Potentiated DNA Damage Response in Circulating Breast Tumor Cells Confers Resistance to Chemotherapy

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Background: Circulating tumor cells (CTCs) are responsible for cancer metastasis and predict prognosis for breast cancer. Whether CTCs and primary tumor cells (PTCs) respond to chemotherapy differently is not known. Here, we show that CTCs of breast cancer are more resistant to chemotherapy than PTCs.

Results: CTCs repair DNA damage more efficiently than primary tumor cells (PTCs) due to checkpoint pre-activation.

Conclusion: CTCs are more resistant to chemotherapy than PTCs.

Significance: Inhibition of DNA checkpoints may reverse chemoresistance in CTCs.

Circulating tumor cells (CTCs) are seeds for cancer metastasis and are predictive of poor prognosis in breast cancer patients. Whether CTCs and primary tumor cells (PTCs) respond to chemotherapy differently is not known. Here, we show that CTCs of breast cancer are more resistant to chemotherapy than PTCs because of potentiated DNA repair. Surprisingly, the chemoresistance of CTCs was recapitulated in PTCs when they were detached from the extracellular matrix. Detachment of PTCs increased the levels of reactive oxygen species and partially activated the DNA damage checkpoint, converting PTCs to a CTC-like state. Inhibition of checkpoint kinases Chk1 and Chk2 in CTCs reduces the basal checkpoint response and sensitizes CTCs to DNA damage in vitro and in mouse xenografts. Our results suggest that DNA damage checkpoint inhibitors may benefit the chemotherapy of breast cancer patients by suppressing the chemoresistance of CTCs and reducing the risk of cancer metastasis.

Circulating tumor cells (CTCs), detached from primary tumors and still alive in circulation (1–3), are suggested to be potential seeds for hematogenous cancer metastasis (4, 5). CTCs from breast cancer patients have been shown to be able to initiate metastasis in a xenograft assay (6). In clinics, enumeration and phenotyping of CTCs have emerged as novel biomarkers to estimate the risk for metastatic relapse or disease progression in various types of cancers, including breast cancer (7, 8). Several recent studies have shown that advanced cancer patients with CTC counts remaining high after systemic anti-cancer therapy have poor clinical outcome (9–13), suggesting that the chemotherapy sensitivity of CTCs may reflect the efficiency of systemic therapy (14).

Accumulating evidence has suggested that CTCs are biologically different from primary tumor cells (PTCs). It was reported that the gene expression profile of CTCs from patients with metastatic colorectal cancers was different from that of PTCs by genes related to enhanced migratory, invasive, adhesive, and anti-apoptotic properties (15, 16). In addition, differential HER2 gene amplification and expression were also noted between CTCs and PTCs in breast cancer patients, which may underlie their different sensitivity to anti-Her2 therapy (17, 18). Moreover, CTCs from breast cancer exhibit migratory mesenchymal or stem-cell like phenotypes that are rarely detected in PTCs (19, 20). Genome sequencing of CTC revealed pre-existing mutations in the PIK3CA gene and newly acquired mutations in the genes of estrogen receptor (ESR1), PIK3CA, and fibroblast growth factor receptor 2, etc. (21). In light of their distinct biological features, CTCs may respond differently to chemotherapy.
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chemotherapies as do PTCs. Recently, it was reported that CTCs were sensitive to paclitaxel and resistant to doxorubicin upon exposure to a panel of drugs (21). However, the chemosensitivity of CTCs has not been well characterized, and the mechanisms that underlie differential chemoresistance of CTCs as compared with PTCs remain elusive.

The distinct biology of CTCs may result from two different mechanisms. CTCs could be enriched from a minority of PTCs that are intrinsically resistant to chemotherapy. Also, the detachment of cancer cells from primary tumors, especially from ECM support, may lead to dramatic biological changes by undergoing excessive stress that induces either anoikis-associated apoptosis (22) or an oxidative burst with massive production of various reactive oxygen species (ROS) (23). Therefore, in response to detachment stress, CTCs that remain alive may gain survival advantage and are thus more resistant to chemotherapy than PTCs.

