Cancer associated fibroblast secreted miR-432-5p targets CHAC1 to inhibit ferroptosis and promote acquired chemoresistance in prostate cancer

Sun HongBin
dyyu1235@njmu.edu.cn
Nanjing First Hospital, Nanjing Medical University

Jun Zhao
Nanjing First Hospital, Nanjing Medical University

Jijie Shen
Nanjing First Hospital, Nanjing Medical University

Liang Mao
Nanjing First Hospital, Nanjing Medical University

Tianli Yang
Nanjing First Hospital, Nanjing Medical University

Jinyu Liu
Nanjing First Hospital, Nanjing Medical University

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Abstract

Ferroptosis is a novel form of programmed cell death in which the accumulation of intracellular iron promotes lipid peroxidation, leading to cell death. Cancer-associated fibroblasts (CAF) are an integral part of the tumor microenvironment and play an important role in tumorigenesis. To understand the potential mechanisms of chemoresistance in prostate cancer, we investigated the role of CAF-derived exosomes in ferroptosis. CAF exosomes inhibited erastin-induced lipid reactive oxygen species (ROS) accumulation in prostate cancer cells and reduced erastin induced damage to mitochondria, thereby inhibiting ferroptosis in prostate cancer cells. miR-432-5p inhibits ferroptosis in prostate cancer cells by targeting CHAC1 to reduce glutathione (GSH) depletion in cells thereby inhibiting ferroptosis. miR-432-5p inhibition enhances the drug sensitivity of PC (prostate cancer) cells in vivo. We found that exosomal miR-432-5p secreted by CAF targets CHAC1 via the ferroptosis pathway, thereby promoting chemoresistance in PC. This study provides a new approach for docetaxel resistance.

Introduction

Prostate cancer has surpassed lung and bronchial cancers as the most common malignancy among men and the second leading cause of cancer-related deaths in men, with an estimated 268,490 new cases and 34,500 deaths in the United States by 2022(1). For patients with metastatic prostate cancer, docetaxel-based chemotherapy has been used as one of the first-line treatments for metastatic prostate cancer(2). Although some patients with metastatic prostate cancer respond to docetaxel treatment, others are intolerant or resistant to this treatment(3, 4). Therefore, it is crucial to investigate the mechanisms of docetaxel resistance in patients with metastatic prostate cancer to develop more effective treatments.

Death is the irreversible termination of life activity. Cell death occurs frequently in normal tissues and is necessary to maintain tissue function and morphology. Cell death is divided into programmed cell death and necrosis, the former being regulated by genes involved in apoptosis, autophagy, and scorch death(5, 6). However, cancer cells can evolve multiple strategies to limit or evade genetically controlled cell death, thereby accelerating cancer cell proliferation and malignant transformation(5, 7, 8). In 2012, Dixon et al. (9) first introduced the new concept of ferroptosis, which is an iron-dependent pattern of cell death due to lipid peroxidation and massive accumulation of reactive oxygen species (ROS). Recent studies have shown that ferroptosis play an important role in the development of many diseases and in tumor drug resistance; however, the molecular mechanisms underlying chemoresistance in prostate cancer are still poorly understood(10, 11).

Tumor development is not merely an alteration of oncogenes in the tumor cells themselves; the tumor tissue is an abnormal organ containing multiple cellular components and extracellular matrix(12). Angiogenic cells, infiltrating immune cells, and cancer-associated fibroblasts (CAF) are the major cell types in the tumor microenvironment(13). CAFs are the main components of tumor microenvironment stromal cells and play an important role in cancer(14, 15). CAFs can not only promote tumor
development, invasion and metastasis by secreting various cytokines or metabolites to inhibit the function of immune cells, but also have the ability to shape the extra-tumor stroma and form a barrier for drug or therapeutic immune cell penetration, thus preventing the deep penetration of drugs and immune cells into tumor tissues and reducing the effectiveness of tumor treatment(16–18).

Recent studies have shown that extracellular vesicles contain almost all bioinformatic components and are involved in the process of tumor development(19, 20). Exosomes are extracellular vesicles (EV), approximately 30–150 in diameter, which include proteins, DNA, mRNA, miRNA, IncRNA, and lipids(21, 22). The cells can influence the function and status of recipient cells by releasing exosomes. Recent studies have shown that exosomes derived from CAFs can influence tumor proliferation, migration and invasive activity as well as promote tumor drug resistance(23). miR-148b-3p upregulation in CAF exosomes promotes proliferation, metastasis and drug resistance in bladder cancer(24). miR-522 secreted by CAFs can inhibit ferroptosis in cancer cells thereby promoting chemoresistance in gastric cancer(25). However, the potential role of CAFs-derived exosomes in ferroptosis in prostate cancer cells has not yet been determined.

