Received: 12 July 2023 | Revised: 6 November 2023 | Accepted: 9 November 2023

DOI: 10.1111/cas.16036

ORIGINAL ARTICLE

Cancer Science WILEY

Cancer cell-derived exosomal miR-20a-5p inhibits CD8$^+$ T-cell function and confers anti-programmed cell death 1 therapy resistance in triple-negative breast cancer

Weina Li[1] | Guohui Han[2] | Feng Li[3] | Peng Bu[4] | Yating Hao[2] | Li Huang[2] | Xiangdong Bai[2]

[1]Department of Radiotherapy, Shanxi Province Cancer Hospital/Shanxi Hospital Affiliated to Cancer Hospital, Chinese Academy of Medical Sciences/Cancer Hospital Affiliated to Shanxi Medical University, Taiyuan, China

[2]Department of Breast Surgery, Shanxi Province Cancer Hospital/Shanxi Hospital Affiliated to Cancer Hospital, Chinese Academy of Medical Sciences/Cancer Hospital Affiliated to Shanxi Medical University, Taiyuan, China

[3]Department of Biochemistry and Molecular Biology, Shanxi Province Cancer Hospital/Shanxi Hospital Affiliated to Cancer Hospital, Chinese Academy of Medical Sciences/Cancer Hospital Affiliated to Shanxi Medical University, Taiyuan, China

[4]Department of Pathology, Shanxi Province Cancer Hospital/Shanxi Hospital Affiliated to Cancer Hospital, Chinese Academy of Medical Sciences/Cancer Hospital Affiliated to Shanxi Medical University, Taiyuan, China

Correspondence
Xiangdong Bai, 3 New Worker's Road, Xinghualing District, Taiyuan, Shanxi 030000, China.
Email: hemonbai@sina.com

Funding information
Natural Science Foundation of Shanxi Province, Shanxi Provincial Health Commission, Grant/Award Number: 2020060, 202103021224431 and 20210302123340

Abstract

Circulating miRNAs (cirmiRNAs) can be packaged into the exosomes, participating in intercellular communication, which affects the malignant progression and therapy resistance of triple-negative breast cancer (TNBC). Currently, immune checkpoint inhibitors that regulate T-cell function, especially antibodies against programmed cell death 1 (PD-1) or its ligand PD-L1, are emerging as new promising therapy for TNBC patients. However, only very limited patients showed complete or partial response to anti-PD-1 treatment. Dysfunction of CD8$^+$ T cells is one of the key reasons for the immune escape of TNBC. The regulation of exosome-derived cirmiRNAs on CD8$^+$ T cells in TNBC deserves more investigation. Here, the cirmiR-20a-5p level was significantly upregulated in the plasma of TNBC patients and culture supernatant of TNBC cells. High abundance of cirmiR-20a-5p was correlated with a worse prognosis of TNBC. cirmiR-20a-5p was secreted in the form of exosomes by TNBC cells. Exosomal cirmiR-20a-5p was internalized into CD8$^+$ T cells and resulted into the dysfunction of CD8$^+$ T. A mechanism study uncovered that cirmiR-20a-5p targeted the nuclear protein ataxia-telangiectasia (NPAT) and decreased NPAT expression in CD8$^+$ T cells. An in vivo xenograft mouse model showed that cirmiR-20a-5p conferred TNBC to anti-PD-1 treatment resistance. Collectively, these findings indicated that cirmiR-20a-5p released by TNBC cells via exosome promotes cancer cell growth and leads to the immunosuppression by inducing CD8$^+$ T cell dysfunction. This study suggests that targeting cirmiR-20a-5p might be a novel strategy for overcoming the resistance of TNBC to anti-PD-1 immunotherapy.

KEYWORDS
cirmiR-20a-5p, exosome, immunotherapy, TNBC

Abbreviations: 3'-UTR, 3'-untranslated region; cirmiRNAs, circulating miRNAs; EVs, extracellular vesicles; HNSCC, head and neck squamous cell carcinoma; miRNA, microRNA; NPAT, nuclear protein ataxia-telangiectasia; PD-1, programmed cell death 1; RLU, luminescence units; TNBC, triple-negative breast cancer.

Weina Li and Guohui Han contributed equally to this work.

1 | INTRODUCTION

As the leading cause of cancer-related death among women, breast cancer is classified into three subtypes based on the expression levels of estrogen, progesterone receptor and HER2 overexpression.[1,2] Triple-negative breast cancer (TNBC), which accounts for 15% of all breast cancer cases, typically appears with high-grade invasive ability and poorer prognosis compared with other breast cancer subtypes.[3–5] Chemotherapy has been considered as the only viable systemic treatment option for TNBC. However, recent studies showed that TNBC is more likely to benefit from immunotherapy due to its rich infiltration of lymphocytes and higher immunogenicity.[6–9] Thus far, treatment of anti-programmed cell death 1 (PD-1) monoclonal antibody combined with chemotherapy has shown an impressive pathological complete rate in patients with TNBC.[10–12] Unfortunately, based on the clinical experience of PD-1 therapy in other cancers, a major of patients inevitably acquire therapy resistance after a period of treatment. Thus, it is urgent to explore promising biomarkers to improve the prognosis and understand the mechanisms for immunotherapy resistance of TNBC.

Non-coding microRNA (miRNA) molecules play important roles in regulating gene expression by binding the 3'-untranslated region (UTR) of mRNA, which leads to mRNA degradation or translation inhibition.[13–15] Given the critical function of miRNAs in cell proliferation, differentiation, and migration, dysregulated miRNAs are involved in cancer progression and treatment resistance.[16–18] In addition to the residence in intracellular components, miRNAs can be secreted by cells or tissues and exist stably in body fluids within extracellular vesicles (Evs). Exosomes are a type of membrane-bound EV with complex cargos, including miRNA, protein, mRNA, and lipid.[19,20] Therefore, exosomes act as important message transmitters between neighboring and distant cells, resulting in the direct regulation of target mRNAs.[21] It is well documented that tumor cells highly express certain miRNAs and package these miRNAs into exosomes to promote tumor metastasis by reshaping the microenvironment.[22] Additionally, recent studies found that tumor cell-derived exosomal miRNAs can modulate the function of immune cells, such as T cells and Treg, which finally induces tumor immune invasion.[23] Our previous study reported that overexpressed miR-20a-5p in TNBC promoted cell proliferation.[24] The potential role and expression pattern of cirmiR-20a-5p in TNBC have not been fully elucidated.

This study was performed to investigate the regulation of TNBC-cell-derived exosomes on T-cell function, and TNBC cell growth, and evaluate the therapeutic outcomes of exosomal miRNA on PD-1 resistance. cirmiR-20a-5p was significantly enriched in the serum of TNBC patients and the culture medium of TNBC cells. Further study showed that cirmiR-20a-5p released by TNBC cells via exosomes inhibited T-cell function and contributed to PD-1 treatment resistance.