In this study, we compared the chemosensitivity of CTCs with that of PTCs from metastatic breast cancer patients. Furthermore, we evaluated whether detachment of PTCs may recapitulate drug resistance in CTCs and investigated the underlying mechanisms.

Experimental Procedures

Patients and Clinical Specimens—Blood and primary tumor samples were obtained from 60 cases of patients with metastatic breast cancer at Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University. Pathological diagnosis, including estrogen receptor, PR, and Her2 status of primary tumors, was verified by two different pathologists. Among these patients, 55 cases underwent chemotherapy (epirubicin, 90 mg/m²; paclitaxel, 75 mg/m²; and cyclophosphamide, 600 mg/m³). All patient samples with informed consents were collected according to the legal mandates and the Ethics Boards of the Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University.

Enumeration and Phenotypic Assessment of CTC—Enumeration of CTC in the whole blood of breast cancer patients was done in the CellSearch® system according to the manufacturer’s instruction as described previously (7). Apoptotic CTCs were detected by incorporating CTC assay with specific mAbs, M30 CytoDEATH™ fluorescein (ALX-804-590, Alexis Biochem), and analyzed with the fourth filter of the CellSearch® system; data were expressed as the percentage of apoptotic CTCs per 7.5 ml of blood.

Isolation of CTCs—To isolate human CTCs (24), 20–50 ml of fresh blood from metastatic breast cancer patients was layered over Ficoll–Paque (1.077, density) and centrifuged at 400 g for 2 h at room temperature. The interphase cells, containing lymphocytes, monocytes, and tumor cells, were resuspended at 5 × 10⁷ cells in 300 μl of solution containing 100 μl of FcR blocking reagent (130-059-901, Miltenyi Biotec, Bergisch Gladbach, Germany), 100 μl of CD45 microbeads (130-045-801, Miltenyi Biotec), and 100 μl of CD15 microbeads (130-091-058, Miltenyi Biotec). After depletion of CD45⁺ and CD15⁺ cells by magnetic separation with autoMACS™ Pro Separator, 100 μl of CD326 (EpCAM) microbeads (130-095-500, Miltenyi Biotec) per 5 × 10⁷ cells was added for 30 min of incubation at 4 °C. The magnetic CD326⁺ and CD326⁻ cell fractions were eluted as the EpCAM⁺ and EpCAM⁻ CTCs. The purity of epithelial cells was determined by immunofluorescent staining with anti-cytokeratin antibody (10 μg/ml, ab41825, Abcam), which was over 95%. For a cell to be identified as a CTC, it had to meet two criteria as follows: (i) positive staining for a tumor-specific marker by immunocytochemistry (cytokeratin) and (ii) positive scoring upon review by the cytopathologist.

Mouse CTCs were obtained from the blood specimens of the animals by cardiac puncturing as described previously (25). Nucleated cells from mouse blood were enriched in erythrocytes lysis buffer and the epithelial cells were isolated by magnetic antibody cell separation (Miltenyi Biotec) and resuspended in 0.1 ml of PEB (1 × PBS, 0.1 mM EDTA, and 0.5% BSA fraction V) (Sigma) as described previously.

Cell Cultures and Treatment—MCF-7 and MDA-MB-231 breast cancer cell lines were obtained from the American Type Culture Collection (ATCC) and cultured according to the manufacturer’s instruction. PTCs were isolated from breast cancer samples, as described previously (26), and were maintained in RPMI 1640 medium (Gibco) supplemented with 10% serum. Suspension culture was performed in the Ultra Low Attachment Surface Products (Corning, NY). After testing a range of culture conditions, we found that CTCs proliferated better as tumor spheres when cultured in ultralow attachment plates (Corning) containing tumor sphere medium consisting of RPMI 1640 medium (with phenol red) supplemented with EGF (20 ng/ml, Life Technologies, Inc.), basic FGF (20 ng/ml, Life Technologies, Inc.), B27 (10 ml, Life Technologies, Inc.), and 1× antibiotic/antimycotic (Life Technologies, Inc.). Cells were cultured in a humid 37 °C incubator with 5% CO₂. Our CTC culture condition is similar as described previously (21).

