Molecular Therapy
Oncology
Original Article

TROP2 is highly expressed in triple-negative breast cancer CTCs and is a potential marker for epithelial mesenchymal CTCs

Qingyu Liao,[1,4] Ruiming Zhang,[1,4] Zuli Ou,[1,4] Yan Ye,[1] Qian Zeng,[1] Yange Wang,[1] Anqi Wang,[3] Tingmei Chen,[1] Chengsen Chai,[1] and Bianqin Guo[1,2]

[1]Key Laboratory of Clinical Laboratory Diagnostics (Ministry of Education), College of Laboratory Medicine, Chongqing Medical University, Chongqing 400016, China; [2]Department of Clinical Laboratory, Chongqing University Cancer Hospital, Chongqing 40030, China; [3]CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, CAS Key Laboratory of Standardization and Measurement for Nanotechnology, CAS Center for Excellence in Nanoscience, National Center for Nanoscience and Technology, Beijing100190, China

Circulating tumor cells (CTCs) are the seeds of distant metastases of malignant tumors and are associated with malignancy and risk of metastasis. However, tumor cells undergo epithelial-mesenchymal transition (EMT) during metastasis, leading to the emergence of different types of CTCs. Real-time dynamic molecular and functional typing of CTCs is necessary to precisely guide personalized treatment. Most CTC detection systems are based on epithelial markers that may fail to detect EMT CTCs. Therefore, it is clinically important to identify new markers of different CTC types. In this study, bioinformatics analysis and experimental assays showed that trophoblast cell surface antigen 2 (TROP2), a target molecule for advanced palliative treatment of triple-negative breast cancer (TNBC), was highly expressed in TNBC tissues and tumor cells. Furthermore, TROP2 can promote the migration and invasion of TNBC cells by upregulating EMT markers. The specificity and potential of TROP2 as an EMT-associated marker of TNBC CTCs were evaluated by flow cytometry, immunofluorescence, spiking experiments, and a well-established CTC assay. The results indicated that TROP2 is a potential novel CTC marker associated with EMT, providing a basis for more efficacious markers that encompass CTC heterogeneity in patients with TNBC.

INTRODUCTION

Breast cancer (BC) is the most common cancer in women and the second leading cause of cancer-related deaths after lung cancer. Triple-negative BC (TNBC) accounts for 15%–20% of all BC cases,[1] which is negative for immunohistochemistry (IHC)-based assessment of estrogen receptor (ER), progesterone receptor, and human epidermal growth factor receptor 2 (HER2) amplification. Due to the lack of effective targeted therapies, TNBC is the most aggressive subtype of BC, with early recurrence, a high incidence of visceral metastasis, and short survival.[2,3] Therefore, there is an urgent need for more rapid, effective, and less invasive methods for early diagnosis and monitoring.

Compared with tissue biopsy, liquid biopsy is a good tool for tumor diagnosis, response monitoring, and prognosis assessment. The advantages of liquid biopsy include noninvasive sampling and reproducibility.[4] Liquid biopsy involves the detection of circulating biomarkers such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and extracellular vesicles and circulating microRNAs.[5,6] CTCs are tumor cells that are shed from solid tumors and circulate in the peripheral blood; they carry genomic, transcriptomic, proteomic, and metabolomic information, which is important for oncology.[5,7] Detection of CTCs primarily involves three technical aspects: separation, enrichment, and identification. Conventional enrichment assays mainly rely on the epithelial cell adhesion molecule (EpCAM), such as the Food and Drug Administration–approved CellSearch system.[8]

Previous studies have showed that heterogeneous CTC populations, such as subgroups undergoing epithelial-mesenchymal transition (EMT) with low or undetectable levels of EpCAM, may be highly aggressive and invasive.[9,10] In general, enrichment of CTCs based on the EpCAM antibody alone makes it difficult to capture different types of CTCs.[11] Moreover, tumor heterogeneity leads to decreased detection sensitivity, which poses a challenge for detecting CTCs.[12] Because EMT and the acquisition of chemoresistance are associated with the loss of epithelial markers and features, research to identify different CTC subtypes and efficacy monitoring–related markers is ongoing.[13,14] It is important to discover novel markers to capture

Received 26 September 2023; accepted 5 January 2024;
https://doi.org/10.1016/j.omton.2024.200762.

[4]These authors contributed equally

Correspondence: Chengsen Chai, Key Laboratory of Clinical Laboratory Diagnostics (Ministry of Education), College of Laboratory Medicine, Chongqing Medical University, Chongqing 400016, China.
E-mail: chengsenchai@cqmu.edu.cn
Correspondence: Bianqin Guo, Key Laboratory of Clinical Laboratory Diagnostics (Ministry of Education), College of Laboratory Medicine, Chongqing Medical University, Chongqing 400016, China.
E-mail: guobianqin121@cqu.edu.cn

more types of CTC subgroups and to define alternative markers for more comprehensive and efficient CTC detection assays.

Bioinformatics analysis revealed that trophoblast cell surface antigen 2 (TROP2), a calcium-transducing transmembrane protein, is expressed at low levels in normal tissues and high levels in many malignant tumors, particularly TNBC.[15,16] TROP2 is involved in several oncogenic signaling pathways that lead to tumor development, invasion, and metastasis.[17] High expression of TROP2 in BC, especially TNBC, is associated with poor prognosis, making it a potential therapeutic target in cancer treatment.[15] Based on clinical trials data, an anti-TROP2 antibody-drug conjugate (ADC; sacituzumab govitecan; Trodelvy) has been approved to treat metastatic TNBC.[18] In some clinical trials (particularly BC), increased numbers of CTCs and CTCs with mesenchymal features have been associated with a higher disease stage, metastasis, treatment response, and poor clinical prognosis.[10,13,19,20] However, TROP2 reportedly enhances EMT and promotes tumor metastasis.

In this study, we aimed to investigate the correlation between TROP2 expression levels and the EMT-related biological behavior of BC cells. In addition, we explored the applicability and efficacy of TROP2 as a novel marker of CTCs in TNBC compared to classical EMT markers.

RESULTS

TROP2 is highly expressed in TNBC and is related to poor prognosis

The tumor antigen TROP2, encoded by the *TACSTD2* gene, is one of the most significantly differentially expressed proteins between normal and tumor tissues, based on the analysis of uniformly processed RNA-sequencing data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) in the RNAseqDB database (https://github.com/mskcc/RNAseqDB).[21] As shown in Figure 1A, the transcriptional expression of TROP2 from the *TACSTD2* gene was significantly higher in BC tissues compared to their adjacent normal breast tissues. Furthermore, the protein expression of TROP2 was higher in TNBC than in the other BC subtypes (Figure 1B). Subsequently, IHC results of BC tissue sections showed that TROP2 was significantly upregulated in TNBC and positively correlated with higher TNM staging (Figures 1C and 1D). In addition, the Kaplan-Meier plot showed that patients with TNBC with higher *TACSTD2* expression had a worse prognosis (Figure 1E). These data indicate that increased expression of TROP2 in TNBC is strongly associated with a worse prognosis in patients with TNBC.

TROP2 affects expression of tumor progression markers

Subsequently, ER + BC cell line MCF-7; $HER2^{+}$ BC cell line SK-BR3; TNBC cell lines MDA-MB231, MDA-MB453, MDA-MB468, and BT549 cells; and normal mammary epithelial cells MCF-10A were used to further verify TROP2 by qRT-PCR. The results were generally consistent with the database. Compared to MCF-10A, TROP2 was more highly expressed in MDA-MB468 and MDA-MB231 cells, but expression was lower in BT549 cells (Figure 2A). High expression of TROP2 has been shown to contribute to tumor proliferation and migration. To evaluate whether TROP2 affects the biological behavior of BC cells, TROP2 was overexpressed in BT549 cells and knocked down in MDA-MB231 cells using the corresponding lentivirus (Figure 2B). EMT and cell proliferation markers in these cells were examined by qRT-PCR. TROP2 overexpression resulted in increased mRNA expression of vimentin, cyclinD1, c-myc, and sox2. In contrast, TROP2 knockdown resulted in decreased mRNA expression of these markers (Figure 2C).

