Received: 9 August 2022 | Accepted: 31 October 2022

DOI: 10.1111/1759-7714.14728

ORIGINAL ARTICLE

WILEY

Molecular profiles of single circulating tumor cells from early breast cancer patients with different lymph node statuses

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Funding information
National Natural Science Foundation of China, Grant/Award Numbers: 32071440, 61871403, 82001422, 82150209; Natural Science Foundation of Jiangsu Province Youth Project, Grant/Award Number: BK20200274

Abstract

Background: Characterization of early breast cancer circulating tumor cells (CTCs) may provide valuable information on tumor metastasis.

Methods: We used immunomagnetic nanospheres to capture CTCs from the peripheral blood of eight early breast cancer patients and then performed single-cell RNA sequencing using our proposed bead-dd-seq method.

Results: CTCs displayed obvious tumor cell characteristics, such as the activation of oxidative stress, proliferation, and promotion of metastasis. CTCs were clustered into two subtypes significantly correlated with the lymph node metastasis status of patients. CTCs in subtype 1 showed a strong metastatic ability because these CTCs have the phenotype of partial epithelial-mesenchymal transition and enriched transcripts, indicating breast cancer responsiveness and proliferation. Furthermore, DNA damage repair pathways were significantly upregulated in subtype 1. We performed in vitro and in vivo investigations, and found that cellular oxidative stress and further DNA damage existed in CTCs. The activated DNA damage repair pathway in CTCs favors resistance to cisplatin. A checkpoint kinase 1 inhibitor sensitized CTCs to cisplatin in mouse models of breast cancer metastasis.

Conclusion: The present study dissects the molecular characteristics of CTCs from early-stage breast cancer, providing novel insight into the understanding of CTC behavior in breast cancer metastasis.

KEYWORDS
circulating tumor cells, DNA damage repair, early breast cancer, metastasis, single-cell RNA sequencing

Shuyun Pang and Shu Xu these authors contributed equally to this work.

INTRODUCTION

Breast cancer has become the most frequently diagnosed cancer type worldwide in women.[1] Although early screening and targeted drug therapy have reduced the progression and mortality of breast cancer, metastasis is still the main cause of death.[2,3] Hematogenous metastasis mediated by circulating tumor cells (CTCs) is a crucial mechanism of distant metastasis of breast cancer. CTCs enter the blood circulation from the primary site and then colonize the new site.[4,5] It is possible to employ a noninvasive approach to monitor CTC numbers in real time.[6] Hence, CTCs have become a potential biomarker for evaluating and monitoring metastasis or tumor recurrence. It has been proved that CTC enumeration is correlated with breast cancer progression.[7,8] More importantly, the cellular molecular characteristics of CTCs in metastatic breast cancer using single-cell RNA sequencing (scRNA-seq) provide new biomarkers for monitoring tumor metastasis.[9,10]

With the support of scRNA-seq, the molecular profile of CTCs has made considerable progress for metastatic breast cancer, while the CTCs in nonmetastatic breast cancers are not investigated comprehensively. The prevalence of CTCs in early breast cancer is low (~ 21.5%).[8] The appearance of CTCs in blood refers to a worse prognosis in early breast cancer. CTCs found in early-stage tumors also indicate the potential to form metastases.[8] Accordingly, the molecular landscape of CTCs in early breast cancer is meaningful.

Lymph node metastasis (LNM) is an important prognostic indicator of breast cancer, indicating that the cells in tumors have metastatic potential.[11] CTCs are related to LNM in early breast cancer,[8] illustrating that LNM is a factor affecting the metastatic potential of CTCs. CTCs from patients with LNM or non-LNM (NLNM) could be in different physiological states. Therefore, molecular investigation of CTCs in patients with early breast cancer with LNM and NLNM is helpful to further understand the molecular mechanism of metastasis.

In the present study, RNA analysis from single CTCs was implemented in treatment-free patients with early breast cancer with LNM or NLNM. The differences between CTCs from early breast cancer and primary tumor tissues were dissected. Based on differential expression patterns, the CTCs were classified into two subtypes that were correlated with the lymph node status of patients. Epithelial-mesenchymal transition (EMT) and stemness characteristics were extracted from different subtypes of CTCs. Antioxidant and DNA damage repair were the main survival pathways in the subtype of CTCs associated with LNM to resist the unfavorable environment. We believe that the results from single-cell molecular profiling of CTCs in early breast cancer should be helpful for inhibiting breast cancer metastasis.

RESULTS

ddATP incorporation during cDNA poly-A tailing could effectively increase the amounts of cDNA library

To achieve effective complementary DNA (cDNA) library construction of single CTCs derived from patients with early breast cancer, a bead-seq method was used because it is sensitive and simple for single-cell library construction.[12,13] However, we found that the amplicons from bead-seq were insufficient for downstream sequencing, although PCR was used to amplify the cDNA. This is because CTCs are different from normal cells, and the RNA level in a single CTC derived from a primary breast cancer patient is extremely low. The protocol of bead-seq should therefore be modified for cDNA library construction of single CTCs.

The excessive poly(A) tails supply many inappropriate positions for anchored primers, and extension should thus be blocked if the bases "VN" in the 3′ end of the anchored primers are not hybridized with the cDNA, leading to the low efficiency of second-strand DNA synthesis (Figure S1a). We proposed adding 2′,3′-dideoxyadenosine triphosphate (ddATP) to limit the length of the poly(A) tail. As shown in Figure S1b, the incorporation of 1% ddATP spiked in deoxyadenosine triphosphate effectively improved the yields of two-strand synthesis (Figure S1b). The scRNA-seq method based on poly(A) tailing with additional 1% ddATP is termed bead-dd-seq (Figure S1c). Compared to conventional bead-seq, bead-dd-seq gave increased library abundance in the case of the EEF1G and RSP18 genes (Figure S1d). Meanwhile, we found that bead-dd-seq has a sequencing coverage better than bead-seq (Figure S1e).

Single CTC expression profiles are heterogeneous

Whole blood and primary tumor tissues were isolated from four primary breast cancer patients with NLNM and four primary breast cancer patients with LNM. None of the enrolled patients received any treatment. Whole blood was processed by an immunomagnetic nanospheres platform to enrich CTCs.[14,15] Cells positive for epithelial cell adhesion molecule (EpCAM) and calcein-acetoxymethyl ester (calcein-AM) but negative for protein tyrosine phosphatase receptor type C were identified as CTCs. Ninety cells were identified and micromanipulated for further scRNA-seq, and 39 of these 90 cells were successfully sequenced (Figures 1a and S2). Bulk transcriptomes of primary breast cancers were detected from the eight patients and a separate cohort of 18 patients (Figure 1a, Tables S1 and S2). To further eliminate potential leukocyte contamination, an expression threshold was used to define CTCs, including breast cancer-related markers (ESR1, PGR, ERBB2, ERBB3, BRCA1, BRCA2, RAB7A, CDH1, EPCAM, KRT7, KRT8, KRT18, AR, JUN) and leukocyte

