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ORIGINAL ARTICLE

Identification of miR-146a is Associated with the Aggressiveness and Suppresses Proliferation via Targeting CDKN2A in Breast Cancer

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Abstract

There is emerging evidence that some microRNAs can promote or suppress several human cancer development and progression. However, the profile and molecular mechanism of microRNAs for human breast cancer is poorly unknown. We used bioinformatics approaches to find new candidate diagnostic and therapeutic miRNAs in human breast cancer via analysis of TCGA RNA sequencing data and publicly GEO microarray data, in order to provide theoretical basis for the future investigations of breast cancer. Decreased expression miR-146a was identified as a key regulator of human breast cancer development and progression. Interestingly, we founded that miR-146a expression levels dependent on tumor size and pathological grading in breast cancer patients, but not associated with other factors including age, T classification. Kaplan-Meier survival analysis showed that patients with high miR-146a expression had a longer survival rate than those low miR-146a expressions. In vitro assays of over-expression miR-146a induces cell cycle arrest and inhibits MDA-MB-231 cell proliferation. Furthermore, luciferase reporter gene assays demonstrated that miR-146a directly combine the 3-untranslated region of CDKN2A mRNA. In conclusion, we demonstrated miR-146a play an important role in breast cancer development and progression.

Keywords miR-146a · Biomarker · Breast cancer

Introduction

Breast cancer is the most common invasive cancer in females worldwide. The global incidence of breast cancer has been on the rise since the late 1970s. The cause of breast cancer has not yet fully understood, the study found that breast cancer has certain regularity, with women at high risk for breast cancer risk of breast cancer. Risk factors refers to the various risk factors associated with breast cancer, while most of the patients with breast cancer have risk factors are called risk factors of breast cancer [1–3]. Family history is a risk factor for breast cancer; breast cancer family history refers to the first-degree relatives (mother, daughter, sister) in patients with breast cancer. In recent years found that mammary gland density has become a risk factor for breast cancer. A risk factor for breast cancer and early menarche (< 12 years old), late menopause (> 55); Unmarried, childless, late childbirth, not lactation; Breast benign disease failed to timely diagnosis and treatment; Confirmed by biopsy (a biopsy) hospital patients with atypical hyperplasia of mammary gland; Chest exposure to high dose of radiation; [4–7]. Long-term use of exogenous estrogen; Postmenopausal obesity; Long-term excessive drinking; and carry mutations in genes associated with breast cancer. Need to explain is breast cancer susceptibility gene have done a lot of European and American countries, is now known to have a BRCA 1, BRCA 2, and p53 and PTEN, associated with these mutations called hereditary breast cancer, breast cancer accounts for 5% ~ 10% of all breast cancer [8–11]. However, mechanisms and factors affecting ovarian cancer growth and metastasis remain to be completely elucidated.

MicroRNA (miRNA) is a kind of endogenous, length of about 20 to 24 small RNA nucleotides which it has many important regulating roles in the cell. Each miRNA can have multiple target genes, and several miRNAs can also adjust the same gene. The regulation of this complex networks either through a miRNA to regulate the expression of multiple genes, also can be a combination of several micrornas to

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regulate the expression of certain genes [12–15]. Micrornas in cell differentiation, biological development and disease play a huge role in the process of development, more and more attention among researchers [16]. As for further in-depth study of the mechanism of miRNAs, and using the latest such as high flux of miRNAs chip technology to study the relationship between miRNA and diseases, will make people understanding higher eukaryotic gene expression regulation of the network to a new level [17]. It will also make the miRNA may become a new biological marker of disease diagnosis, may also makes the molecular drug targets, or the molecular simulation for new drug research and development, this could be for the treatment of human diseases provides a new means [18].

To conduct a comprehensive characterization of miRNAs in human breast cancer, we analyzed TCGA breast cancer and normal miRNA-seq data and GEO datasets including GSE40267 and GSE19783. Decreased expression miR-146a was identified as a key regulator of human breast cancer development and progression. Interestingly, Kaplan-Meier survival analysis showed that the overall survival rates for the low miR-146a group were shorter than those in the low miR-146a group. Over-expression miR-146a inhibits MDA-MB-231 cell proliferation and induces cell cycle arrest. Furthermore, luciferase reporter gene assays demonstrated that miRNAs directly targeted the 3-untranslated region of CDKN2A mRNA. Taken together, these results showed miR-146a play an important role in breast cancer development and progression.

