-29a) has been assessed in breast cancer cells. Herein, we analyze the underlying mechanisms of its role in cell cycle progression in breast cancer cells. Methods: We applied real-time polymerase chain reaction (PCR) to detect the expression of miR-29 in breast cancer cell lines. Then one of the cell lines, MDA-MB-453, was transfected with mimics of miR-29a. The cell cycle was analyzed by fluorescence-activated cell sorting after staining the cells with propidium iodide. Real-time PCR, luciferase assay and western blot were used together to verify the regulation of the predicted target, cell division cycle 42 (CDC42) by miR-29a. Results: MiR-29s were decreased in our selected mammary cell lines, among which miR-29a was the dominant isoform. Overexpression of miR-29a caused cell cycle arrest at the G0/G1 phase. We further found that miR-29a could target the expression of CDC42, which is a small GTPase associated with cell cycle progression. Conclusion: We suggest that miR-29a exerts its tumor suppressor role in breast cancer cells partially by arresting the cell cycle through negative regulation of CDC42.

Keywords: miR-29s, breast cancer, cell cycle, CDC42

1 Introduction

In women, breast cancer (BC) represents the most commonly diagnosed cancer, which is also the second leading cause of cancer-related death after lung cancer [1]. During the last few decades, progress has been made in its prognosis due to efficient and exact early diagnosis, as well as successful radical surgery and adjuvant therapy [2-3]. However, the current determination of prognosis of BC still remains unsatisfactory, and the 5-year survival rate upon the occurrence of metastasis declines to less than 25% [4-6]. Actually, the mechanism underlying the development and progression of BC is far from clearly understood. Hence, gathering more and precise knowledge about its progression would facilitate the generation of novel diagnostic and therapeutic targets. Now there is a large body of evidence showing that a class of small non-coding RNAs, microRNAs (miRNAs), play important parts in BC progression [7-10].

The microRNA-29 (miR-29) family, which is composed of miR-29a, -b and -c, has been shown to be dysregulated and crucially involved in various types of human cancers [11-14], incuding BC [15,16]. Specifically, Wu et al. showed that miR-29a was significantly decreased in different types of BC, and overexpression of miR-29a resulted in cell growth defects. They speculated that the growth inhibitory role of miR-29a might be mediated by its direct targeting B-Myb transcription factor, which is closely associated with tumorigenesis [17]. However, whether other molecular explanations exist, which could further characterize the function of miR-29a in BC cells, has not been well addressed.

Cell division cycle 42 (CDC42) is a well-known member of the Ras homolog (Rho) family. It regulates crucial cellular processes, including cell cycle, and cell cytoskeleton organization [18,19]. The negative regulation of CDC42 by miR-133 has been reported in gastric cancer cells, which correlates with cell proliferation and migration defects [20]. In the current study, we have endeavored to evaluate the role of miR-29a in breast cancer cells and have constructed the relationship of miR-29a with CDC42. Our

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findings might highlight miR-29a as a novel therapeutic target for BC treatment.

2 Materials and methods

2.1 Cell culture

MCF-10A, MDA-MB-453, MDA-MB-231, T47D and MCF-7 cells were obtained from American Type Culture Collection. Cells were maintained in their proper media and placed in a humidified incubator with 5% CO_2 at 37°C.

2.2 Reagents

Control microRNA and miR-29a mimics were purchased from Genepharma. Fetal bovine serum was from GIBCO. The SuperSignal Substrate Western blotting detection system was from Pierce. B-actin antibody and CDC42 antibody were purchased from Santa Cruz. Luciferase Assay Kit was purchased from Promega. Lipofectamine2000 reagent was purchased from Invitrogen.

2.3 Cell transfection

MDA-MB-453 cells were transfected with control or miR-29a mimics by using lipofectamine2000. After 24 hours, cells were used in different experiments. Transfection of MDA-MB-453 cells for luciferase assay is described in detail below.

2.4 Quantitative RT-PCR

Total RNA was extracted using Invitrogen Trizol Reagent. The total level of miR-29s or CDC42 was quantified by qRT-PCR by TaKaRa SYBR Green Real-time PCR Master Mix Kit. Fold changes of miRNA and/or mRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method using the levels of U6B (for miRNA) or gapdh (for mRNA) as the internal controls.