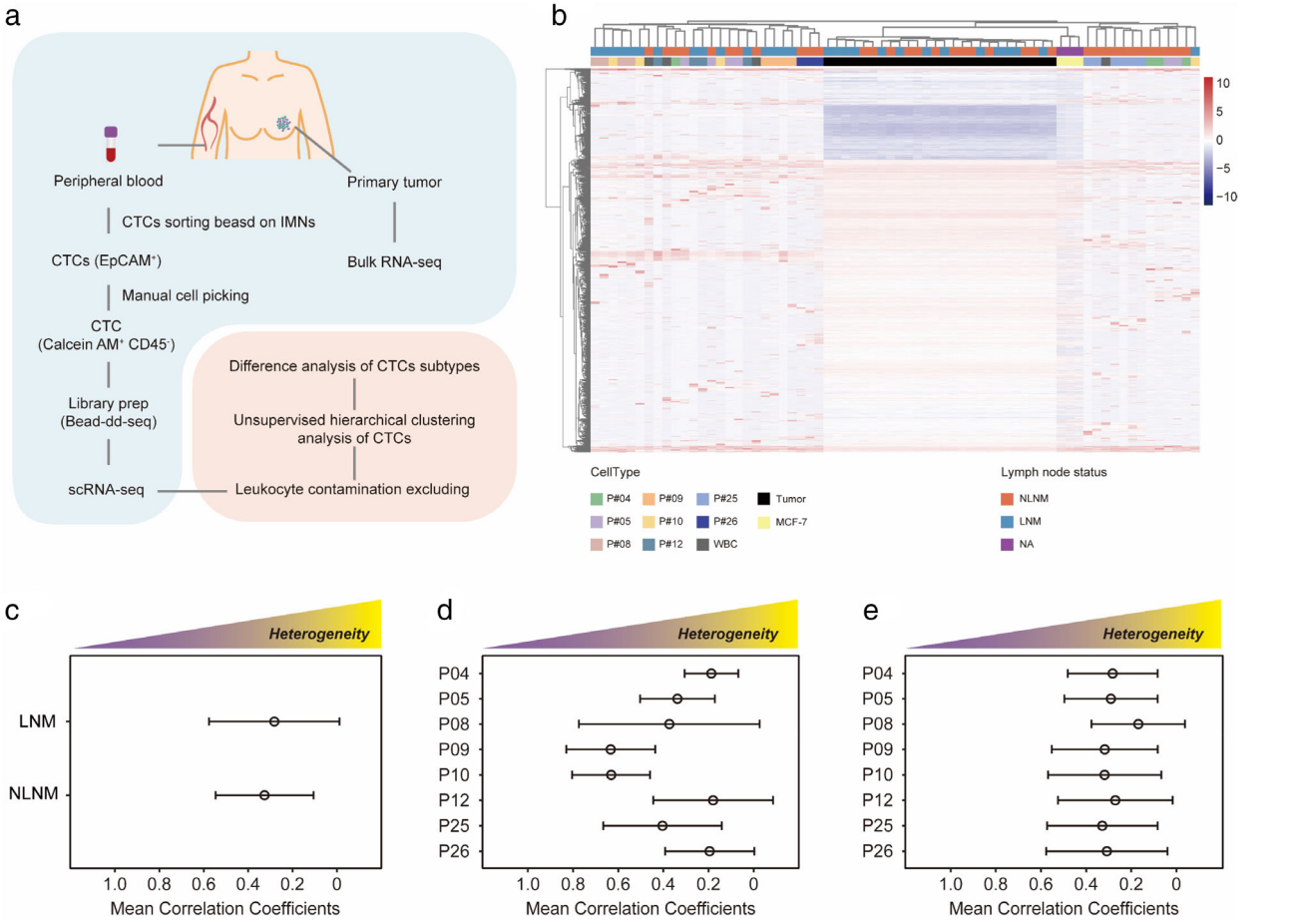

FIGURE 1 scRNA-seq of breast CTCs. (a) An overview of the workflow for single CTC sequencing and analysis. IMNs, immunomagnetic nanospheres. (b) Heatmap of unsupervised hierarchical clustering analysis of single CTCs, primary tumors, single cells identified as leukocytes and single cells from MCF-7 cells. LNM, lymph node metastasis; NLNM, nonlymph node metastasis; NA, missing value. (c–e) Heterogeneity of CTCs from patients with LNM and without LNM (NLNM) (C), CTCs from each individual (d), and CTCs among individuals (e). Heterogeneity was calculated by the mean correlation coefficients from the expression data.

markers (PTPRC, FCGR3B, FCGR3A). Four cells were excluded due to leukocyte contamination (red circles in Figure S3a). In addition, copy number variation analysis was performed to identify the origin of CTC. The results revealed that there were obvious gain and loss of copy numbers in CTCs (Figure S3b). Hence, the remaining 35 cells were defined as classified CTCs (Figure S3a).

Unsupervised hierarchical clustering analysis of single breast CTCs, single leukocytes, primary tumor tissues, and single MCF-7 cells showed that the transcriptional signatures of all tumor tissues were similar, while single CTCs were highly heterogeneous (Figure 1b). There was no difference in the heterogeneity of single CTCs between NLNM and LNM (average correlation coefficient from 0.3250 to 0.2814) (Figure 1c). Interestingly, the heterogeneity of single CTCs varied greatly within patients, whereas this was not observed among patients (average correlation coefficient from 0.1686 to 0.3274) (Figure 1d and Figure S3c). The heterogeneity of CTCs among patients was higher than that within patients (correlation coefficient 0.2874 vs. 0.3686) (Figure 1e).

CTCs exhibit more oxidative stress activation and negative cell cycle regulation than tumor tissues

To characterize CTCs, we investigated the expression of breast cancer-related genes (ERBB2, ERBB3, RAB7A, GAPDH, JUN, CDH1), epithelial genes (EPCAM, CDH1, KRT8, KRT18, KRT19, ITGA6, ITGA3, FLOT2), mesenchymal genes (CDH2, CDH11, FN1, VIM, SERPINE1), cancer stem cell-related (CSC-related) genes (ALDH7A1, CD44, KIT, NES, POU5F1, KLF4, SOX2, MYC), and proliferation-related genes (MKI67, BIRC5, AURKA, MYBL2, CCNB1, CCND1) in single CTCs (Figure 2a). Epithelial markers were expressed in 82.86% of CTCs (29/35). Almost all CTCs (94.29%) expressed stem cell-related markers, including CD44, KIT, and SOX2. In addition, 88.57% of CTCs (31/35) expressed markers related to cell proliferation.

Differential expression analysis was used to identify genes that were upregulated in CTCs compared to primary tumors (Figure 2b). A total of 1409 genes were upregulated

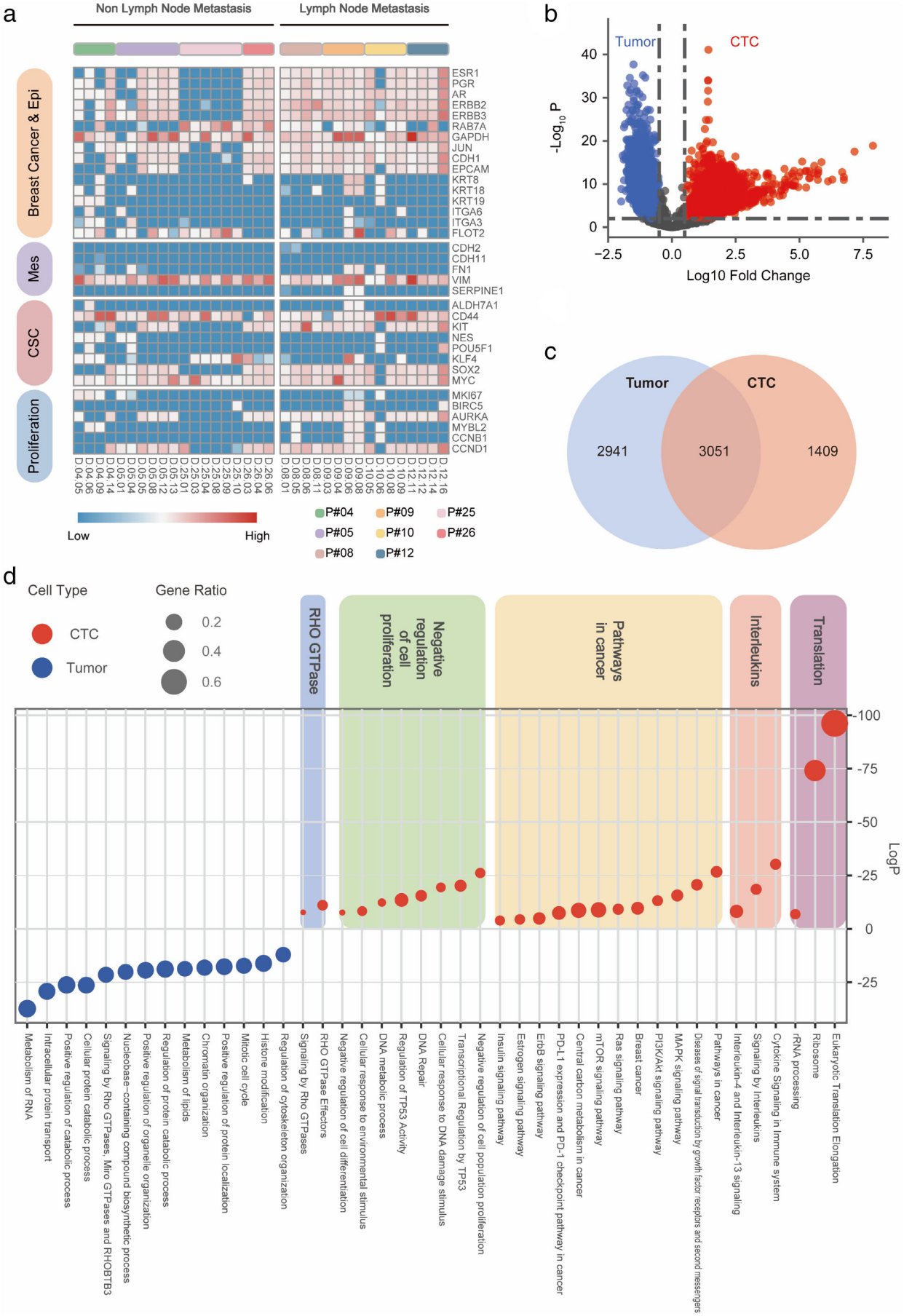

FIGURE 2 Legend on next page.