Materials and Methods

RNA Sequencing Analysis

Breast cancer miRNA expression data were downloaded from the TCGA Data Portal (Data Portal (https://gdc-portal.nci.nih.gov/search/s) and GEO dataset (https://www.ncbi.nlm.nih.gov/geo/). Raw data files were normalized at transcript and gene level by means of the robust multiaverage method (RMA workflow). Limma of R/bioconductor was used for screening of the DEGs (settings: $P < 0.05$,|log2(Fold Change)| > =1).

RNA Isolation and Quantitative Real-Time PCR

The breast cancer and adjacent samples of this study control were enrolled from March 2012 to May 2013 at The First People's Hospital of Changde. Total miRNA and mRNA were isolated from tumor cells using the TRIzol reagent (Invitrogen), according to the protocol of the manufacturer. Reverse transcription of miRNA was carried out with the Sangon microRNA First Strand cDNA Synthesis, mRNA was reverse transcribed into cDNA using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Otsu, Japan). Quantitative real-time PCR was performed using THUNDERBIRD SYBR® qPCR Mix (Toyobo). PCR thermal cycling conditions were 94 °C for 2 min, which could activate DNA polymerase, followed by 30 cycles (94 °C for 30 s, 56 °C for 30 s, and 72 °C for 10 min). All qRT-PCR assays and independent experiments were performed in triplicate respectively. *RECK* mRNA expression was normalized to U6 (endogenous control).

Cell Proliferation and Cell Cycle Assays

Cells were seeded in 96-well plates at a density of 5000 cells/well in DMEM containing 10% FBS. After they had adhered, cell growth was monitored for 48 or 72 h, and then assessed using MTT assay. Briefly, 20 μl of MTT solution (5 mg/ml in PBS) was added into triplicate wells and cells were incubated for 4–6 h in an incubator. Absorbance at 490 nm was read with a microplate reader. MDA-MB-231 cells transfected with ctrl or miR-146a mimics were harvested 48 h after transfection by trypsinization. After the staining with Propidium iodide (PI), the cells were analyzed with a flow cytometry.

Luciferase Reporter Assays

The full-length 3-untranslated region (UTR) ofCDKN2A was amplified and cloned downstream Renilla luciferase in a psiCHECK2 vector. HEK293 Cells were seeded in 24-well plates cultured overnight and then cotransfected with 100 ng of psiCHECK2-CDKN2A-WT, psiCHECK2-CDKN2A-mut, and 200 nmol/L of miR-146a or negative control by using Lipofectamine 2000. Luciferase assays were performed 48 h after transfection using the dual-luciferase reporter assay system (Promega). After 48 h, cells were lysed and analyzed for Renilla and Firefly luciferase activity using the Dual luciferase assay system (Promega). Each transfectant was assayed in triplicates. Activity of Renilla luciferase was normalized to Firefly luciferase.

Statistical Analysis

All results are reported as mean ± SEM. Each experiment was performed in triplicates and was repeated at least three times. The results obtained in two experimental groups were compared by two-tailed unpaired Student's t tests. $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered statistically significant, and are indicated in Figurers by one, two, and three asterisks, respectively.

Results

MiR-146a is Specifically Down-Expressed in Human Breast Cancer

To screen differently expressed miRNAs which play piovtal role in human breast cancer progression, 908 miRNA-seq data sets and corresponding clinical data were downloaded from the publicly available TCGA databases. By comparing the normal tissue group, we identified 252 differentially expressed miRNAs, 139 in GSE25631 datasets using p-value <0.05 and |log2 FC (fold change) | >1 as the screening criteria. Then the differentially expressed genes were visualized in the hierarchical clustering heat map (Fig. 1a). We selected decreased expression miR-146a because these miRNAs may be useful for early diagnosis biomarkers or therapeutic targets (Fig. 1b). Then, we detected the expression levels of three miRNAs in 17 pairs of breast and matched adjacent tissue by qRT-PCR. We founded that miR-146a is significantly decreased in patients with breast cancer (Fig. 1c). MiR-146a expression levels in breast cancer cell lines of MCF-10A, MCF-7, MDA-MB-468, and MDA-MB-231 was detected by qRT-PCR. MiR-146a levels were shown to be significantly decreased in MDA-MB-231 cells but increased in MCF-10A cells (Fig. 1d).