2 | METHODS

2.1 | Cell lines and reagents

TNBC cell lines SKBR3, MDA-MB-231, HCC-1937, BT-20, and normal MCF10A cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). McCoy's 5A or RPMI-1640 medium supplemented with 10% fetal bovine serum was used for the culture of these TNBC cells at 37°C with 5% CO_2. I-miR-20a-5p mimics are small, chemically synthesized double-stranded RNAs that mimic a high level of mature miR-20a-5p and enable miRNA functioIHsa-miR-20a-5p antagomir is a chemically modified oligonucleotide that hybridizes with mature miR-20a-5p, which strongly competes with miR-20a-5p to prevent its complementary pairing with the target genes, thereby inhibiting miRNAs from functioning. Both miR-20a-5p mimics and antagomir were obtained from Ribobio and transfected into the cells using lipofectamine 3000 reagent.

2.2 | Clinical samples

Fifty TNBC tissue specimens, fresh plasma samples from healthy donors and TNBC patients, were collected at the Shanxi Province Cancer Hospital. Samples were maintained in liquid nitrogen before use. $CD8^+$ T cells infiltrated in TNBC tissues were detected by immunohistochemistry (IHC) staining with antibody specifically targeting CD8 (MA1-145, 1:50 dilution; ThermoFisher Scientific). The number of $CD8^+$ cells in a randomly selected five fields was counted. The ethical committee of Shanxi Province Cancer Hospital approved this study. Informed consent was obtained from all participants.

2.3 | RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

RNA was extracted from tissues, plasma or exosomes with Trizol solution (Beyotime). Reverse transcription was performed with the PrimeScript RT Kit (Takara) following the protocol. Quantification of miRNA and nuclear protein ataxia-telangiectasia (NPAT) was determined using the SYBR Green Master Mix (BioRad). U6 RNA (U6) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected as the normalization control for miRNA or mRNA levels, respectively. The primer sequences were as follows: miR-20a-5p forward, 5′-GCCCGCTAAAGTGCTTATAGTG-3′, reverse, 5′-CCAGTGCAGGGTCCGAGGT-3′; NPAT forward, 5′-GCATG CAAAGTTCCCCAAGG-3′, and reverse, 5′-TGGCCACTTGGTCG AGTAAC-3′.

2.4 | Western blot

Cells were lysed with NP-40 buffer (Yeason) in the presence of protease inhibitor. Proteins were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (15%) and transferred onto nitrocellulose membranes (Millipore) using a semidry blotter (BioRad). After blocking with 5% non-fat milk, primary antibodies against NPAT (ab99436; Abcam), CD63 (Santa), tris buffered saline with tween (TST101) (Q99816, Solarbio;) or GAPDH were applied at 4°C overnight. After TBST washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature on the shaker. The signals were visualized using an enhanced chemiluminescence kit.

2.5 | Exosome isolation

Exosomes were extracted from serum or cell culture medium via gradient centrifugation. Briefly, the plasma or medium was centrifugated at 4000×*g* to remove the cells or debris. Supernatants were collected and centrifugated at 12,000×*g* to exclude the components larger than exosomes. Exosomes were purified by centrifugating the supernatants at 120,000×*g* for 2 h at 4°C. Exosomes were suspended in phosphate buffered saline (PBS) and stored at −80°C before further analysis.

2.6 | $CD8^+$ T-cell isolation and incubation with exosomes

Isolation of $CD8^+$ T cells from human peripheral blood mononuclear cells (PBMCs) was performed using the Easy-Sep™ $CD8^+$ T cell Isolation Kit (Stemcell Technologies). Exosomes derived from TNBC cells were added into a 12-well plate and co-cultured with CD3/28 bead (MBS-C001; AcroBiosystems) preactivated $CD8^+$ T cells. Further analysis was performed after incubation for 24 h.

2.7 | T-cell-mediated tumor killing assay

To analyze the killing of TNBC cells, 1×10^4 MDA-MB-231 cells were seeded into 96-well plates. The next day, exosomes from human breast cancer cell line MDA-MB-231 (MDA-MB-231) and 5000 T cells that were pre-activated with 100 ng/mL CD3 antibody and 10 ng/mL IL-2 were added to the wells. After co-culturing for 5 days, Cell-Titer Glo reagent was added into the plates and incubated for 10 min at room temperature. Luminescence units were measured using MD SpectraMax i3.

2.8 | ELISA

The levels of tumor necrosis factor-alpha (TNF-α), interferon gamma (IFNg), granzyme B and perforin released by $CD8^+$ T cells were determined by enzyme-linked immunosorbent assay (ELISA) kit (#Ab285312, 174,443, 235,635, 46,048) following the protocol.

2.9 | miRNA target screen and luciferase reporter assay

The possible binding targets of miR-20a-5p were screened using the miRNA targets prediction database (miRDB) website (https://mirdb.org). The wild-type or mutated 3′-UTR sequences of NPAT1 containing the putative binding site of miR-20a-5p were constructed into the p-MIR luciferase reporter vector (p-MIR). $CD8^+$ T cells were co-transfected with p-MIR-NPAT and miR-20a-5p using lipofectamine 3000.

2.10 | In vivo tumor growth

NOD/SCID gamma (NSG) mice (7–8 weeks) were provided by Charles River Laboratories and maintained in a ventilated caging system with freely available food and sterile water. MDA-MB-231 cells were transfected with the lentivirus expressing miR-20a-5p or control vector and stable cells were obtained via G-418 screening. After acclimatized for 1 week, mice were injected intravenously with 0.5 million human PBMCs to reconstitute the immune system. Then, 5×10^6 TNBC cells with stably expressed miR-20-5p mimics or antagomir were subcutaneously injected into the right flanks. Mice were randomly grouped when tumor volume reached to around 200 mm^3 and treated with isotype immunoglobulin G (IgG) or anti-PD-1 monoclonal antibody (500 μg) intraperitoneally twice a week. During this period, tumor volume (*V*) was measured every 3 days and calculated following the for½a: $1/2\times ab^2$ (where *a* and *b* are the maximum lengths of the minor axis and major axis, respectively). At the end of the assay, exosomal miR-20a-5p levels in the plasma were analyzed via collecting the blood from the tail vein. Mice carrying tumor volume up to 2000 mm^3 or body weight loss more than 20% were euthanized humanely. The experiments were approved by the Ethics Committee of Shanxi Province Cancer Hospital.

2.11 | Statistical analysis

All results were presented as the mean ± SD and analyzed using SPSS software. Analysis of two or more groups was performed using S'udent's *t* test or one-way ANOVA. The survival curve is presented as the Kaplan–Meier plotter and analyzed via the log-rank Test. *p* value less than 0.05 was statistically significant.