in CTCs (Figure 2c). CTCs exhibit interaction with blood components. The expression of PF4 and PPBP genes illustrates the existence of platelet adhesion on the CTCs (Figure S4).[16] The CXCR4 gene expressed in CTCs was an important chemokine receptor for CTC metastasis and homing (Figure S4).[17,18] CXCL12 secreted from endothelial cells attracts CXCR4-expressing CTC, thus facilitating their further intravasation.[19] In addition, antioxidation is also a feature of CTC. HBB and SOD2 could neutralize and reduce intracellular reactive oxygen species (ROS) respectively to maintain normal oxidation levels (Figure S4).[20–22]

Next, we identified the key pathways of CTCs and primary tumors. A total of 28 pathways were enriched in CTCs. Most of them were related to translation, interleukins, pathways in cancer, negative regulation of cell proliferation and Rho GTPase (Figure 2d). Active translational processes implied that there was substantial protein synthesis in CTCs. Furthermore, ribosomal protein expression was also significantly upregulated in CTCs (Figure S4) and was associated with CTC epithelial phenotype maintenance and proliferation metastasis.[23] We observed that cancer-related pathways, including the PI3K-AKT signaling pathway and the R-MAPK signaling pathway, were also upregulated in CTCs. Activation of these pathways indicates the maintenance of proliferative function in CTCs.[24] Simultaneously, CTCs exhibited negative cell cycle regulation, including activation of TP53 signaling, responding to multiple DNA damage stimuli, and DNA repair. CTCs undergo various harsh conditions in blood, leading to the production of ROS.[20,25,26] ROS can cause DNA damage.[27] The negative regulation of the cell cycle might be one of the manifestations of CTCs in harsh environments.

CTCs clustered into two subtypes with distinct EMT phenotypes

CTC clustering can be divided into two main subtypes by nonlinear dimensionality reduction analysis, consistent with the results of unsupervised hierarchical clustering (Figures 3a and 1b). We observed that CTCs in subtype 1 expressed breast cancer therapy-related markers, whereas CTCs in subtype 2 did not (Figure 4a). This is consistent with previously reported results that breast cancer therapy-related genes have been expressed in parts of the CTCs,[10,28] indicating that cells with therapy resistance phenotype exist in CTCs. CTC subtypes were related to the lymph node metastasis status of patients ($p = 0.0011$) (Figure 3b), and 93.75% (15/16) of CTCs isolated from patients with luminal B breast cancer were clustered into subtype 1 (Figure 3c). Moreover, most of the patients with CTCs in subtype 1 had a high expression of Ki67 in primary tumors (Table S1). These results indicated that CTCs in subtype 1 might be derived from clones in primary tumors with metastatic potential. This illustrates that CTCs of patients with early breast cancer might have different phenotypes, which might be related to the outcome of patients.

Characteristics of CTC subtypes were determined by differential expression analysis. There were 1594 genes upregulated in subtype 1, including genes participating in stemness maintenance, EMT, DNA repair, and anti-apoptosis (Figure 3d and Figure S5a,b). Multiple tumor-related kinase pathways were enriched in subtype 1, including the PI3K-Akt signaling pathway, MAPK signaling pathway, ErbB signaling pathway, platinum drug resistance, and Jak–STAT signaling pathway (Figures 3e–i and Figure S5f). CTCs in subtype 1 exhibited characteristics similar to ER-positive breast cancers, such as CTCs positively expressing ER, CCND1, MTOR, MYC, PI3K and CDK4/6 (Figure 4a,d).[29] We also investigated the cell cycle score in CTCs. The majority of CTCs in subtype 1 and subtype 2 were in the G0/G1 phase. CTCs in subtype 1 had more cells in the G2/M phase than those in subtype 2 (Figure 4e). Transcription factor (TF) target gene analysis revealed several TFs regulating the cell cycle and stemness in subtype 1, including TP53, E2F1, BRCA1, ESR1, SP1, MYC, and KLF4 (Figure S5c–e). These results showed that CTCs in subtype 1 had tumor proliferative and antiapoptotic properties. CTCs in subtype 1 might have higher malignancy and possibly be associated with stronger metastatic potential.

CTCs in subtype 2 had 627 upregulated genes, including genes involved in anti-apoptosis, EMT and migration (Figure 3d and Figure S5a,b,f). Subtype 2 was composed of two relatively independent subclusters, one of which was contributed by P#25 (Figure 3a). We observed refined subtypes existing in ER/PR/ERBB2 negative CTCs based on Figure 3a. This phenomenon was also observed in scRNA-seq of CTCs from metastatic breast cancer and the bulk RNA-seq of CTC from early-stage breast cancer.[10,30] In addition, we believe that the sequencing data of CTCs from P#25 may be influenced by intrapatient characteristics. Further analysis requires more CTCs to extend the dataset of subtype 2. The commonality among CTCs in subgroup 2 is weak. Alternatively, CTCs in subtype 2 did not express ErbB2, but cellular localization, plasma membrane adhesion, and Rho GTPase signaling pathways were enriched in subtype 2. Activation of Rho GTPases and actin organization in CTCs facilitated resistance to fluid shear stress (FSS) and a reduction in FSS-induced plasma membrane damage.[31] In addition, O-glycosylated mucins, including MUC4 and MUC16, were expressed in subtype 2 CTCs (Figure S6).

FIGURE 2 Differentially expressed genes and the corresponding signaling pathways in CTCs versus primary tumors. (a) Heatmap of the expression levels of genes related to breast cancer, mesenchyme, stemness, and proliferation in single CTCs. Epi, epithelial (breast cancer-related) genes; Mes, mesenchymal genes; CSC, cancer stem cell-related genes. (b, c) Volcano plot (b) and Venn diagram (c) show the differentially expressed genes between CTCs and primary tumors ($p < 0.01$ and fold change >2). (d) Signaling pathways enriched in CTCs versus primary tumors.

