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Original Articles

Reprogramming carcinoma associated fibroblasts by AC1MMYR2 impedes tumor metastasis and improves chemotherapy efficacy

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ABSTRACT

Carcinoma associated fibroblasts (CAFs) produce a nutrient-rich microenvironment to fuel tumor progression and metastasis. Reactive oxygen species (ROS) levels and the inflammation pathway cooperate to transform CAFs. Therefore, elucidating the mechanism mediating the activity of CAFs might identify novel therapies. Abnormal miR-21 expression was reported to be involved in the conversion of resident fibroblasts to CAFs, yet the factor that drives transformation was poorly understood. Here, we reported that high miR-21 expression was strongly associated with lymph node metastasis in breast cancer, and the activation of the miR-21/NF-κB was required for the metastatic promoting effect of CAFs. AC1MMYR2, a small molecule inhibitor of miR-21, attenuated NF-κB activity by directly targeting VHL, thereby blocking the co-precipitation of NF-κB and ß-catenin and nuclear translocation. Taxol failed to constrain the aggressive behavior of cancer cells stimulated by CAFs, whereas AC1MMYR2 plus taxol significantly suppressed tumor migration and invasion ability. Remodeling and depolarization of F-actin, decreased levels of β-catenin and vimentin, and increased E-cadherin were also detected in the combination therapy. Furthermore, reduced levels of FAP-α and α-SMA were observed, suggesting that AC1MMYR2 was competent to reprogram CAFs via the NF-κB/miR-21/VHL axis. Strikingly, a significant reduction of tumor growth and lung metastasis was observed in the combination treated mice. Taken together, our findings identified miR-21 as a critical mediator of metastasis in breast cancer through the tumor environment. AC1MMYR2 may be translated into the clinic and developed as a more personalized and effective neoadjuvant treatment for patients to reduce metastasis and improve the chemotherapy response.

Introduction

Metastasis accounts for the majority of deaths for patients with breast cancer [1]. Most studies of tumor cells metastasis focus on genetic or phenotypic alterations of the cancer cell itself. However, there is growing evidence that tumor–stroma interaction in the cancer microenvironment is considered to be a significant determinant of cancer progression and metastasis [2,3]. Cancer associated fibroblasts (CAFs), characterized by the expression of α-smooth muscle actin (α-SMA) and fibroblast activation protein alpha (FAP-α), are one of the most representative cellular constituents of tumor stroma and fuel tumor development and metastasis by mutually interacting with tumor cells [4–6]. Thus, elucidating the mechanism mediating the activity of CAFs may lead to the development of novel therapies that target the tumor microenvironment [7].

Several intracellular signaling pathways were reported to regulate the activity of CAFs and promote cell growth and epithelial–mesenchymal transition (EMT), including the activation of TGF-β signaling, PDGF signaling, and PI3K/AKT/Girdin and the deregulation of ROCK and JAK signaling [8–11]. Moreover, ROS levels and signaling by inflammatory cytokines had also been demonstrated to activate CAFs and modulate the tumor microenvironment, consequently sustaining EMT and drug resistance in breast cancer

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[12,13]. Therefore, a comprehensive molecular understanding of the signaling pathways involved in CAF activation and mechanism of CAF- mediated metastasis might identify more effective targeted therapies for metastatic breast cancer patients.

High-throughput profiling studies have uncovered microRNA (miRNA)-mediated pathways in the regulation of cancer metastasis [14,15]. Recent research highlighted the importance of miRNA deregulation in the tumor microenvironment [16,17]. MiR-21 is well known as the first onco-miRNA to be identified. Deregulation of miR-21 has been correlated with advanced breast cancer stages, lymph node metastasis, and poor survival [18,19]. Furthermore, high miR-21 expression in tumor stroma was proved to drive the normal fibroblasts (NFs) to CAF transformation, thereby promoting tumor growth and metastasis, suggesting that miR-21 may contribute to the cellular crosstalk in the tumor microenvironment [20]. Our previous study demonstrated that AC1MMYR2 (AMR), a specific small-molecule inhibitor of miR-21, was capable of realizing EMT process reversal and impairing high dose paclitaxel-induced tumor metastasis in breast cancer [21,22]. However, whether and how AMR eliminated CAF-induced metastasis has not been deeply investigated [23].

In the current study, we found that CAFs contributed to tumor metastasis by elevating the miR-21/NF-κB axis in both MDA-MB-231 and MCF-7 cells. AMR treatment impaired CAF-induced EMT and enhanced paclitaxel (taxol) response by elevating the miR-21 target Von Hippel–Lindau (VHL), thereby attenuating NF-κB pathway activity. Moreover, AMR inhibited β-catenin nuclear translocation and co-immunoprecipitated with NF-κB. In parallel, AMR treatment reduced cell growth and ablated pulmonary metastasis in a xenograft mouse model of breast cancer. Our findings identified miR-21 as a critical mediator of metastasis in breast cancer through the tumor environment, and high stromal miR-21 expression may serve as a clinical molecular predictor of poor chemotherapy response for breast cancer patients. AC1MMYR2 may be translated into the clinic and developed as a personalized and effective neoadjuvant treatment for patients with breast cancer to reduce metastasis and improve chemotherapy response.

Materials and methods

Cell lines and culture conditions

Human breast cancer cells MDA-MB-231 and MCF-7 were obtained from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units per mL penicillin and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 humidified incubator. Cells in logarithmic growth phase or at 80% confluence were used for experiments. Breast cancer cell lines were treated with the conditioned media (CM) of NFs or CAFs for 72 h. An equal volume of complete culture medium was used as the control.