MiR-146a Expression is Correlated with Breast Cancer Progression and Poor Prognosis

To determine the relationship between miR-146 expression and human breast cancer clinical data, the miR-146a expression was examined in 37 breast cancer and 37 matched normal tissue by qRT-PCR analysis. Interestingly, we founded that miR-146a expression levels dependent on tumor size ($P = 0.025$) and Pathological grading ($P = 0.012$) in breast cancer patients, but not associated with other factors including age, T classification as shown in Tables 1 and 2. Kaplan-Meier survival analysis showed that the overall survival rates for the low miR-146a group were shorter than those in the low miR-146a group (Fig. 2). These data showed that miR-146a might function as tumor suppressor during breast cancer progression.

MiR-146a Inhibits Cell Proliferation and Induces Cell Cycle Arrest

To understand the biologic function of miR-146a, miR-146a mimics were transfected into MDA-MB-231 cells. Transfection efficiency was verified by qRT-PCR (Fig. 3a). Ectopic miR-186 expression slowed down the propagation of the MDA-MB-231 cells as analyzed using CCK-8 assay.

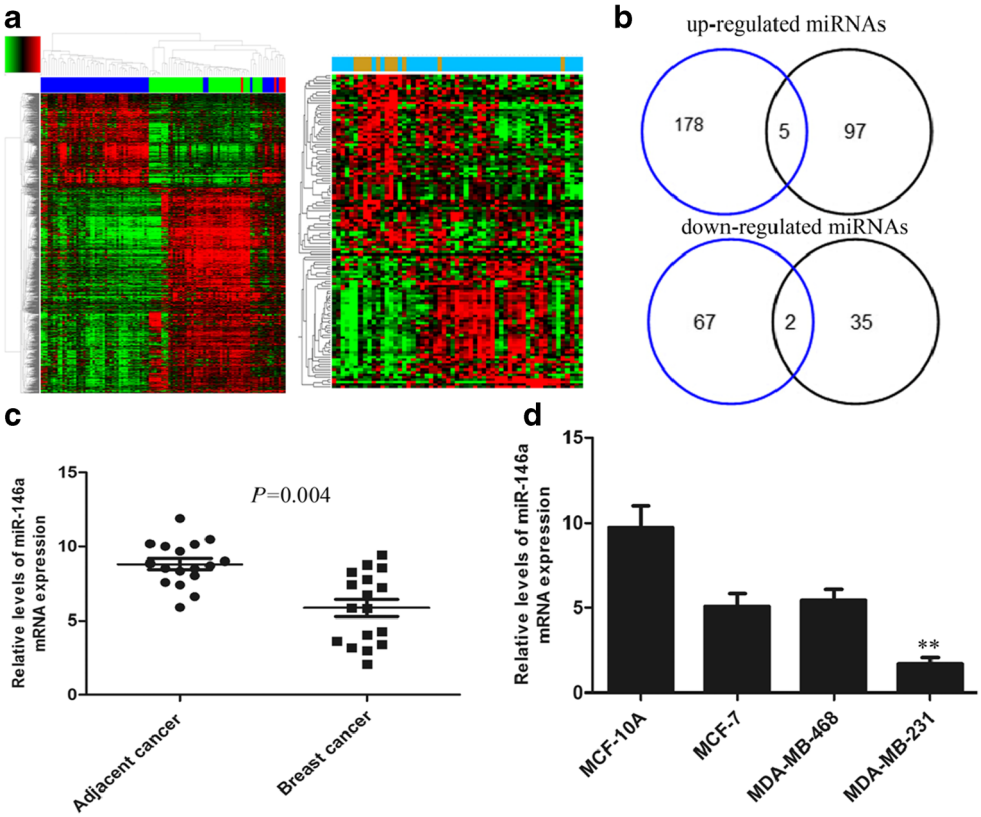

Fig. 1 miR-1d6a is down regulated in human breast cancer. **a** Hierarchical clustering analysis of miRNAs that were differentially expressed in breast cancer and normal tissue from TCGA miRNA-seq and GEO dataset. **b** Overlap of deregulated miRNAs in TCGA data and GEO datasets. **c** miR-146a expression was analyzed by qRT-PCR in breast cancer and matched adjacent tissues ($N = 17$). (D) Analyses of miR-1825 expression levels in cell line of MCF-10A, MCF-7, MDA-MB-468, and MDA-MB-231. $^{**}P < 0.01$

Table 1 Correlation between the clinicopathologic features and expression of miR-146a

Characteristics	miR-146a(%)		p-Value
	Low expression	High expression	
Age (y)			0.365
< 60	20(86.9)	3(13.1)	
≥ 60	11(78.5)	3(21.5)	
Clinical stage			0.0032
I	10(83.3)	2(16.7)	
II	13(92.8)	1(7.2)	
III	8(18.2)	3(81.8)	
Histological type			0.015
Malignant	31(83.7)	6(16.3)	
Normal	4(10.9)	33(89.1)	
Pathological grading			0.057
1	11(91.6)	1(8.4)	
2	16(88.8)	2(11.2)	
3	5(66.6)	3(33.4)	
T classification			0.52
T1	17(85.0)	3(15.0)	
T2	4 (100.0)	0(0)	
T3	10(70.0)	3(30.0)	
N classification			0.86
N0	7(77.7)	2(22.3)	
N1–3	24(86.6)	4(13.4)	

