European Review for Medical and Pharmacological Sciences 2019; 23: 708-715

Long non-coding RNA Tubulin Alpha 4B (TUBA4B) inhibited breast cancer proliferation and invasion by directly targeting miR-19

A.-X. LIU[1,2], F. YANG[3], L. HUANG[4], L.-Y. ZHANG[5], J.-R. ZHANG[1], R.-N. ZHENG[6]

[1]Department of Oncology, Zhujiang Hospital, Southern Medical University, GuangZhou City, P.R. China
[2]Department of Oncology, Shenzhen Second People's Hospital, Shenzhen City, P.R. China
[3]Department of Basic medical, XiangNan University, ChenZhou City, P.R. China
[4]Department of Oncology, The First Affiliated Hospital of Gannan Medical University, Ganzhou City, P.R. China
[5]Medical Department of Chongqing Bishan District People's Hospital, Chongqing City, P.R. China
[6]Department of Oncology, Dongguan People's Hospital, Southern Medical University, Dongguan City, Guangdong Province, P.R. China

Abstract. – OBJECTIVE: Long non-coding RNA (lncRNA) is a significant member of the non-coding RNA family. New evidence has shown that it plays a pivotal role in the processes of tumor genesis and development. According to previous verification, the lncRNA Tubulin Alpha 4B (TUBA4B) is a tumor-associated molecule, but how TUBA4B expresses and functions in breast cancer is still not clear.

PATIENTS AND METHODS: We conducted this study to examine what expression and biological role TUBA4B plays in breast cancer. The expression of TUBA4B was measured in breast cancer samples and cell lines. CCK8 assays and transwell assays were used for evaluating the effects of TUBA4B on breast cancer cell proliferation and invasion. Luciferase reporter assays were used for identifying the direct target of TUBA4B.

RESULTS: According to the results, TUBA4B was largely downregulated in breast cancer samples and cell lines. The functional analysis demonstrated that breast cancer cells proliferation and invasion could be inhibited by overexpression of TUBA4B. The results of Luciferase reporter assays indicated that TUBA4B directly targeted miR-19, which could rescue the effects of TUBA4B on breast cancer cells.

CONCLUSIONS: It is suggested that TUBA4B was downregulated in breast cancer and suppressed proliferation and invasion of breast cancer by targeting miR-19.

Key Words
TUBA4B, MiR-19, Breast cancer, Proliferation, Invasion.

Introduction

Breast cancer is regarded as the most common malignant cancer in women all over the world due to its high morbidity[1]. It is the main cause of death-related cancer among female around the world[2]. It is estimated that there were about 252,710 newly diagnosed breast cancer cases and 40,610 deaths connected to breast cancer in America in the year of 2017[3]. In the past decade, the medical world has greatly improved the diagnostic and therapeutic strategy of breast cancer, but it is still difficult to improve the prognosis of advanced breast cancer[4]. Hence, the medical world desperately needs a profound elucidation of the biological mechanism for breast cancer. Long non-coding RNA (lncRNA) plays an important role in the non-coding RNA family. With transcripts of more than 200 nucleotides in length, it is in lack of protein-coding potential[5]. Because of its non-coding characteristic, lncRNA was once defined as transcriptional "junk"[6]. Nevertheless, emerging evidence shows that lncRNA plays a pivotal role in many biological processes across life[7,8]. Many types of research have illustrated that lncRNA was unusually expressed in numerous cancers such as breast cancer, osteosarcoma and lung adenocarcinoma[9-11]. Studies[12-14] in the past also indicated that dysregulated lncRNA was connected to the prognosis and influenced biological behavior of cancers. Recently, the lncRNA Tubulin Alpha 4B (TUBA4B) has been

Corresponding Author: Jiren Zhang, MD; e-mail: Zhangjiren@126.com

found unusually expressed and possibly acting as a potential tumor suppressor in epithelial ovarian cancer and non-small cell lung cancer[15,16]. It is still mysterious to the public what gene expression and exact role TUBA4B plays in breast cancer. In this research, we dug deep into the expression pattern, phenotype and the underlying mechanisms of TUBA4B on breast cancer.

Materials and Methods

Clinical Samples Collection

38 breast cancer patients who had mammectomy in Zhujiang Hospital, Southern Medical University, took part in this study. After the surgery, we received paired adjacent benign tissues and the breast cancer tissues. The tissues were identified by two individual pathologists and maintained in liquid nitrogen waiting for extracting RNA. With all patients' consent, the study got the approval from the authority in the Ethics Review Board of Zhujiang Hospital, Southern Medical University.