In this study, miR-432-5p secreted by CAFs promoted prostate cancer cell proliferation and reduce sensitivity to docetaxel. miR-432-5p expression in prostate cancer directly inhibits ChaC glutathione-specific γ-glutamylcyclo-transferase1(CHAC1). CHAC1 has γ-glutamylcyclo-transferase activity, and its decreased expression can promote glutathione (GSH) accumulation, thus activating glutathione peroxidase 4 (GPX4) to prevent lipid peroxide (lipid-ROS) accumulation and inhibit ferroptosis. Therefore, this study identifies that exosomes derived from CAFs can inhibit ferroptosis in prostate cancer cells and provides a new approach for enhancing the sensitivity of prostate cancer chemotherapy.

**Methods**

**Tumor tissue**

Tumor tissues of all patients with prostate cancer in this study were obtained from the Nanjing Hospital of Nanjing Medical University. This study was approved by the Ethics Committee of Nanjing Hospital, Nanjing Medical University.

**Cell culture**

Human prostate cancer cell lines (22RV1, PC-3, 22rv1, and LNCap) were purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China. 22RV1, 22rv1, and LNCap cells were cultured in RPMI 1640 medium (Gibco, USA) containing 10% fetal bovine serum, (FBS) (Gibco, USA) and 1% penicillin and streptomycin (Gibco, USA). PC-3 cells were cultured in F-12K medium (Gibco, USA). All cells were cultured in a constant temperature incubator at 37°C, and 5% CO₂. Cell status was observed daily, with fluid changes every 1–2 days, and cells were grown until they reached 90% fusion and passaged.

**Isolation and culture of cancer-associated fibroblasts**
We collected 12 cases of clinical prostate cancer tissues and corresponding paracancerous tissues stored in serum-free medium at 4°C and separated within two hours. Tissue pieces were cut to 2 mm under aseptic conditions and collected into centrifuge tubes. Tumor tissue dissociation solution (Metenyi) was added and digested for 30 min at 37°C, according to the manufacturer's instructions (26). The digestion was then terminated by adding medium containing 10% serum and centrifuging at 1000 rpm for 5 min. The supernatant was then discarded and resuspended in 1640 medium containing 10% FBS and add to the culture dish. After 24 hours of incubation, the medium was partially changed until fibroblasts appeared, and then it was changed every three days. Cells were grown to 70–80% confluence for passaging.

**Extraction of exosomes**

CAFs were cultured using exosome-free serum (System Bioscience), the supernatant was collected, and exosomes were extracted by ultracentrifugation. Briefly, the supernatant was centrifuged at 10,000g for 30 min to remove debris, followed by centrifugation at 110,000g (Beckman Coulter) for 70 min. Then, the supernatant was discarded to collect exosomes from the sediment and resuspended in phosphate-buffered saline (PBS); all steps were performed at 4°C.

**Characterization of exosomes**

The morphology of the exosomes and mitochondria was examined using transmission electron microscopy. Briefly, exosomes or cells were fixed with 2.5% glutaraldehyde at 4°C overnight. Samples were rinsed in PBS (three times for 15 min each), fixed with 1% osmium tetroxide for 2–3 h, and rinsed in PBS. This was followed by gradient ethanol dehydration (30,50,70,90,95,100%) for 10 min/per step. Subsequent embedding and curing were performed overnight in an oven at 37°C and 24 h at 60°C. Sections (100 nm) were obtained using a Lycra ultrathin sectioning machine and double-stained with 3% uranyl acetate-lead citrate. The sections were observed under a transmission electron microscope (TEM, Hitachi, Japan).

**Nanoparticle tracking analysis (NTA)**

A nanoparticle tracker (Particle Metrix, Germany) was used to analyze exosome particle size, concentration and distribution. The sample cell was first washed with deionized water. The instrument was cleaned with polystyrene microspheres (100 nm) calibrated with PBS and then diluted with PBS for detection. Data were analyzed using ZetaView (8.04.02 software).

**Exosome labeling**

The PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich) was used to label the exosomes. The PKH26 dye was first diluted with diluent C according to the manufacturer's instructions, the exosome samples were mixed 1:1 with the dye solution for 5 min, and the residual dye was neutralized using a medium containing 10% FBS. Labeled exosomes were extracted by ultracentrifugation.