3 | RESULTS

3.1 | Expression of circulating miR-20a-5p is increased in plasma and predicts poor prognosis

Increasing abundance of miR-20a-5p has been found in the malignancy of multiple cancers, including TNBC. To determine the correlation between tissue-expressed miR-20a-5p and circulating miR-20a-5p, cirmiR-20a-5p levels in the plasma of TNBC patients were examined. As indicated in Figure 1A, a scatter plot analysis showed a positive correlation between expression of TNBC-derived miR-20a-5p and plasma cirmiR-20a-5p from TNBC patients before therapeutic treatment. To further verify the upregulated cirmiR-20-5p in TNBC, plasma was obtained from healthy donors, TNBC patients or patients with relapsed TNBC. The results showed that the plasma cirmiR-20a-5p expression in TNBC and relapsed TNBC patients was significantly higher compared with that derived from healthy donors (Figure 1B). These data indicated the presence of cirmiR-20a-5p in TNBC and its possible correlation with TNBC progression. Additionally, miR-20a-5p was significantly enriched in the culture medium of TNBC cells in contrast with that of the normal MCF10A cells (Figure 1C). A patient-derived xenograft (PDX) model was established by transferring tumor tissues from TNBC patients into an immune system-deficient NSG mouse. The plasma cirmiR-20a-5p level in the peripheral blood was detected. As indicated, plasma cirmiR-20a-5p was significantly increased in PDX models compared with the normal group without tumors, suggesting that cirmiR-20a-5p was secreted by TNBC tissues.

To determine the clinical value of cirmiR-20a-5p in TNBC, patients were divided into cirmiR-20a-5p-high or -low groups with the median cirmiR-20a-5p expression value as the cut-off. A high level of plasma miR-20a-5p was significantly correlated with a poorer prognosis of TNBC patients (Figure 1E). These results indicate that cirmiR-20a-5p is highly enriched and might participate in TNBC progression.

3.2 | miR-20a-5p is released by TNBC cells in the form of exosomes

Increasing evidence has reported that miRNA is released from cancer cells via exosomes.[25] To test whether cirmiR-20a-5p was secreted from TNBC cells through exosomes, the exosomes were isolated from the culture medium of MDA-MB-231 cells by sequential centrifugation. As shown in Figure 2A, exosomes from TNBC cells showed a typical cup-shape morphology with a 100-nm diameter. The specific markers of exosomes including TSG101 and CD63 were confirmed by western blot (Figure 2B). In addition, exosomes from MCF10A and TNBC cells were isolated and miR-20a-5p levels in these cell-derived exosomes were determined. The data showed

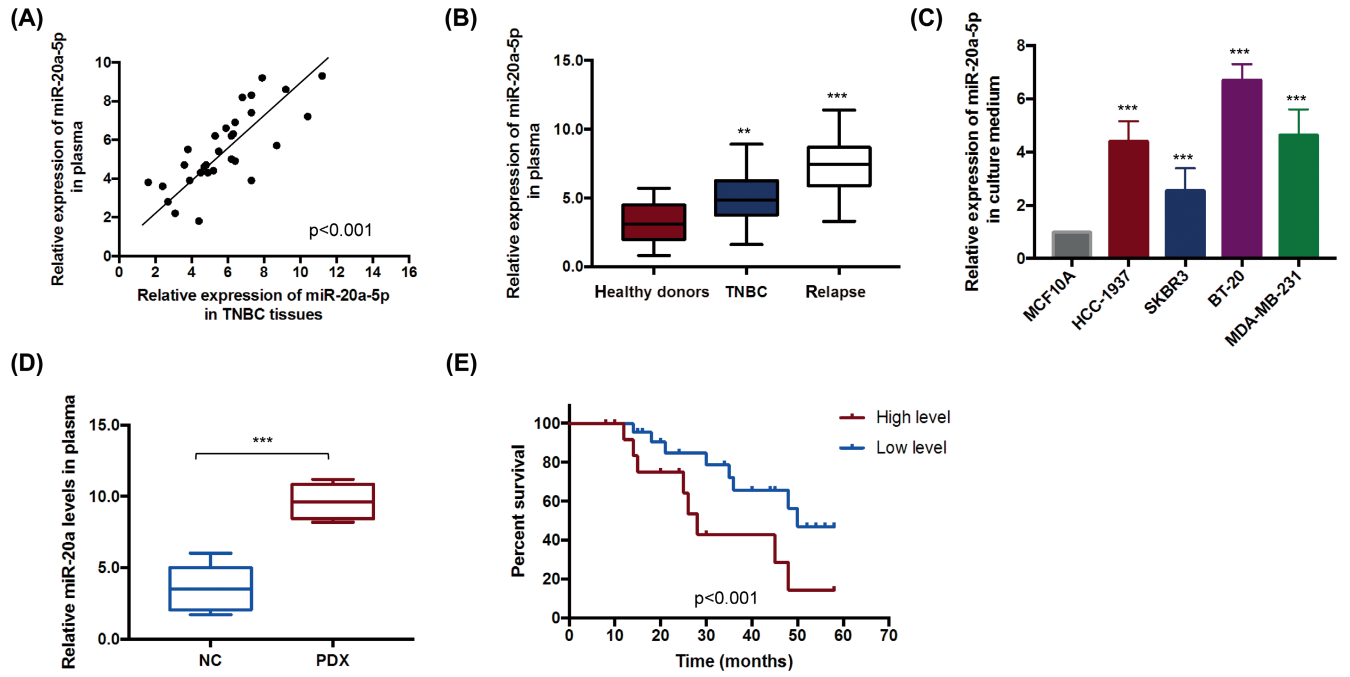

FIGURE 1 cirmiR-20a-5p is increased in TNBC. (A) The levels of cirmiR-20a-5p in plasma derived from TNBC patients and miR-20a-5p in TNBC solid tissues were detected by RT-qPCR. The correlation of cirmiR-20a-5p and tissue residual miR-20a-5p was analyzed. (B) cirmiR-20a-5p abundance in the plasma of healthy donors, TNBC or patients with relapsed TNBC was determined and a higher level of cirmiR-20a-5p was found in advanced TNBC. (C) The culture medium of normal MCF10A and TNBC cells were collected. The cirmiR-20a-5p level was significantly increased by the malignant TNBC cell lines. (D) The PDX model was established by implanting TNBC tissues into NSG mouse. The plasma expression of cirmiR-20a-5p was obviously higher in PDX mice than those without tumors. (E) TNBC patients were divided into cirmiR-20a-5p high or low groups based on the median expression of cirmiR-20a-5p. High cirmiR-20a-5p was significantly correlated with the lower survival rate of TNBC.