TROP2 promotes invasion by upregulating mesenchymal markers in TNBC cells

There is evidence that TROP2 promotes the invasion in gastric cancer.[22] To explore whether TROP2 has the same function in TNBC, TCGA database analysis of gene expression was performed to validate our hypothesis that the *TACSTD2* gene is significantly associated with EMT-related molecules or signaling pathways. TROP2 was positively correlated with EMT markers and the transforming growth factor β (TGF-β) EMT-related signaling pathways (Figure 3A). Western blotting results showed that overexpression or knockdown of TROP2 induced up- and downregulation of N-cadherin and vimentin, respectively (Figure 3B). Furthermore, wound healing assay results revealed that overexpression of TROP2 significantly promoted the migration of BT549 cells compared to the control. In contrast, knockdown of TROP2 by small hairpin RNA (shRNA) significantly attenuated the migration of MDA-MB231 cells (Figures 3C and 3D). Transwell assay results of the migration and invasion capacity of cells demonstrated that overexpression of TROP2 significantly increased the migration and invasion ability of BT549 cells. In contrast, the knockdown of TROP2 reduced the capacity of migration and invasion in MDA-MB231 cells (Figures 3E and 3F). The collective findings demonstrate that TROP2 promotes the invasion and migration of TNBC cells by upregulating EMT, suggesting that TROP2 can be used as an EMT-related marker in combination with other classical CTC assay markers to improve CTC detection rates.

Expression of TROP2 in TNBC cells and blood cells

TROP2 expression was examined in TNBC cell lines by qRT-PCR. Compared with MCF-10A cells, TROP2 expression was detected in BC cell lines, particularly in the TNBC cell lines MDA-MB468, MDA-MB231, and MDA-MB453 (Figure 2A), suggesting that TROP2 may be a suitable marker for the detection of BC cells. In addition, the TROP2-targeted ADC has been used for the treatment of some types of solid tumors. Sacituzumab govitecan, an ADC targeting TROP2, has recently been approved for the treatment of advanced palliative TNBC. TROP2 may, therefore, be a novel marker for the detection of CTCs in TNBC. In addition, TROP2 belongs to the same TACSTD gene family as the classical tumor marker EpCAM. To assess the specificity of TROP2 as a CTC marker, flow cytometry and cell immunofluorescence (IF) were used to identify TROP2 and EpCAM expression in TNBC cells and MCF-10A (Figures 4A and 4B). Their expression was then analyzed in peripheral blood mononuclear cells (PBMCs) from healthy controls by IF to determine their specificity as novel biomarkers for CTC (Figure 4C). Western blot

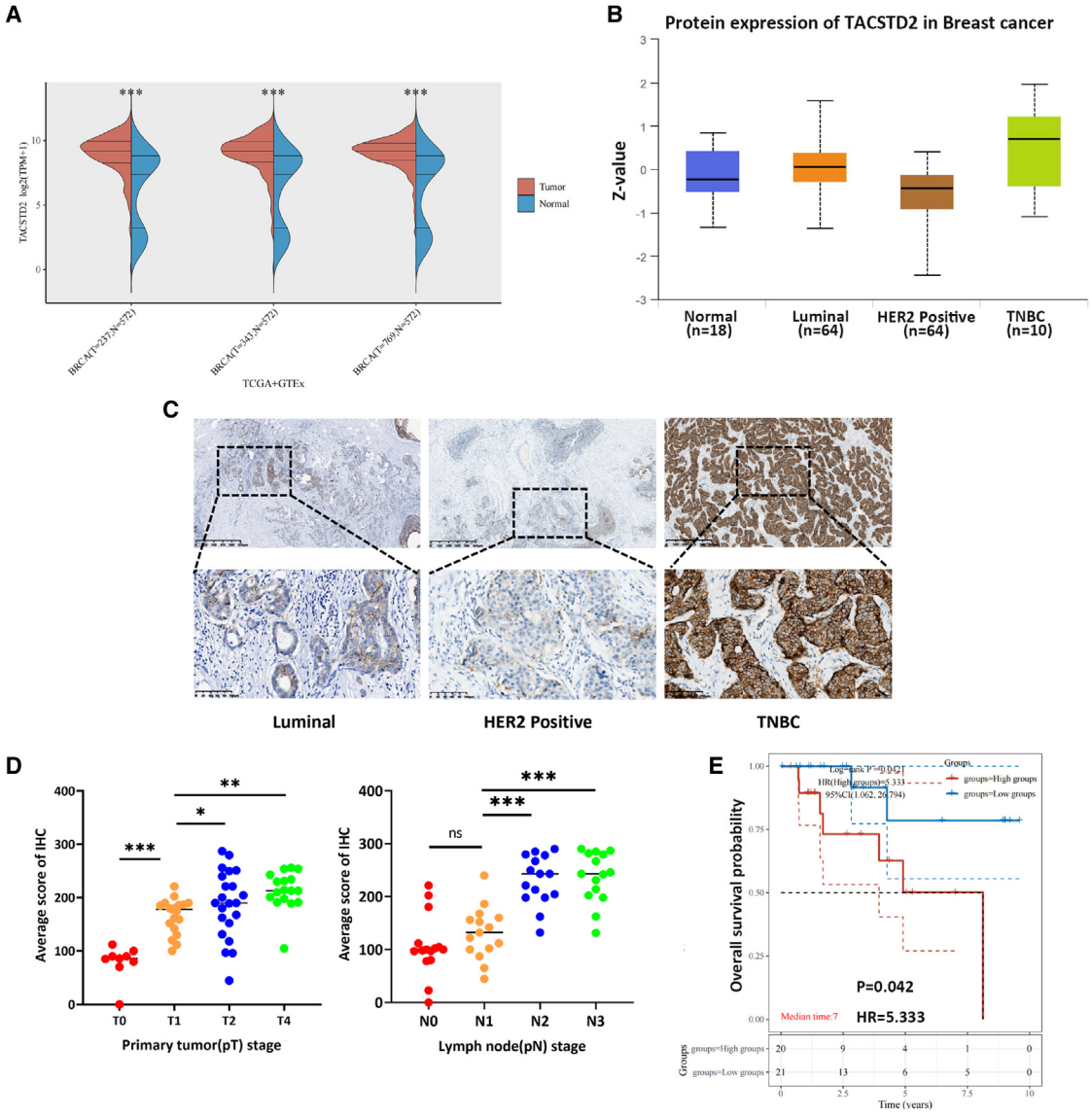


Figure 1. TROP2 is highly expressed in TNBC and is related to poor prognosis

(A) Analysis of the TCGA and GTEx databases revealed the high expression of TROP2 in BC tissues compared to normal tissues. (B) In the CPTAC database, TROP2 was expressed at higher levels in TNBC than in other BC types. (C) IHC staining of pathological tissue from clinical patients showed higher TROP2 expression in TNBC tissue than in luminal and HER2^{+} BC tissue. Longer scale bars, 625 μm; shorter scale bars, 100 μm. (D) TROP2 IHC score is correlated with higher TNM staging. (E) Kaplan-Meier survival analysis of TROP2 from TCGA dataset. The data suggest that high TROP2 expression is associated with poor prognosis in patients with TNBC. HR denotes the hazard ratio of the low-expression sample relative to the high expression sample. HR > 1 and <1 indicate that the gene is a risk factor and a protective factor, respectively. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

results also showed low or absent expression of TROP2 in MCF-10A cells and PBMCs (Figure S2).

Subsequently, the potential applicability of TROP2 for tumor cell capture and identification was then tested using a spiking assay. Defined numbers of three types of TNBC cells were added to 5 mL of healthy human peripheral whole blood to capture tumor cells by negative enrichment and identify them by IF. After negative enrichment of spiking assay, in the single-positive identification for creatine kinase (CK) antibody, the recovery of MDA-MB468, MDA-MB453, and MDA-MB231 cells was 19.3%, 29.6%, and 25.6%, respectively. In the double-positive test for CK and TROP2 antibodies, the recovery was 16.9%, 30.2%, and 25.3%, respectively (Figure 4D). In combination with the previously described high expression of TROP2 in TNBC and its ability to influence EMT, the findings suggest that TROP2 has the potential to be an adjunctive marker for identifying