**Exosome phagocytosis**
First, the cells were placed in a 24-well plate, and PC-3 and 22RV1 cells were grown evenly in a well plate. After the cells adhered to the wall, exosomes labeled with PKH26 were added, and the cell crawl sheets were removed after 24 h for paraformaldehyde fixation, followed by staining of the cytoskeleton with phalloidin, labeling of the nucleus with DAPI, and observation under a fluorescence microscope (Zeiss, Germany).

**EdU cell proliferation assay**

PC-3 and 22RV1 cells were spread evenly in 24-well plates containing cell crawlers, and when the cell density reached 70–80%, complete medium containing EdU solution (Beyotime, China) was added and incubated for 2 h at 37°C. The EdU medium was removed, wash with PBS three times (5 min each time), fixed with 4% paraformaldehyde at room temperature for 15 min, and washed with PBS three times (5 min each time). PBS containing 0.3% TritonX-100 was added, and the cells were incubated at room temperature for 10 min. The permeation solution was removed, and cell were washed with PBS, stained with DAPI, and observed under a fluorescence microscope.

**Colony formation assay**

Approximately 500 cells were inoculated into 6-well plates and incubated in a constant temperature incubator at 37°C with 5% CO2 for 2 weeks. The culture medium was removed, cells were washed with PBS, 4% paraformaldehyde was added to fix the cells, paraformaldehyde was discarded, and crystalline violet (Beyotime, China) staining was performed to observe the number of cell colonies.

**Detection of lipid-ROS levels**

ROS levels in the cells were measured using an ROS assay kit (KeyGEN BioTECH, China). According to the manufacturer’s instructions, briefly, the cells were plated the day before the assay to ensure that the number of cells at the time of the assay was less than 5 × 10^5/ml. The culture medium was removed and serum-free culture medium containing 10 µM DCFH-DA was added and incubated for 30 min at 37°C in a cell incubator protected from light. Fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 525 nm, and the average fluorescence intensity of each group was determined.

**Cell death detection**

Briefly, cells were first evenly grown in 12-well plates, collected after drug treatment, resuspended in flow tubes (50,000 cells/tube), incubated with 2 µl of PI reagent (KeyGEN BioTECH, China) for 10 min and analyzed for dead cells using a flow cytometer (BECKMEN COULTER, USA).

**Mitochondrial membrane potential (MMP) detection**

First, the cells were evenly grown in a six-well plate and incubated for 15 min with the appropriate amount of TMRE working solution (Beyotime, China) according to the manufacturer’s instructions. The residual TMRE was removed by washing with PBS, and labeled cells were collected. The cells were
detected using a flow cytometer at an excitation wavelength of 550 nm and an emission wavelength of 575 nm.

**Glutathione (GSH) assay**

First, cells overexpressing CHAC1 were spread in 12-well plates and treated with Erastin and CAF Exos for 48 h. The culture medium was removed, and cells were washed three times with PBS and collected. Using the GSH assay kit (KeyGEN BioTECH, China), reagents were added according to the manufacturer's instructions and the OD value was detected using an enzyme marker at 420 nm.

**Immunofluorescence**

CAF and normal fibroblast (NF) were grown in 24-well plates lined with cell crawlers, and the cell crawlers were collected when the cell density reached 70–80%. Then, 4% paraformaldehyde was used to fix the cells for 15 min, and they were then permeabilized with 0.05% Triton X-100 for 10 min. Anti-α-SMA antibody (Proteintech, China), anti-FAP antibody (Proteintech, China), and anti-FSP1 antibody (CST, USA) were added and maintained overnight at 4°C. The antibody was removed by washing with PBS three times, FITC goat anti-rabbit antibody was added and incubated for 2 h in closed cells, and the nuclei were stained with DAPI and observed under a fluorescence microscope.

**RNA extraction and quantitative RT-PCR assay**

Total RNA was extracted using TRIzol reagent (Vazyme) according to the manufacturer's instructions. miR-432-5p detection was performed using microRNA probes. All experiments were performed in triplicate. U6 was used as the miRNA internal reference, and the mRNA level internal reference was used to compare GAPDH. The expression levels of all the genes were normalized using the $\text{Ct}(2^{-\Delta\Delta\text{CT}})$ method. The primers used in this study are listed in Table S1.

**miRNA target prediction**

miRNA target prediction and analysis were performed using TargetScan (http://www.targetscan.org/), miRDB (http://www.mirdb.org/), miRBase (http://www.mirbase.org/) and miRanda (http://www.microrna.org/) algorithms.

