Expression of miR-146a-5p in breast cancer and its role in proliferation of breast cancer cells

WEI GAO1*, JING HUA2*, ZHAOYANG JIA3, JIPING DING1, ZENGWEI HAN4, YUN DONG1, QING LIN3 and YUAN YAO1

1Department of Radiation Oncology, 9th People’s Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 201999; 2Department of Emergency, Shanghai Tongji Hospital, Tongji University School of Medicine, Shanghai 200065; 3Department of Radiation Oncology, Shanghai Tenth People’s Hospital of Tongji University, Shanghai 200072, P.R. China

Received July 18, 2017; Accepted March 7, 2018

DOI: 10.3892/ol.2018.8589

Abstract. In the present study, the expression level of microRNA-146a-5p (miR-146a-5p) in breast cancer tissue and cell lines was investigated and its effects on proliferation of breast cancer cells. miR-146a-5p expression was detected by reverse transcription-quantitative polymerase chain reaction in breast cancer tissues, paraneoplastic tissue (collected by The Department of Oncology of Changhai Hospital from January 2014 to June 2015), breast cancer cell line MCF-7 and normal breast epithelial cell line MCF 10A. Bioinformatics analysis was conducted to forecast target genes of miR-146a-5p, which was further verified by fluorescent reporter gene detection. The results demonstrated the expression level of miR-146a-5p in breast cancer tissue was significantly higher, compared with paraneoplastic tissue (P<0.01), and the expression level of miR-146a-5p in MCF-7 cells was significantly higher, compared with MCF 10A cells (P<0.01). Overexpression of miR-146a-5p in MCF-7 cells can promote the proliferation, and low expression miR-146a-5p in MCF-7 can inhibit the proliferation. BRCA1 was further identified as a target gene of miR-146a-5p by bioinformatics analysis and fluorescent reporter gene detection. It was concluded that miR-146a-5p is expressed in breast cancer tissue and breast cancer cell line and may regulate the proliferation of MCF-7 via BRCA1.

Introduction

Breast cancer derived from mammary gland epithelial tissue has had an increasing morbidity rate since the 1980s (1). Breast cancer is also the most common malignant tumor in China, and accounts for 12.2% of the novel diagnosed cases and 9.6% of mortalities (2). A microRNA (miRNA) is a small non-coding single strand RNA containing ~22 nucleotides, with a function of combining the 3’untranslated region (UTR) of target genes. With the combination of intracellular proteins to form a RNA interference silencing ribonucleoprotein complex, the target mRNA is bound to and degraded via complete complementary sequence specific binding; however, it can inhibit translation by incomplete complementary combination, to regulate the expression of gene at the post transcriptional level (3,4). At present, nearly 1,000 miRNA sequences have been cloned in vivo, including those involved in: The development of the organism (5); cell differentiation and proliferation (6); cell apoptosis (7); tumorigenesis (8); inflammation (9); and numerous other physiological and pathological processes (3). It is speculated that 1/3 of gene expression may be regulated by miRNAs (10).

Numerous studies have indicated that miRNAs serve an important part in genesis and progression of breast cancer (11-15). In the previous experimental study (16), it was determined that the expression of miR-146a-5p in breast cancer tissue was significantly higher, compared with paraneoplastic tissue. In the present study, we further investigated the expression of miR-146a-5p in breast cancer tissue and its association with the proliferation of breast cancer cells to search for the target gene and understand the molecular mechanism underlying miR-146a-5p, which regulates the proliferation of breast cancer cells (17), providing a novel strategy for the prevention and treatment of breast cancer.

Materials and methods

Breast cancer tissue and paraneoplastic tissue. The surgical specimens of carcinoma tissue and paraneoplastic tissue (<3 cm from the edge of tumor specimens) were collected from 32 clinical diagnosed female patients with breast cancer from January 2014 to June 2015 in The Department of Oncology, Changhai Hospital (Shanghai, China). The median age of the patients was 49 years, and the age ranged from 27-69 years. Any patient underwent chemotherapy, immunotherapy or...
radiotherapy were excluded. All specimens were frozen in liquid nitrogen rapidly and stored at -80°C.