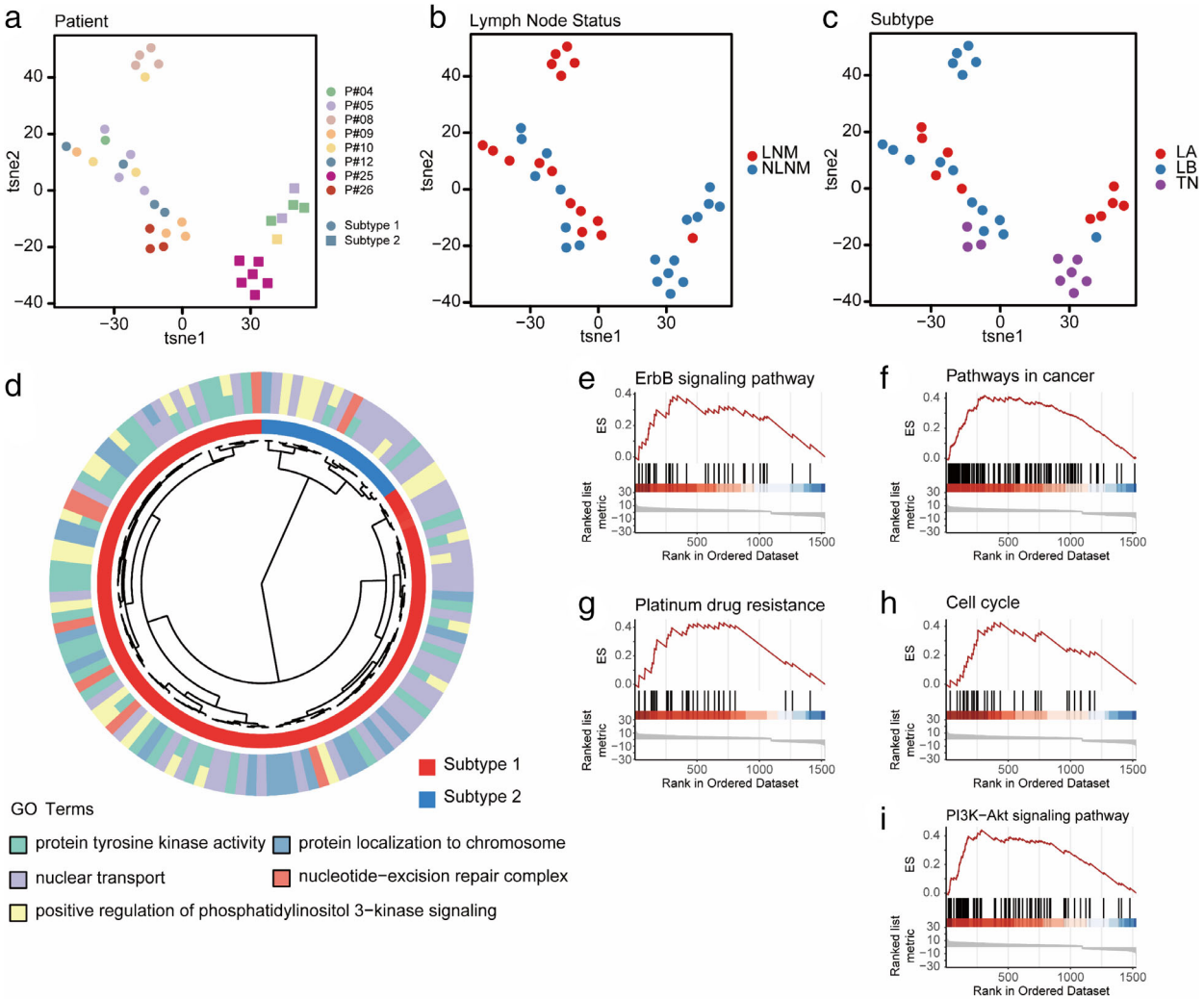

FIGURE 3 Gene signatures and signaling pathways in CTCs. (a) *t*-SNE plot of CTCs from eight patients and subtype analysis by unsupervised hierarchical clustering analysis. (b, c) *t*-SNE plots show the relationship between the two subtypes of CTCs and lymph node status (b) and the two subtypes of CTCs and molecular subtypes of patients (c). LA, luminal A breast cancer; LB, luminal B breast cancer; TN, triple-negative breast cancer. (d) Gene Ontology (GO) pathways in the two subtypes by gene set enrichment analysis (GSEA; FDR < 0.05). (e–i) GSEA plots of the ErbB signaling pathway (e), pathway in cancer (f), platinum drug resistance (g), cell cycle (h), and PI3K-AKT signaling pathway (i) in CTCs.

Mucins play an important role in tumor invasion and immune escape because they can mediate the binding of CTCs to leukocytes and platelets and thus promote metastasis.[32,33]

EMT is an important process in which tumor cells escape from epithelial primary foci and enter the blood circulation system. Almost all CTCs in subtype 1 expressed epithelial markers and simultaneously expressed mesenchymal markers, such as CD44, VIM, and SOX2, indicating that these CTCs were in the intermediate phases of EMT. We calculated the epithelial and mesenchymal scores of all CTCs (Figure 4b,f and Figure S6) and found that almost all CTCs exhibited coexisting epithelial and mesenchymal phenotypes. The phenotype of CTCs in subtype 1 was relatively inclined to the epithelial type. The phenotype of CTCs in subtype 2 was inclined to the mesenchymal type (Figure 4e). Based on the expression of three markers (CDH1, VIM, and CD44), CTCs in subtype 1 were considered to have a partial EMT (pEMT) (CDH1+/VIM+) phenotype, while CTCs in subtype 2 were considered to have a pEMT/mesenchymal (pEMT/M) (CDH1−/VIM+/CD44+) phenotype (Figure 4g).[34]

The CTCs undergoing EMT were accompanied by the upregulation of stemness genes. We therefore investigated the expression of stemness genes in CTCs. Stemness genes were completely different between subtypes 1 and 2, but all CTCs expressed CD44. EpCAM, CD44, and MET were expressed in the subtype 1 CTCs. Some of these CTCs expressed CD47 (Figure 4c). This finding suggested that subtype 1 would have a metastasis-initiating cell (MIC) phenotype. In addition, CTCs in subtype 1 expressed the stem cell transcription factor SOX2 (Figure 4c). There were two stem expression patterns in CTCs of subgroup 2. One pattern is a high expression of FLOT2 and KDM1A in the CTCs from P#25, and the other pattern is the expression of NES and TP63 in the remaining

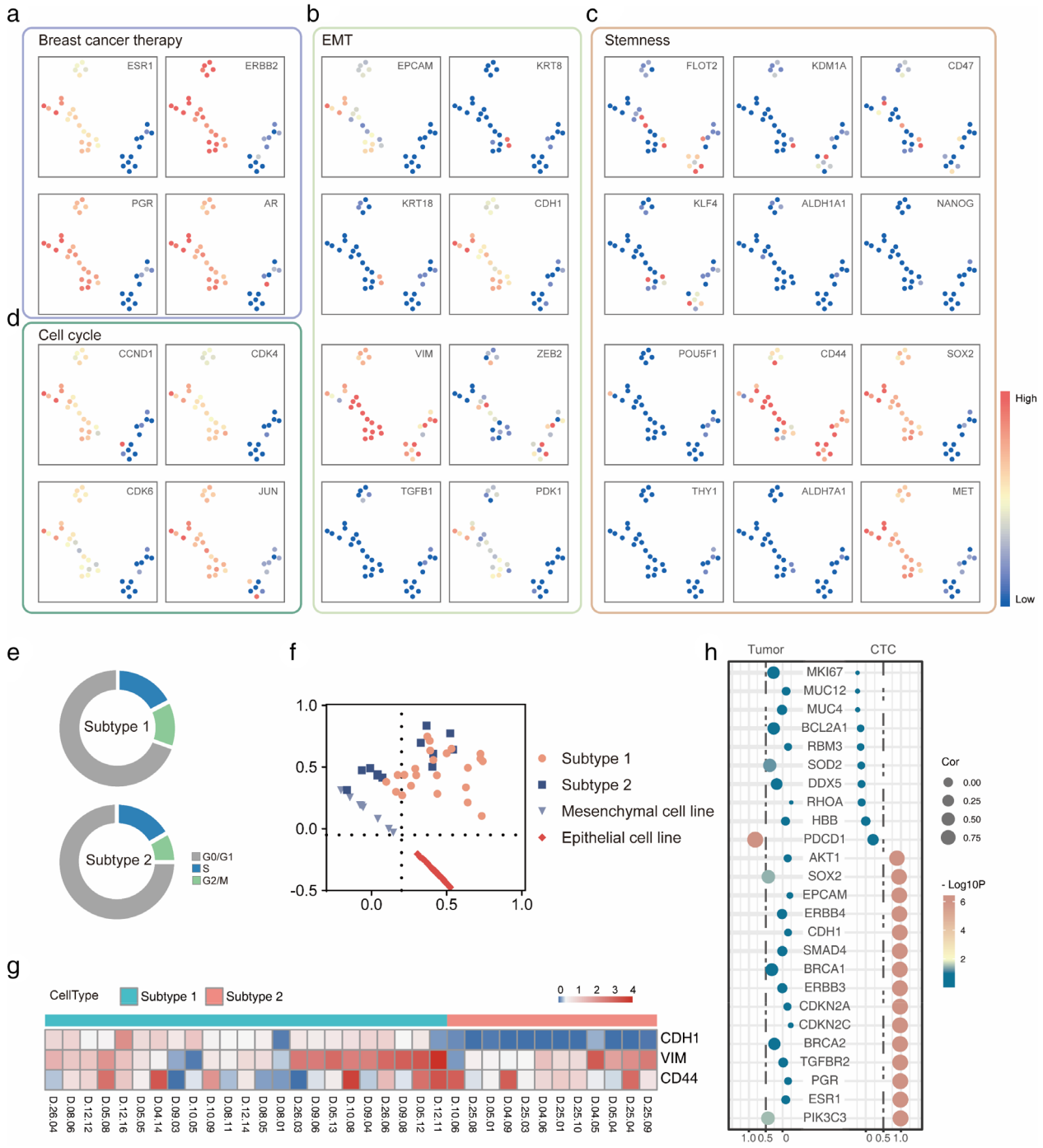

FIGURE 4 Gene expression profiles and characterizations of the two CTC subtypes. (a–d) Gene expression in CTCs for breast cancer therapy-related genes (a), EMT-related genes (b), stemness-related genes (c), and cell cycle-related genes (d). (e) Pie charts display the percentage of CTCs in G0/G1 phase (gray), S phase (green), and G2/M phase (blue) in subtype 1 and subtype 2. (f) Scatterplots showing EMT score of CTC subtypes, epithelial breast cancer cell lines and mesenchymal breast cancer cell lines. Sequencing data of breast cancer cell lines are derived from the Cancer Cell Line Encyclopedia (CCLE). (g) Heatmap showing the expression of CHD1, VIM, and CD44 in single CTCs. (h) Correlation coefficient between the PDL1 gene and breast cancer-related genes in single CTCs and primary tumors.