**Western blotting**

Protein expression of FAP, α-SMA, and FSP1 was assessed by protein blot analysis and samples were normalized to GAPDH or ACTIN. Samples were blocked with 5% skim milk powder for 2 h at room temperature and incubated at 4°C with anti-FAP (1:1000, Proteintech), anti-α-SMA (1:2000, Cell Signaling Technology), anti-FSP1 (1:1000, Cell Signaling Technology), anti-Calnexin (1:1000, Proteintech), anti-TSG101 (1:1000, Proteintech), anti-CD63 (1:1000, Proteintech), anti-CD9 (1:1000, Proteintech), anti-GAPDH (1:5000, Proteintech), anti-NFR2 (1:1000, Proteintech), anti-ACSL4 (1:1000, Proteintech), anti-SLC7A11 (1:1000, Proteintech), anti-CHAC1 (1:500, Proteintech), anti-GPX4 (1:1000, Proteintech) and anti-ACTIN (1:5000, Proteintech) antibodies overnight. Next, membranes were imaged with horseradish
peroxidase-conjugated secondary antibodies (anti-rabbit or anti-mouse, 1:5000, Proteintech), and protein blots were imaged using a gel imaging system (Tanon) and quantified using the ImageJ software.

**Luciferase reporter gene assay**

Following the steps of plasmid transfection, CHAC1-MUT, -WT were co-transfected into prostate cancer cell lines using Lipofectamine 2000 with miR-432-5p mimics and miR-432-5p NC, respectively, and routinely cultured for 36–48 h with fluid exchange. After repeated blowing, transfer into EP tubes and centrifuge at 12000rpm for 15min at room temperature, and the supernatant was collected and transferred to new EP tubes. Then, 200µl of lysate was added to the 24-well plate, and after a sufficient amount of time the lysate was collected into an EP tube at 12000rpm, centrifuged for 15 min, and the supernatant was collected and transferred into a new tube. An equal volume of luciferase detection reagent (Vazyme) was added and detected using chemiluminescence.

**Nude mice with xenograft tumors**

Five-week-old male BALB/c nude mice were randomly assigned to 3 groups (n = 6). Briefly, 5 × 10^6 cell from one mouse were injected subcutaneously with lentivirus-infected or negative control PC-3 cells and CAFs. Tumor-implanted mice were injected with docetaxel (5 µg/ g) every 5 days from day 10 onwards, and subcutaneous tumor dimensions were examined every 5 days using Vernier calipers, excised on day 30, weighed, photographed, and subjected to subsequent immunohistochemistry analysis and other experiments.

**Statistical analysis**

Statistical data were analyzed using SPSS 19.0 statistical software (IBM, USA) and GraphPad Prism 7 (GraphPad Software, USA). Differences between two groups were performed using the Student’s t-test, and comparisons between more than two groups were performed using one-way ANOVA. Statistical significance was set at p < 0.05. All data are expressed as the mean ± standard deviation (SD) of at least three independent replicates.

**Results**

**Identification of CAF and CAF-derived exosomes**

First, we observed the cell morphology of the extracted NFs and CAFs using a light microscope. NFs and CAFs were both long and shuttle-shaped and could grow in multiple layers with a disorderly arrangement, which is typical of fibroblast morphology (Supplementary Fig. 1). Subsequently, we identified the surface markers α-SMA, FAP, and FSP1 of CAFs by immunofluorescence and western blotting, and found that α-SMA, FAP and FSP1 were positive in CAFs, demonstrating their successful isolation (Fig. 1A, B). To further investigate the functions of NF and CAF-derived exosomes, they were extracted by ultracentrifugation and identified by transmission electron microscopy, particle size analysis, and western blot analysis. Transmission electron microscopy showed that the exosomes had a
round or oval vesicle structure (Fig. 1C). Particle size analysis showed that the diameter of the exosomes was approximately 100 nm, and most exosomes were between 30–150 nm in diameter (Fig. 1D). Western blot analysis showed positive expression of exosome markers TSG01, CD63, and CD9 and negative expression of the endoplasmic reticulum marker calnexin, indicating successful extraction of exosomes (Fig. 1E).

**CAF-derived exosomes promote the proliferation of prostate cancer cells**

To test the role of CAF-derived exosomes in regulating prostate cancer cell proliferation, isolated CAF exosomes were co-cultured with prostate cancer cell lines. We labeled exosomes with PKH26 and co-cultured them with 22RV1 and PC-3 cells. PKH26-labeled exosomes were detected in both cell lines after 12 h. The results indicated that exosomes derived from CAF were efficiently taken up by prostate cancer cells (Fig. 2A). It was found that CAF-derived exosomes could promote the clonogenic ability of prostate cancer cells by a clonogenisis assay (Fig. 2B, C). Further we examined the effect of exosomes on the proliferation ability of prostate cancer cells using CCK-8 and EdU assays. The results showed that the proliferative ability of prostate cancer cells treated with CAF-derived exosomes was higher than that of the normal and NF-derived exosome groups (Fig. 2D-F). In addition, the wound healing assay and Transwell assays showed that CAF exosomes could promote the migration ability of prostate cancer cells (Supplementary Fig. 2).