**Cell culture.** Human breast cancer cell line MCF-7, normal breast epithelial cells MCF 10A and human embryonic kidney cell line, 293T, were purchased from Shanghai Bioleaf Biotech Co., Ltd. (Shanghai, China). Cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% FBS in a 5% CO₂ incubator at 37°C. The medium was replaced every 2-3 days. When the cells were at 70-80% confluency, the transfection experiment was carried out.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from breast cancer tissue, paraneoplastic tissue, human breast cancer cell line MCF-7 and normal breast epithelial cells MCF 10A. RT-qPCR was used to detect the expression of miR-146a-5p. Primers of miR-146a-5p and β-actin U6 were designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). RT primers were of an miRNA specific stem loop structure. Oligo(dT)20 were used as RT primers for reverse transcription. The relative expression of miRNA and U6 were detected by using in SYBR Premix Ex Taq enzyme (Takara Biomedical Technology (Beijing) Co., Ltd., Beijing, China) and specific qPCR primer. The primer sequences were as follows: miR-146a-5p forward, 5'-cagtcctcagttttccagga-3', and reverse, 5'-gtctgtatctcgtcgagg-3'; U6 forward, 5'-tctggctcgagacac-3', and reverse, 5'-aaccctgcatatgtggt-3'.

RT-qPCR reaction conditions: 95°C for 10 min, then 95°C 15 sec, annealing temperature (55°C) depending on the primer for 20 sec and 72°C for 30 sec, for 40 cycles, this was following with 72°C for 10 min. The 2ΔΔCq method was used for quantification (18).

**Transfection and experimental groups.** The logarithmic growth phase cells were divided into blank control group, mock group, over expression group and suppression group. The blank control group had no intervention, whilst the mock, over expression group and suppression group. The transfection concentration was 50 nM. The RT-qPCR by detecting the miR-146a-5p expression level of each group.

**MTT.** MTT was used to detect the proliferation rate at 1 day (D1), 2 days (D2) and 3 days (D3) following transfection. The cells in the logarithmic growth phase were seeded into a 96 well plate (5x10³ cells/well). A total of 20 µl 5 µg/µl MTT solution was added into each well at D1, D2 and D3 following transfection, then 150 µl dimethyl sulfoxide (Henan Tianfu Chemical Co., Ltd., Henan, China) was also added into each well following a 4-h incubation at room temperature, value the optical density of each group at detection wavelength of 490 nm. The proliferation ratio of the other groups were calculated based on comparison with the blank control group value and the experiment was repeated three times.

**Bioinformatics prediction.** Using TargetScan (http://www.targetscan.org/vert_71/), PicTar (http://www.pictar.org), MiRBase (http://www.mirbase.org) and BibiServ (https://bibiserv.cbi.tieidelberg.de/index.html) software to predict the potential target gene of miR-146a-5p. The results were screened according to the matching condition between miRNA seed sequence and target sequence, RNA double chain free energy and miRNA target sequence conservation among different species.

**Dual fluorescence reporter gene assay.** The 3'UTR region of BRCA1 gene sequence that can interact with 3'UTR region of miR-146a-5p was cloned into the same region in reporter gene Luciferase of plasmid pG3. If miR-146a-5p can act on the target gene sequence, then the translation process of report gene luciferase will be inhibited, the amount of luciferase protein with activity will be reduced, thus the catalytic substrate luminescence signal will be reduced; therefore, the inhibitory effect of miR-146a-5p on the target gene sequence can be reflected indirectly by the luminescence signal strength. Pre-miR-146a-5p was amplified with the 293T cell genome as a template, subcloned into pSuper plasmid H1 RNA promoter into multiple downstream cloning sites, and the recombinant pSuper plasmid expressing pre-miR-146a-5p was constructed. Cell lysis following transfection into 293T cells for 24 h, then the luciferase detection kit (Promega Corporation, Madison, WI, USA) was used to detect the relative expression of luciferase.

**Statistical analysis.** SPSS 13.0 (SPSS, Inc. Chicago, IL, USA) statistical software was applied to determine to statistical significance. Data are presented as mean ± standard deviation. Two groups were compared by Student’s t-test and the comparison between multiple groups was conducted by one-way analysis of variance followed by Student-Neuman-Keuls post hoc comparisons.

**Results**

**Expression of miR-146a-5p in breast cancer and breast cancer cell lines.** The results of RT-qPCR demonstrated that the expression of miR-146a-5p in breast cancer tissue was 3.2±0.3 times more, compared with paraneoplastic tissue (Fig. 1A, P<0.01), the expression in MCF-7 cell lines was 2.9±0.3 times more, compared with MCF 10A cells (Fig. 1B, P<0.01).

miR-146a-5p mimic and inhibitor transfection into MCF-7 cells. According to the miR mimic and inhibitor transfection introduction, the transfection concentration was 50 nM. The results indicated that the expression of miR-146a-5p in MCF-7 cells transfected with miR-146a-5p mimic was 80±7 times more, compared with original expression (Fig. 2A, P<0.01), whilst cells transfected with miR-146a-5p inhibitor decreased
Effect of miR-146a-5p on the proliferation of breast cancer cell line MCF-7.