Cell Culture

We purchased human breast cancer cell lines from the American Type Culture Collection (ATCC, Manassas, VA, USA), and MCF-7, ZR-75-1, MDA-MB-231 and HCC-1937 were all included in the purchase. It is in the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) where the normal breast epithelial cell line MCF-10A was obtained. MCF-7, ZR-75-1, MDA-MB-231, HCC-1937 and MCF-10A were maintained in Dulbecco's Modified Eagle Medium/high glucose medium (DMEM; Gibco, Grand Island, NY, USA), with additional 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). The environment for culturing all the cells was at 37°C and 5% CO_2 in a humidified incubator.

Reverse Transcription and Quantitative Real Time-Polymerase Chain Reaction

When extracting total RNA of clinical tissues and cell lines, we employed TRIzol reagent (Invitrogen, Carlsbad, CA, USA). On Applied Biosystems Real Time-Polymerase Chain Reaction (RT-PCR) platform (Applied Biosystems, Foster City, CA, USA), Reverse Transcription and quantitative Real Time-PCR were used to measure the RNA expression of TUBA4B and miR-19. As for internal control, GAPDH and U6 came into play. The following is a list of the sequences of specific primers: TUBA4B, forward 5'-GCAGTTATGCAGCTGGTCAT-3', reverse 5'-AGTTGCGATGCGGCAGTGC-3'; GAPDH, forward 5'-TATCGTGATGCTAGTCCGATG-3', reverse 5'-TGCAGCTAGCTGCATCGATCGG-3'.

Cell Transfection

We bought the pcDNA-TUBA4B (TUBA4B) and pcDNA-control (lnc-ctr) from GenePharma (Shanghai, China) while miR-19 mimic (miR-19) and miR-19 mimic control (miR-ctr) were from Ribobio (Guangzhou, China). After issuing the manufacturer's protocol, cell transfection was done successfully by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Cell Counting Kit-8 Assays

To access the proliferative capacity of breast cancer cells, Cell Counting Kit-8 (CCK8; Dojindo, Kumamoto, Japan) was put into use. Shortly, after transfected, the cells were seeded and digested on 96 well plates. Then, we added the CCK8 reagents into the plate for 4-hour incubation. With the help of a microplate reader, we measured the absorbance on 450 nm. The CCK8 was individually operated three times.

Cell Invasion Assays

To evaluate the invasive capability of breast cancer cells, we used transwell inserts (Corning, Lowell, MA, USA). The chambers were carpeted with Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA) before use. We placed the lower chamber in 400 μl of Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) added with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and covered the upper chamber with $2x10^5$ cells in 300 μl of serum-free medium. When incubation finished, the cells invaded through the lower membrane, and methanol and 0.1% crystal violet were used to stain the cells. Equipped with a light microscope, we counted the number of the cells sticking to the lower chambers.

Luciferase Reporter Assays

Putative miR-19 binding site or the mutant site was contained in the fragment of TUBA4B that was synthesized and inserted into the pmirGLO vector (Promega, Madison, WI, USA). We co-transfect the cells with these vectors with miR-19 mimic or miR-19 mimic control. Dual-Lucifer-

ase Reporter Assay System (Promega, Madison, WI, USA) was put into practice to measure the Luciferase activity. We adopted Renilla Luciferase activity as normalized control.

Statistical Analysis

All the data were presented as mean ± standard deviation (SD). To analyze statistics, we used SPSS 21.0 (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA). Student' s *t*-test (2-tailed) was used for analyzing the data. From a statistical perspective, $p < 0.05$ was considered significantly different.

Results

LncRNA TUBA4B Was Greatly Decreased in Breast Cancer

We employed Reverse Transcription and quantitative Real Time-PCR in breast cancer tissues and adjacent non-tumor tissues for examining the expression of lncRNA TUBA4B. According to the result, lncRNA TUBA4B was greatly down-regulated in breast cancer tissues than adjacent non-tumor tissues (Figure 1A). Moreover, Reverse Transcription and quantitative Real Time-PCR in breast cancer cell lines can also determine the expression profiles of lncRNA TUBA4B. According to the results, lncRNA TUBA4B was significantly decreased in breast cancer cell lines (including MCF-7, ZR-75-1, MDA-MB-231 and HCC-1937) than normal breast epithelial cell line MCF-10A (Figure 1B). The results indicated that lncRNA TUBA4B was markedly decreased in breast cancer specimens and cell lines.