**Differentially expressed miRNAs in exosomes secreted by NF and CAF**

To investigate the mechanism by which CAF-derived exosomes affect prostate cancer progression, an miRNA microarray assay was performed to identify differentially expressed miRNAs in exosomes secreted by NF versus CAF (Fig. 3A). The number of differentially expressed miRNAs is shown in the Supplementary Material. To validate the results of miRNA microarrays analysis, we obtained results through the EVmiRNA database showing that miR-432-5p was highly expressed in microvesicles secreted by fibroblasts (Fig. 3B). It was also highly expressed in microvesicles secreted by prostate cancer cell (Fig. 3C). In addition, we examined the expression of miR-432-5p in 12 pairs of NF and CAF cells and their secreted exosomes by real-time PCR, and the results showed that miR-432-5p levels were significantly increased in CAF and CAF-secreted exosomes (Fig. 3D, E). Meanwhile, we detected the expression of miR-432-5p in four prostate cancer cell lines as well as secreted exosomes by real-time PCR, and the results showed that the miR-432-5p levels in CAF and CAF-derived exosomes were dominant (Fig. 3F, G).

**CAF- secreted exo-miR-432-5p suppresses ferroptosis of PC cells**
We transfected Cy3-labeled miR-432-5p into CAF and subsequently co-cultured CAF cells with 22RV1 and PC-3 cells. Twenty-four hours later Cy3-miR-432-5p was detected in 22RV1 and PC-3 cells, indicating that miR-432-5p could enter prostate cancer cells (Fig. 4A). We found that miR-432-5p in exosomes could effectively inhibited erastin-induced lipid-ROS accumulation and ferroptosis in prostate cancer cells (Fig. 4B, C). The membrane potential (MMP) of mitochondria in 22RV1 and PC-3 cells treated with erastin was significantly increased, and CAF exosomes partially reversed the damage to mitochondria by erastin(Fig. 4D). It could be observed by transmission electron microscopy that the mitochondria of the cells became smaller, the membrane density thickened and the mitochondrial cristae disappeared after erastin treatment, which was alleviated by CAF exosomes (Fig. 4E).

**CHAC1 is a target gene of miR-432-5p in PC**

To investigate the mechanism of miR-432-5p in PC ferroptosis, four online software programs (TargetScan, miRcode, miRBase and miRDB) were used to predict the 107 potential target genes of miR-432-5p (Fig. 5A). We analyzed these potential genes using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis as well as gene ontology (GO) functional enrichment analysis, and the results showed that these genes function in cancer pathway enrichment (Fig. 5B) as well as in the nucleus (Fig. 5C). We finally selected CHAC1, a gene associated with ferroptosis, as the target of our study, and bioinformatics analysis suggested that it contains a binding site for miR-432-5p in the CHAC1 3'UTR region (Fig. 5D). Dual luciferase reporter gene analysis revealed that miR-432-5p mimics significantly inhibited luciferase activity in the CHAC1 wild-type (Wt) vector compared to the mutant (Mut) vector (Fig. 5E).

**CAF-secreted exosome miR-432-5p inhibits PC ferroptosis by targeting CHAC1**

To verify that miR-432-5p regulates PC ferroptosis through CHAC1, we examined the changes in CHAC1 mRNA levels in 22RV1 and PC-3 cells after CAF exosome treatment by real-time quantitative PCR and found that CHAC1 expression levels were significantly reduced in 22RV1 and PC-3 cells after CAF exosome treatment (Fig. 6A). We also examined the changes in CHAC1 protein expression levels in both cell lines after CAF exosome treatment, and the experimental results showed that the protein expression levels of CHAC1 were significantly reduced in 22RV1 and PC-3 cells after CAF exosome treatment. We then examined the expression of ferroptosis-related proteins in prostate cancer cells treated with PBS, NF exosomes, and CAF exosomes, and found that the expression levels of SLC7A11 and GPX4 were decreased while those of NFR2 and ACSL4 were increased in the CAF exosome-treated group (Fig. 6B-E). To further verify this, we constructed a CHAC1 overexpression plasmid and transfected the overexpression plasmid into 22RV1 and PC-3 cells and treated them with miR-432-5p mimics to verify whether overexpression of CHAC1 could restore the inhibitory effect of miR-432-5p on ferroptosis in prostate cancer cells. The experimental results showed that miR-432-5p inhibited erastin-induced GSH depletion, and overexpression of CHAC1 reversed this effect (Fig. 6F). Meanwhile, the effect of miR-432-
5p on mitochondrial damage by inhibiting erastin-induced lipid ROS accumulation, ferroptosis and MMP decrease in prostate cells could also be reversed by overexpression of CHAC1 (Fig. 6G-I).