Fluorescence-activated cell sorting (FACS) was applied to analyze changes of DNA content throughout various phases of the cell cycle. As shown in Fig. 3c, both miR-186-overexpressing MDA-MB-231 cells displayed a significant increase in the percentages of cells in G1-phase but decreased proportions of S-phase cells.

Table 2 Association between MIR-146a expression and tumor subtypes in carcinomas of breast

Tumor type	MIR-146a low expression		(%)	*P* value
Estrogen receptor				**0.063**
Negative	20	14	70.0	
Positive	17	8	47.0	
Progesterone receptor				**0.037**
Negative	21	15	71.4	
Positive	16	7	43.7	
ERBB/HER2				**0.052**
Negative	18	13	72.2	
Positive	19	9	47.3	
Triple negative				**0.039**
Yes	18	13	72.2	
No	19	6	56.2	

The *ER*, *PR* and *HER2* status of breast cancer and that relate to *MIR-146a* expression

MiR-146a Repressing CDKN2A Expression by Binding to 3′-UTR

To understand miR-146a suppressive effect of miR-186 on the proliferation in breast cancer, two bioinformatic tool for miRNA targe a (PicTar and TargetScan Release) were used to predict conserved miR-146a-binding sites targeting the 3′ UTR of CDKN2A (Fig. 4a). CDKN2A gene generates several transcript variants which differ in their first exons. At least three alternatively spliced variants encoding distinct proteins have been reported, two of which encode structurally related isoforms known to function as inhibitors of CDK4 kinase. A putative miR-146a binding site was found in the 3′UTR of CDKN2A which was highly conserved in some species (Fig. 4b). MiR-146a mimic significantly reduced the expression Of CDKN2A protein in MDA-MB-231 cells (Fig. 4c).

To determine whether CDKN2A was regulated by miR-146a through direct binding to its 3′ UTR, we generated the 3′UTR of CDKN2A containing the putative miR-146a binding sites into a luciferase reporter construct. we also constructed a mutated 3′UTR bearing six mismatched nucleotides in the seed sequence of miR-146a (Fig. 4d). Luciferase reporter assays showed that miR-146a mimics transfection caused a remarkable decrease in relative luciferase activity in the presence of wild-type CDKN2A 3′ UTR. However, the luciferase activity with the mutated 3′ UTR was not affected by miR-146a (Fig. 4e). Taken together, we concluded that miR-186 was able to directly target the 3-UTR of the genes of cell-cycle regulator.