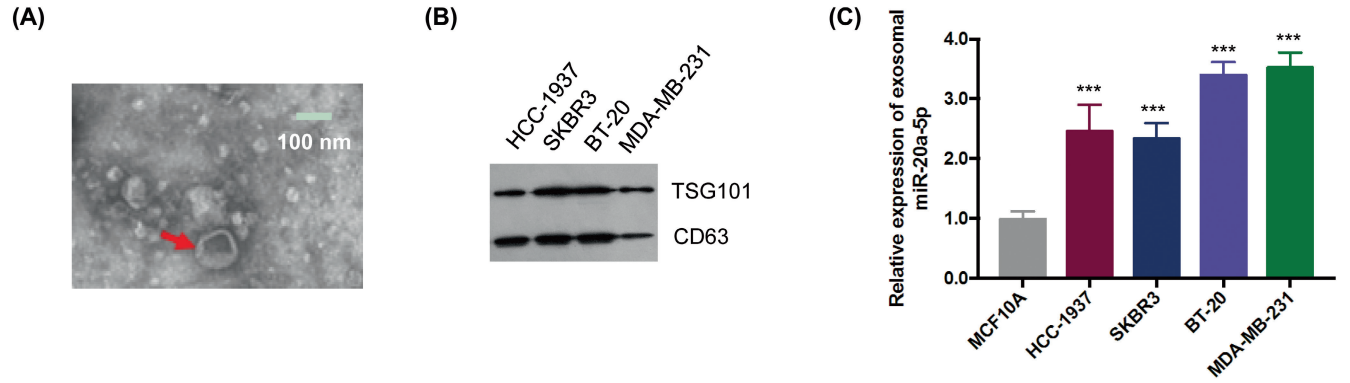

FIGURE 2 cirmiR-20a-5p is released by TNBC cells in the form of exosomes. (A) The exosomes detected by the transmission electron microscopy. (B) The expression of exosome markers CD63 and TSG101 in purified exosomes were detected by western blot. (C) Exosomes were extracted from the normal cell line MCF10A and TNBC cells. The cirmiR-20a-5p in the exosomes were detected by RT-qPCR. Enhanced exosomal cirmiR-20a-5p was found in TNBC cell lines.

a significant higher abundance of miR-20a-5p in the exosomes from TNBC cells compared with that of the normal cells MCF10A (Figure 2C), suggesting that cirmiR-20a-5p was released by TNBC cells in the form of exosomes.

3.3 | TNBC-derived exosomal miR-20a-5p inhibits CD8$^+$ T-cell function

Increasing studies reported the regulation of circulating RNA on cancer immunotherapy. To explore the relationship of cirmiR-20a-5p and T-cell function, plasma cirmiR-20a-5p and TNBC-infiltrated CD8$^+$ T cells were explored. The data suggested a significantly negative correlation between cirmiR-20a-5p and the infiltration of CD8$^+$ T cells in TNBC tissues (Figure 3A). To determine the function of exosomal miR-20a-5p in immune response, exosomes derived from TNBC cell lines and normal MCF10A cells were isolated and incubated with CD8$^+$ T cells, which were first activated by anti-CD3/CD28 antibody. CD8$^+$ T cells incubated with the exosomes derived from TNBC cells produced significantly lower levels of IFNg, TNF-α, perforin and granzyme B than those incubated with the exosomes from MCF10A (Figure 3B). These data demonstrate that TNBC cell-derived exosomes inhibited the function of CD8$^+$ T cells.

Because the components of exosomes are complicated, to determine whether miR-20a-5p mediated the inhibitory effects of exosomes on CD8$^+$ T cells, antagomir of miR-20a-5p was transfected into TNBC cells to deplete the expression of miR-20a-5p. Exosomes were isolated from these cells and the exosomal miR-20a-5p expression level was significantly decreased with miR-20a-5p antagomir (Figure 3C). Exosomes derived from MCF10A or TNBC cells with depleted miR-20a-5p were incubated with CD8$^+$ T cells. The cytokine levels released by CD8$^+$ T cells were significantly decreased with exosomes from TNBC cells, while increased cytokine levels were observed with the addition of miR-20a-5p-deficient exosomes (Figure 3D,E). The T-cell-mediated killing assay indicated that exosomal miR-20a-5p attenuated the cytotoxicity of T cells against TNBC cells (Figure 3F). These results indicate that TNBC cell-released exosomal miR-20a-5p inhibited CD8$^+$ T-cell function.

3.4 | miR-20a-5p targets and inhibits NPAT in CD8$^+$ T cells

To understand how miR-20a-5p affected CD8$^+$ T cells function, the possible binding targets of miR-20a-5p were predicted with the miRDB database, and NPAT was chosen for further analysis because of its role in regulating T cells.[26] NPAT is a highly expressed cell cycling gene in immature CD8$^+$ T cells that is essential for rapid cell proliferation.[26] According to the prediction, miR-20a-5p bound the 3′-UTR of NPAT (Figure 4A). To further verify this prediction, miR-20a-5p mimics were transfected into MDA-MB-231 and BT-20 cells (Figure 4B). As indicated, the luciferase activity of TNBC cells expressing the wild-type but not mutated 3′-UTR of NPAT was significantly decreased with miR-20a-5p overexpression (Figure 4C,D). In addition, this assay was repeated by incubating T cells with TNBC-derived exosomes. The results suggest that the luciferase activity of T cells carrying wild-type luciferase reporter vector was reduced when treated with exosomes from TNBC cells (Figure 4E). No inhibition was observed with the mutated 3′-UTR of NPAT, which did not contain the binding position of miR-20a-5p (Figure 4E). Moreover, the sharp decrease in luciferase activity was lost when miR-20a-5p was downregulated in the exosomes (Figure 4E). To detect whether the binding of miR-20a-5p affected the mRNA level of NPAT, CD8$^+$ T cells were co-cultured with the exosomes secreted from TNBC cells expressing control vector or miR-20a-5p mimics. No obvious changes were found for NPAT mRNA expression levels between all groups (Figure 4F). In contrast, NPAT protein abundance was decreased when treated with exosomes from TNBC cells and recovered with downregulation of miR-20a-5p (Figure 4G). These results suggest that exosomal miR-20a-5p may target and suppress NPAT in CD8$^+$ T cells.