**Docetaxel inhibits PC ferroptosis by promoting CAF secretion of miR-432-5p**

To determine the response to chemotherapy-induced injury, we treated CAFs with different concentrations of docetaxel and investigated the effect of docetaxel on CAF cell viability. We chose 100 nmol/L docetaxel as the sublethal dose of CAF. The expression of miR-432-5p in CAF and CAF exosomes was detected by real-time quantitative PCR, and the results showed no significant change in miR-432-5p in CAF treated with docetaxel (Fig. 7A), while miR-432-5p expression in CAF exosomes increased significantly (Fig. 7B), and the results indicated that docetaxel stimulated the secretion of miR-432-5p by CAF. We isolated exosomes from docetaxel-treated CAF and co-cultured them with PC, and the results of PCR showed that docetaxel-treated exosomes inhibited CHAC1 mRNA expression more than the control (Fig. 7C). Exosomes isolated from docetaxel-treated CAF were found to further enhance the proliferation of prostate cancer cells compared to normal CAF-derived exosomes by clonogenesis and EdU assays (Fig. 7D-I). In addition, docetaxel-treated exosomes effective reduced lipid-ROS production and erastin-induced ferroptosis (Fig. 7J, K). The addition of docetaxel to the culture medium could greatly contribute to PC mortality, whereas the addition of docetaxel-treated CAF exosomes reduced cell mortality (Fig. 7L). We also found by transmission electron microscopy that docetaxel-treated CAF exosomes attenuated the damage to mitochondria by docetaxel (Fig. 7M). These in vitro experiments demonstrated that CAF exosomes can reduce the sensitivity of PC to docetaxel chemotherapy.

**In vivo role of miR-432-5p in regulating PC growth and chemosensitivity**

Finally, we assessed the role of miR-432-5p in influencing tumor growth and chemotherapeutic efficacy. We knocked down miR-432-5p in CAF by using an shRNA-containing lentivirus and implanted these fibroblasts subcutaneously into mice mixed with PC-3 cells. Docetaxel was then injected into these tumor-bearing mice every 5 days starting on day 10, and the tumors were harvested, imaged and weighed on day 30 (Fig. 8A). We found that knockdown of miR-432-5p in CAFs was inhibited tumor growth and enhance sensitivity to docetaxel (Fig. 8B-D). The expression of CHAC1 protein in tumor tissues was detected by Western Blot. The results showed that the level of CHAC1 protein in miR-432-5p knockout group was significantly higher than that in CAFs group (Fig. 8E, F). Ki-67, a marker of proliferation, was detected by immunohistochemical assay, which showed a significant decrease in Ki-67 expression after inhibition of miR-432-5p, indicating that inhibition of miR-432-5p could enhance PC cell sensitivity to docetaxel. Meanwhile, the number of CHAC1-positive cells increased after inhibition of miR-432-5p expression, and the targeted inhibition of CHAC1 by miR-432-5p was confirmed in vivo (Fig. 8G, H).

**Discussion**
Ferroptosis is a new programmed non-apoptotic regulated cell death, which is an iron-dependent pattern of cell death caused by massive accumulation of lipid peroxide(27). Recent studies have suggested that ferroptosis may be an adaptive process critical for cancer cell eradication(28). Activation of ferroptosis-related pathways may be effective in combating malignant tumor progression and may enhance the sensitivity of tumor cells to chemotherapy(29, 30). Tumor progression is not strictly dependent on the epigenetic modifications in cancer cells, but is also influenced by the components of the tumor microenvironment. Stromal cells in the tumor microenvironment usually contribute to tumor progression and drug resistance(31, 32). However, a recent study showed that CD8 T cells induced ferroptosis in tumor cells by suppressing SLC7A11 expression in cancer immunotherapy(33). Zhou et al. also showed that knockdown of hnRNP L in prostate cancer cells promoted T cell-mediated ferroptosis(34). However, the role of other stromal cells, especially CAFs, which constitute the majority of the tumor microenvironment, in regulating ferroptosis in prostate cancer cells has not been well studied.