The Proliferation and Invasion of Breast Cancer Cells Were Depressed by LncRNA TUBA4B

To further determine TUBA4B's potential function in breast cancer, we proceeded with phenotype analysis in breast cancer cell lines. ZR-75-1 and MCF-7 cell lines revealed lower endogenous expression of TUBA4B and were selected for the pc-TUBA4B transfection. After that, ZR-75-1 and MCF-7 manifested a dramatic growth of TUBA4B (Figure 2A, D). CCK8 assays were applied to calculate TUBA4B's effects on cell proliferation. As a result, treatment with pc-TUBA4B remarkably depressed the breast cancer cell proliferation in comparison with the control groups (Figure 2B, E). Transwell inserts covered with Matrigel matrix were applied to assess breast cancer cell's invading ability. The consequence demonstrated that lncRNA TUBA4B's overexpression was able to remarkably restrain ZR-75-1 and MCF-7 cells' invasive capability (Figure 2C, F). These outcomes suggested that breast cancer cells' proliferation and invasion could be suppressed by lncRNA TUBA4B.

LncRNA TUBA4B Directly Targeted MiR-19

We browsed a bioinformatic website, Starbase (http://starbase.sysu.edu.cn/), to investigate substantial molecule, which was capable of interacting with TUBA4B. It was indicated from the prediction algorithm that miR-19 was an underlying target of TUBA4B (Figure 3A). We then transfected the breast cells with miR-19 and the transfection efficiencies were validated (Figure 3B, C). The expression of lncRNA TUBA4B in

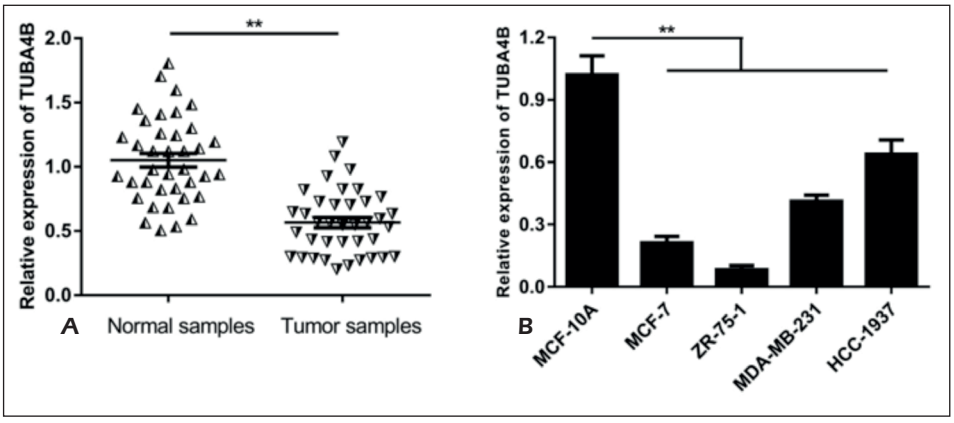

Figure 1. LncRNA TUBA4B was remarkably decreased in breast cancer. **A**, The ex-pression of TUBA4B was significantly decreased in breast cancer tissues than adja-cent normal tissues. **B**, The expression of TUBA4B was markedly decreased in breast cancer cells than normal breast epithelial cells. $^{**}p<0.01$.