Discussion

The global incidence of breast cancer has been on the rise since the late 1970s. The cause of breast cancer has not yet fully understood, the study found that breast cancer has certain regularity, with women at high risk for breast cancer risk of breast cancer. However, the profile and molecular mechanism of microRNAs for human breast cancer is poorly unknown. Micrornas in cell differentiation, biological development and disease play a huge role in the process of development, more and more attention among researchers.

MiRNAs participate in a series of important process in the process of life, including the early development, cell proliferation, apoptosis, cell death, fat metabolism and cell differentiation [16, 18]. MicroRNAs have the potential to be used as targets for treatment of different cancers. Another role for miRNA in cancers is to use their expression level for prognosis. In addition, a study shows that a decrease in the level of two miRNAs and significant correlation between chronic lymphocytic leukemia, suggests there may be potential relationship between miRNAs and cancer [19, 20].

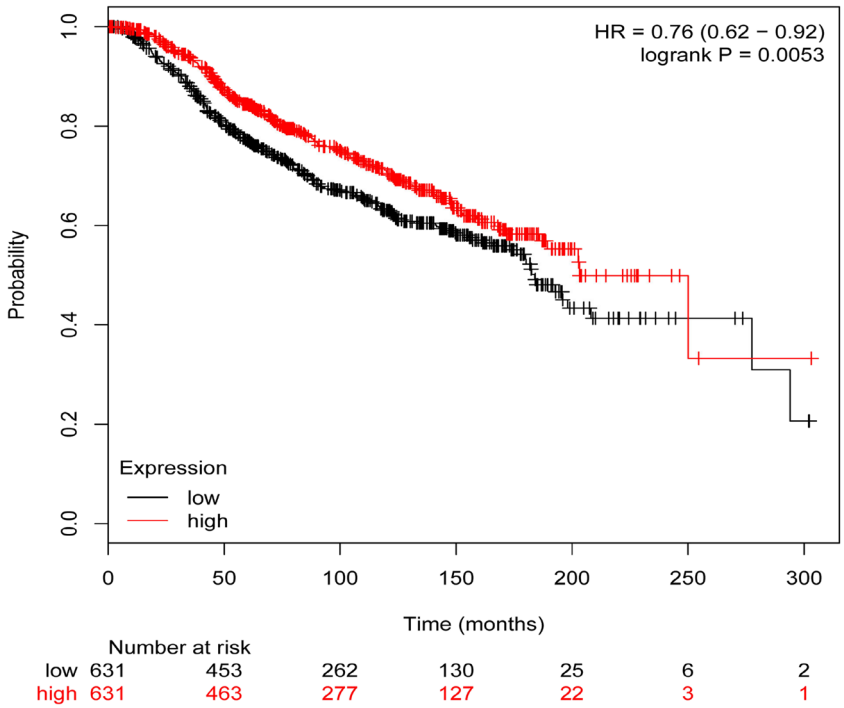

Fig. 2 Kaplan–Meier plots of miR-146 expression in breast cancer patients. Overall survival rate was performed by log-rank test

