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MAL2 promotes proliferation, migration, and invasion through regulating epithelial-mesenchymal transition in breast cancer cell lines

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ABSTRACT

Background: Breast cancer is one of the most common malignant tumors in women. However, the underlying molecular mechanisms of breast cancer are still far to clear. With the development of sequencing technology, we discovered that MAL2 is overexpressed in tumor tissues. But the major function of MAL2 in breast cancer has not to be well confirmed.

Materials and methods: We downloaded and analyzed the MAL2 expression in The Cancer Genome Atlas (TCGA) database. Real-time quantitative polymerase chain reaction (RT-qPCR) was conducted to detect the expression of MAL2 in 35 breast cancer patients. Then, we performed proliferation, colony formation, migration, invasion and western blot assays to investigate the role of MAL2 in breast cancer cell lines (MDA-MB-231 and BT-549).

Results: In our research, we found that MAL2 is remarkably overexpressed in breast cancer tissues compared to adjacent non-cancer tissues by RT-qPCR (T: $N = 5.28 \pm 4.34$:1.82 ± 1.11, $P < 0.001$) and high expression of MAL2 has worse overall survival in TCGA cohort ($P = 0.0032$). Knocked down MAL2 could decrease the ability of proliferation, migration, and invasion of breast cancer cell lines. Our Western Blot assay results investigated that MAL2 could regulate EMT.

Conclusion: In this study, we demonstrated the function of MAL2 in breast cancer cell lines and it might act as an oncogene in breast cancer.

1. Introduction

Breast cancer is one of the most malignant tumors detected in females worldwide. Studies show that the estimated number of newly diagnosed breast cancer patient in 2018 was 268,670, with an estimated 41,400 deaths [1,2]. With the advance of techniques and knowledge, breast cancer could be grouped by category of estrogen receptors (ER-), progesterone receptors (PR-), and HER2 (HER2-) genes [3,4]. A substantial amount of treatments is used to cure breast cancer, such as surgery, endocrine, and targeted therapies. However, the prognosis is not always satisfactory. Over the past decades, considerable articles have been made in exploring the underlying mechanisms of breast cancer [5–7]. Although there are farther more mechanisms about breast cancer that yet need to be revealed.

MAL2, a member of the MAL proteolipid family, plays a significant role in the transcytosis, an intracellular transport pathway used to deliver membrane-bound proteins. Many studies have investigated that MAL2 could influence tumorigenesis in various cancers and correlate with poor prognosis [8–10]. Byrne JA et al. discovered that MAL2 is upregulated in high-grade serous ovarian

Abbreviations: EMT, Epithelial-to-mesenchymal transition; RT-qPCR, Quantitative reverse transcription PCR; CCK8, counting kit-8.

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carcinomas compared with serous borderline tumors [11]. However, the potential mechanism between MAL2 and breast cancer is far too clear.

In our research, eight pairs of breast cancer tissue and adjacent non-tumor tissue were selected to do RNA sequencing in our unpublished article and results showed that MAL2 is significantly higher in tumor compared to normal tissue. Then another 35 paired breast cancer tissue samples and adjacent normal tissues were performed to RT-qPCR assay to validate the dysregulation of MAL2. In vitro experiments and western blot were performed to explore the role of MAL2 in breast cancer. The present study aimed to investigate the relationship between MAL2 gene and breast cancer.

2. Materials and methods

2.1. Patients and breast tissue samples

We obtained 35 breast cancer patients who underwent primary surgical resection at the Department of Thyroid and Breast Surgery, The First Affiliated Hospital of Wenzhou Medical University. Patients signed informed consents and research protocols for the use of tissues. This research was thoroughly approved by and conducted in accordance with the ethical standards of the Institutional Review Board of First Affiliated Hospital of Wenzhou Medical University (approval no. 2012-57). All fresh tissues were snap-frozen in liquid nitrogen immediately and stored at −80 °C. Breast cancer mRNA expression data were downloaded from the TCGA data portal (https://tcga-data.nci.nih.gov/docs/publications/tcga/).

2.2. Cell cultures and growth conditions

MDA-MB-231, BT-549, MDA-MB-468 and MCF-7 cells were taken in this study. All cells were bought from Shanghai Cell Biology, Institute of the Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 and MCF-7 were cultured in Dulbecco's Modified Eagle's Medium DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco, Grand Island, NY, USA). MDA-MB-468 was cultured in L-15 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco, Grand Island, NY, USA). BT-549 was cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco, Grand Island, NY, USA) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2 mM of L-glutamine, 20 ng/ml of epidermal growth factor and 10% FBS (Gibco, Grand Island, NY, USA). MDA -MB-468 was incubated in a standard cell culture incubator (Thermo, Waltham, MA, USA) at 37 °C without CO_2. The others were incubated in a humidified incubator at 37 °C with 5% CO_2.