Isolation and culture of CAFs

CAFs were acquired from breast cancer patients who underwent mastectomy at Tianjin Medical University Cancer Institute and Hospital (TMUCIH). NFs were isolated from tissue at least 2 cm distal to the outer margin of the cancer mass. The investigation and the use of specimens were approved by the Institutional Review Board of TMUCIH.

Tissues were diced into 1 mm^3 and digested with collagenase type I (1 mg/mL; Sigma) and hyaluronidase (125 units/mL; Sigma) for 6 h in DMEM without FBS at 37 °C. After filtering the undigested tissues, the stromal fraction was centrifuged at 1000 rpm for 5 min. The separated stromal cell-enriched supernatant was suspended in DMEM with 10% FBS, and the cells were cultured on tissue culture plates.

Immunofluorescence and histochemical analysis

Frozen tumors were embedded in OCT, and 6–8 μm sections were cut. These sections were then fixed in acetone for 30 min. Cells were seeded on the coverslips and cultured for 48 h. Cells were fixed for 15 min with 4% paraformaldehyde. The cells or sections were permeabilized with 0.25% Triton-X 100 for 10 min, followed by blocking with 3% BSA for 1 h. Immunofluorescence staining was conducted with antibodies against FAP-α, α-SMA, vimentin, ß-catenin (1:100 dilutions, Abcam), NF-κB and E-cadherin (1:100 dilutions, Cell Signaling Technology). For immunohistochemical staining, the sections were incubated with pan-cytokeratin and α-SMA (1:200 dilutions, Abcam) overnight at 4 °C. The cells were washed with phosphate-buffered saline (PBS) and incubated with Alexa Fluor 488 or Alexa Fluor 546 (Life Technologies) secondary antibodies. For stress fiber formation detection, cells were stained

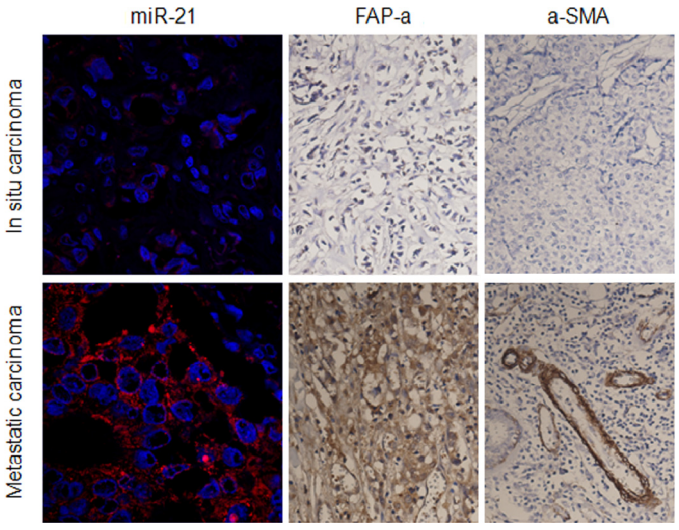

Fig. 1. High expression of miR-21, FAP-α and α-SMA associated with tumor metastasis. MiR-21, FAP-α and α-SMA were detected by in-situ hybridization and immunohistochemistry in lymph node metastasis and in situ carcinoma, respectively.

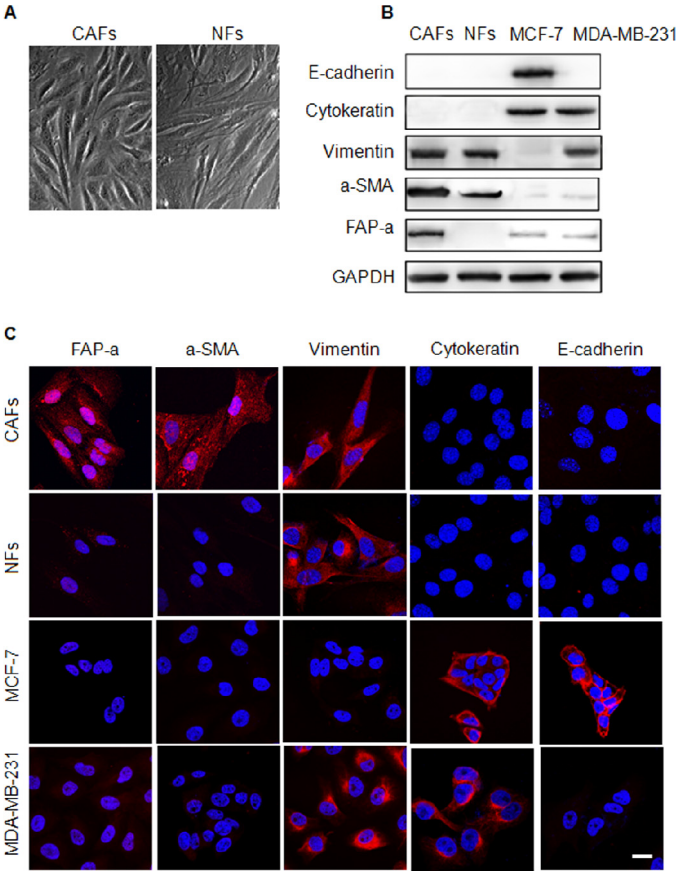

Fig. 2. Myofibroblastic properties of extracted fibroblasts isolated from breast cancer tissues. (A) Morphological features of CAFs and NFs. The expressions of FAP-α, α-SMA, the mesenchymal marker vimentin and the epithelial marker cytokeratin, E-cadherin in CAFs, NFs, MCF-7 and MDA-MB-231 breast cancer cells were detected by Western blotting (B) and immunofluorescence staining (C). Scale bar: 20 μm.