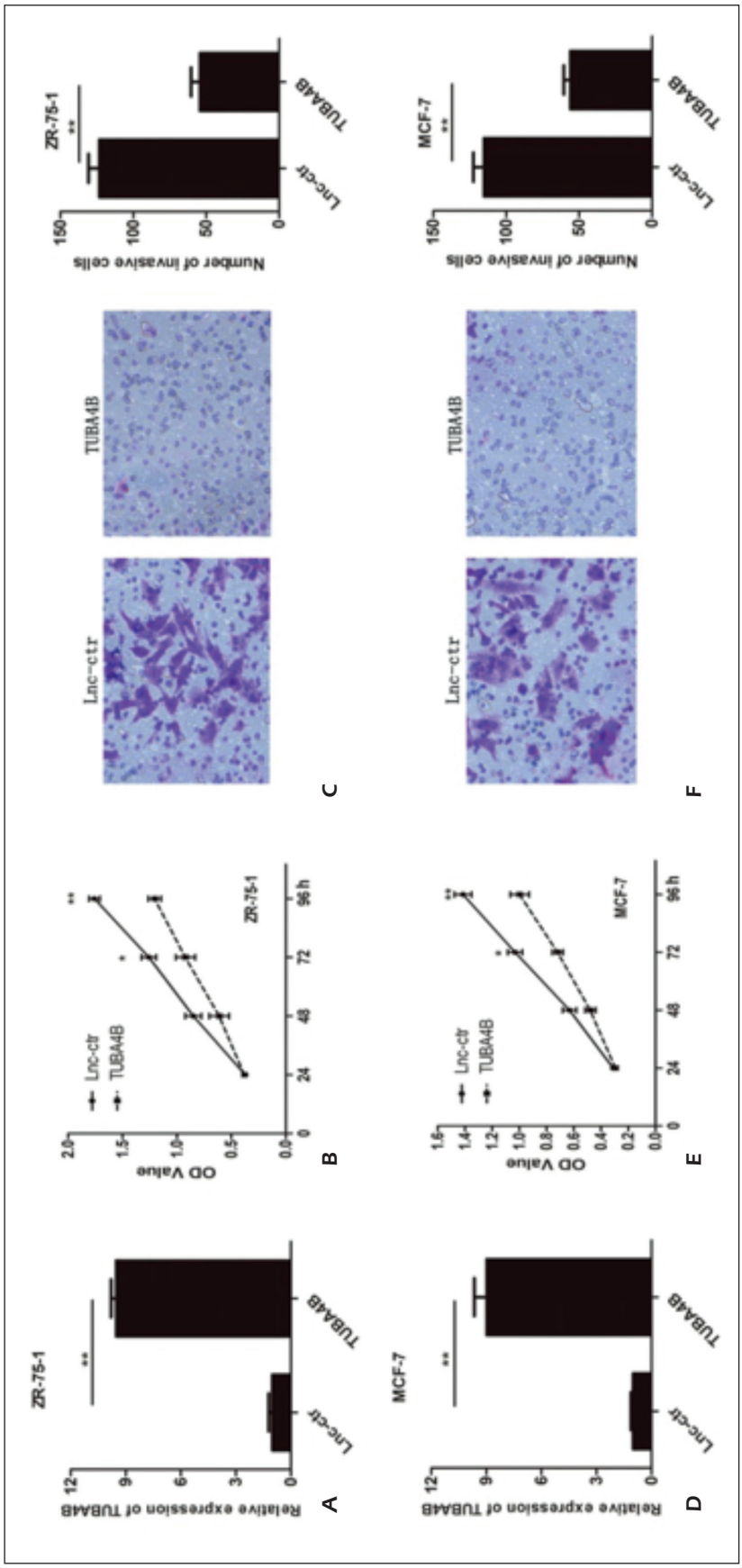

Figure 2. The phenotype analysis of lncRNA TUBA4B on breast cancer. **A**, qRT-PCR showed the transfection of TUBA4B in ZR-75-1 cells. **B**, CCK8 assays re-vealed that TUBA4B significantly inhibited ZR-75-1 cells proliferation. **C**, Transwell assays uncovered that TUBA4B remarkably suppressed ZR-75-1 cells invasion. **D**, qRT-PCR showed the transfection of TUBA4B in MCF-7 cells. **E**, CCK8 assays revealed that TUBA4B markedly inhibited MCF-7 cells proliferation. **F**, Transwell assays uncovered that TUBA4B markedly suppressed MCF-7 cells invasion. $^{**}p<0.01$.