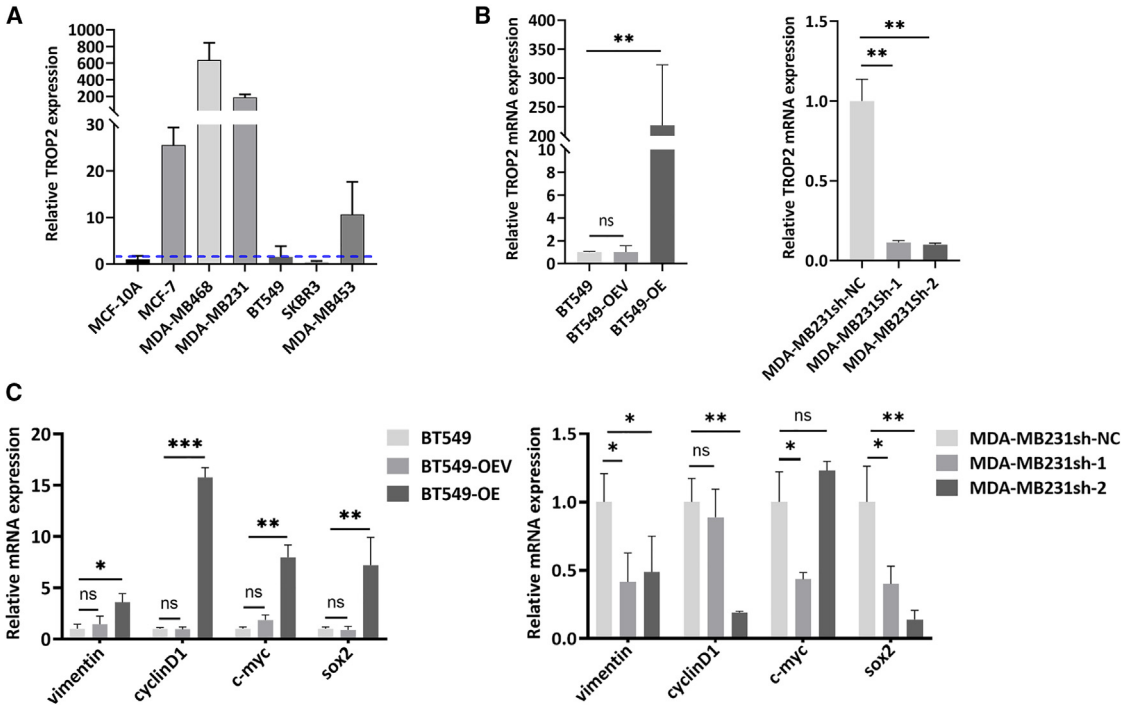


Figure 2. TROP2 expression affects the expression of tumor progression markers

(A) Differential mRNA expression levels of TROP2 in different BC cell lines by qRT-PCR. (B) Overexpression of TROP2 in BT549 cells and knockdown of TROP2 in MDA-MB231 cells using lentivirus. (C) Overexpression or knockdown of TROP2 is followed by changes in the expression of markers related to EMT, cell-cycle progression, and cell stemness. Results are presented as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

TNBC CTCs, thus assisting in the detection of more metastatic potential CTCs.

High expression of TROP2 in TNBC CTCs and its potential for detecting mesenchymal-associated CTCs

The well-established CanPatrol CTC assay platform was used to validate the clinical applicability of TROP2 as a candidate molecule for novel CTC markers in blood samples from patients with TNBC. The workflow of the assay platform is shown in Figure 5A. Next, the spiking assay was used to verify the TROP2 expression results of standard TNBC cell lines detected by the RNA *in situ* hybridization (RNA-ISH) assay in the CanPatrol CTC assay platform; the steps of the spiking assay were described previously. TROP2 was highly expressed in the different TNBC cells (Figure 5B), which was generally consistent with qRT-PCR results shown in Figure 2A, demonstrating the feasibility of clinical blood CTC detection by TROP2 using the CanPatrol CTC assay platform. Furthermore, the results showed that the expression level of TROP2 was positively correlated with the mesenchymal marker vimentin, especially in MDA-MB231 and MDA-MB468 cells (Figure S3). The CanPatrol CTC assay platform was then used to count and type CTCs from a total of 39 blood samples, including luminal, $HER2^+$ BC patients and TNBC. The ratios of different types of CTCs to total CTCs in luminal, $HER2^+$ BC, and TNBC are shown in Figure S4. More hybrid epithelial mesenchymal CTCs were evident in patients with TNBC. In addition, TROP2 was more highly expressed in CTC patients with TNBC and was low in $HER2^+$ patients, although TROP2 expression levels were also high in CTCs of luminal BC patients, and consistent with the Clinical Proteomic Tumor Analysis Consortium (CPTAC) database information (Figure 1B) and BC cell qRT-PCR results (Figure 2A). Specifically, we analyzed the typical cases of each BC subtype. TROP2 expression levels in CTCs from a patient with TNBC (P3030) were significantly higher than that from a $HER2^+$ patient with BC (P4187). Although higher TROP2 expression was detected in CTCs from a patient with luminal BC (P2997), it was significantly lower than that in patients with TNBC (Figure 5C). More interesting, we found that in patients with TNBC, TROP2 was not expressed or was expressed at low levels in epithelial CTCs and was highly expressed with increasing levels of EMT in hybrid epithelial mesenchymal CTCs. The expression level of TROP2 was positively correlated with the expression level of mesenchymal markers, as shown in Figure 5D, demonstrating that TROP2 has the potential to detect mesenchymal-associated CTCs in patients with TNBC.

DISCUSSION

BC is the most common cancer in women, according to statistics from the World Cancer Research Fund International (https://www.wcrf.org/cancer-trends/worldwide-cancer-data/). Approximately 15%–20% of all BCs are TNBC.[1] Compared with other BC subtypes, TNBC is the most difficult subtype to treat. Rapid and effective early

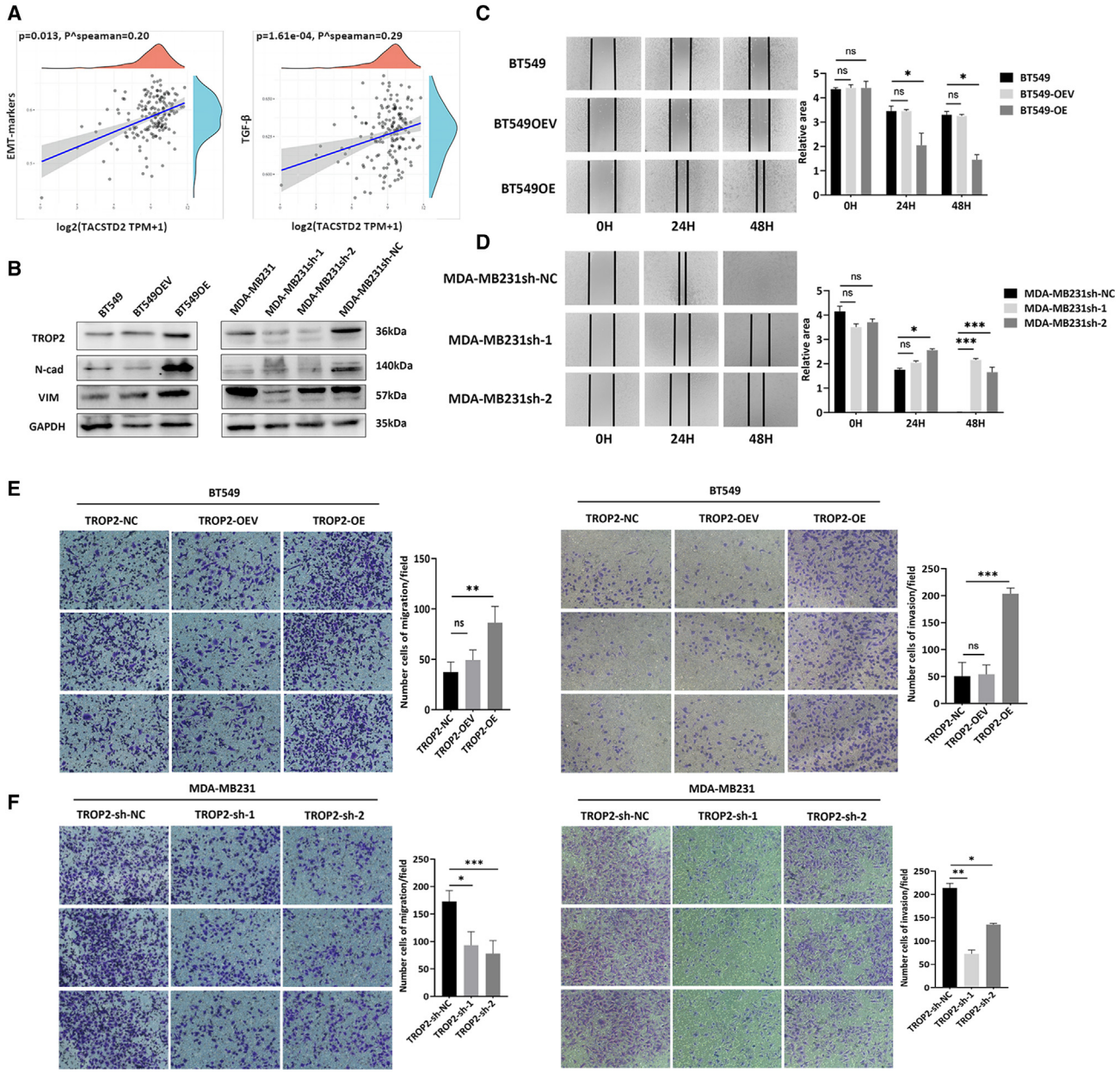
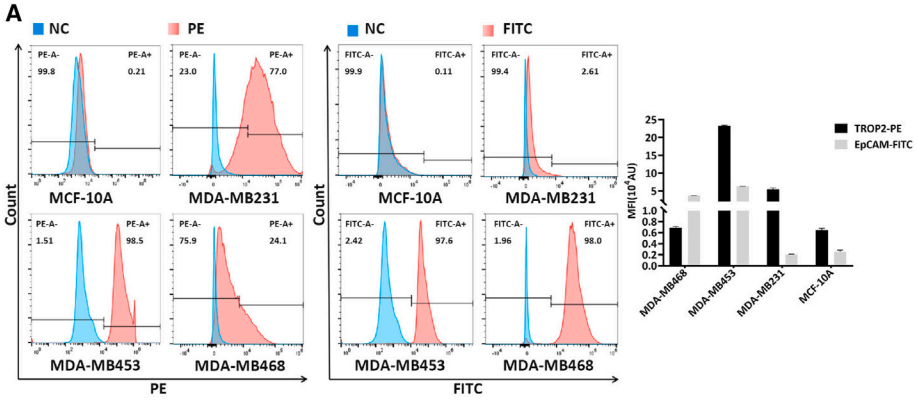