The proliferation (OD 490) of MCF-7 cells increased gradually following D3. The proliferation of the overexpression group significantly increased following D3 compared with the blank control group. **P<0.01 vs. the blank control group.**

Figure 2. Mimic miR-146a-5p and inhibitor miR-146a-5p transfected MCF-7 cells. (A) Transfected with mimic miR-146a-5p, the expression of miR-146a-5p in MCF-7 cells was significantly upregulated, compared with blank control group. **P<0.01. (B) Transfected with inhibitor miR-146a-5p, the expression of miR-146a-5p in MCF-7 cells was significantly downregulated, compared with the blank control group. **P<0.01. miR-146a-5p, microRNA-146a-5p.

Figure 3. Effects on the proliferation of MCF-7 following transfection of mimic miR-146a-5p (P<0.05 vs. the blank control group) and inhibitor miR-146a-5p (P<0.01 vs. the blank control group). miR-146a-5p, microRNA-146a-5p; OD, optical density.

Figure 4. Bioinformatics analysis revealed that the 3’ untranslated region of tumor suppressor gene BRCA1 existed at the acting site of miR-146a-5p. miR-146a-5p, microRNA-146a-5p.

Figure 5. The luciferase activity of transfected miR-146a-5p group was significantly reduced compared with the control group. **P<0.01.
miRNA is a type of non-coding small single stranded RNA containing 20-24 nucleotides involved in gene transcription and expression (19). miRNA is not encoding protein, but has the ability to direct degrade the target mRNA or inhibit its translation by complete or incomplete complementary combination with the target mRNA (20). Numerous studies have demonstrated that miRNA is involved in the process of tumor growth, metastasis and angiogenesis by regulating the expression of oncogenesis, migration and other associated genes (21-23).

In 2005, Iorio et al (24) first reported the change of miRNAs expression profile in human breast cancer and determined that there were 29 kinds of miRNAs with an expression disorder, in which the expression of miRNA-21 and miRNA-155 were significantly upregulated, whilst the expression of miRNA-10b, miRNA-125b and miRNA-145 were significantly reduced. Additionally, a number were associated with the clinical and pathological features of breast cancer, including estrogen and progesterone receptor expression and vascular invasion. In 2007, Blenkiron et al (25) determined that there were 133 kinds of miRNA expression in normal breast tissue and/or cancer tissues, a number of which were associated with molecular subtype of breast cancer. Following this, the role of miRNAs in breast cancer gained more attention.

In the previous work, it was determined that the expression of miR-146a-5p in breast cancer tissue was significantly lower, compared with paraneoplastic tissue (16). It was confirmed through RT-qPCR in the present study and also discovered that the expression of miR-146a-5p in breast cancer cell line MCF-7 was also significantly higher, compared with control cells. High expression of miR-146a-5p in MCF-7 could significantly promote the proliferation, and low expression of miR-146a-5p could significantly inhibit it. BRCA1 was preliminarily confirmed as the target gene of miR-146a-5p by bioinformatics prediction and fluorescence reporter gene detection.

BRCA1 is a tumor suppressor gene, which frequently mutated in hereditary breast cancer (26). BRCA1 serves an important role in DNA repair, cell cycle control, transcriptional activation, ubiquitin and other processes (27,28). In the present study, it was determined that the proliferation of MCF-7 cells was enhanced following high expression of miR-146a-5p, whilst the proliferation of MCF-7 cells decreased following low expression of miR-146a-5p. It was hypothesized that the decreased expression of BRCA1, in consequence of the high expression of miR-146a-5p, weakened the anti-tumor ability and caused the increase of tumor cell proliferation. Following miR-146a-5p low expression, the expression of BRCA1 was increased, which caused the inhibition of tumor and the decrease of the proliferation ability.

In conclusion, high expression of miR-146a-5p in breast cancer and breast cancer cell lines may regulate the proliferation of MCF-7 by regulating the expression of BRCA1.

Acknowledgements

Not applicable.

Funding

The present study was supported by the natural science foundation of Science and Technology Commission of Shanghai Municipality (grant no. 14ZR1408800).

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GW, HJ and YY designed this study. JZ, DJ, HZ, DY and LQ performed the experiments and interpreted the data. GW and HJ analyzed and interpreted the data. GW and HJ performed the histological examination of the breast and were major contributors towards the writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was supported by the natural science foundation of Science and Technology Commission of Shanghai Municipality (grant no. 14ZR1408800).
References

1. Rodriguez-Barrueco R, Nekritz EA, Bertucci F, Yu J, Sanchez-Garcia F, Zeleke TZ, Gorbatenko A, Birnbaum D, Ezhkova E, Cordon-Cardo C, et al: miR-424(322)/503 is a breast cancer tumor suppressor whose loss promotes resistance to chemotherapy. Genes Dev 31: 553-566, 2017.

2. Fan L, Strasser-Weippl K, Li JI, St Louis J, Finkelstein DM, Yu KD, Chen WQ, Shao ZM and Goss PE: Breast cancer in China. Lancet Oncol 15: e279-e289, 2014.