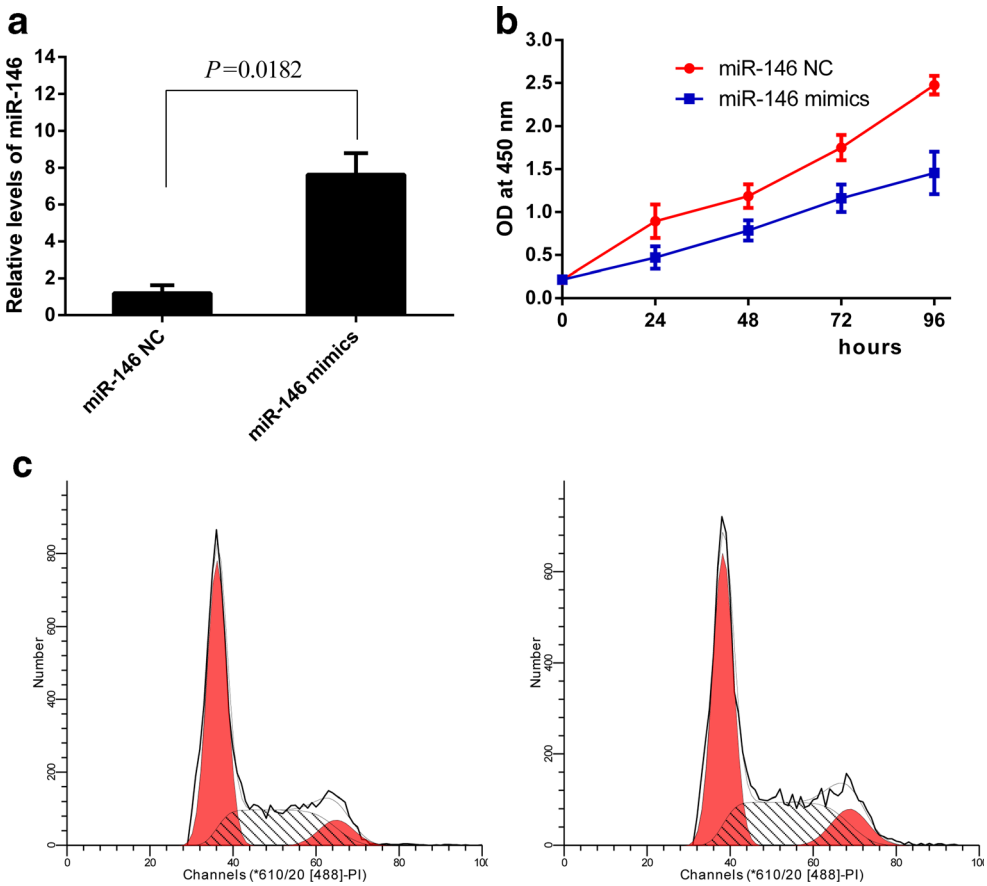

Fig. 3 Over-expression of miR-146a inhibited MDA-MB-231 cell proliferation induces cell cycle arrest. **a** Transfection efficiency of miR-146a was verified by qRT-PCR. **a** CCK-8 assays revealed cell growth curves of MDA-MB-231 cells. **c** Fluorescence-activated cell sorting determination of proportion of indicated cells in distinct cell-cycle phases