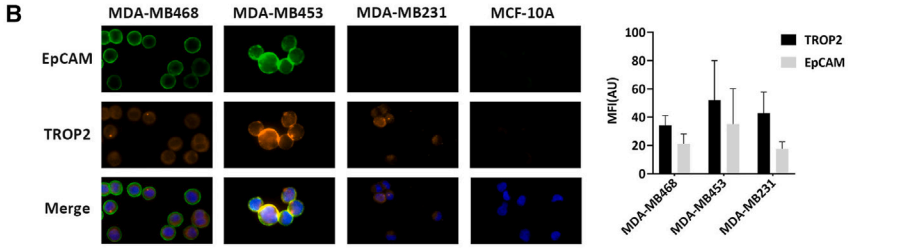
Figure 3. TROP2 promotes invasion by upregulating mesenchymal markers in TNBC cells

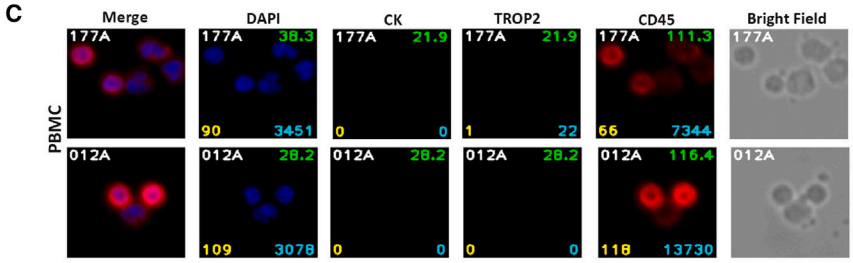
(A) Positive correlations between *TACSTD2* and EMT-related molecules and the TGF-β pathway by Spearman correlation analysis. (B) Overexpression or knockdown of TROP2 affects the protein levels of the mesenchymal-associated markers N-cadherin and vimentin. (C and D) The migration ability of BC cells is increased after overexpression of TROP2 (C) and decreased after knockdown of TROP2 (D) as assessed by the wound healing assay. (E and F) The migration and invasion abilities of BC cells were increased and decreased, respectively, after overexpression or knockdown of TROP2 as assessed by the transwell assay. Results are presented as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

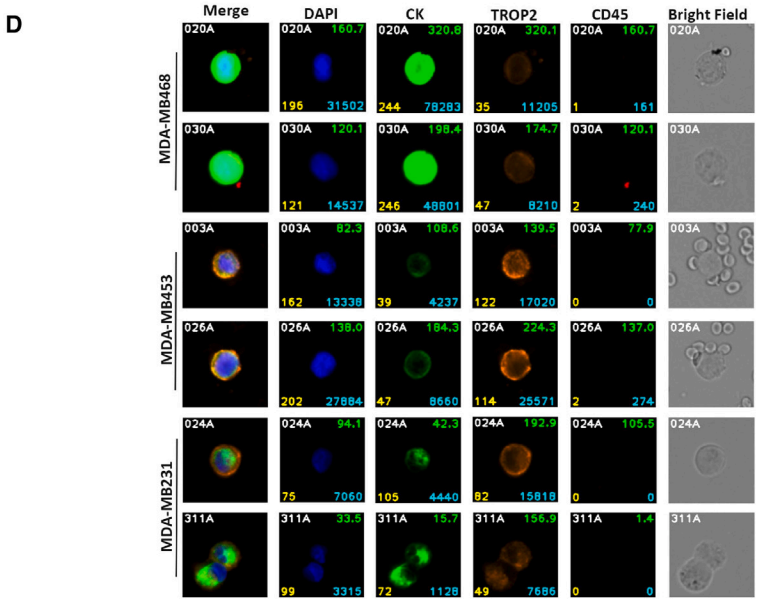
diagnosis and monitoring of the outcome of curative treatment are urgently needed.[23]

Liquid biopsy is an effective tool for early diagnosis, minimal residual disease monitoring, and prediction of treatment efficacy.[24] Liquid biopsy includes the detection of CTCs, ctDNA, extracellular vesicles, and other circulating tumor biomarkers such as microRNAs.[6,25–27] CTCs carry multi-omics information and have rich clinical value. CTCs in early, locally advanced, or metastatic BC have shown independent prognostic value in several clinical trials and meta-analyses.[28] CTC count and molecular subtype correlate with prognosis and treatment monitoring. For example, the presence of epithelial









(legend on next page)

and mesenchymal subtypes of CTCs is dynamic during therapy, with higher rates of mesenchymal CTCs during tumor progression.[29] However, the phenotypic heterogeneity of CTCs poses challenges in laboratory detection.[25,28] In addition, the properties of CTCs that lead to their successful metastatic dissemination remain unclear. Consequently, research has sought to detect various types of CTCs and to determine which type has more metastasis characteristics.[30]

EMT is a highly conserved mechanism associated with cell migration, invasion, and dissemination during the development of cancer metastasis. EMT is a dynamic process that imparts mesenchymal properties to epithelial cells, facilitating the spread of tumor cells and metastasis.[13,31–33] However, EMT also leads to the downregulation of epithelial proteins, such as EpCAM,[34] which is commonly used to enrich and detect CTCs, resulting in a subpopulation of CTCs that express low levels of EpCAM. These cells are poorly captured by EpCAM-dependent approaches, such as CellSearch. Thus, it is possible that CTCs with more metastatic phenotypes may be missed.[35] Therefore, the search for novel biomarkers of CTCs to overcome missed detection is an urgent one.

TROP2 was first described in 1981 as a protein highly expressed on the surface of trophoblast cells.[36] TROP2 is a transmembrane glycoprotein and calcium signaling transducer, which is limited expressed in normal human tissues and overexpressed in a variety of malignant tumors, including BC. TROP2 is involved in tumor development, invasion, and metastasis.[16,37] In addition, retrospective studies have linked TROP2 expression to worse prognosis in patients with BC.[38,39] In this study, through database and patient tumor tissue IHC analyses revealed higher expression of TROP2 in TNBC than in other subtypes. In addition, the expression was associated with higher levels of pathological staging. The previous and presently documented characteristics of TROP2 make the protein an attractive therapeutic target in cancer therapy. Sacituzumab govitecan is an ADC that has been approved for the treatment of metastatic TNBC.[18,40] However, based on the mechanism of action of ADCs, it is expected that the targeted antigen will be overexpressed on the cancer cell membrane to achieve antitumor activity.[41] Drawing on the findings of previous clinical cohort studies of ADC drugs, the first-in-human trial of HER2-ADC ado-trastuzuma (T-DM1) was performed in BC patients with obvious HER2 overexpression, which was indirectly demonstrated by IHC or RNA-ISH detection. However, T-DM1 displayed markedly less activity in patients with low or negligible levels of HER2.[42,43] ADC drugs are very expensive; biomarkers are needed to aid physicians in clinical decision making and accurate prediction of therapy to increase clinical benefit to patients. These findings confirm the need for a predictive biomarker to select patients for ADC treatment.