The effects of ferroptosis on tumors have been extensively studied in recent years. Ali et al. (35) suggested that use of the ferroptosis inducer erastin may serve as a novel approach for the treatment of advanced prostate cancer. Although the exact molecular mechanism of ferroptosis remains unknown, upregulation of CHAC1 expression levels is widely considered to be a marker of early ferroptosis and is associated with GSH degradation and the onset of ferroptosis(9). This finding is consistent with the results of the present study. In prostate cancer cells, we observed that CHAC1 overexpression increased intracellular lipid peroxide levels and decreased GPX4 protein levels, leading to the onset of ferroptosis. Previous studies have shown that CHAC1 increases the sensitivity of prostate cancer cells to docetaxel through its effects on endoplasmic reticulum stress and ferroptosis in prostate cancer cells(36). However, that study did not indicate a direct cause of the reduced expression of CHAC1 in prostate cancer cells. In the current study, we found that miR-432-5p in CAF-derived exosomes caused a reduction in CHAC1 expression by binding to CHAC1 in prostate cancer cells, thereby inhibiting ferroptosis in prostate cancer cells.

Chemotherapy is the main treatment for advanced cancer, and docetaxel is the first-line chemotherapeutic drug for metastatic prostate cancer(37, 38). However, resistance to docetaxel is becoming more increasingly severe in prostate cancer treatment(3, 39). Chemotherapy resistance is usually associated with DNA damage repair, mutations in molecules that regulate apoptosis, and elevated GSH levels(40, 41). Here, we show that changes in ferroptosis-related signaling pathways may provide new ideas for reversing chemoresistance. Exosomes can promote the development of chemoresistance in tumor cells, and a deeper understanding of the mechanisms of resistance will help improve treatment outcomes and prognosis.

In this study, we demonstrated that a CAF-derived exosomes-mediated decrease in CHAC1 expression levels inhibited cellular glutathione depletion leading to decreased ferroptosis levels in cancer cells, thereby decreasing chemotherapy sensitivity. Further studies are needed to understand the impact of ferroptosis in therapeutic strategies for prostate cancer, particularly targeted therapies. Our study showed that CAFs are involved in regulating ferroptosis in prostate cancer cells through the secretion of
miR-432-5p. Knockdown of miR-432-5p in CAFs can improve the sensitivity of prostate cancer to chemotherapy; therefore, inhibition of specific miRNA secretion in CAFs is a new idea for the clinical treatment of prostate cancer, especially for metastatic prostate cancer. However, none of the ferroptosis-related genes involved in this study are a classical pathways of tumorigenesis development. Since miR-432-5p plays a key role in mediating the downregulation of CHAC1 and lipid-ROS, leading to the inhibition of cell death, we considered miR-432-5p to be a tumor driver. Although miR-432-5p is upregulated in CAF, it is also widely expressed in several other normal tissues. Given the more complex expression and regulation of these genes in humans, the mechanisms involved need to be explored future.

**Abbreviations**

CAF: cancer associated fibroblast; ROS: reactive oxygen species; GSH: glutathione; CHAC1: ChaC glutathione-specific γ-glutamylcyclo-transferase1; GPX4: glutathione peroxidase 4; NF: normal fibroblast; SD: standard deviation;

**Declarations**

**Ethics approval and consent to participate**

All procedures were permitted by the Ethics Committee for the use of Experimental Animals of Nanjing First Hospital, Nanjing Medical University. This study was directed according to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

J Z and HB S participated in study design; J Z, TL Y and L M involved in data collection; J Z, and JJ S performed research; JJ S, JY L and L M performed the software; J Z, and L M involved in methodology and data analysis; J Z, JJ S and HB S involved in manuscript preparation. All authors read and approved the final manuscript.

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References


**Supplementary Figures**

Supplementary Figures 1 and 2 are not available with this version.

**Figures**
Figure 1

Identification of NF and CAF. **A** Immunofluorescence staining for FAP, α-SMA, and FSP1 expression in NF and CAF. Scale bar = 20 μm. **B** Western blot analysis of protein expression levels of α-SMA, FAP, and FSP in 6 pairs of NF and CAF. **C** TME images of exosomes extracted from NF and CAF cultures. Scale bar = 100 nm. **D** NTA found that the majority of NF and CAF-derived exosomes were from 30 to 150 nm in diameter. **E** Western blot analysis of Calnexin, TSG101, CD63 and CD9 expression in exosomes.
CAF-Exos promotes the proliferation of PCa cells. **A** PCa cells take fluorescence images of exosomes labeled with PKH26. Scale bar = 50 μm. **B** The ability of PBS, NF-Exos and CAF-Exos to clone PCa cell lines PC-3 and 22RV1 was tested by clone formation experiment. **C** Quantitative analysis result of clone formation experiment. **D** EdU experiment was used to detect the effects of PBS, NF-Exos and CAF-Exos on the proliferation of PCa cells, Scale bar = 100 μm. **E** Quantitative analysis of EdU experimental results. **F** CCK8 experiment was used to detect the cell proliferation ability of PC-3 and 22RV1 cells co-cultured with NF-Exos and CAF-Exos. * p ≤ 0.05 ** p ≤ 0.01
Figure 3