For in vitro chemotherapy, cells were exposed to epirubicin (1 μg/ml, Sigma) or cisplatin (10 μg/ml, Sigma) for 2 h and were placed in fresh media for another 1–2 h for recovery after stringent washing, followed by evaluation of cell survival and apoptosis. In some experiments, Chk1 inhibitor (Chir-124, 250 nm, Selleckchem), Chk2 inhibitor (Chk2 inhibitor II, 10 μM, Sigma), Chk1/2 inhibitor (debrombomohymanidislisine (DBH), 3 μM, Calbiochem), N-acetylcysteine (NAC, 10 mM, Sigma), or nordinhydroguaiatic acid (NDGA, 5 μM, Sigma) was added to the cultures 2 h prior to chemotherapy.

Tumor Xenografts—All procedures of animal experiments were approved by the Animal Care and Use Committee of Sun Yat-Sen University and conformed to the legal mandates and national guidelines for the care and maintenance of laboratory animals. 2 × 10⁶ MDA-MB-231 cells stably expressing luciferase or enhanced GFP were injected into the mammary fat pads of 6-week-old BALB/c-nu mice. After tumors were detected, tumor size was measured in two dimensions with calipers, and tumor volume was calculated as length (mm) × width (mm) × depth (mm) × 0.5 up to 60 days. Treatment began when tumors reached ~0.5 cm. Intravenous injection with or without the Chk1 inhibitor (Chir-124, 10 mg/kg) (Selleckchem) or Chk2 inhibitor (Chk2 inhibitor II, 1 mg/kg) (Sigma) 2 h preceding intravenous injection with cisplatin (5 mg/kg) was performed in a weekly cycle. Mice were subjected to a total of four cycles of therapy. To obtain CTC in the mice, approxi-
mately, 0.8-ml blood samples were obtained via cardiac puncture from mice anesthetized with 1.5% isoflurane for CTC isolation after the above-mentioned treatment. To evaluate *in vivo* metastasis, mice were anesthetized and given 150 mg/kg of D-luciferin in PBS by intraperitoneal injection for bioluminescence imaging with a charge-coupled device camera (IVIS; Xenogen). Paraffin sections (4 μm) of the lung and liver tissues were used for immunohistochemistry with PCNA antibody (sc-7907, 1:200, Santa Cruz Biotechnology). Briefly, immunohistochemistry was performed on paraffin sections according to standard LSAB protocol (Dako, Carpinteria, CA), using primary antibodies against PCNA antibody. Isotype-matched IgG was used as negative controls. The percentages of positive tumor cells were calculated per field of view, with at least 20 viewfields per section evaluated at \( \times 400 \) magnification.

**Statistical Analysis**—The cumulative changes of live and apoptotic CTCs were expressed as described previously (27) by the parameter ΔAUC; the detected numbers of M30-negative and M30-positive CTCs were separately plotted in relation to time, and the area under the curve (AUC) of longitudinal graphs was calculated (by the trapezoidal rule) following a procedure that is commonly adopted to evaluate cumulative changes of serological tumour markers. The difference between live and apoptotic CTC concentration-time area was calculated in all patients according to Equation 1,

\[
\Delta AUC = M30_{\text{negative CTC AUC}} - M30_{\text{positive CTC AUC}} \quad \text{(Eq. 1)}
\]

Progression-free survival was measured as the time between the baseline CTC assessment (*i.e.* the initiation of treatment) and the documentation of the first radiographic disease progression or death. Patients who were alive and progression-free at the time of analysis were censored by using the time between the baseline CTC assessment and their most recent follow-up evaluations. The progression-free survival between groups defined by \( < = 0 \) or \( > 0 \) ΔAUC was compared with the Kaplan-Meier method, and differences were tested with the log-rank test.

Other results are expressed as mean values ± S.D., and mean values of at least three experiments are shown. Statistical analysis was done by one-way analysis of variance, and comparisons among groups were done by the independent sample \( t \)-test or Bonferroni multiple comparison \( t \)-test, using SPSS for Windows version 13.0 (SPSS, Chicago). A \( p \) value <0.05 in all cases was considered statistically significant.