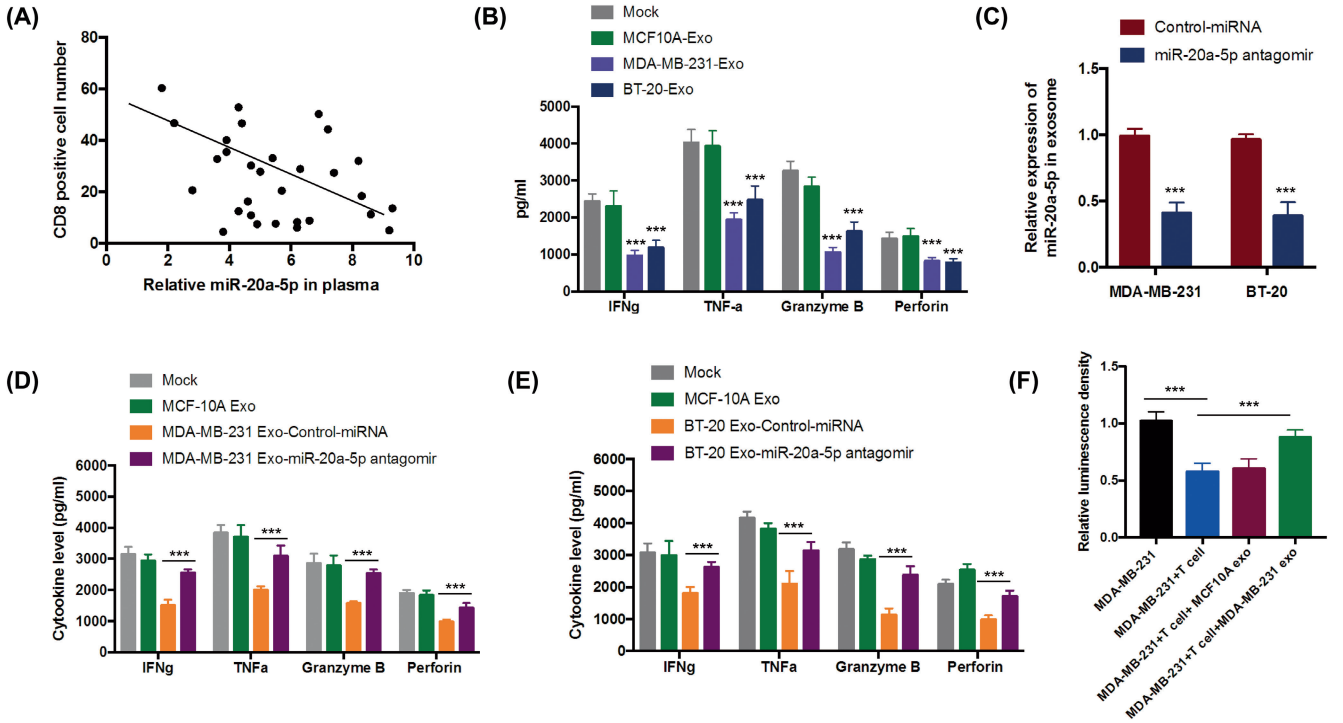

FIGURE 3 TNBC-derived exosomal miR-20a-5p inhibits the function of $CD8^+$ T cells. (A) The relationship of plasma cirmiR-20a-5p and $CD8^+$ T cells in TNBC tissues was determined. (B) $CD8^+$ T cells were co-cultured with exosomes isolated from MCF10A or TNBC cell line MDA-MB-231 and BT-20 cells. The secretion of IFNg, TNF-α, granzyme B and perforin by $CD8^+$ T cells was detected by ELISA. (C) MDA-MB-231 and BT-20 cells were transfected with control vector or miR-20a-5p antagomir, and the expression of cirmiR-20a-5p in exosomes released by these cells was determined. (D, E) $CD8^+$ T cells were co-cultured with exosomes derived from MCF10A or TNBC cells expressing control miRNA or depleted miR-20a-5p. The cytokine levels were assessed after treatment. (F) The T-cell mediated killing of TNBC cells was determined by adding the exosomes in the co-culture assay.

3.5 | Tumor-derived exosomal miR-20a-5p inhibits tumor growth and confers anti-PD-1 treatment resistance

To further explore the role of cirmiR-20a-5p in the immunotherapy of TNBC, NSG mouse was received human peripheral blood mononuclear cells (hPBMCs) to reconstruct the humanized immune system. MDA-MB-231 cells stably expressing miR-20a-5p or scramble vector were implanted into the right flank of the mouse. Exosomal cirmiR-20a-5p in the peripheral blood was significantly higher in mice bearing tumors with overexpressed miR-20a-5p than the control mice (Figure 5A). This result indicated that cirmiR-20a-5p can be secreted by TNBC cells and presented in the peripheral blood. Considering the regulation of cirmiR-20a-5p on $CD8^+$ T cells, the enrichment of $CD8^+$ T cells in tumors was detected by fluorescence activating cell sorter (FACS) analysis. More $CD8^+$ T cells were found in the mock cell-derived tumor tissues than cirmiR-20a-5p overexpressed tissues (Figure 5B). Reduced expression of MHC-I class molecules, including HLA-A, HLA-B, and HLA-C, was also found in tumors with overexpressed miR-20a-5p (Figure S1A). Moreover, tumors with highly expressed miR-20a-5p showed obviously decreased NPAT levels (Figure 5C).

The promising therapeutic efficacy of PD-1 monoclonal antibody is emerging in TNBC-related clinical trials. To further determine whether cirmiR-20a-5p affected anti-PD-1 therapy, mice bearing cirmiR-20a-5p overexpressed MDA-MB-231 cells or mock cells were administrated with Opdivo. The xenograft mice with a high level of cirmiR-20a-5p showed resistance to anti-PD-1 treatment (Figure 5D). Consistently, exosomal miR-20a-5p in PD-1 therapy-resistant TNBC patients was determined. The results showed that compared to patients that were sensitive to anti-PD-1 antibodies, those with PD-1 resistance showed relative higher levels of exosomal miR-20a-5p (Figure 5E). To further demonstrate the involvement of miR-20a-5p in PD-1 therapy of TNBC, mice were implanted with miR-20a-5p down-regulated MDA-MB-231 cells, which were then administrated with Opdivo. As shown in Figure S1B, depletion of miR-20a-5p further inhibited the tumor growth compared to the group with anti-PD-1 antibody alone. Tumors with miR-20a-5p antagomir showed significantly reduced exosomal miR-20a-5p (Figure S1C). These findings suggest the potential involvement of cirmiR-20a-5p in anti-PD-1 therapy resistance of TNBC.

4 | DISCUSSION

TNBC, a global public health issue for females, remains the leading cause of cancer-associated mortality world-wide.[3,4] Due to the lack of specific targets and limited therapeutic options, how to improve