711

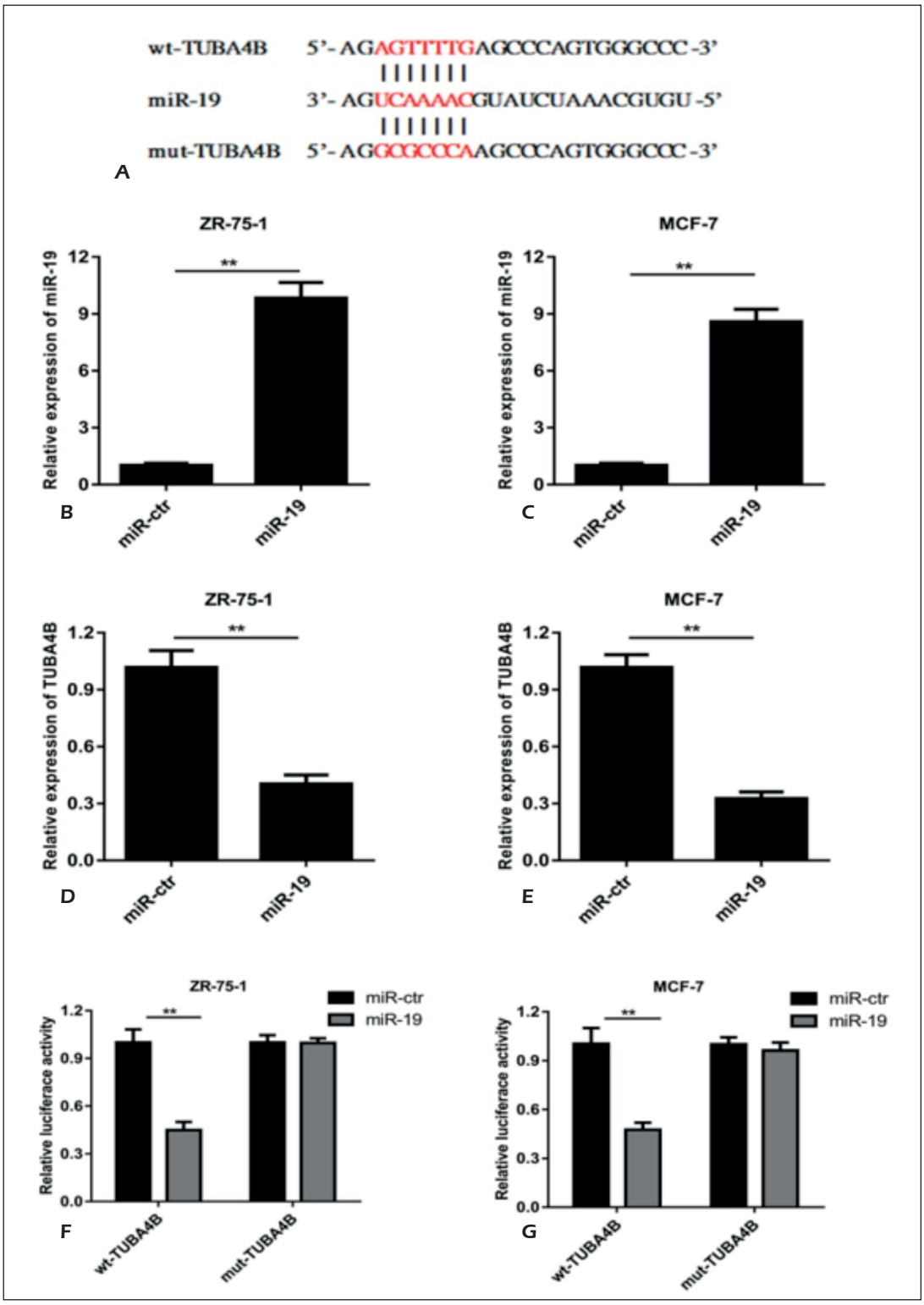

Figure 3. LncRNA TUBA4B directly targeted miR-19. **A**, A potential binding site ex-isted between TUBA4B and miR-19. **B**, qRT-PCR demonstrated the transfection of miR-19 in ZR-75-1 cells. **C**, qRT-PCR revealed the transfection of miR-19 in MCF-7 cells. **D**, The expression of TUBA4B in ZR-75-1 was significantly inhibited by miR-19. **E**, The level of TUBA4B in MCF-7 was markedly suppressed by miR-19. **F-G**, Luciferase reporter assay demonstrated that miR-19 was directly targeted by TU-BA4B in ZR-75-1 and MCF-7 cells. $^{**}p<0.01$.

breast cells, which transfected with miR-19 were measured. Along with the high level of miR-19, TUBA4B s expression decreased sharply (Figure 3D, E). These results indicated the interaction between TUBA4B and miR-19. With the help of Luciferase reporter assays, we could verify the interaction between TUBA4B and miR-19. We constructed the wild-type (wt) plasmids with putative miR-19 binding sites or mutant (mut) plasmids without putative mirR-19 binding sites. The plasmids were then co-transfected with miR-19 or miR-ctr into the ZR-75-1 cells. As a result, Luciferase activity could be lessened by miR-19 in the wt plasmid groups instead of the mut plasmids groups (Figure 3F). The assays in MCF-7 cells came to the same conclusion (Figure 3G). From the outcome, miR-19 was indicated to be a direct target of lncRNA TUBA4B.