**Results**

**CTCs Are More Resistant to Chemotherapy than PTCs**—Although CTCs from breast cancer may have undergone dynamic epithelial mesenchymal transition characterized by down-regulation of epithelial markers, they can be captured by antibody mixtures with EpCAM or EGF receptor (19, 20), plus depletion of leukocyte by immunomagnetically targeting both the common leukocyte antigen CD45 and the granulocyte marker CD15 (24). In this study, we used magnetic beads coupled with anti-EpCAM antibody for positive selection, and beads with anti-CD45 and anti-CD15 antibodies for negative selection to isolate CTCs from 50 ml of peripheral blood from five patients with metastatic breast carcinomas, in which CTC enumerations were over 530 cells/ml of blood determined by CellSearch® system (Table 1). The corresponding PTCs were isolated from tumor biopsies of the same patients. The isolated CTCs and PTCs were treated with the following two commonly used chemotherapeutic drugs for breast cancer, respectively: epirubicin at 1 μg/ml or cisplatin at 10 μg/ml for 2 h, and then recovered in fresh media for another 2 h, followed by evaluation of cell survival and apoptosis. Treatment with epirubicin or cisplatin reduced the colony formation of PTCs in soft agar by ~87% (\( p < 0.001 \)) and 89% (\( p < 0.001 \)), respectively, and in CTCs by only 11.4% (\( p > 0.05 \)) and 17.3% (\( p > 0.05 \)), respectively (Fig. 1A), suggesting that CTCs are more resistant to the growth inhibition of chemotherapy *in vitro*. To evaluate apoptotic cell death of CTCs and PTCs treated with epirubicin or cisplatin, we examined the expression of M30 determined by immunostaining. In contrast to PTCs that showed increased M30 immunostaining after treatment with epirubicin or cisplatin, we only slightly enhanced (Fig. 1B). Together, these data suggest that CTCs are more resistant to the cytotoxic effect of chemotherapy *in vitro*.

To evaluate the clinical relevance of the above findings, we examined the apoptosis of CTCs and PTCs that were freshly isolated from 55 metastatic breast cancer patients (Table 2), before and after four cycles of chemotherapy. Chemotherapy increased the percentage of M30+ PTCs by 3.5-fold (\( p < 0.01 \), Fig. 1C) in the biopsies taken from primary tumors but increased M30+ CTCs only by 1.6-fold (\( p < 0.05 \), Fig. 1C). Next, we further correlated the changes of apoptotic M30+ CTCs and PTCs upon chemotherapy with the progression of distant metastasis determined by computerized tomography scanning or magnetic resonance imaging (Fig. 1D). Although all the patients showed markedly increased proportion of M30+ PTCs in the primary tumors following chemotherapy (Fig. 1D), only the patients responded to chemotherapy (PR + SD) showed a significant increase in the percentage of M30+ CTCs (Fig. 1D), whereas the nonresponders (PD) demonstrated a stable M30+ CTC level (Fig. 1D and Table 2). In addition, we correlated the cumulative changes of live/apoptotic CTCs (27) with patient.

**TABLE 1**

| Case no. | Primary tumor site | Pathological classification | ER | PR | Metastatic sites | Grade | CTC counts |
|----------|-------------------|-----------------------------|----|----|----------------|-------|------------|
| 1        | 50                | IDC                         | +  | −  | Brain, liver    | III   | 8785       |
| 2        | 48                | IDC                         | −  | −  | Liver, bone     | III   | 7585       |
| 3        | 54                | IDC                         | −  | −  | Liver, bone     | III   | 5785       |
| 4        | 42                | IDC                         | +  | −  | Liver, lung     | III   | 4885       |
| 5        | 40                | IDC                         | +  | −  | Liver, lung     | III   | 3985       |

*Quantification was by CellSearch® system (Veridex LLC, Raritan, NJ) per 7.5 ml of blood.

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survival and found that live CTCs were associated with poor survival (Fig. 1E and Table 2). Therefore, in agreement with the in vitro findings, CTCs in vivo are more resistant to chemother-apy-induced cell death.