2.5 Cell counting

MDA-MB-453 cells were transfected with control or miR-29a mimics for 48 hours. After that, cells were trypsinzed and seeded into 24-well plates at a density of 6000 cells. Then cells were counted by a blood-cell-counting chamber for the next 4 days. Experiments were repeated at least three times independently.

2.6 Cell cycle assay

The transfected MDA-MB-453 cells were harvested, fixed with cold 70% ethanol overnight at −20°C, and incubated in the dark with RNase (100mg/ml) and propidium iodide (50 mg/ml) for 1 hour at 37°C. A total of 30000 nuclei were examined by flow cytometry.

2.7 Western blotting

Breast cancer cells were lysed with RIPA lysis buffer with a protease inhibitor mixture. After quantification, equal amounts of protein were separated by 10% SDS-PAGE, transferred to Millipore polyvinylidene difluoride membranes, immunoblotted with primary antibodies, and visualized with horseradish-peroxidase-coupled secondary antibodies.

2.8 Dual-luciferase reporter assays

The 3'-untranslated region (3'-UTR) of CDC42 containing the predicted binding site for miR-29 was amplified and cloned into pGL3 vector. Forty-eight hours after transfection, luciferase activity was detected using a dual-luciferase reporter assay system and normalized to Renilla activity.

2.9 miR-29 target prediction

Candidate targets of miR-29 were predicted by miRBase (http://www.mirbase.org/) and TargetScan (http://www. Targetscan.org/).

2.10 Statistical analysis

Statistical analysis was performed using SPSS18.0. Values were expressed as mean ± standard deviation (s.d.). Differences between groups were calculated using the Student's t-test. P value < 0.05 was considered as statistically significant.

3 Results

3.1 Downregulation of miR-29 in BC cell lines

To examine the relative expression levels of miR-29, including −a, −b and −c, real-time PCR was performed in four human BC cell lines (MDA-MB-453, MD-MB-231, T47D and MCF-7), and in the normal breast epithelium cell line MCF-10A. As shown in Figure 1, all miR-29 showed significantly downregulation in BC cell lines when compared to that of the control MCF-10A cells. Notably, among the miR-29 members, miR-29a represented as the dominant isoform, which showed the most decreased expression in MDA-MB-453 cells. The downregulation of miR-29 in BC cell lines was consistent with the previous findings [17], and suggested that miR-29 might function in a similar way to suppress BC progression.

3.2 miR-29a negatively modulate MDA-MB-453 cell growth in vitro

To verify the biological roles, especially the growth inhibitory activities of miR-29a in BC cells, cell growth and cell cycle profile were investigated in MDA-MB-453 cells. As shown in Figure 2A, after synthesized mimics of miR-29a was transfected into the MDA-MB-453 cells, the indicated expression level of miR-29a was significantly elevated. Meanwhile, MDA-MB-453 cells overexpressed with miR-29a displayed slower cell growth rate than control cells (Figure 2B). Importantly, compared to the control

Figure 1: Relative levels of miR-29 in normal mammary epithelia and breast cancer cells. (A) Comparison of miR-29 isoforms. **(B)** Relative levels of miR-29 were normalized against the level in MCF-10A cell; U6B was used as an internal control.

cells, overexpression of miR-29a led to cell cycle arrested at the G0/G1 phase (Figure 2C). These results indicated that the growth inhibiting activity of miR-29a could be interpreted by the cell cycle progression defects by these miRNAs, among which miR-29a took the major part in the cellular growth process.

3.3 miR-29a targets CDC42

To probe the potential mechanisms of miR-29 in the BC cell growth, we searched the literature and miRNA database, TargetScan (http://www.targetscan.org/) for potential targets of miR-29. We therefore hypothesized that miR-29 might target CDC42 to inhibit BC cell growth. To confirm our hypothesis, we applied the pMIR-REPORT System. A fragment representing the binding sequences within the 3'-UTR of CDC42 DNA was inserted into the luciferase reporter vector. As shown in Figure 3A, in miR-29a overexpressed groups, the activity of luciferase was significantly inhibited, among which miR-29a exhibited the most decreased luciferase expression. Consistently, western blot confirmed that CDC42 mRNA and protein levels were also decreased in the miR-29a overexpressed cells (Figure 3B). The above findings suggest that miR-29a might all regulate BC cell growth by targeting CDC42.