The Suppressive Functions of LncRNA TUBA4B Were Mediated by MiR-19

To explore the suppressive functions of LncRNA TUBA4B on breast cancer whether mediated by miR-19, we conducted function-rescued experiments. The breast cancer cell line ZR-75-1 was co-transfected with lnc-ctr, TUBA4B, miR-19 or miR-ctr. The results of quantitative Real Time-PCR revealed that the expression of TUBA4B could be increased by pc-TUBA4B but decreased by miR-19 in ZR-75-1 cells (Figure 4A). Moreover, the results of CCK8 assays found that the repressive effects on breast cancer cells' proliferation (caused by lncRNA TUBA4B) could be attenuated by overexpression of miR-19 (Figure 4B). As demonstrated by the results of transwell assays, lncRNA TUBA4B could inhibit the invasive capability of breast cancer cell but the effect was receded by miR-19 (Figure 4C). Therefore, we concluded that the suppressive functions of lncRNA TUBA4B on breast cancer were mediated by miR-19.

Discussion

It was certified from rising research that lncRNA was linked to the participation in most physiological and pathological processes, which also indicated that lncRNA exerted pivotal impacts on cancer development and progression. LncRNA could control cancer cells proliferation, apoptosis, autophagy and invasion. For example, lncRNA highly upregulated in liver cancer (HULC) could increase the survival rate and cell cycle and decrease the apoptosis of prostate cancer cells under irradiation. The knockdown of HULC accelerated PC3 and DU-145 cells autophagy by interacting with Beclin-1 and mTOR[17]. Yang et al[18] measured the expression of lncRNA MAGI2-AS3 in breast cancer specimens and cell lines. It was demonstrated that MAGI2-AS3 was decreased in breast cancer and remarkably suppressed cell growth by silencing Fas and Fas ligand signaling. The research from Fu et al[19] revealed that lncRNA colon cancer associated transcript 2 (CCAT2) was closely related to the prognosis in patients with pituitary adenomas. It could regulate pituitary ad-

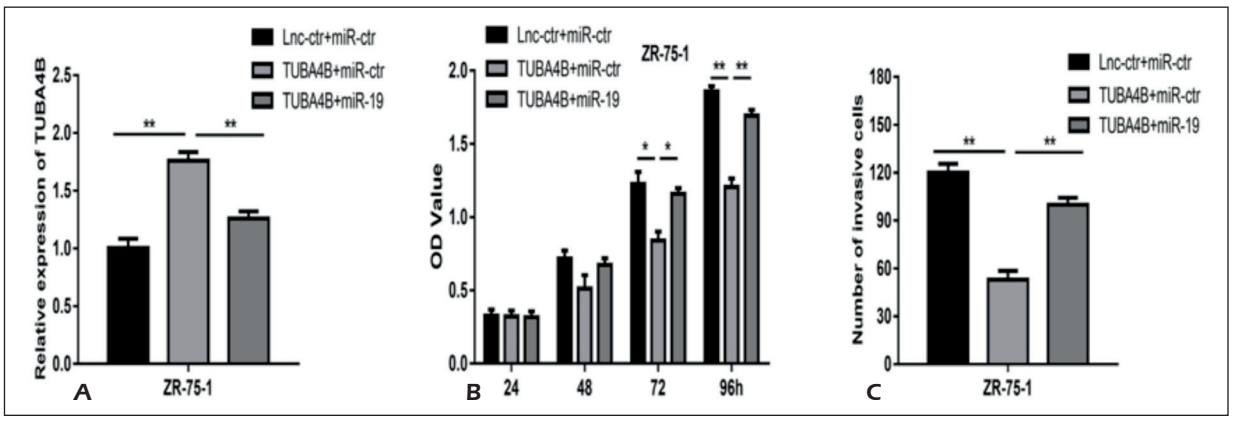

Figure 4. The suppressive functions of lncRNA TUBA4B were mediated by miR-19. **A**, The expression of TUBA4B could be increased by pc-TUBA4B but decreased by miR-19 in ZR-75-1 cells. **B**, MiR-19 could attenuate the repressive effects of TUBA4B on ZR-75-1 cells proliferation. **C**, MiR-19 could rescue the suppressive effects of TUBA4B on ZR-75-1 cells invasion. $^{**}p<0.01$.