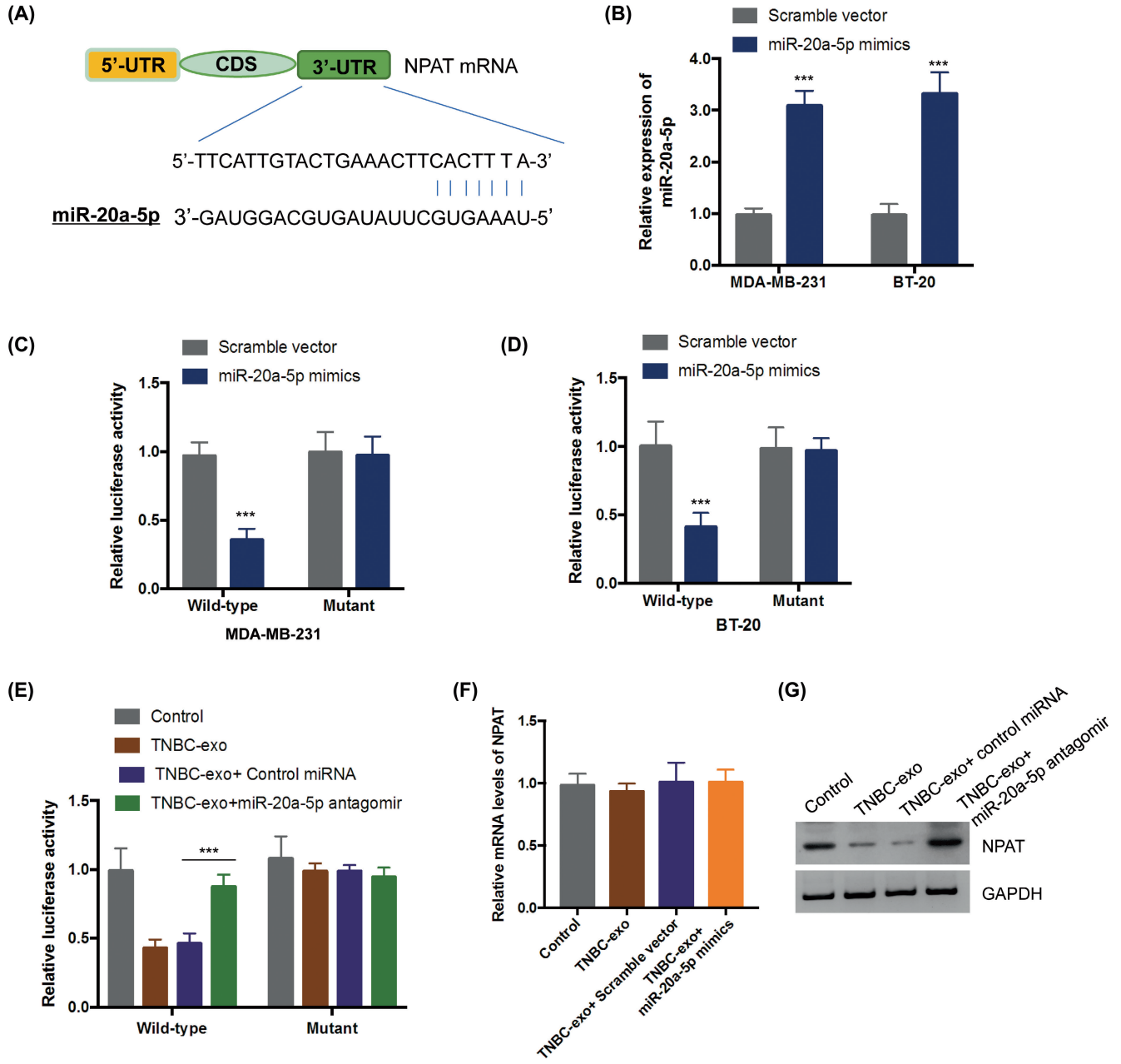

FIGURE 4 miR-20a-5p targets and inhibits NPAT in CD8$^+$ T cells. (A) Predicted binding site between miR-20a-5p and the 3′-UTR of NPAT. (B) Cells were transfected with scramble vector or miR-20a-5p mimics, and the overexpression of miR-20a-5p was validated by RT-qPCR. (C, D) TNBC cells were co-transfected with luciferase reporter plasmid containing wild-type (WT) or mutated 3′-UTR of NPAT and miR-20a-5p mimics. The relative luciferase activity was determined by normalizing to that of *Renila*. (E) CD8$^+$ T cells were transfected with luciferase reporter vector expressing WT or mutated 3′-UTR of NPAT. T cells were incubated with the exosomes from MCF10A, TNBC cells, or TNBC cells with down-regulated miR-20a-5p followed by luciferase activity analysis. (F, G) Relative mRNA (F) or protein (G) levels of NPAT in CD8$^+$ T cells that co-cultured with TNBC-derived exosomes.

the response of patients to the current therapies and overcome the therapeutic resistance is still a challenge. Our study found that exosomal miR-20a-5p could be released by TNBC cells and transferred to CD8$^+$ T cells to inhibit T-cell function by targeting NPAT, which consequently conferred anti-PD-1 therapy resistance. These findings suggest the vital role of TNBC-derived exosomal miR-20a-5p in promoting immune escape and immunotherapy resistance by inducing CD8$^+$ T-cell dysfunction.

cirmiRNAs are aberrantly expressed in cancers and involved in tumor progression and drug resistance.[27,28] However, the biological molecular function of cirmiRNAs in TNBC needs more investigation. In the current study, cirmiR-20a-5p, which is derived from miR-20a-5p, was highly enriched in TNBC-derived exosomes. Apart from TNBC, miR-20a-5p was reported to be highly expressed in other cancers, such as head and neck squamous cell carcinoma, ovary carcinoma, and hepatocellular carcinoma.[29] Similarly, expression of