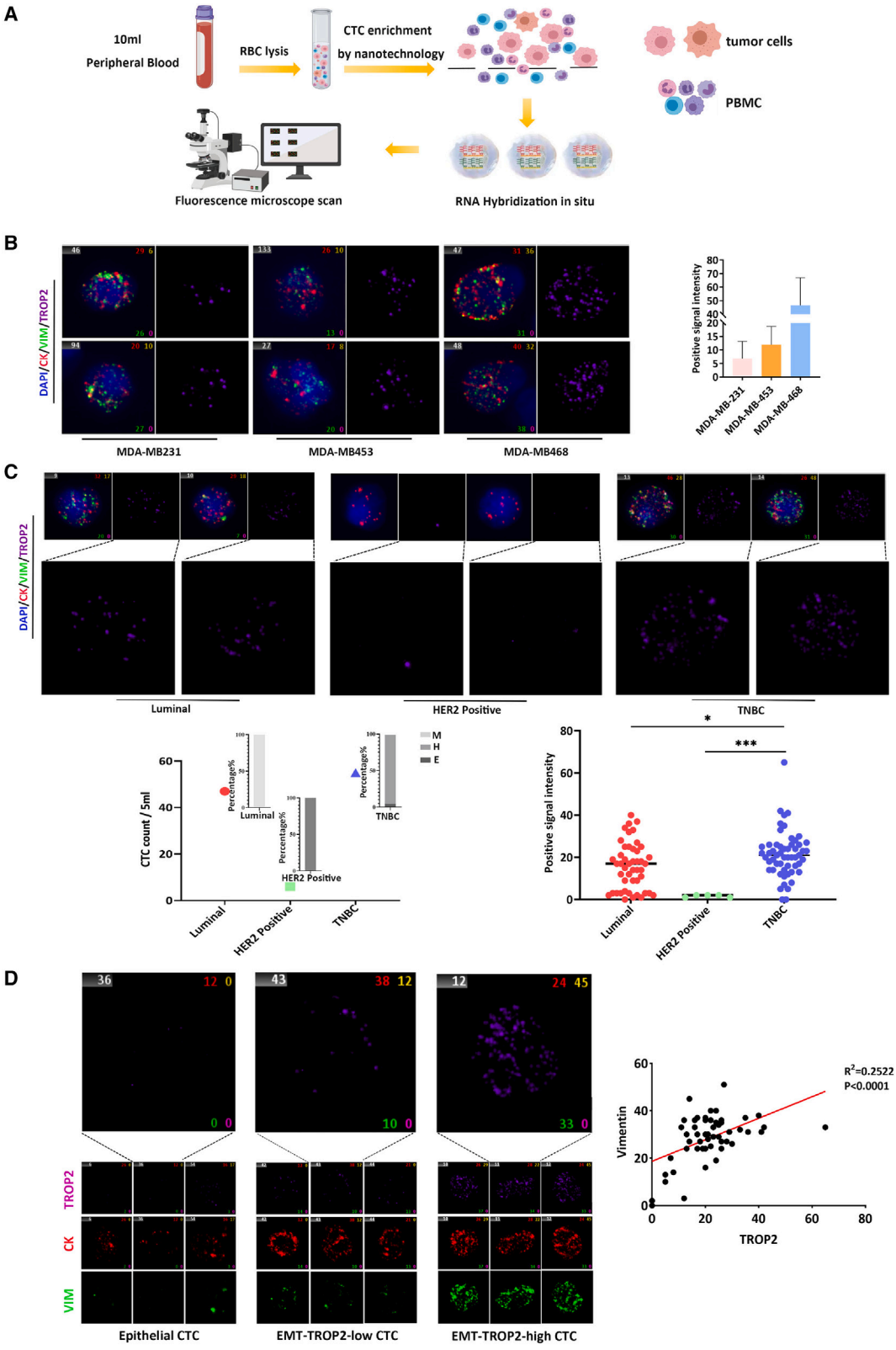
Current CTC assays have significantly advanced cancer prognosis, response to anticancer treatment, and predictions of sensitivity and resistance.[44] Biomarkers in CTC are used as target molecules for predicting and monitoring clinical cancer therapy, including HER2, ER, androgen receptor variant 7, immune checkpoint inhibitors programmed cell death protein 1/programmed death-ligand 1, and others.[45,46] Similarly, TROP2 ADC drugs need to be diagnosed to assist in clinical decision making. In clinical cohort studies of ADCs targeting TROP2, the method for measuring TROP2 expression levels is mainly IHC. However, due to the heterogeneity of tumors, especially TNBC, the most heterogeneous subtype, liquid biopsy techniques that include CTC assays to detect the expression of TROP2 in patients would more accurately identify patients with high or low TROP2 expression.

TROP2 is a widely expressed glycoprotein encoded by the *TACSTD2* gene. TROP2 belongs to the EpCAM family which is a classical marker of CTCs.[47] In the present study, we observed that TROP2 was overexpressed in TNBC cells, but not in normal mammary cells. These findings indicate that TROP2 may be a suitable novel marker for the identification of CTCs in TNBC. The specificity of TROP2 as a CTC marker was highlighted by its absence in PBMCs from healthy controls. In addition, TROP2 is associated with poor prognosis in multiple cancers. TROP2 may serve as an independent prognostic biomarker in patients with glioblastoma and oral squamous cell carcinoma.[48,49] Furthermore, previous studies have shown that the upregulation of TROP2 plays a critical role in human cancer growth and that tumor development is quantitatively driven by TROP2 expression levels.[50,51] Moreover, TROP2 high expression is a suitable marker for stratifying patients with a high risk of colorectal cancer.[52] It has also been indicated that the expression of TROP2 acted as an efficient predictor of the response to precision cancer therapy.[53] These results indicate that TROP2 has the potential to serve as a biomarker for CTCs, which could be useful for efficacy monitoring and prognosis prediction in patients with TNBC.

Previous studies have also considered that TROP2 has important roles in tumor metastasis and promotion of EMT.[22,39,47] In the present study, *TACSTD2* expression was significantly and positively correlated with EMT markers and affected the migration and invasion of TNBC cells. TROP2 remained highly expressed in MDA-MB231 cells in which the epithelial marker EpCAM was downregulated or even absent. This finding suggests that TROP2 can be used in combination with EpCAM to detect CTCs. However, the CanPatrol CTC assay platform revealed that the expression of TROP2 was higher in both

Figure 4. Expression of TROP2 in TNBC cells and blood cells

(A and B) Flow cytometry assay and IF comparison of EpCAM and TROP2 expression in TNBC cells (MDA-MB468, MDA-MB453, and MDA-MB231) and MCF-10A normal breast cells. The bar graph shows mean fluorescence intensity ± SD. (C) The specificity of TROP2 as a potential marker for CTCs in TNBC, as assessed by the IF assay of the expression of EpCAM, CD45, and TROP2 in human PBMCs. (D) The potential applicability of TROP2 to capture and identify tumor cells, as assessed by the spiking assay using the novel marker TROP2 and classical marker CK.



(legend on next page)

CTCs from TNBC and EMT CTCs, suggesting that TROP2 may be a potential marker for EMT-associated CTCs in TNBC. Combined with classical CTC markers, this could improve the detection rate of CTCs and reduce the rate of missed detections. In addition, the higher expression of TROP2 in TNBC CTCs is consistent with the theory that it could be a good therapeutic target for TNBC.

A limitation of this study is the small number of patients. Confirmation of TROP2 as a novel marker of EMT-associated CTCs in patients with TNBC requires larger-scale studies.

In conclusion, TROP2 is particularly highly expressed in TNBC and promotes BC cell migration and invasion. The specificity and applicability of TROP2 as a potential biomarker of EMT CTCs in TNBC was validated in cell lines and clinical blood specimens. Future studies are needed to provide more data on liquid biopsy associated with anti-TROP2 ADC companion diagnosis and detection of more types of CTCs.

MATERIALS AND METHODS

Cell lines and culture

MCF-7, BT549, MDA-MB231, MDA-MB468, MDA-MB453, and SK-BR3 human BC cells lines and HEK-293FT human cells were obtained from the American Type Culture Collection (Rockville, MD). MCF-10A cells were purchased from Cell Signaling Technology (Danvers, MA). BT549 cells were cultured in RPMI 1640 medium (Basal Media, Shanghai, China) supplemented with 10% fetal bovine serum (FBS; Gibco, Franklin Lakes, NJ) and 1% penicillin-streptomycin. MDA-MB231, MDA-MB468, MDA-MB453, and SK-BR3 cells were cultured in DMEM (Basal Media) supplemented with 10% FBS and 1% penicillin-streptomycin. MCF-7 cells were cultured in modified Eagle's medium (Basal Media) supplemented with 10% FBS and 1% penicillin-streptomycin. MCF-10A cells were cultured in MCF-10A specific medium (Procell Life Science & Technology, Wuhan, China). All of the cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

Gene and pathway correlation analysis

RNA-sequencing expression[54] profiles and corresponding clinical information for TNBC were downloaded from the TCGA dataset (https://portal.gdc.cancer.gov/). The gene set variation analysis tool in the R software package was used, with method = 'ssgsea' as the parameter. The correlation between gene and pathway scores was analyzed using Spearman's correlation. All of the analysis methods and R packages were implemented in R version 4.0.3. Statistical significance was set at $p < 0.05$.