CTCs (Figure 4c). ALDH was detected only in a small number of CTCs, showing that CTCs might lack the characteristics of epithelial stem cells. Other known CSC-regulated genes, including STAT3, Notch 1, and Notch 2, were also expressed in subsections of CTCs (Figure S6).

The PD-L1 gene correlates with breast cancer-related genes in CTCs

We observed that CTCs in subtype 1 expressed PD-L1, which helped tumor cells escape immune surveillance. CTCs

with high expression of PD-L1 are associated with the risk of metastatic breast cancer recurrence.[35] We found that the expression of PD-L1 was significantly associated with the PIK3C3, ESR1, BRCA1, CDH1, EpCAM, and AKT1 genes in CTCs, while this phenomenon was not found in the primary tumors (Figure 4h). This finding indicates that CTCs with proliferative properties tend to express PD-L1 to acquire the ability to escape immune surveillance. Thus, it is possible to use CTCs as potential biomarkers for evaluating immunotherapy in early breast cancer.

Oxidative stress leads to DNA damage repair activation in CTCs

As described above, negative regulation of the cell cycle, activation of TP53, and anti-apoptotic processes existed in the CTCs of early breast cancer in our data (Figure 2d and Figure S5f). This finding might be the terminal performance of early breast cancer cells escaping from local sites and then forming CTCs. CTCs might be detached from the extracellular matrix (ECM) and stimulated by the shear force of blood fluid. To verify this hypothesis, we used a microfluidic system driven by a peristaltic pump to generate FSS and simulate the physical environment of CTCs in the blood vessel (Figure S7a). The FSS is controlled at 15 dyn/cm^2. MCF-7 and MDA-MB-231 cells were put into microfluidics to simulate the behavior of CTCs. We found that cells treated with FSS had a slightly higher proportion of apoptotic cells (Figure S7b,c) and stronger migration ability than those from static culture (Figure S7d).

Alternatively, reactive oxygen species (ROS) in cells were increased after 2 h of FSS treatment (Figure 5a,b). A high level of ROS was observed until 12 h after FSS treatment, thus FSS would cause oxidative stress in cells and then lead to the generation of ROS, which is recognized as a type of DNA damage mediator. As a result, longer DNA tailing was detected in FSS-treated cells in the comet assay (Figure 5c,d and Figure S7f). In addition, we observed the activation of ataxia-telangiectasia-mutated protein (ATM), ataxia telangiectasia and Rad3-related protein (ATR), checkpoint kinase 1 (CHK1), and checkpoint kinase 2 (CHK2) in FSS-treated cells (Figure 5e). As shown in Figure 5f, DNA damage caused a decreased proportion of cells in G2/M phase but an increased proportion of cells in G0/G1 phase after FSS treatment. This finding indicates that FSS caused DNA damage in cells and then led to cell cycle arrest. We used N-acetylcysteine (NAC) to inhibit oxidative stress, and the results showed that NAC could induce a decline in the phosphorylation of histone H2AX in Serine 139 (γH2AX), phosphorylation of ATM (pATM) and phosphorylation of ATR (pATR) in cells treated with FSS (Figure S7g), hence the DNA damage caused by FSS could be neutralized by antioxidants, and the DNA damage caused by FSS was mediated by ROS.

Additionally, the activation of DNA repair could rescue the cells from apoptosis caused by ROS, therefore we speculated that the inhibition of the DNA damage repair pathway could stimulate cells to undergo apoptosis, thus reducing the occurrence of metastasis. From the genes upregulated in CTCs, we selected ATM, ATR, and CHK1, which participate in the DNA repair pathway mediated by DNA double-strand breaks (DSBs) (Figure S4). Then, KU-55933 (ATM inhibitor), VE-821 (ATR inhibitor), and prexasertib (CHK1 inhibitor) were employed to treat the cells as specified in Figure 5. The FSS-treated cells were more sensitive to these inhibitors than static-cultured cells (Figure 5g,h), indicating that the inhibition of DNA damage repair could induce CTC apoptosis.

DNA damage repair is one of the main mechanisms of resistance to platinum drugs. To determine whether CTCs would be resistant to platinum drugs, cisplatin (CDDP) was chosen. The results showed that the comet tail length of FSS-treated cancer cells was shorter than that of static-cultured cells when exposed to CDDP for 2 h, followed by culturing the cells for 2 h in the absence of the drug (Figure 5c,d and Figure S7g). However, we did not find any significant effect of CDDP on the tail length in FSS-treated cells (Figure 5c,d and Figure S7g). We then investigated whether the apoptosis of cells occurred after exposure to CDDP. As shown in Figure 5i, CDDP increased apoptosis in static-cultured cells but not in FSS-treated cells. Additionally, we found that apoptosis was increased after the FSS-treated cells were exposed to CDDP in combination with VE-821 or prexasertib (Figure 5j). This finding suggests that DNA repair activated by FSS could reduce the cell apoptosis caused by CDDP, and the inhibition of ATR or CHK1 activity by VE-821 or prexasertib could increase the sensitivity of CTCs to CDDP.

To further investigate the efficacy of the CHK1 inhibitor prexasertib on CTC-induced metastasis in vivo, 4T1 cells labeled with luciferase (4T1/Luc) were injected into the tail vein of Balb/c mice, followed by the administration of CDDP and prexasertib after 6 h. Prexasertib was injected intraperitoneally every 12 h during the first 3 days in a week (for 2 weeks per test). CDDP was injected intraperitoneally once every 4 days (for 2 weeks per test). The administration of CDDP was 6 h later than that of prexasertib if two drugs were administered on the same day (Figure 6a). After 2 weeks of treatment, either the combination of two agents (CDDP and prexasertib) or the individual agents showed a significant reduction in metastatic seeding, but the combination medication was better than a single agent (Figure 6b,c); this finding was confirmed by the hematoxylin–eosin (HE) staining results (Figure 6d). Consequently, the combination of platinum-based antineoplastic drugs and cell cycle checkpoint inhibitors had a better effect on inhibiting the formation of tumor metastasis.

DISCUSSION

Lymph node status is an important prognostic factor for breast cancer.[36] The relationship of lymph node metastasis