Detachment of PTCs from ECM recapitulates the chemoresistance of CTCs—CTCs differ from PTCs by losing attachment to ECM that supports tumor cells in the stroma of breast cancer. It has been demonstrated that nonadherent culture conditions were critical for CTC proliferation ex vivo (21). Therefore, we investigated whether detachment from ECM may recapitulate chemoresponse of breast cancer CTCs by comparing the chemosensitivity of breast cancer cells in suspension versus in adherent cultures. Primary breast cancer cells were isolated from eight breast cancer samples and plated in suspension culture or adherent culture (Table 3). Cells were treated with epirubicin at 1 μg/ml or cisplatin at 10 μg/ml for 2 h before being plated for soft agar assay. The colony formation of cancer cells in adherent culture was tremendously reduced (p < 0.001) but that of cancer cells in suspension culture was only slightly reduced (p < 0.05, Fig. 2A and B). Based on CellTiter-Glo assay that detects cell viability by quantifying the presence of ATP, suspended primary breast cancer cells and MDA-MB-231 cells were more resistant to epirubicin and cisplatin than adherent cells, with a 3.1-fold increase in IC50 for epirubicin (p < 0.001, Fig. 2C) and a 3.9-fold increase for cisplatin (p < 0.001, Fig. 2D). The similar results were found in MDA-MB-231 cells (p < 0.001, Fig. 2D).

We further evaluated whether suspended breast cancer cells are resistant to chemotherapy-induced apoptosis. Determined by annexin V staining, treatment with cisplatin at 10 μg/ml
increased the apoptosis of adherent primary breast cancer cells by 7.5-fold ($p < 0.001$, Fig. 2E) but only by 1.8-fold ($p > 0.05$, Fig. 2E) in suspended cells. The similar results were found in MDA-MB-231 cells ($p < 0.001$, Fig. 2F). Assays for caspase 3 activity also showed that epirubicin and cisplatin dramatically enhanced caspase 3 activity in adherent PTCs and MDA-MB-231 cells but not in the suspended ones (Fig. 2, G and H).

It was reported that CTCs displayed markers of cancer stem cells (CSCs) (28); however, there is no increased expression of stem cell-related signatures in CTC cultures (21), so we explored the possibility that CTCs may resist chemotherapy through increased expression of ABCG2, an ABC transporter responsible for drug efflux in CSCs (26, 29). CSCs were isolated from the primary tumors of six metastatic breast cancer patients using FACS sorting with CD44+ (CD24−) phenotype (26), and CTCs from the same patients were also obtained. In contrast to the CSCs from primary tumors with enhanced epirubicin efflux, CTCs did not exclude more epirubicin than the non-CSC portion of PTCs (Fig. 2I). Quantitative RT-PCR also demonstrated that ABCG2 expression in CTCs, but not CTCs, was considerably increased as compared with non-CTCs (Fig. 2I), suggesting that the mechanisms for chemoresistance in CTCs is different from that in CSCs.
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Table 3: CTC counts and clinicopathological features in eight metastatic breast cancer patients

| Case no. | Age  | Tumor size | Pathological classification | ER | PR | Metastatic sites | Grade | CTC counts |
|----------|------|------------|-----------------------------|----|----|----------------|-------|------------|
| 1        | 45   | 3.6        | IDC                         | +  | −  | Lung, brain     | III   | 62         |
| 2        | 38   | 4.2        | IDC                         | −  | −  | Brain, bone     | III   | 53         |
| 3        | 44   | 3.6        | IDC                         | +  | −  | Liver, bone     | III   | 73         |
| 4        | 40   | 4.1        | IDC                         | +  | −  | Liver          | III   | 57         |
| 5        | 51   | 5.2        | IDC                         | +  | −  | Liver          | III   | 46         |
| 6        | 38   | 4.5        | IDC                         | +  | +  | Lung, bone     | III   | 70         |
| 7        | 40   | 3.5        | IDC                         | +  | −  | Liver, bone     | III   | 73         |
| 8        | 58   | 4.3        | IDC                         | +  | −  | Liver          | III   | 68         |

*a Quantification was by CellSearch® system per 7.5 ml of blood.