Differential expression of miRNA in exosomes. **A** Thermogram showed that miRNA was differentially expressed in NFs and CAFs exosomes. **B,C** The expression of miR-432-5p in microvesicles secreted by cells and tissues was obtained by EVmiRNA database. **D** Expression of miR-432-5p in NFs and CAFs. **E** Expression of miR-432-5p in NF-Exos and CAF-Exos. **F,G** Expression of miR-432-5p in PCa cell line and secreted exosomes. **p < 0.01*** p < 0.001.
miR-432-5p inhibits ferroptosis in PCa cells. A The CAFs of miR-432-5p mimics labeled with Cy3 was co-cultured with PC-3 and 22RV1, and red fluorescence was expressed in PC-3 and 22RV1 cells, Scale bar = 100 μm. B-D miR-432-5p in CAF-Exos inhibited the ferroptosis of PCa cells induced by Erastin, and miR-432-5p secreted by CAFs inhibited the accumulation of ROS (B), reduced the cell death induced by Erastin (C) and decreased the abnormal decrease of MMP (D). E Typical images of mitochondrial morphological changes were taken by transmission electron microscope. Black arrows represent abnormal mitochondria. Scale bar = 2 μm. * p < 0.05 ** p < 0.01 *** p < 0.001
Figure 5

Predicting the target gene of miR-432-5p in PCa cells. **A** Four miRNA target gene prediction websites, TargetScan, miRcode, miRbase and miRDB, were used to predict the target gene of miR-432-5p. **B,C** KEGG and GO bioinformatics analysis. **D** miR-432-5p has a binding site with the 3'UTR region of CHAC1. **E** Double luciferase reports the test results. * p < 0.05 ** p < 0.01 *** p < 0.001.
Figure 6

miR-432-5p inhibits ferroptosis in PCa cells by down-regulating the expression of CHAC1. **A** The expression of CHAC1 mRNA in PCa cell line after exosomes treatment. **B** Western Blot was used to verify the expression of ferroptosis-related proteins in 22RV1 cells. **C** Image J software for quantitative analysis of western blot. **D** Western Blot was used to verify the expression of ferroptosis-related proteins in PC-3 cells. **E** Quantitative analysis of western blot by Image J software. **F-I** Overexpression of CHAC1 can reverse the inhibition of CAF-Exos on ferroptosis in PCa cells, which is manifested by the decrease of GSH content (F), the increase of ROS level (G), the increase of cell mortality (H) and the decrease of mitochondrial membrane potential (I). * p < 0.05 ** p < 0.01 *** p < 0.001.
**Figure 7**

DTX promotes the secretion of miR-432-5p by CAF. **A** The effect of DTX on cell viability. **B** The expression of miR-432-5p in CAF-Exos increased after DTX treatment. **C** DTX-CAF-Exos significantly down-regulated the expression of CHAC1 mRNA in PC-3 and 22RV1 cells. **D, E** The ability of CAF-Exos and DTX-CAF-Exos to clone PCa cell lines PC-3 and 22RV1 and their quantitative analysis were detected by clone formation experiment. **F-I** EdU experiment was used to detect the effects of CAF-Exos and DTX-CAF-Exos on the proliferation of PCa cells and quantitative analysis. Scale bar= 100 μm. **J** DTX-CAF-Exos inhibited the accumulation of ROS induced by Erastin. **K** DTX-CAF-Exos inhibits the death of PCa cells induced by Erastin. **L** DTX-CAF-Exos is helpful for PCa cells to acquire chemical resistance. **M** DTX-CAF-Exos can reduce the mitochondrial damage caused by chemotherapy in PCa cells. Black arrows represent abnormal mitochondria. Scale bar = 2 μm. * p < 0.05** p < 0.01*** p < 0.001.
Figure 8

Role of miR-432-5p in regulating PCa growth and DTX sensitivity in vivo. A Establishing an experimental design diagram of an animal model. B The changes of tumor diameter in each group. C Measurement of tumor weight. D Images of each group of tumors. E Western Blot was used to detect the expression of CHAC1 protein in tumor tissues. F Image J software quantitatively analyzes the gray value. G Immunohistochemical analysis of the distribution of CHAC1 and Ki67 in tumor tissues. Scale bar = 200 μm. H Quantitative analysis of Ki67 positive cells. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

Supplementary Files

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