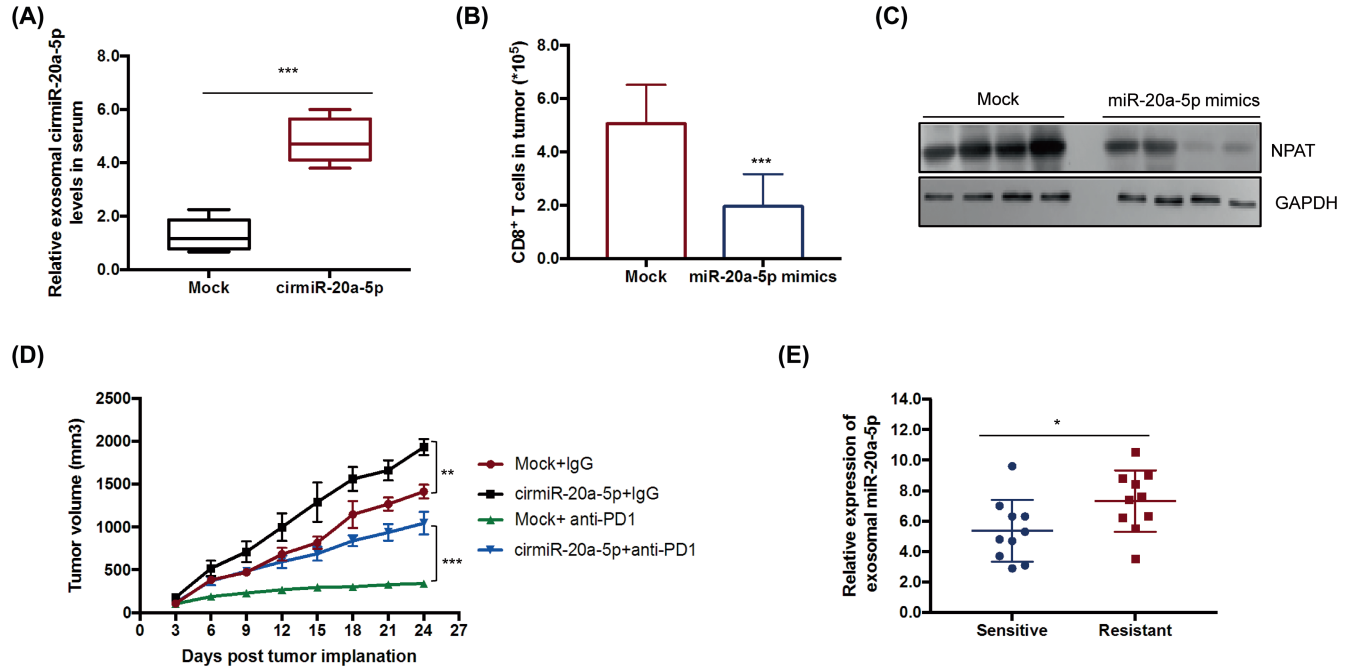

FIGURE 5 Tumor=derived exosomal miR-20a-5p inhibits tumor growth and confers anti-PD-1 treatment resistance. (A) MDA-MB-231 cells were transfected with lentivirus expressing control vector or miR-20a-5p mimics. Stable cell line was subcutaneously injected into the NSG mice to generate the tumor model. Relative levels of cirmiR-20a-5p in serum exosomes were detected by RT-qPCR. (B) $CD8^+$ T cells in tumors were analyzed by FACS at the end of the in vivo study. (C) The protein levels of NPAT in isolated tumors were detected by western blot. NPAT expression was decreased in tumor with overexpressed miR-20a-5p. (D) NSG mice were received hPBMC and MDA-MB-231 cells with stably expressed control vector or miR-20a-5p. Opdivo or IgG were administrated twice a week when tumor volume reached 200 mm^3. Tumor volume in each group was presented. (E) The expression level of exosomal miR-20a-5p in TNBC patients who were sensitive or resistant to PD-1 therapy was compared.

circulating miR-20a-5p was also found to be upregulated in smoldering myeloma and renal cell carcinoma.[29] In this study, a higher cirmiR-20a-5p level was also found in cervical cancer tissues than in adjacent non-cancerous tissues (Figure S2A). Compared with normal cell H8, expression of exosomal miR-20a-5p was significantly increased in cervical cancer cells (Figure S2B), therefore overexpression of cirmiR-20a-5p is not a unique characteristic of TNBC, which also suggests the critical involvement of cirmiR-20a-5p in cancer development.

Increased exosomal miR-20a-5p was significantly correlated with poor survival of TNBC patients, suggesting cirmiR-20a-5p as a possible biomarker for TNBC prognosis. Recent studies uncovered that tumor cell-derived exosomes could be uptaken by the tumor-infiltrated $CD8^+$ T cells, which consequently reduced the cytotoxicity of $CD8^+$ T and resulted into immune escape.[30] Exploring the immune-regulating function of tumor secreted exosomal miRNAs is therefore critical for understanding resistance to immune therapy. In the present study, exosome-delivered cirmiR-20a-5p inhibited $CD8^+$ T-cell activation. TNBC cells with high levels of exosomal miR-20a-5p showed resistance to anti-PD-1 treatment in the human immune system reconstituted mouse model. These results provide novel insights about the regulatory function of exosomal miR-20a-5p in $CD8^+$ T cells and the potential immune therapy response of TNBC patients.

The discovery of exlsome in"the 'iological fluids of cancer patients indicated the potential clinical significance of exosome as a diagnostic readout. In principle, exosomes are extracellular vehicles and secreted by various cells that can transfer functional molecules including proteins, lipids, and non-coding RNAs between donor and recipient cells.[31] The delivery of cellular components by exosomes changes the fate of neighboring cells, suggesting the possibility of exosome-based target therapy in cancer treatment. Immune therapies with antibodies targeting the checkpoints PD-(L) 1 have been the first-line standard treatment option for hot tumors owing to the high infiltration of cytotoxic lymphocytes.[32] Blocking PD-1 combined with chemotherapy has emerged promising efficacy for TNBC.[33] Although PD-L1 expression works as a potential biomarker for identifying patients who can benefit from anit-PD-1 therapy, other factors that may combine with this immune checkpoint therapy need further study. Our results illustrate that exosomal miR-20a-5p promotes tumor growth and confers resistance to PD-1 mAb therapy in the TNBC xenograft mouse model, suggesting that patients with higher levels of exosomal miR-20a-5p might respond poorly to anti-PD-1 treatment. Additionally, more clinical samples are needed to verify the correlation between cirmiR-20a-5p expression in peripheral blood and the clinic response of these patients to PD-1 mAb therapy.

In conclusion, exosomal cirmiR-20a-5p was upregulated in TNBC and correlated with the poor prognosis of TNBC patients.

cirmiR-20a-5p released via exosomes by TNBC cells was uptaken by $CD8^+$ T cells and led to the dysfunction of $CD8^+$ T by targeting NPAT. Increased cirmiR-20a-5p enhanced the resistance of TNBC to anti-PD-1 therapy. These results suggest that targeting cirmiR-20a-5p may be a novel strategy for improving the immunotherapy efficacy of TNBC patients.

AUTHOR CONTRIBUTIONS

Weina Li: Data curation; investigation; methodology; validation; writing – original draft; writing – review and editing. **Guohui Han:** Data curation; investigation; methodology; validation; writing – original draft; writing – review and editing. **Feng Li:** Methodology; writing – review and editing. **Peng Bu:** Validation; writing – original draft; writing – review and editing. **Yating Hao:** Data curation; methodology; writing – original draft; writing – review and editing. **Li Huang:** Visualization; writing – original draft; writing – review and editing. **Xiangdong Bai:** Funding acquisition; investigation; project administration; writing – original draft; writing – review and editing.

ACKNOWLEDGMENTS

This work was supported by the Natural Science Foundation of Shanxi Province (20210302123340, 202103021224431) and the Shanxi Provincial Health Commission (2020060).

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available from the corresponding author.

ETHICS STATEMENT

Approval of the research protocol by an institutional reviewer board: This study was approved by the Ethics Committee of Shanxi Province Cancer Hospital.

Informed consent: Informed consents were provided by all the participants.

Registry and Registration: N/A.

Animal studies: The animal study was approved by the Ethics Committee of Shanxi Province Cancer Hospital.