enoma progression by affecting the downstream genes including SRY-box 2 and delta-like non-canonical Notch ligand 1 (DLK1). Therefore, we have reasons to put in faith that lncRNA was essential to pathological processes of cancer cells. The distinct lncRNA, TUBA4B, was first discovered by microarray in clinical non-small cell lung cancer specimens by a research group from China. They verified the aberrant expression of TUBA4B by Reverse Transcription-quantitative Polymerase Chain Reaction, suggesting that it may act importantly in lung cancer development[20]. Then, another research group continued to explore the clinical association and biological roles of TUBA4B in on-small cell lung cancer. The expression of TUBA4B was found to be associated with TNM stage, metastatic status and survival times of non small cell lung cancer patients. The phenotype analysis revealed that the up-regulation of TUBA4B markedly inhibited decreased lung cancer cell proliferation[16]. Next, Zhu et al[21] unveiled the impacts of TUBA4B in epithelial ovarian cancer and found that it suppressed the proliferation of ovarian cancer cells significantly. We conducted this study, first looked into the expression pattern of TUBA4B in breast cancer and found that it was decreased in breast cancer samples/breast cancer cell lines. We presumed TUBA4B might serve as a tumor suppressor in breast cancer. With the help of pcDNA in ZR-75-1 and MCF-7 cell lines, we made an ectopic expression of TUBA4B in breast cancer cells to study cellular phenotype changes. The phenotype evaluation demonstrated that TUBA4B is able to depress, to a great extent, the growth and progression of ZR-75-1 and MCF-7 cells. As far as we're concerned, this work is the first one to determine the expression and the biological effects of TUBA4B in breast cancer, which might help to better understand this lncRNA. Serving as competitive endogenous RNA (ceRNA) to silence miRNAs is one of the functional mechanisms of lncRNA. We employed an online bioinformatics algorithm to pool the downstream miRNA targets of TUBA4B and miR-19 was brought in front of us. From previous findings, we knew that miR-19 could suppress the endogenous tissue factor expression in MCF-7 cells and downregulates tissue factor expression in MDA-MB-231 cells, which indicated miR-19 act importantly in breast cancer[22]. Moreover, some studies[23,24] demonstrated that miR-19 could promote cancer cell viability migration, invasion and epithelial-mesenchymal transition. In this work, Luciferase reporter assays were used to vilify the interaction and miR-19 was initially identified as a direct target of TUBA4B. What's more, we showed that the effects of TUBA4B on breast cancer cells could be mediated by miR-19 and the regulatory way lcnRNA TUBA4B/miR-19 was initially showed. We might need more studies in the future to explore the downstream signaling pathways which TUBA4B /miR-19 may regulate.

Conclusions

We found that lncRNA TUBA4B was decreased in breast cancer. Breast cancer cell proliferation and invasion were inhibited by TUBA4B by means of miR-19. We conducted this study to better comprehend the molecular biological mechanism of breast cancer and to represent new molecular targets for therapy in the future.

Conflict of Interests

The authors declare that they have no conflict of interest.

References

1) Mamounas EP, Kuehn T, Rutgers EJT, Von MG. Current approach of the axilla in patients with early-stage breast cancer. Lancet 2017; 17: 31451-314514
2) Harbeck N, Gnant M. Breast cancer. Lancet 2017; 389: 1134-1150.
3) DeSantis CE, Ma J, Goding Sauer A, Newman LA, Jemal A. Breast cancer statistics, 2017, racial disparity in mortality by state. CA Cancer J Clin 2017; 67: 439-448.
4) Zhang W, Shi S, Jiang J, Li X, Lu H, Ren F. LncRNA MEG3 inhibits cell epithelial-mesenchymal transition by sponging miR-421 targeting E-cadherin in breast cancer. Biomed Pharmacother 2017; 91: 312-319.
5) Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. Cell 2009; 136: 629-641.
6) Palazzo AF, Lee ES. Non-coding RNA: what is functional and what is junk? Front Genet 2015; 6: 2.
7) Quinn JJ, Chang HY. Unique features of long non-coding RNA biogenesis and function. Nat Rev Genet 2016; 17: 47-62.
8) Ulitsky I, Bartel DP. lincRNAs: genomics, evolution, and mechanisms. Cell 2013; 154: 26-46.
9) Gradia DF, Mathias C, Coutinho R, Cavalli IJ, Ribeiro E, de Oliveira JC. Long non-coding RNA TUG1 expression is associated with different subtypes in human breast cancer. Noncoding RNA 2017; 3(4): E26.