Chemotherapy-induced DNA Damage Is Efficiently Repaired in CTCs—DNA damage is an essential mechanism that a large group of chemotherapeutic drugs exert their cytotoxic effects on cancer cells. DNA-damaging drugs, including doxorubicin, epirubicin, cis-, and carboplatin (30), generate DNA double strand break (DSB), although efficient DNA repair may confer resistance to the cytotoxicity of these drugs in tumor cells (30).

It has been reported that breast CTCs were resistant to doxorubicin, a DNA-damaging agent, but sensitive to olaparib, a DNA-damaging drug, which may lead to mild DNA damage (32). It has been suggested that efficient DNA damage repair in CTCs is due to detachment.

We have also compared the efficiency of DNA damage repair in CSC or non-CSC portions of PTC with that in CTCs. Whereas 15 min of drug exposure resulted in less DNA damage in CSCs compared with non-CSCs, CTCs showed a similar level of DNA damage with non-CSCs, and DNA damage was not efficiently repaired in either non-CSCs or CSCs at 1–2 h following chemotherapy (Fig. 3E). These results suggest that enhanced efficiency of DNA damage repair in CSCs after chemotherapy is due to detachment.

Elevated ROS in CTCs Partially Activates DNA Damage Response—It has been reported that loss of matrix attachment induces oxidative stress in mammary epithelial cells by increasing the production of ROS (23), which may lead to mild DNA damage (32). In this context, CTCs may have increased production of ROS due to detachment and thus result in higher basal levels of DNA damage and pre-activation of DNA checkpoints. Indeed, ROS production in the CTCs from metastatic breast cancer patients, detected by cell-permeating carboxy-H₂DCFDA (5-(and-6)-carboxy-2',7’-dichlorodihydrofluorescein diacetate), was significantly higher as compared with PTCs from the same patients (p < 0.01, Fig. 4A and Table 1), suggesting that higher ROS production in CTCs may result from detachment. To further evaluate the oxidative DNA lesions in breast cancer cells, we examined the accumulation of 8-oxo-dG, which are the most abundant oxidized DNA bases under oxidative stress. The signal of 8-oxo-dG was more intense in the nuclei of CTCs than that of PTCs from patients (Fig. 4B and Table 1). We then checked whether this accumulation of ROS in CTC or suspended breast cancer cells was due to increased levels of antioxidant proteins, including thioredoxin 1 and thioredoxin 2. To our surprise, compared with the adherent MCF-7, the expression of both antioxidant proteins, thioredoxin 1 and thioredoxin 2, had increased in the sus-
pended MCF-7 cells (Fig. 4C). The mRNA level of thioredoxin in CTCs was also higher than that in PTCs (Fig. 4D). This may be explained by rapid expression of antioxidant proteins induced by elevated basal ROS in suspended cells. Therefore, higher ROS production in CTCs due to detachment is associated with higher basal levels of oxidative DNA damage.
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FIGURE 2. Detachment from ECM induces chemoresistance in breast cancer cells. A and B, anchorage-independent colony formation in primary cancer cells (PTC) (A) or MDA-MB-231 cells (B) treated with increasing concentrations of epirubicin and cisplatin for 2 h was determined by soft agar assays. Untreated cells were set as 100%. Bars correspond to mean ± S.D. *, p < 0.05; **, p < 0.01, and ***, p < 0.001 versus untreated cells. C and D, CellTiter-Glo assays for PTC (C) or MDA-MB-231 cells (D) treated as in A. Bars correspond to mean ± S.D. Untreated cells were set as 100%. ***, p < 0.001 versus adherent cells. E and F, annexin V− cells in PTC (E) or MDA-MB-231 cells (F) without treatment, or withdrawal of cisplatin for 1 h (1hR) or 2 h (2hR). Bars correspond to mean ± S.D. *, p < 0.05; **, p < 0.01, and ***, p < 0.001 versus suspended cells. G and H, caspase 3 activity in PTC (G) or MDA-MB-231 cells (H) treated as in A. RU, relative fluorescent unit. Bars correspond to mean ± S.D. **, p < 0.01, and ***, p < 0.001 versus suspended cells. I, capacity of absorbing epirubicin determined by FAM. J, quantitative RT-PCR analysis for ABCG2 expression normalized to β-actin.