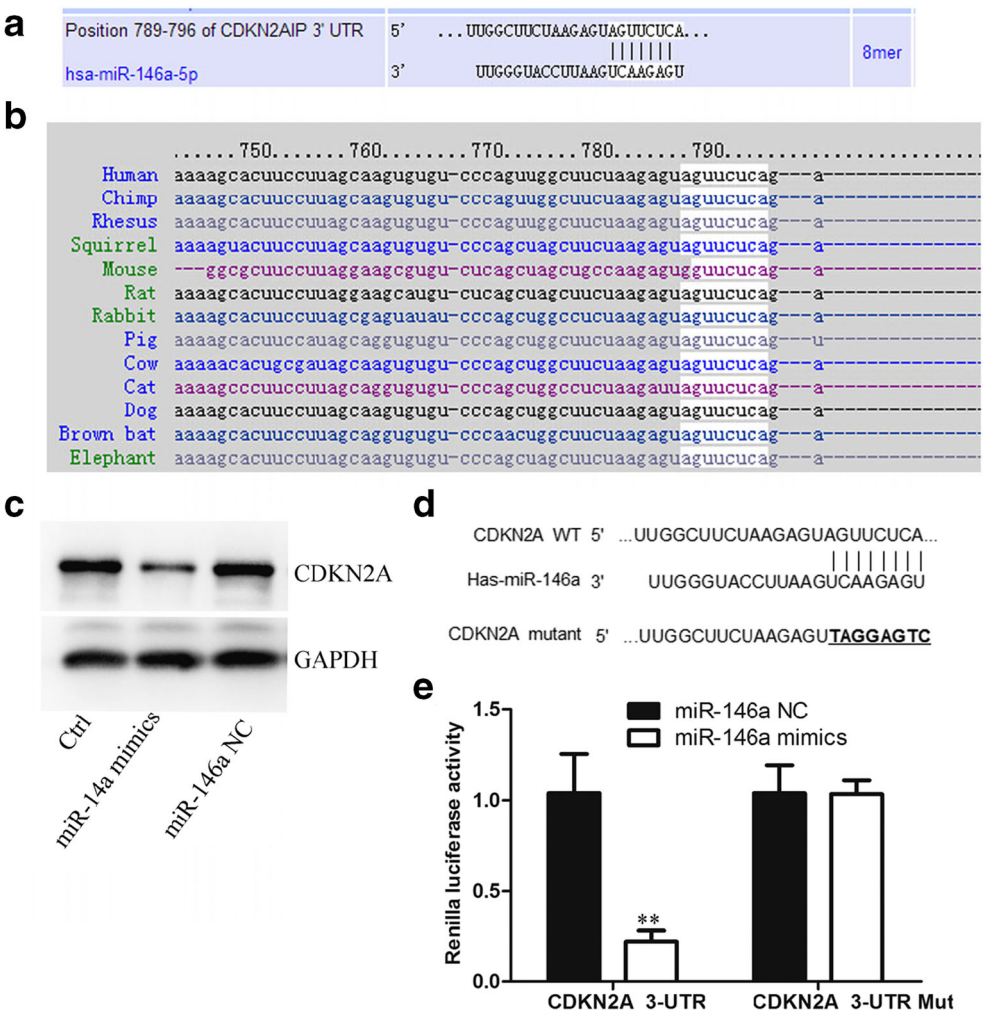

Fig. 4 CDKN2A is a direct target of miR-146a. **a** The wild-type binding site of CDKN2A 3′ UTR for miR-146a (**b**) The complementary sequences and evolutionary conservation of the miR-146a binding site in CDKN2A 3′ UTR from different mammalian species (**c**) Western blot assays of CDKN2A protein after transfection with miR-146a mimic, mimic control in MDA-MB-231 cells (**d**) The wild-type or mutant binding sites of CDKN2A 3′ UTR for miR-146a (**e**) The effect of miR-146a on luciferase intensity controlled by the wild-type or mutant 3′ UTR of CDKN2A was determined by luciferase assay

In this study, by comparing the normal tissue group, we identified 549 differentially expressed miRNAs, 77in GSE25631 datasets using p-value <0.05 and |log2 FC (fold change)| >1 as the screening criteria. Then the differentially expressed genes were visualized in the hierarchical clustering heat map. We selected decreased expression miR-146a because these miRNAs may be useful for early diagnosis biomarkers or therapeutic targets. We founded that miR-146a expression levels dependent on tumor size and Pathological grading in breast cancer patients, but not associated with other factors including age, T classification as shown in Table 1. Kaplan-Meier survival analysis showed that the overall survival rates for the low miR-146a group were shorter than those in the low miR-146a group. These data showed that miR-146a might function as tumor suppressor during breast cancer progression. MiR-146a inhibits cell proliferation and induces cell cycle arrest.

We predict conserved miR-146a-binding sites targeting the 3′ UTR of CDKN2A. CDKN2A gene generates several transcript variants which differ in their first exons. At least three alternatively spliced variants encoding distinct proteins have been reported, two of which encode structurally related isoforms known to function as inhibitors of CDK4 kinase [21, 22]. A putative miR-146a binding site was found in the 3′ UTR of CDKN2A which was highly conserved in some species. Luciferase reporter assays showed that miR-146a mimics transfection caused a remarkable decrease in relative luciferase activity in the presence of wild-type CDKN2A 3′ UTR. However, the luciferase activity with the mutated 3′ UTR was not affected by miR-146a (Fig. 4e). Taken together, we concluded that miR-186 was able to directly target the 3-UTR of the genes of cell-cycle regulator. In conclusion, we demonstrated miR-146a plays an important role in breast cancer development and progression.

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Compliance with Ethical Standards

Conflict of Interest No potential conflicts of interest were disclosed.

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