4 Discussion

As described previously, miR-29 functions as tumor suppressor in BC progression [15-17]. In other types of human cancers, the roles of miR-29 remain controversial. In clear cell renal cell carcinoma, miR-29 was all downregulated, and restoration of all mature members of miR-29 could inhibit cell proliferation, migration and invasion [21]. However, in a study of pancreatic cancer, miR-29a was found to be increased and could upregulate the expression of pro-inflammatory factors and epithelial-mesenchymal transition (EMT) markers [22]. Hence, the particular roles of miR-29 might be interpreted by its different family members.

In our study, we characterized miR-29a as the dominant isoform of the miR-29 family in our tested BC cells, and we confirmed that miR-29 levels were remarkably decreased in collected BC cell lines. Thereafter, we applied several methods to enrich the functions of miR-29a, and we concluded that miR-29a acted similarly to inhibit cell growth, which might be further explained by the negative modulation of cell cycle progression. A previous study
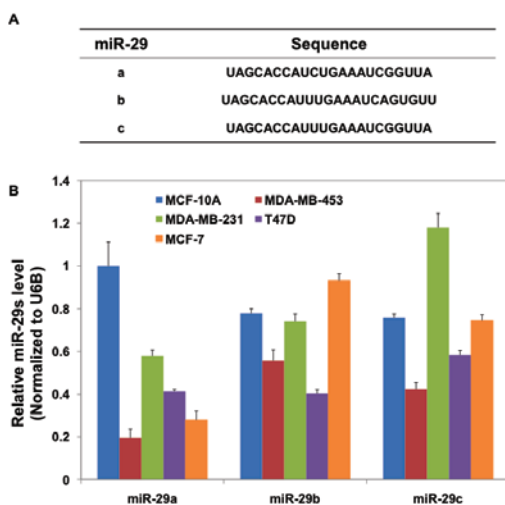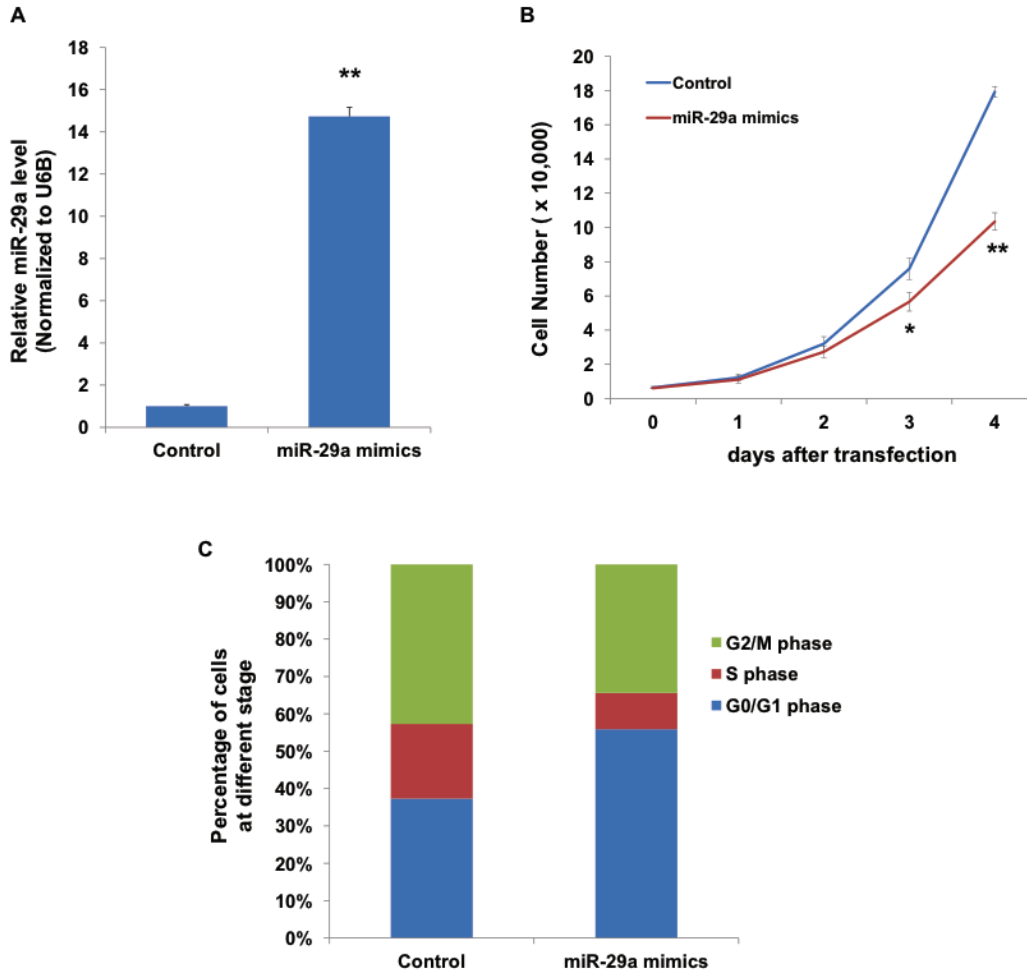
Figure 2: Effects of miR-29a on MDA-MB-453 cell viability. (A) MDA-MB-453 cells were transfected with miR-29a mimics or controls and subjected to qRT-PCR analysis. **(B)** Effect of miR-29a on MDA-MB-453 cell proliferation was measured by cell counting assay. *: $P<0.05$. **: $P<0.01$. **(C)** Effect of miR-29a on MDA-MB-453 cell cycle was measured by flow cytometry. G0/G1 arrest was increased by miR-29a overexpression. The results represent three independent experiments.
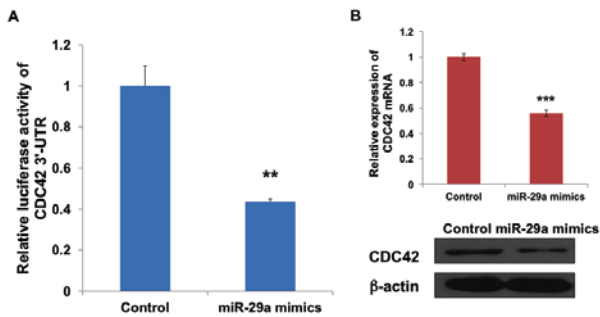