2.3. Cell transfection

MDA-MB-231 and BT-549 were transfected through Lipofectamine RNAiMAX transfection reagent (Invitrogen). 100,000 BC cells were plated 24 h before transfection. MAL2 was silenced by siRNA. The siRNA sequences used in the study are: MAL2 siRNAs target the following sequences: The siRNA sequences used in the study are: MAL2 siRNAs target the following sequences: MAL2 siRNA-1, Forward 5′- GCAUGAUUUGCAUUGCAAUTT -3′and Reverse 5′- AUUGCAAUGCAAAUCAUGCTT -3′; MAL2 siRNA-2, Forward 5′-GCCUUUAUGACGACAGCUUTT -3′ and Reverse 5′- AAGCUGUCGUCAUAAAGGCTT -3′; Both siRNAs were provided by Gene-Pharma (Shanghai, People's Republic of China).

2.4. RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

According to the manufacturer's instructions (Invitrogen, USA), total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc, USA). The isolated RNA was measured at 260/280 nm using spectrophotometry (Thermo, San Jose, CA, USA). All the RNA samples were stored at −80 °C. Real-time reactions were run and analyzed by using the ABI 7500 quantitative PCR System (Applied Biosystems, Foster City, CA, USA). The relative expression of mRNA was calculated using the comparative cycle threshold (CT) $(2-\Delta\Delta CT)$ method with GAPDH as the endogenous control to normalize the data. The sequences of the primers used were:

MAL2 Forward: 5′- CTCCTGAGTGATAACCAGTATA -3′ and Reverse: 5′- CTCCTGAGTGATAACCAGTATA-3'; GADPH Forward: 5′-GTCTCCTCTGACTTCAACAGCG-3′ and Reverse: 5′-ACCACCCTGTTGCTGTAGCCAA-3′

2.5. Invasion and migration assay

For cell invasion assays, cells were collected in the medium containing 10% FBS. Invasion of cells was measured in Matrigel (BD Bioscience, Franklin Lakes, USA). A total of 40,000 cells (~250 μl) transfected with si-NC, si-MAL2 were transferred into the upper chamber and 600 μl medium containing 20% FBS was added to the lower chamber. Then positioned the plate into the incubator with 5% CO2 atmosphere. After 24 h, the membrane was carefully extracted and fixed with 4% paraformaldehyde and stained with 0.4% crystal violet solution for 15 min. Motility assays were similar to invasion assay. Cell migration and invasion were counted in five random fields and images captured under a microscope at a magnification of × 20.

2.6. Western blot analysis

Protein extraction buffers (radioimmunoprecipitation assay and phenylmethanesulfonyl fluoride; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were added to the cells, and the protein concentrations were quantified using a bicinchoninic acid (BCA) kit. The samples were then homogenized, centrifuged at 12,000×*g* at 4 °C for 30 min, then subjected to ultra-sonication (each action 5 s, intermittent 6 s and each specimen 5 times). The supernatant was removed via centrifugation at 4000×*g* at 4 °C for 20 min. The sample was used to perform quantification of the protein expression with BCA. According to the manufacturer's protocol, 20 μg from each sample were separated by 10% SDS-polyacrylamide gel electrophoresis (BioRad Laboratories, Inc., Hercules, CA, USA) and then transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA). PVDF Membranes were then incubated with blocking buffer (PBS with 5% non-fat milk and 0.1% Tween-20) for 1 h at room temperature. Then membranes were subsequently washed three times with Tris-buffered saline/Tween 20 for 5 min each and incubated overnight with primary antibodies at 4 °C. The primary antibodies used in this study were as follows: N-cadherin (cat no. 13116; Cell Signaling Technology, Inc., Danvers, MA, USA), vimentin (cat no. 5741; Cell Signaling Technology, Inc.), E-cadherin (cat no. 3195; Cell Signaling Technology, Inc.), and human β-actin (cat no. 3700; Cell Signaling Technology, Inc.) Then incubated with the goat anti-rabbit immunoglobulin G secondary antibody (Alexa Fluor 488; cat. no. ab150077; 1:1000 dilution; Thermo Fisher Scientific, Inc.) for 2 h in room temperature. The enhanced chemiluminescent (ECL)kit (Beyotime Institute of Biotechnology) was used to detect the results of western blot analysis. The relative level of each protein was deduced from the ratio of the mean value of each band to that of β-actin. The relative densities were quantified with a digital imaging analyzer, ImageJ 1.4.1.

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