10) Wang Y, Kong D. LncRNA GAS5 represses osteosarcoma cells growth and metastasis via sponging MiR-203a. Cell Physiol Biochem 2018; 45: 844-855.

11) Rani S, Gately K, Crown J, O'Byrne K, O'Driscoll L. Global analysis of serum microRNAs as potential biomarkers for lung adenocarcinoma. Cancer Biol Ther 2013; 14: 1104-1112.

12) Chang ZW, Jia YX, Zhang WJ, Song LJ, Gao M, Li MJ, Zhao RH, Li J, Zhong YL, Sun QZ, Qin YR. LncRNA-TUSC7/miR-224 affected chemotherapy resistance of esophageal squamous cell carcinoma by competitively regulating DESC1. J Exp Clin Cancer Res 2018; 37: 56.

13) Fu X, Zhu X, Qin F, Zhang Y, Lin J, Ding Y, Yang Z, Shang Y, Wang L, Zhang Q, Gao Q. Linc00210 drives Wnt/beta-catenin signaling activation and liver tumor progression through CTNNBIP1-dependent manner. Mol Cancer 2018; 17: 73.

14) Ma H, Ma T, Chen M, Zou Z, Zhang Z. The pseudogene-derived long non-coding RNA SFTA1P suppresses cell proliferation, migration, and invasion in gastric cancer. Biosci Rep 2018; 38: BSR20171193.

15) Zhu FF, Zheng FY, Wang HO, Zheng JJ, Zhang Q. Downregulation of lncRNA TUBA4B is associated with poor prognosis for epithelial ovarian cancer. Pathol Oncol Res 2018; 24: 419-425.

16) Chen J, Hu L, Wang J, Zhang F, Chen J, Xu G, Wang Y, Pan Q. Low expression LncRNA TUBA4B is a poor predictor of prognosis and regulates cell proliferation in non-small cell lung cancer. Pathol Oncol Res 2017; 23: 265-270.

17) Chen C, Wang K, Wang Q, Wang X. LncRNA HULC mediates radioresistance via autophagy in prostate cancer cells. Braz J Med Biol Res 2018; 51: e7080.

18) Yang Y, Yang H, Xu M, Zhang H, Sun M, Mu P, Dong T, Du S, Liu K. Long non-coding RNA (lncRNA) MAGI2-AS3 inhibits breast cancer cell growth by targeting the Fas/FasL signalling pathway. Hum Cell 2018; 31: 232-241.

19) Fu D, Zhang Y, Cui H. Long noncoding RNA CCAT2 is activated by E2F1 and exerts oncogenic properties by interacting with PTTG1 in pituitary adenomas. Am J Cancer Res 2018; 8: 245-255.

20) Wang Y, Xu G, Chen W, Pan Q, Huang K, Pan J, Zhang W, Chen J. Detection of long-chain non-encoding RNA differential expression in non-small cell lung cancer by microarray analysis and preliminary verification. Mol Med Rep 2015; 11: 1925-1932.

21) Zhu FF, Zheng FY, Wang HO, Zheng JJ, Zhang Q. Downregulation of lncRNA TUBA4B is associated with poor prognosis for epithelial ovarian cancer. Pathol Oncol Res 2018; 24: 419-425.

22) Zhang X, Yu H, Lou JR, Zheng J, Zhu H, Popescu NI, Lupu F, Lind SE, Ding WQ. MicroRNA-19 (miR-19) regulates tissue factor expression in breast cancer cells. J Biol Chem 2011; 286: 1429-1435.

23) Sun Z, Liu Q, Hong H, Zhang H, Zhang T. miR-19 promotes osteosarcoma progression by targeting SOCS6. Biochem Biophys Res Commun 2018; 495: 1363-1369.

24) Li J, Yang S, Yan W, Yang J, Qin YJ, Lin XL, Xie RY, Wang SC, Jin W, Gao F, Shi JW, Zhao WT, Jia JS, Shen HF, Ke JR, Liu B, Zhao YQ, Huang WH, Yao KT, Li DJ, Xiao D. MicroRNA-19 triggers epithelial-mesenchymal transition of lung cancer cells accompanied by growth inhibition. Lab Invest 2015; 95: 1056-1070.