IHC

BC and TNBC samples were obtained from Chongqing University Cancer Hospital, the ethics committee had approved the relevant research. Sixty TNBC tissue sections were included in the study. The tissue samples were sectioned at a 4-μm thickness, deparaffined in xylene, dehydrated, and antigen retrieval was blocked by 10% goat serum. The tissue sections were incubated with TROP2 primary antibody (1:50, Absin, Shanghai, China), overnight at 4°C, followed by horseradish peroxidase secondary antibody (1:50, Biosharp, Anhui, China) for 30 min at room temperature and then stained with diaminobenzidine. IHC staining was observed with a FluoView FV1000 microscope (Olympus, Tokyo, Japan). TROP2 expression was scored using the semiquantitative H-score method, which considers both the staining intensity and the percentage of cells at that intensity.

RNA extraction and qRT-PCR

Total RNA was extracted from the cells using TRIzol reagent (Biosharp). For reverse transcription, 1 μg of total RNA was reverse transcribed using Hifair III reverse transcriptase (YEASEN, Shanghai, China). qPCR was performed using the Hieff qPCR SYBR Green Master Mix (YEASEN). Relative RNA expression of selected genes was normalized to β-actin. All of the experiments were performed according to the manufacturer's instructions. The primer sequences for the detected genes are listed in Table S1.

Western blotting

The cells were lysed in radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China) with PMSF (Solarbio, Beijing, China). Extracted protein concentration was measured using bicinchoninic acid protein assay kit (Beyotime), and then separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes according to the standard methods. The membranes were blocked with 5% skim milk and incubated with specific primary antibodies against TROP2 (1:1,000, Absin), N-cadherin (1:1,000, Proteintech, Wuhan, China), vimentin (1:1,000, Proteintech), and glyceraldehyde 3-phosphate dehydrogenase (1:1,000, Proteintech) at 4°C overnight. The membranes were incubated with secondary antibody (1:10,000, Biosharp) at room temperature for 1 h. The proteins were visualized by enhanced chemiluminescence assay (Millipore Corporation, Burlington, MA).

Plasmids, transfection, and lentivirus infection

The TROP2 overexpression and corresponding control vectors were obtained from Genecopoeia (Rockville, MD). All of the transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Lentivirus produced by transfected HEK-293FT were used to

Figure 5. High expression of TROP2 in TNBC CTCs and its potential for detecting mesenchymal-associated CTCs

(A) Flowchart of CanPatrol CTC assay platform based on RNA-ISH. (B) Detection of TROP2 and vimentin expression levels in different TNBC cells by RNA-ISH assay. The bar graph shows mean positive signal intensity ± SD. (C) Typical images of CTCs based on the RNA-ISH. TROP2 expression in CTCs from patients with TNBC was significantly higher than that from other BC subtypes by CanPatrol CTC assay. (D) Typical images of TROP2 expression in CTCs from TNBC and correlation between TROP2 and vimentin. Low or negligible expression of TROP2 was observed in epithelial CTCs. Expression was higher in EMT CTCs. E, epithelial CTC; M, mesenchymal CTC; H, hybrid epithelial-mesenchymal CTC. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

infect BT549 cells. MDA-MB231 cells were infected by shRNA specific to TROP2 lentiviruses (Tsingke Biotechnology, Beijing, China). All of the experiments were performed according to the manufacturer's instructions. Infected cells were selected using puromycin (2 μg/mL) for 2 weeks.

Wound healing assay

Cells from different groups were plated in 6-well plates. Each cell monolayer that developed was scratched with a sterile pipette tip, and the floating cells were removed using PBS. The remaining adherent cells were cultured in serum-free medium to allow for cell growth and wound healing. After culturing for 0, 24, and 48 h, images were captured using by phase-contrast microscopy (Olympus).

Transwell assay

Each Transwell chamber was coated with 200 μL Matrigel (1:8 dilution; BD Bioscience, San Jose, CA) for 2 h at 37°C. For migration assays, the chambers were not coated with Matrigel. For both assays, Transwell chambers were placed on a 24-well plate with 500 μL 1640 or DMEM medium containing 10% FBS in the lower chamber according to the manufacturer's protocols. Cells were harvested, resuspended (2×10^4) in 1640 or DMEM serum-free medium, and added to the upper chamber. After culturing for 24 h, the invading or migrating cells on the opposite side of the membrane were fixed, stained with crystal violet, and imaged by microscopy.

Flow cytometry

Cells from the different BC cell lines were detached with 0.25% trypsin and resuspended as single cells at a concentration of 1×10^6 in PBS. Each suspension was incubated with phycoerythrin anti-TROP2 (1:100, BioLegend, San Diego, CA) and fluorescein isothiocynate anti-EpCAM (1:100, BioLegend) for 1 h at 4°C protected from light. The cells were washed 3 times with PBS and resuspended in 500 μL PBS. $TROP2^+$ or $EpCAM^+$ cells were evaluated by flow cytometry using Attune NxT Flow Cytometer (Invitrogen).

IF assay

Cells were resuspended and incubated in 4% paraformaldehyde for 10 to 15 min, detached with 0.25% trypsin, washed with PBS, placed on slides pretreated with poly-L-lysine solution (Solarbio), and permeabilized with 1% Triton X-100 in PBS. Nonspecific binding was blocked with 5% bovine serum antibody (Solarbio) after 2 washes. The cells were then incubated with TROP2 antibody (1:50, BioLegend), EpCAM (1:50, BioLegend), and CD45 (1:50, BioLegend) for 2 h at room temperature and washed 3 times with PBS. Finally, the slides were coverslipped with a solution containing DAPI and an antiquenching sealant. Marker levels were determined by fluorescence or confocal laser scanning microscopy.

Extraction of PBMCs and IF staining

Fresh ethylenediaminetetraacetic acid-stabilized blood samples were collected from healthy volunteers. Human whole-blood mononuclear cell separation medium (Stemcell Technology, Vancouver, BC, Canada) was used to isolate PBMCs. The cells were obtained using the Ficoll-Paque technique.[55] IF staining was performed as described above.

Spiking assay

A total of 2,000 BC cells were spiked into 5 mL blood samples from healthy volunteers. The tumor cells were obtained by negative enrichment using the EasySep Direct Human CTC Enrichment Kit (Stemcell Technology) as described by the manufacturer. IF staining was performed according to the protocol described above, except that the captured cells were incubated with phycoerythrin anti-TROP2 and fluorescein isothiocyanate anti-CK8/18/19 antibodies (a gift from Professor Hu, National Center for Nanoscience and Technology, Beijing, China). The percentage of captured CK^+ and $TROP2^+$ cells was determined using fully automated scanning fluorescence microscopy. A sample flowchart is shown in Figure S1.

RNA-ISH

TROP2, CK8/18/19, vimentin, and Twist mRNA expression levels in TNBC cells and CTCs were detected using an RNA-ISH assay (Surexam, Guangzhou, China). An mRNA-specific probe was used to capture the corresponding mRNA. A fluorescence microscope (ZEISS, Jena, Germany) was used for image analysis. Capture probes were designed based on the NCBI database and synthesized by Invitrogen. The probe sequence of TROP2 is shown in Table S2. The other probe sequences have been previously described.[56]

Isolation and identification of CTCs from peripheral blood

Blood samples were obtained from the Chongqing University Cancer Hospital, the ethics committee had approved the relevant research, and CTCs from blood samples were enriched and identified using the CanPatrol CTC assay platform (Surexam) as previously described.[57]

Statistical analyses

Statistical analyses were performed by Student's t test and one-way ANOVA using GraphPad Prism 9 software (GraphPad, Boston, MA) to evaluate the significance of two and multiple groups. The correlation between two variables was tested by Spearman's rank correlation analysis using GraphPad Prism 9 software. Each experiment was performed independently at least three times. Results are presented as mean ± SD. Differences with $p < 0.05$ were considered statistically significant.