3. Cheng Y, Dong L, Zhang J, Zhao Y and Li Z: Recent advances in microRNA detection. Analyst, 2018.

4. Rapado-González O, Majem B,Muiñelo-Romay L, Álvarez-Castro A, Santamaría A, Gil-Moreno A, López-López R and Suárez-Cunqueiro MM: Human salivary microRNAs in breast cancer. J Cancer 9: 638-649, 2018.

5. Horák M, Novák J and Bienertova-Vasku J: Muscle-specific microRNAs in skeletal muscle development. Dev Biol 410: 1-13, 2016.

6. Harding RL and Vellman SG: MicroRNA regulation of myogenic satellite cell proliferation and differentiation. Mol Cell Biochem 412: 181-195, 2016.

7. Nakagawa R, Leyland R, Meyer-Hermann M, Lu D, Turner M, Arbore G, Phan TG, Brink R and Vigorito E: MicroRNA-155 controls affinity-based selection by protecting c-MYC+ B cells from apoptosis. J Clin Invest 126: 377-388, 2016.

8. Saito Y, Nakaoka T and Saito H: microRNA-34a as a therapeutic agent against human cancer. J Clin Med 4: 1951-1959, 2015.

9. Saito Y, Nakaoka T and Saito H: microRNA-34a as a therapeutic agent against human cancer. J Clin Med 4: 1951-1959, 2015.

10. Lewis BP, Burge CB and Bartel DP: Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15-20, 2005.

11. Ezhkova E, Cordon-Cardo C, et al: miR-424(322)/503 is a breast cancer tumor suppressor whose loss promotes resistance to chemotherapy. Genes Dev 31: 553-566, 2017.

12. Moshkov R, Tanha HM, Naeini MM, Ghaedi K, Sanati MH, Moshkov R and Bagheri F: Functional SNP in stem of miR-146a affects Her2 status and breast cancer survival. Cancer Biomark 17: 213-222, 2016.

13. Fujita K, Takanori N and Yuki K: miR-213 promotes cell viability and migration of breast cancer cells. Mol Cancer 15: 9884-9888, 2018.

14. Takahashi RU, Miyazaki H and Ochiya T: The roles of micromRNAs in breast cancer. Cancer(Basel) 7: 598-616, 2015.

15. Wang B, Teng Y and Liu Q: MicroRNA-153 regulates NRF2 expression and is associated with breast carcinogenesis. Clin Lab 62: 39-47, 2016.

16. Moshkov R, Tanha HM, Naeini MM, Ghaedi K, Sanati MH, Moshkov R and Bagheri F: Functional SNP in stem of miR-146a affects Her2 status and breast cancer survival. Cancer Biomark 17: 213-222, 2016.

17. Majer A, Blanchard AA, Medina S, Booth SA and Myal Y: Claudin 1 expression levels affect miRNA dynamics in human basal-like breast cancer cells. DNA Cell Biol 35: 328-339, 2016.

18. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.

19. Schanza LM, Seles M, Stotz M, Fosselteder J, Hutterer GC, Pichler M and Stiegelbauer V: MicroRNAs associated with von hippel-lindau pathway in renal cell carcinoma: A comprehensive review. Int J Mol Sci 18: 2017.

20. Kagiya T: MicroRNAs: Potential biomarkers and therapeutic targets for alveolar bone loss in periodontal disease. Int J Mol Sci 17: E1317, 2016.

21. Janssen MD and Lund AH: MicroRNA and cancer. Mol Oncol 6: 590-610, 2012.

22. Lin S and Gregory RI: MicroRNA biogenesis pathways in cancer. Nat Rev Cancer 15: 321-333, 2015.

23. Ohtsuka M, Ling H, Doki Y, Mori M and Calin GA: MicroRNA processing and human cancer. J Clin Med 4: 1651-1667, 2015.

24. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, et al: MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65: 7065-7070, 2005.

25. Blenkiron C, Goldstein LD, Thorne NP, Spiteri I, Chin SF, Dunning MJ, Barbosa-Morais NL, Teschendorff AE, Green AR, Ellis IO, et al: MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. Genome Biol 8: R214, 2007.

26. Schuyck H, Korach L, Laitman Y, Bernstein-Molho R and Friedman E: Mutational analysis of candidate genes in Israeli male breast cancer cases. Breast Cancer Res Treat, 2018.

27. Drost R and Jonkers J: Opportunities and hurdles in the treatment of BRCA1-related breast cancer. Oncogene 33: 3753-3763, 2014.

28. Narod SA and Foulkes WD: BRCA1 and BRCA2: 1994 and beyond. Nat Rev Cancer 4: 665-676, 2004.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.