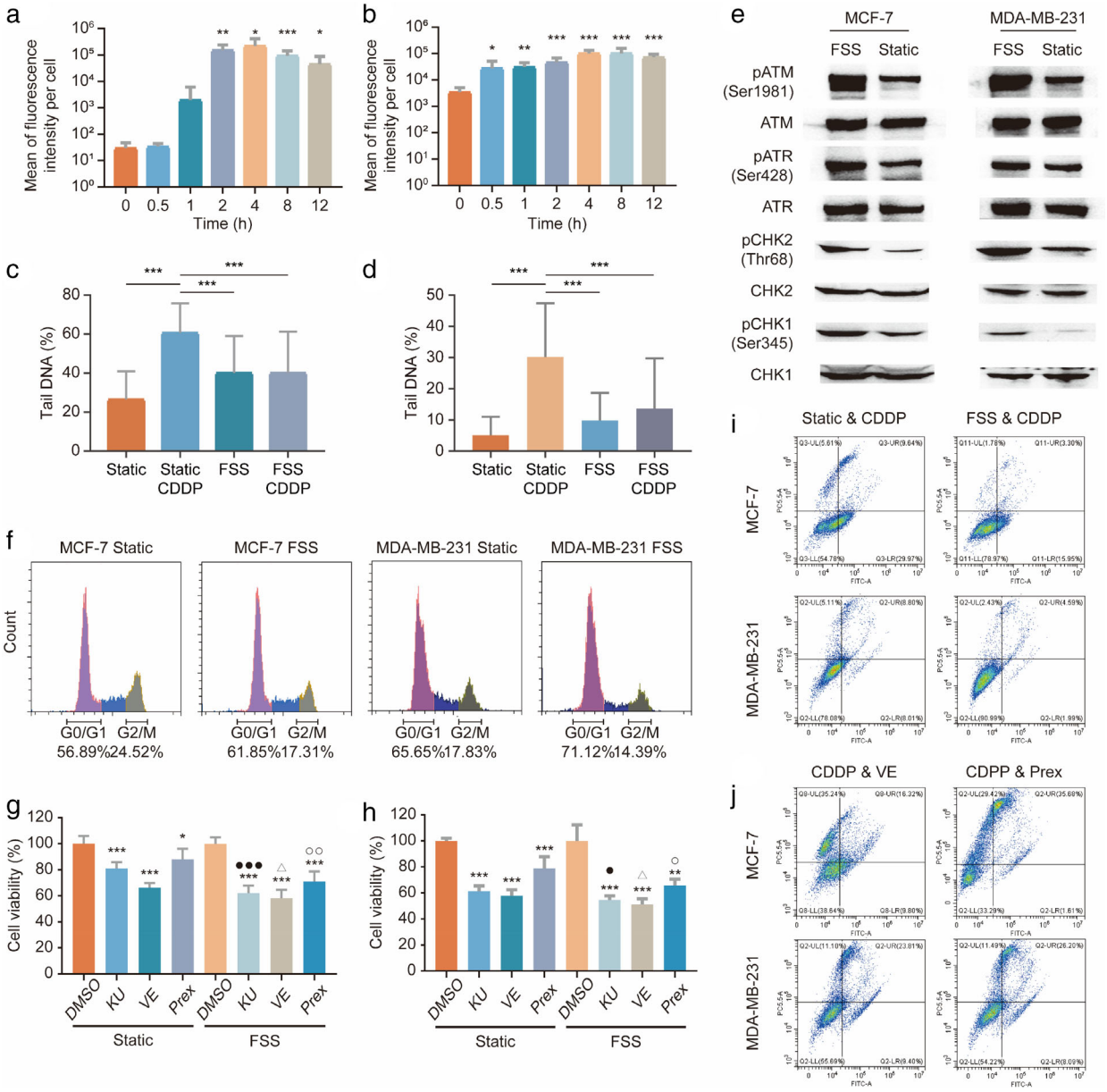

FIGURE 5 In vitro test for investigating the effect of FSS on oxidative stress-induced cellular DNA damage. (a, b) ROS levels in MCF-7 cells (a) and MDA-MB-231 cells (b) determined by H2DCFDA after FSS treatment for 0, 0.5, 1, 2, 4, 8, and 12 h. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus cells after FSS treatment for 0 h. (c, d) DNA damage by comet assay in MCF-7 cells (c) and MDA-MB-231 cells (d) after subjecting cells to FSS treatment or static culture and then treatment with and without CDDP. Tail length is used to measure the extent of DNA damage. ***$p < 0.001$. (e) Western blots of p-ATM (Ser-1981), ATM, p-ATR (Ser-428), ATR, p-Chk1 (Ser-345), Chk1, p-Chk2 (Thr-68), and Chk2 in MCF-7 and MDA-MB-231 cells after FSS treatment or static culture. (f) FACS analysis of cell cycle populations in MCF-7 cells and MDA-MB-231 cells after FSS treatment for 24 h. (g, h) Cell viability assay by CCK8 in MCF-7 (g) and MDA-MB-231 (h) under static culture or FSS treatment treated with 10 μM KU-55933 (KU), 5 μM VE-821 (VE), and 20 nM prexasertib (Prex) for 48 h. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus DMSO. ●, $p < 0.05$, and ●●●, $p < 0.001$ versus KU-55933 in static culture. △, $p < 0.05$ versus VE-821 in static culture. ○, $p < 0.05$ and ○○, $p < 0.01$ versus prexasertib in static culture. (i) Cell apoptosis and necrosis using YO-PRO-1/propidium iodide (PI) for staining FSS-treated or static MCF-7 cells and MDA-MB-231 cells that were cultured with 1 μg/mL CDDP. (j) FACS analysis of cell apoptosis and necrosis using YO-PRO-1/PI for staining FSS-cultured MCF-7 and MDA-MB-231 cells that were treated with 1 μg/mL CDDP combined with 1 μM VE-821 (VE) or 10 nM prexasertib (Prex).

and distant metastasis has always been a concern, therefore the distant metastasis contributed by lymph node metastasis via blood-derived metastasis has attracted attention. Recently, mice-based studies indicated that the active tumor cells in lymph node metastasis locus invade lymphatic vessels to generate CTCs and then cause distant metastasis.[37,38] At the same time, another study found that the tumor cells in distant metastasis and in lymph node metastasis do not have the same evolutionary lineage,[11] therefore the contribution of lymph node metastasis to distant metastasis needs further discussion. Based on the results that lymph node metastasis was related to the CTCs positivity,[8] we

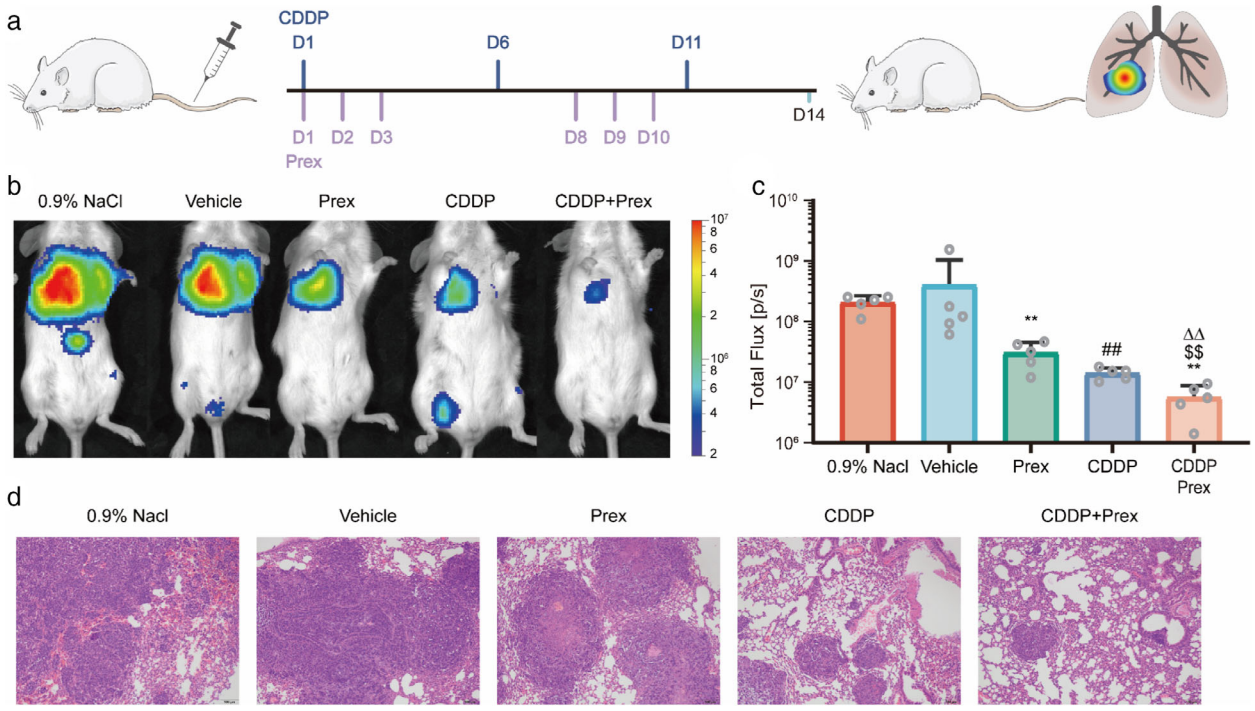

FIGURE 6 In vivo test for investigating the effect of the Chk1 inhibitor on the response of CDDP-induced CTC apoptosis. (a) Schematic of the test. 4T1 cells expressing luciferase were injected into the tail vein of Balb/c mice to generate single CTCs. Mice were treated with 0.9% NaCl, vehicle (5% DMSO, 40% PEG300, 5% Tween 80, and 50% ddH_2O), prexasertib (10 mg/kg) dissolved in vehicle (purple line at the bottom), CDDP (3 mg/kg) (blue line on the top), and the combination of prexasertib and CDDP. (b) Whole animal bioluminescent signals acquired after treatment. (c) Quantification analysis of the bioluminescent signals from the chests of mice. $^{**}p < 0.01$ versus vehicle and $\#\#p < 0.01$ versus 0.9% NaCl. \$\$, $p < 0.01$ versus prexasertib. $\triangle\triangle$, $p < 0.01$ versus CDDP. (d) HE staining images of lungs (×100) from tumor-bearing mice. Prex, prexasertib

hypothesized that lymph node metastasis could reflect the presence of CTCs which have the potential to generate metastases. To prove this, we characterized the CTCs from early-stage breast cancer patients with and without lymph node metastasis by scRNA-seq. The CTCs were clustered into two subtypes significantly correlated with lymph node status. CTCs from patients with lymph node metastasis are distributed in subtype 1, exhibiting the capability associated with metastasis potential such as proliferation, stemness, and immune escape. This illustrates that patients with lymph node metastasis are more likely to generate CTCs able to generate metastasis.