DATA AND CODE AVAILABILITY

Please contact the corresponding author for all data requests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omton.2024.200762.

ACKNOWLEDGMENTS

This work was supported by the Foundation of Chongqing Municipal Education Commission (grant no. HZ2021006), the Natural Science Foundation of Chongqing (grant no. CSTB2023NSCQ-MSX0845

and CSTC2021JCYJ-MSXMX1221), and the Chongqing Talents Program for Innovative and Entrepreneurial Pioneers (grant no. CQYC20210309587). In addition, we thank Prof. Zhiyuan Hu and Dr. Ting Li for their advice and support.

AUTHOR CONTRIBUTIONS

B.G., T.C., and C.C. designed the initial experimental plan. Q.L. performed most of the experiments and wrote the manuscript under the supervision of T.C. and C.C. B.G. provided important assistance in the collection of blood samples. R.Z. and Z.O. collected some of the clinical cases information. Y.Y. and A.W. performed some of the cell experiments. Q.Z. and Y.W. helped to perform some of the CTC enrichment experiments. Q.L. and B.G. analyzed the experimental data. The final manuscript was approved by all of the authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Siegel, R.L., Miller, K.D., Wagle, N.S., and Jemal, A. (2023). Cancer statistics, 2023. CA A Cancer J. Clin. *73*, 17–48.
2. Zhao, S., Ma, D., Xiao, Y., Li, X.M., Ma, J.L., Zhang, H., Xu, X.L., Lv, H., Jiang, W.H., Yang, W.T., et al. (2020). Molecular Subtyping of Triple-Negative Breast Cancers by Immunohistochemistry: Molecular Basis and Clinical Relevance. Oncol. *25*, e1481–e1491.
3. Asleh, K., Riaz, N., and Nielsen, T.O. (2022). Heterogeneity of triple negative breast cancer: Current advances in subtyping and treatment implications. J. Exp. Clin. Cancer Res. *41*, 265.
4. Lone, S.N., Nisar, S., Masoodi, T., Singh, M., Rizwan, A., Hashem, S., El-Rifai, W., Bedognetti, D., Batra, S.K., Haris, M., et al. (2022). Liquid biopsy: a step closer to transform diagnosis, prognosis and future of cancer treatments. Mol. Cancer *21*, 79.
5. Nikanjam, M., Kato, S., and Kurzrock, R. (2022). Liquid biopsy: current technology and clinical applications. J. Hematol. Oncol. *15*, 131.
6. Ozawa, P.M.M., Jucoski, T.S., Vieira, E., Carvalho, T.M., Malheiros, D., and Ribeiro, E.M.d.S.F. (2020). Liquid biopsy for breast cancer using extracellular vesicles and cell-free microRNAs as biomarkers. Transl. Res. *223*, 40–60.
7. Yap, T.A., Lorente, D., Omlin, A., Olmos, D., and de Bono, J.S. (2014). Circulating tumor cells: a multifunctional biomarker. Clin. Cancer Res. *20*, 2553–2568.
8. Riethdorf, S., O'Flaherty, L., Hille, C., and Pantel, K. (2018). Clinical applications of the CellSearch platform in cancer patients. Adv. Drug Deliv. Rev. *125*, 102–121.
9. Singh, M., Yelle, N., Venugopal, C., and Singh, S.K. (2018). EMT: Mechanisms and therapeutic implications. Pharmacol. Ther. *182*, 80–94.
10. Zhang, Q., Rong, Y., Yi, K., Huang, L., Chen, M., and Wang, F. (2020). Circulating tumor cells in hepatocellular carcinoma: single-cell based analysis, preclinical models, and clinical applications. Theranostics *10*, 12060–12071.
11. Shen, Z., Wu, A., and Chen, X. (2017). Current detection technologies for circulating tumor cells. Chem. Soc. Rev. *46*, 2038–2056.
12. Deng, Z., Wu, S., Wang, Y., and Shi, D. (2022). Circulating tumor cell isolation for cancer diagnosis and prognosis. EBioMedicine *83*, 104237.
13. Yu, M., Bardia, A., Wittner, B.S., Stott, S.L., Smas, M.E., Ting, D.T., Isakoff, S.J., Ciciliano, J.C., Wells, M.N., Shah, A.M., et al. (2013). Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science (New York, N.Y.) *339*, 580–584.
14. Thege, F.I., Gruber, C.N., Cardle, I.I., Cong, S.H., Lannin, T.B., and Kirby, B.J. (2019). anti-EGFR capture mitigates EMT- and chemoresistance-associated heterogeneity in a resistance-profiling CTC platform. Anal. Biochem. *577*, 26–33.
15. Cortesi, M., Zanoni, M., Maltoni, R., Ravaioli, S., Tumedei, M.M., Pirini, F., and Bravaccini, S. (2022). TROP2 (trophoblast cell-surface antigen 2): a drug target for breast cancer. Expert Opin. Ther. Targets *26*, 593–602.
16. Liu, X., Deng, J., Yuan, Y., Chen, W., Sun, W., Wang, Y., Huang, H., Liang, B., Ming, T., Wen, J., et al. (2022). Advances in Trop2-targeted therapy: Novel agents and opportunities beyond breast cancer. Pharmacol. Ther. *239*, 108296.
17. (2021). TROP2 ADC Intrigues in NSCLC. Cancer Discov. *11*, OF5.
18. Bardia, A., Mayer, I.A., Diamond, J.R., Moroose, R.L., Isakoff, S.J., Starodub, A.N., Shah, N.C., O'Shaughnessy, J., Kalinsky, K., Guarino, M., et al. (2017). Efficacy and Safety of Anti-Trop-2 Antibody Drug Conjugate Sacituzumab Govitecan (IMMU-132) in Heavily Pretreated Patients With Metastatic Triple-Negative Breast Cancer. J. Clin. Oncol. *35*, 2141–2148.
19. Gradilone, A., Naso, G., Raimondi, C., Cortesi, E., Gandini, O., Vincenzi, B., Saltarelli, R., Chiapparino, E., Spremberg, F., Cristofanilli, M., et al. (2011). Circulating tumor cells (CTCs) in metastatic breast cancer (MBC): prognosis, drug resistance and phenotypic characterization. Ann. Oncol. *22*, 86–92.
20. Orrapin, S., Udomruk, S., Lapisatepun, W., Moonmuang, S., Phanphaisarn, A., Phinyo, P., Pruksakorn, D., and Chaiyawat, P. (2022). Clinical Implication of Circulating Tumor Cells Expressing Epithelial Mesenchymal Transition (EMT) and Cancer Stem Cell (CSC) Markers and Their Perspective in HCC: A Systematic Review. Cancers *14*, 3373.
21. Wang, Q., Armenia, J., Zhang, C., Penson, A.V., Reznik, E., Zhang, L., Minet, T., Ochoa, A., Gross, B.E., Iacobuzio-Donahue, C.A., et al. (2018). Unifying cancer and normal RNA sequencing data from different sources. Sci. Data *5*, 180061.
22. Zhao, W., Jia, L., Kuai, X., Tang, Q., Huang, X., Yang, T., Qiu, Z., Zhu, J., Huang, J., Huang, W., and Feng, Z. (2019). The role and molecular mechanism of Trop2 induced epithelial-mesenchymal transition through mediated β-catenin in gastric cancer. Cancer Med. *8*, 1135–1147.
23. Bianchini, G., De Angelis, C., Licata, L., and Gianni, L. (2022). Treatment landscape of triple-negative breast cancer - expanded options, evolving needs. Nat. Rev. Clin. Oncol. *19*, 91–113.
24. Pantel, K., and Alix-Panabières, C. (2019). Liquid biopsy and minimal residual disease - latest advances and implications for cure. Nat. Rev. Clin. Oncol. *16*, 409–424.
25. Ignatiadis, M., Sledge, G.W., and Jeffrey, S.S. (2021). Liquid biopsy enters the clinic - implementation issues and future challenges. Nat. Rev. Clin. Oncol. *18*, 297–312.
26. Lee, Y., Ni, J., Beretov, J., Wasinger, V.C., Graham, P., and Li, Y. (2023). Recent advances of small extracellular vesicle biomarkers in breast cancer diagnosis and prognosis. Mol. Cancer *22*, 33.
27. Akhlaghipour, I., Taghehchian, N., Zangouei, A.S., Maharati, A., Mahmoudian, R.A., Saburi, E., and Moghbeli, M. (2023). MicroRNA-377: A therapeutic and diagnostic tumor marker. Int. J. Biol. Macromol. *226*, 1226–1235.
28. Ramos-Medina, R., López-Tarruella, S., Del Monte-Millán, M., Massarrah, T., and Martín, M. (2021). Technical Challenges for CTC Implementation in Breast Cancer. Cancers *13*, 4619.
29. Bidard, F.C., Proudhon, C., and Pierga, J.Y. (2016). Circulating tumor cells in breast cancer. Mol. Oncol. *10*, 418–430.
30. Ring, A., Nguyen-Sträuli, B.D., Wicki, A., and Aceto, N. (2023). Biology, vulnerabilities and clinical applications of circulating tumour cells. Nat. Rev. Cancer *23*, 95–111.
31. Alix-Panabières, C., Mader, S., and Pantel, K. (2017). Epithelial-mesenchymal plasticity in circulating tumor cells. J. Mol. Med. *95*, 133–142.
32. Lowes, L.E., and Allan, A.L. (2018). Circulating Tumor Cells and Implications of the Epithelial-to-Mesenchymal Transition. Adv. Clin. Chem. *83*, 121–181.
33. Lawrence, R., Watters, M., Davies, C.R., Pantel, K., and Lu, Y.J. (2023). Circulating tumour cells for early detection of clinically relevant cancer. Nat. Rev. Clin. Oncol. *20*, 487–500.
34. Nieto, M.A., Huang, R.Y.J., Jackson, R.A., and Thiery, J.P. (2016). EMT: 2016. Cell *166*, 21–45.
35. Gabriel, M.T., Calleja, L.R., Chalopin, A., Ory, B., and Heymann, D. (2016). Circulating Tumor Cells: A Review of Non-EpCAM-Based Approaches for Cell Enrichment and Isolation. Clin. Chem. *62*, 571–581.