ORCID

Feng Li https://orcid.org/0000-0002-7935-0892
Xiangdong Bai https://orcid.org/0000-0002-0980-2515

REFERENCES

1. Matsen CB, Neumayer LA. Breast cancer: a review for the general surgeon. *JAMA Surg*. 2013;148:971-979. doi:10.1001/jamasurg.2013.3393
2. Katsura C, Ogunmwonyi I, Kankam HK, Saha S. Breast cancer: presentation, investigation and management. *Br J Hosp Med (Lond)*. 2022;83:1-7. doi:10.12968/hmed.2021.0459
3. Elias AD. Triple-negative breast cancer: a short review. *Am J Clin Oncol*. 2010;33:637-645. doi:10.1097/COC.0b013e3181b8afcf
4. Ismail-Khan R, Bui MM. A review of triple-negative breast cancer. *Cancer Control*. 2010;17:173-176. doi:10.1177/107327481001700305
5. Jamdade VS, Sethi N, Mundhe NA, Kumar P, Lahkar M, Sinha N. Therapeutic targets of triple-negative breast cancer: a review. *Br J Pharmacol*. 2015;172:4228-4237. doi:10.1111/bph.13211
6. Loizides S, Constantinidou A. Triple negative breast cancer: immunogenicity, tumor microenvironment, and immunotherapy. *Front Genet*. 2022;13:1095839. doi:10.3389/fgene.2022.1095839
7. Geurts V, Kok M. Immunotherapy for metastatic triple negative breast cancer: current paradigm and future approaches. *Curr Treat Options Oncol*. 2023;24:628-643. doi:10.1007/s11864-023-01069-0
8. Morganti S, Tolaney SM. Role of immunotherapy in early- and late-stage triple-negative breast cancer. *Hematol Oncol Clin North Am*. 2023;37:133-150. doi:10.1016/j.hoc.2022.08.014
9. Nederlof I, Voorwerk L, Kok M. Facts and hopes in immunotherapy for early-stage triple-negative breast cancer. *Clin Cancer Res*. 2023;29:2362-2370. doi:10.1158/1078-0432.CCR-22-0701
10. Haratake N, Toyokawa G, Tagawa T, et al. Positive conversion of PD-L1 expression after treatments with chemotherapy and nivolumab. *Anticancer Res*. 2017;37:5713-5717. doi:10.21873/anticanres.12009
11. Wakita A, Motoyama S, Nanjo H, et al. PD-L1 expression is a prognostic factor in patients with thoracic esophageal cancer treated without adjuvant chemotherapy. *Anticancer Res*. 2017;37:1433-1441. doi:10.21873/anticanres.11467
12. Rojko L, Reiniger L, Téglási V, et al. Chemotherapy treatment is associated with altered PD-L1 expression in lung cancer patients. *J Cancer Res Clin Oncol*. 2018;144:1219-1226. doi:10.1007/s00432-018-2642-4
13. Ivan M. The ongoing microRNA revolution and its impact in biology and medicine. *J Cell Mol Med*. 2008;12:1425. doi:10.1111/j.1582-4934.2008.00419.x
14. Wilbert ML, Yeo GW. Genome-wide approaches in the study of microRNA biology. *Wiley Interdiscip Rev Syst Biol Med*. 2011;3:491-512. doi:10.1002/wsbm.128
15. Xiao Y, MacRae IJ. Toward a comprehensive view of MicroRNA biology. *Mol Cell*. 2019;75:666-668. doi:10.1016/j.molcel.2019.08.001
16. McGinnis LK, Luense LJ, Christenson LK. MicroRNA in ovarian biology and disease. *Cold Spring Harb Perspect Med*. 2015;5:a022962. doi:10.1101/cshperspect.a022962
17. Tiwari A, Mukherjee B, Dixit M. MicroRNA key to angiogenesis regulation: MiRNA biology and therapy. *Curr Cancer Drug Targets*. 2018;18:266-277. doi:10.2174/1568009617666170630142725
18. Matsuyama H, Suzuki HI. Systems and synthetic microRNA biology: from biogenesis to disease pathogenesis. *Int J Mol Sci*. 2019;21:132. doi:10.3390/ijms21010132
19. Zhang Y, Bi J, Huang J, Tang Y, Du S, Li P. Exosome: a review of its classification, isolation techniques, storage, diagnostic and targeted therapy applications. *Int J Nanomedicine*. 2020;15:6917-6934. doi:10.2147/IJN.S264498
20. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science*. 2020;367:eaau6977. doi:10.1126/science.aau6977
21. Zhang J, Li S, Li L, et al. Exosome and exosomal microRNA: trafficking, sorting, and function. *Genomics Proteomics Bioinformatics*. 2015;13:17-24. doi:10.1016/j.gpb.2015.02.001
22. Li B, Cao Y, Sun M, Feng H. Expression, regulation, and function of exosome-derived miRNAs in cancer progression and therapy. *FASEB J*. 2021;35:e21916. doi:10.1096/fj.202100294RR
23. Agarwal A, Fanelli G, Letizia M, et al. Regulatory T cell-derived exosomes: possible therapeutic and diagnostic tools in transplantation. *Front Immunol*. 2014;5:555. doi:10.3389/fimmu.2014.00555
24. Bai X, Han G, Liu Y, Jiang H, He Q. MiRNA-20a-5p promotes the growth of triple-negative breast cancer cells through targeting

RUNX3. *Biomed Pharmacother*. 2018;103:1482-1489. doi:10.1016/j.biopha.2018.04.165
25. Sun Z, Shi K, Yang S, et al. Effect of exosomal miRNA on cancer biology and clinical applications. *Mol Cancer*. 2018;17:147. doi:10.1186/s12943-018-0897-7
26. Cui Z, Zhao F, Chen X, et al. NPAT supports CD8(+) immature single-positive thymocyte proliferation and thymic development. *J Immunol*. 2022;209:916-925. doi:10.4049/jimmunol.2200214
27. Cheng G. Circulating miRNAs: roles in cancer diagnosis, prognosis and therapy. *Adv Drug Deliv Rev*. 2015;81:75-93. doi:10.1016/j.addr.2014.09.001
28. Zhu L, Zhao L, Wang Q, et al. Circulating exosomal miRNAs and cancer early diagnosis. *Clin Transl Oncol*. 2022;24:393-406. doi:10.1007/s12094-021-02706-6
29. Huang W, Wu X, Xiang S, et al. Regulatory mechanism of miR-20a-5p expression in cancer. *Cell Death Discov*. 2022;8:262. doi:10.1038/s41420-022-01005-5
30. Chen G, Huang AC, Zhang W, et al. Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. *Nature*. 2018;560:382-386. doi:10.1038/s41586-018-0392-8
31. He C, Zheng S, Luo Y, Wang B. Exosome theranostics: biology and translational medicine. *Theranostics*. 2018;8:237-255. doi:10.7150/thno.21945
32. Yi M, Zheng X, Niu M, Zhu S, Ge H, Wu K. Combination strategies with PD-1/PD-L1 blockade: current advances and future directions. *Mol Cancer*. 2022;21:28. doi:10.1186/s12943-021-01489-2
33. Emens LA. Breast cancer immunotherapy: facts and hopes. *Clin Cancer Res*. 2018;24:511-520. doi:10.1158/1078-0432.CCR-16-3001

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Li W, Han G, Li F, et al. Cancer cell-derived exosomal miR-20a-5p inhibits $CD8^+$ T-cell function and confers anti-programmed cell death 1 therapy resistance in triple-negative breast cancer. *Cancer Sci*. 2024;115:347-356. doi:10.1111/cas.16036