On the other hand, we did not find any significant difference in heterogeneity of CTCs between patients with LNM and patients with NLMN. We also observed the similar phenomena that the heterogeneity of CTCs is related to intrapatient tumor characteristics.[39] The heterogeneity of CTCs is well known[6,40,41] and is affected by its own evolution in addition to the tumor origin.[42,43] The strong heterogeneity of CTCs might therefore indicate that there are highly heterogeneous cells in the primary tumor.[42,44] Moreover, high intratumoral heterogeneity in the primary tumor was associated with a worse clinical prognosis and could thus reflect a high degree of tumor malignancy.[45] Hence, we believe that the heterogeneity of CTCs could reflect the clinical prognosis. In the present study, we detected strong heterogeneity of CTCs with metastatic ability in luminal A subtype breast cancer patients (P#4 and P#5). We also found ER^+ and ER^- CTC coexisting in the patients, implying that the patients might be at the risk of developing resistance to intrafraction therapy. Thus, the CTC characterization is helpful for tumor treatment, in particular for real-time monitoring of the response to various treatments.

Moreover, our results indicated that CTCs interact with the blood environment. The highly expressed platelet-related genes in CTCs tell us that CTCs might have the possibility of inducing platelet aggregation. Since transforming growth factor-beta (TGFβ) produced by platelets promotes the EMT of CTCs,[16] the lack of expression of TGFβ in CTCs is reasonable. EMT is an important biological process involved in the formation of CTCs and can endow epithelial tumor cells with the ability to invade.[46,47] As mesenchymal CTCs with strong stemness were found in metastatic cancer patients, completely mesenchymal CTCs are recognized as the main cause for metastasis.

However, recent studies have shown that the completely mesenchymal CTCs are difficult to generate metastases. CTCs with mixed epithelial-mesenchymal phenotypes are necessary for metastasis.[48,49] We found that CTCs in early breast cancer were the mixed epithelial-mesenchymal type, and only few CTCs expressed EMT transcription factors. Among the mixed epithelial-mesenchymal CTCs, pEMT CTCs expressed EGFR, HER2, CD44, CCND1, and NOTCH1 genes, and activated RAS/MAPK and PI3K/AKT

pathways. This suggests that pEMT is a stable phenotype, which make CTCs better adapt to the environment for completing the colonization of distant locus. pEMT CTCs might therefore be precursors to metastasis in early-stage breast cancer and be the predominant medium of metastasis.

In addition, surviving in the blood circulation gives CTCs more potential to form metastases. We found anti-apoptotic pathways in CTCs specifically in subtype 1, including immune escape, antioxidation, and DNA damage repair. Tumor cells surviving in a simulated blood environment can survive under antioxidant and DNA damage repair conditions. Current treatment strategies for cancer aim at the rapid proliferation of tumor cells, but CTCs could not proliferate because the influence of the blood environment.[50] Induction of CTC death might therefore be an essential way to prevent metastasis formation. We proposed that stimulation of oxidative stress and inhibition of DNA damage repair could be a way to inhibit the survival of CTCs. Antioxidant and cell cycle checkpoint therapy may not realize an effective tumor treatment for now due to oxidative stress and DNA damage having a bidirectional impact on tumor cells,[51,52] but it could become a new therapeutic target based on personalized treatment in the future.

We should emphasize that the number of CTCs we collected in the present study was limited and thus it was difficult to classify CTCs into more refined subgroups. This limitation was mainly due to the low detection rate of CTCs originating from nonmetastatic breast cancer and the relatively low rate of obtaining intact CTCs. Although our observations need to be validated in prospective trials, the results from a single-cell transcriptomic profile of CTCs of early breast cancer do provide us with novel insights into understanding CTC behavior in breast cancer metastasis.

AUTHOR CONTRIBUTIONS

All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization: S.Y.P., B.J.Z., and G.H.Z. Data curation: S.Y.P. Methodology: S.Y.P., S.X., and B.J.Z. Investigation: S.Y.P., S.X., and L.L.W. Formal analysis: S.Y.P. Resources: Y.J.L, H.P.W., X.P.M., and Y.N.C. Writing – original draft: S.Y.P., S.X., and B.J.Z. Writing – review and editing: B.J.Z., S.H.W., and G.H.Z. Visualization: S.Y.P. Supervision: G.H.Z. Funding acquisition: S.X. and Y.J.L.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (61871403, 82001422, 82150209, 32071440) and the Natural Science Foundation of Jiangsu Province Youth Project (BK20200274).

DATA AVAILABILITY STATEMENT

All data needed to evaluate the conclusions in the paper are presented in the paper and/or the Supporting Information. Additional data related to this paper may be requested from the authors.

DISCLOSURE

The funding organization(s) played no role in the study design, the collection, analysis, and interpretation of data, the writing of the report, or the decision to submit the report for publication.

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REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: Cancer J Clin. 2021;71(3):209–49.
2. DeSantis C, Siegel R, Bandi P, Jemal A. Breast cancer statistics, 2011. CA: Cancer J Clin. 2011;61(6):409–18.
3. Redig AJ, McAllister SS. Breast cancer as a systemic disease: a view of metastasis. J Intern Med. 2013;274(2):113–26.
4. Labelle M, Hynes RO. The initial hours of metastasis: the importance of cooperative host-tumor cell interactions during hematogenous dissemination. Cancer Discovery. 2012;2(12):1091–9.
5. Massagué J, Obenauf AC. Metastatic colonization by circulating tumour cells. Nature. 2016;529(7586):298–306.
6. Castro-Giner F, Aceto N. Tracking cancer progression: from circulating tumor cells to metastasis. Genome Med. 2020;12(1):31.
7. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004;351(8):781–91.
8. Rack B, Schindlbeck C, Jückstock J, Andergassen U, Hepp P, Zwingers T, et al. Circulating tumor cells predict survival in early average-to-high risk breast cancer patients. J Natl Cancer Inst. 2014;106(5):dju066.
9. Brechbuhl HM, Vinod-Paul K, Gillen AE, Kopin EG, Gibney K, Elias AD, et al. Analysis of circulating breast cancer cell heterogeneity and interactions with peripheral blood mononuclear cells. Mol Carcinog. 2020;59(10):1129–39.
10. Cheng YH, Chen YC, Lin E, Brien R, Jung S, Chen YT, et al. Hydro-Seq enables contamination-free high-throughput single-cell RNA-sequencing for circulating tumor cells. Nat Commun. 2019;10(1):2163.
11. Ullah I, Karthik G-M, Alkodsi A, Kjällquist U, Stålhammar G, Lövrot J, et al. Evolutionary history of metastatic breast cancer reveals minimal seeding from axillary lymph nodes. J Clin Invest. 2018;128(4):1355–70.
12. Matsunaga H, Goto M, Arikawa K, Shirai M, Tsunoda H, Huang H, et al. A highly sensitive and accurate gene expression analysis by sequencing ("bead-seq") for a single cell. Anal Biochem. 2015;471:9–16.
13. Huang H, Goto M, Tsunoda H, Sun L, Taniguchi K, Matsunaga H, et al. Non-biased and efficient global amplification of a single-cell cDNA library. Nucleic Acids Res. 2014;42(2):e12.
14. Wen CY, Wu LL, Zhang ZL, Liu YL, Wei SZ, Hu J, et al. Quick-response magnetic nanospheres for rapid, efficient capture and sensitive detection of circulating tumor cells. ACS Nano. 2014;8(1):941–9.
15. Pang S, Li H, Xu S, Feng L, Ma X, Chu Y, et al. Circulating tumour cells at baseline and late phase of treatment provide prognostic value in breast cancer. Sci Rep. 2021;11(1):13441.
16. Liu Y, Zhang Y, Ding Y, Zhuang R. Platelet-mediated tumor metastasis mechanism and the role of cell adhesion molecules. Crit Rev Oncol Hematol. 2021;167:103502.
17. Teicher BA, Fricker SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. Clin Cancer Res. 2010;16(11):2927–31.
18. Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature. 2001;410(6824):50–6.