36. Lenárt, S., Lenárt, P., Šmarda, J., Remšík, J., Souček, K., and Beneš, P. (2020). Trop2: Jack of All Trades, Master of None. Cancers *12*, 3328.

37. Ripani, E., Sacchetti, A., Corda, D., and Alberti, S. (1998). Human Trop-2 is a tumor-associated calcium signal transducer. Int. J. Cancer *76*, 671–676.

38. Ambrogi, F., Fornili, M., Boracchi, P., Trerotola, M., Relli, V., Simeone, P., La Sorda, R., Lattanzio, R., Querzoli, P., Pedriali, M., et al. (2014). Trop-2 is a determinant of breast cancer survival. PLoS One *9*, e96993.

39. Zhao, W., Kuai, X., Zhou, X., Jia, L., Wang, J., Yang, X., Tian, Z., Wang, X., Lv, Q., Wang, B., et al. (2018). Trop2 is a potential biomarker for the promotion of EMT in human breast cancer. Oncol. Rep. *40*, 759–766.

40. Bardia, A., Hurvitz, S.A., Tolaney, S.M., Loirat, D., Punie, K., Oliveira, M., Brufsky, A., Sardesai, S.D., Kalinsky, K., Zelnak, A.B., et al. (2021). Sacituzumab Govitecan in Metastatic Triple-Negative Breast Cancer. N. Engl. J. Med. *384*, 1529–1541.

41. Chen, Y.F., Xu, Y.Y., Shao, Z.M., and Yu, K.D. (2023). Resistance to antibody-drug conjugates in breast cancer: mechanisms and solutions. Cancer Commun. *43*, 297–337.

42. Fujiwara, H., Odawara, J., Hayama, B., Takanashi, Y., Iwama, K.i., Yamakura, M., Takeuchi, M., and Matsue, K. (2010). Gross hematopyuria presenting as a first symptom due to the bladder infiltration of extranodal Burkitt's lymphoma. J. Clin. Oncol. *28*, e252–e253.

43. Burris, H.A., 3rd, Rugo, H.S., Vukelja, S.J., Vogel, C.L., Borson, R.A., Limentani, S., Tan-Chiu, E., Krop, I.E., Michaelson, R.A., Girish, S., et al. (2011). Phase II study of the antibody drug conjugate trastuzumab-DM1 for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer after prior HER2-directed therapy. J. Clin. Oncol. *29*, 398–405.

44. Harouaka, R., Kang, Z., Zheng, S.Y., and Cao, L. (2014). Circulating tumor cells: advances in isolation and analysis, and challenges for clinical applications. Pharmacol. Ther. *141*, 209–221.

45. Geng, L., Wang, Z., Jia, X., Han, Q., Xiang, Z., Li, D., Yang, X., Zhang, D., Bu, X., Wang, W., et al. (2016). HER2 Targeting Peptides Screening and Applications in Tumor Imaging and Drug Delivery. Theranostics *6*, 1261–1273.

46. Barraud, O., Isnard, C., Lienhard, R., Guérin, F., Couvé-Deacon, E., Martin, C., Cattoir, V., and Ploy, M.C. (2016). Sulphonamide resistance associated with integron derivative Tn6326 in Actinotignum schaalii. J. Antimicrob. Chemother. *71*, 2670–2671.

47. Trerotola, M., Cantanelli, P., Guerra, E., Tripaldi, R., Aloisi, A.L., Bonasera, V., Lattanzio, R., de Lange, R., Weidle, U.H., Piantelli, M., and Alberti, S. (2013). Upregulation of Trop-2 quantitatively stimulates human cancer growth. Oncogene *32*, 222–233.

48. Erber, R., Spoerl, S., Mamilos, A., Krupar, R., Hartmann, A., Ruebner, M., Taxis, J., Wittenberg, M., Reichert, T.E., Spanier, G., and Spoerl, S. (2021). Impact of Spatially Heterogeneous Trop-2 Expression on Prognosis in Oral Squamous Cell Carcinoma. Int. J. Mol. Sci. *23*, 87.

49. Hou, J., Lv, A., Deng, Q., Zhang, G., Hu, X., and Cui, H. (2019). TROP2 promotes the proliferation and metastasis of glioblastoma cells by activating the JAK2/STAT3 signaling pathway. Oncol. Rep. *41*, 753–764.

50. Guerra, E., Trerotola, M., Aloisi, A.L., Tripaldi, R., Vacca, G., La Sorda, R., Lattanzio, R., Piantelli, M., and Alberti, S. (2013). The Trop-2 signalling network in cancer growth. Oncogene *32*, 1594–1600.

51. Bignotti, E., Todeschini, P., Calza, S., Falchetti, M., Ravanini, M., Tassi, R.A., Ravaggi, A., Bandiera, E., Romani, C., Zanotti, L., et al. (2010). Trop-2 Overexpression as an Independent Marker for Poor Overall Survival in Ovarian Carcinoma Patients. Eur. J. Cancer *46*, 944–953.

52. Huebner, K., Erlenbach-Wuensch, K., Prochazka, J., Sheraj, I., Hampel, C., Mrazkova, B., Michalcikova, T., Tureckova, J., Iatsiuk, V., Weissmann, A., et al. (2022). ATF2 loss promotes tumor invasion in colorectal cancer cells via upregulation of cancer driver TROP2. Cell. Mol. Life Sci. *79*, 423.

53. Guerra, E., Trerotola, M., Tripaldi, R., Aloisi, A.L., Simeone, P., Sacchetti, A., Relli, V., D'Amore, A., La Sorda, R., Lattanzio, R., et al. (2016). Trop-2 Induces Tumor Growth Through AKT and Determines Sensitivity to AKT Inhibitors. Clin. Cancer Res. *22*, 4197–4205.

54. Wei, J., Huang, K., Chen, Z., Hu, M., Bai, Y., Lin, S., and Du, H. (2020). Characterization of Glycolysis-Associated Molecules in the Tumor Microenvironment Revealed by Pan-Cancer Tissues and Lung Cancer Single Cell Data. Cancers *12*, 1788.

55. Ueo, H., Sugimachi, K., Gorges, T.M., Bartkowiak, K., Yokobori, T., Müller, V., Shinden, Y., Ueda, M., Ueo, H., Mori, M., et al. (2015). Circulating tumour cell-derived plastin3 is a novel marker for predicting long-term prognosis in patients with breast cancer. Br. J. Cancer *112*, 1519–1526.

56. Dai, S., Shao, X., Wei, Q., Du, S., Hou, C., Li, H., and Jin, D. (2023). Association of circulating tumor cells and IMP3 expression with metastasis of osteosarcoma. Front. Oncol. *13*, 819357.

57. Wu, S., Liu, S., Liu, Z., Huang, J., Pu, X., Li, J., Yang, D., Deng, H., Yang, N., and Xu, J. (2015). Classification of circulating tumor cells by epithelial-mesenchymal transition markers. PLoS One *10*, e0123976.