Figure 3: CDC42 might be a natural target of miR-29s. (A) Dual luciferase assay results. MDA-MB-453 cells were co-transfected with the recombinant firefly-luciferase plasmids containing CDC42 3'-UTR and miR-29a mimics or controls. The firefly luciferase activities were normalized by the Renilla luciferase activities. **: $P<0.01$. **(B)** The expression levels of CDC42 proteins determined by Western blot. The expression levels were represented by the band intensities normalized by those of β-actin.

showed that miR-29a could mechanically target tristetraprolin, which is involved in EMT [23], or P42.3, which was also found to be associated with tumorigenicity [24]. Herein, we showed that miR-29a could also target CDC42, and this targeting might be used to explain the regulation of G0/G1 arrest by miR-29a. Still, direct evidence was lacking to prove whether CDC42 could be responsible for the growth inhibitory activity of miR-29a. Furthermore, how miR-29a/CDC42-mediated signaling works to function in BC cells remains to be explained. In addition, although we showed that miR-29a was the dominant and most deregulated form of miR-29 in the indicated BC cell lines, we are still concerned about the similarity of these miR-29 members in regulating cellular biology. More efforts should be made to understand the common and different roles of these miR-29 members.

Taken together, our current findings support the growth-inhibiting function of miR-29a in BC cells through

cell cycle regulation, and miR-29a could target CDC42 in a post-transcriptional manner.

Conflict of interest statement: Authors state no conflict of interest.

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