19. Jin F, Brockmeier U, Otterbach F, Metzen E. New insight into the SDF-1/CXCR4 axis in a breast carcinoma model: hypoxia-induced endothelial SDF-1 and tumor cell CXCR4 are required for tumor cell intravasation. Mol Cancer Res. 2012;10(8):1021–31.
20. Zheng Y, Miyamoto DT, Wittner BS, Sullivan JP, Aceto N, Jordan NV, et al. Expression of β-globin by cancer cells promotes cell survival during blood-borne dissemination. Nat Commun. 2017;8: 14344.
21. Ma S, Fu A, Lim S, Chiew GGY, Luo KQ. MnSOD mediates shear stress-promoted tumor cell migration and adhesion. Free Radical Biol Med. 2018;129:46–58.
22. Kamarajugadda S, Cai Q, Chen H, Nayak S, Zhu J, He M, et al. Manganese superoxide dismutase promotes anoikis resistance and tumor metastasis. Cell Death Dis. 2013;4(2):e504.
23. Ebright RY, Lee S, Wittner BS, Niederhoffer KL, Nicholson BT, Bardia A, et al. Deregulation of ribosomal protein expression and translation promotes breast cancer metastasis. Science. 2020; 367(6485):1468–73.
24. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74.
25. Giuliano M, Shaikh A, Lo HC, Arpino G, De Placido S, Zhang XH, et al. Perspective on circulating tumor cell clusters: why it takes a village to metastasize. Cancer Res. 2018;78(4):845–52.
26. Park H-A, Brown SR, Kim Y. Cellular mechanisms of circulating tumor cells during breast cancer metastasis. Int J Mol Sci. 2020;21 (14):5040.
27. Srinivas US, Tan BWQ, Vellayappan BA, Jeyasekharan AD. ROS and the DNA damage response in cancer. Redox Biol. 2019;25:101084.
28. Jordan NV, Bardia A, Wittner BS, Benes C, Ligorio M, Zheng Y, et al. HER2 expression identifies dynamic functional states within circulating breast cancer cells. Nature. 2016;537(7618):102–6.
29. Rueda OM, Sammut S-J, Seoane JA, Chin S-F, Caswell-Jin JL, Callari M, et al. Dynamics of breast-cancer relapse reveal late-recurring ER-positive genomic subgroups. Nature. 2019;567(7748): 399–404.
30. Lang JE, Ring A, Porras T, Kaur P, Forte VA, Mineyev N, et al. RNA-Seq of circulating tumor cells in stage II-III breast cancer. Ann Surg Oncol. 2018;25(8):2261–70.
31. Moose DL, Krog BL, Kim T-H, Zhao L, Williams-Perez S, Burke G, et al. Cancer cells resist mechanical destruction in circulation via RhoA/actomyosin-dependent mechano-adaptation. Cell Rep. 2020; 30(11):3864–74.
32. Chaturvedi P, Singh AP, Chakraborty S, Chauhan SC, Bafna S, Meza JL, et al. MUC4 mucin interacts with and stabilizes the HER2 oncoprotein in human pancreatic cancer cells. Cancer Res. 2008;68(7): 2065–70.
33. Chen S-H, Dallas MR, Balzer EM, Konstantopoulos K. Mucin 16 is a functional selectin ligand on pancreatic cancer cells. FASEB J. 2012; 26(3):1349–59.
34. Karacosta LG, Anchang B, Ignatiadis N, Kimmey SC, Benson JA, Shrager JB, et al. Mapping lung cancer epithelial-mesenchymal transition states and trajectories with single-cell resolution. Nat Commun. 2019;10(1):5587.
35. Papadaki MA, Koutsopoulos AV, Tsoulfas PG, Lagoudaki E, Aggouraki D, Monastirioti A, et al. Clinical relevance of immune checkpoints on circulating tumor cells in breast cancer. Cancers. 2020; 12(2):376.
36. de Boer M, van Dijck JAAM, Bult P, Borm GF, Tjan-Heijnen VCG. Breast cancer prognosis and occult lymph node metastases, isolated tumor cells, and micrometastases. J Natl Cancer Inst. 2010;102(6): 410–25.
37. Brown M, Assen FP, Leithner A, Abe J, Schachner H, Asfour G, et al. Lymph node blood vessels provide exit routes for metastatic tumor cell dissemination in mice. Science. 2018;359(6382):1408–11.
38. Pereira ER, Kedrin D, Seano G, Gautier O, Meijer EFJ, Jones D, et al. Lymph node metastases can invade local blood vessels, exit the node, and colonize distant organs in mice. Science. 2018;359(6382):1403–7.
39. Miyamoto DT, Zheng Y, Wittner BS, Lee RJ, Zhu H, Broderick KT, et al. RNA-Seq of single prostate CTCs implicates noncanonical Wnt signaling in antiandrogen resistance. Science. 2015;349(6254):1351–6.
40. Keller L, Pantel K. Unravelling tumour heterogeneity by single-cell profiling of circulating tumour cells. Nat Rev Cancer. 2019;19(10): 553–67.
41. Stewart CA, Gay CM, Xi Y, Sivajothi S, Sivakamasundari V, Fujimoto J, et al. Single-cell analyses reveal increased intratumoral heterogeneity after the onset of therapy resistance in small-cell lung cancer. Nat Cancer. 2020;1:423–36.
42. Lawson DA, Kessenbrock K, Davis RT, Pervolarakis N, Werb Z. Tumour heterogeneity and metastasis at single-cell resolution. Nat Cell Biol. 2018;20(12):1349–60.
43. Zhang C, Guan Y, Sun Y, Ai D, Guo Q. Tumor heterogeneity and circulating tumor cells. Cancer Lett. 2016;374(2):216–23.
44. Tellez-Gabriel M, Heymann MF, Heymann D. Circulating tumor cells as a tool for assessing tumor heterogeneity. Theranostics. 2019;9(16): 4580–94.
45. Childs A, Steele CD, Vesely C, Rizzo FM, Ensell L, Lowe H, et al. Whole-genome sequencing of single circulating tumor cells from neuroendocrine neoplasms. Endocr Relat Cancer. 2021;28(9):631–44.
46. Tiwari N, Gheldof A, Tatari M, Christofori G. EMT as the ultimate survival mechanism of cancer cells. Semin Cancer Biol. 2012;22(3):194–207.
47. Tsai JH, Yang J. Epithelial-mesenchymal plasticity in carcinoma metastasis. Genes Dev. 2013;27(20):2192–206.
48. Fischer KR, Durrans A, Lee S, Sheng J, Li F, Wong STC, et al. Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. Nature. 2015;527(7579):472–6.
49. Xu Y, Lee D-K, Feng Z, Xu Y, Bu W, Li Y, et al. Breast tumor cell-specific knockout of inhibits cancer cell plasticity, dissemination, and lung metastasis in mice. Proc Natl Acad Sci U S A. 2017;114(43):11494–9.
50. Cheung KJ, Padmanaban V, Silvestri V, Schipper K, Cohen JD, Fairchild AN, et al. Polyclonal breast cancer metastases arise from collective dissemination of keratin 14-expressing tumor cell clusters. Proc Natl Acad Sci U S A. 2016;113(7):E854–63.
51. Qiu Z, Oleinick NL, Zhang J. ATR/CHK1 inhibitors and cancer therapy. Radiother Oncol. 2018;126(3):450–64.
52. Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. Nat Rev Drug Discovery. 2013;12(12):931–47.

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: Pang S, Xu S, Wang L, Wu H, Chu Y, Ma X, et al. Molecular profiles of single circulating tumor cells from early breast cancer patients with different lymph node statuses. Thorac Cancer. 2023;14(2):156–67. https://doi.org/10.1111/1759-7714.14728