FIGURE 3. Chemotherapy-induced DNA damages are more efficiently repaired in CTCs than in PTCs. A, γ-H2AX (Ser-139) staining in CTC and PTC without treatment or withdrawal of epirubicin for 1 h (1hR) or 2 h (2hR). B and C, DNA damage tested by comet assay in CTC and PTCs (B) or PTCs (C) treated in suspended (s) or adherent (a) culture. Tail length was used to measure the extent of DNA damage. Bars correspond to mean ± S.D. *, p < 0.05; **, p < 0.01, and ***, p < 0.001 versus untreated cells. D, average level of γ-H2AX (Ser-139) quantified by FAM in PTC treated as in A. *, p < 0.05, and ***, p < 0.001 versus untreated cells. MFI, median fluorescence intensity. E, DNA damage detected by comet assay in CSC, non-CSC, CTC, and CTCs cultured on ECM for 6 h (attached CTC). Tail length was used to measure the extent of DNA damage. Bars correspond to mean ± S.D. *, p < 0.05, and ***, p < 0.001 versus untreated (Un) cells.

To test whether detachment-induced oxidative stress is responsible for DNA checkpoint activation and efficient DNA repair upon chemotherapy, we used NAC, a general ROS scavenger, and NDGA, an ROS inhibitor. Treatment with NAC at 10 mM or NDGA at 5 μM efficiently reduced ROS levels in suspended MCF-7 cells (Fig. 4E) and suppressed phosphorylation of H2AX at Ser-139, ATM at Ser-1981, ATR at Ser-428, Chk1 at Ser-345, and Chk2 at Thr-68 (Fig. 4F). Similarly, partial pre-activation of ATM, Chk2, ATR, and Chk1 was also observed in CTCs but not in PTCs of breast cancer patients and was suppressed after treatment with ROS production inhibitors (Fig. 4G and Table 4). These data suggest that ROS production due to detachment partially pre-activates DNA damage checkpoints in CTCs. Likewise, pre-treatment with NAC and NDGA increased DNA damage and apoptosis in CTCs challenged with epirubicin (Fig. 4, H and I, and Table 4). Different from the effects observed in the CTCs and suspended MCF-7 cells, the ROS inhibitors did not further increase the toxicity of cisplatin in PTCs cultured in adherent conditions or in adherent MCF-7 cells, determined by caspase3 activity (Fig. 4J). Therefore, increased ROS in CTCs is responsible for efficient DNA damage repair and drug resistance upon chemotherapy, which is accompanied by partially activated DNA damage checkpoints.

Partially Activated DNA Damage Response in CTCs Potentiates Their Ability in DNA Damage Repair—To further investigate whether an early DNA damage response in CTCs due to ECM detachment is responsible for their efficient DNA repair after chemotherapy, we examined the activation of DNA damage checkpoints in breast cancer cells upon detachment from ECM. Along with the elevation of basal DNA breakage, moderate phosphorylation of ATM at Ser-1981 and Chk2 at Thr-68 and relatively milder phosphorylation of ATR at Ser-428 and Chk1 at Ser-345 were observed in MCF-7 cells in suspension cultures prior to chemotherapy but not in adherent MCF-7 cells (Fig. 5A). Furthermore, at 1 h after withdrawal of epirubicin, phosphorylation of ATM, Chk2, ATR, and Chk1 was dramatically enhanced in the suspended MCF-7 cells (Fig. 5A),...
suggesting that DNA checkpoints are partially pre-activated prior to chemotherapy but rapidly acquire full activation following chemotherapy. In contrast, DNA checkpoint activation was only mildly increased in the adherent MCF-7 cells following chemotherapy, which subsided to the baseline levels at 2 h after drug withdrawal (Fig. 5A). In addition, we tested the expression level of repair-associated proteins, including RAD51 and ERCC1, in PTCs cultured in suspended and adherent conditions. The protein level of suspended cells was similar to that of adherent cells, although the ERCC1 level of the former was slightly increased after prolonged detachment (6h) (Fig. 5B), which suggested that an increase of expression of the repair proteins might play a limited role in the robust repair of DNA damage in the suspended cells and CTCs. Collectively, these data suggest that partial pre-activation of DNA damage checkpoints in suspended cells due to detachment may be responsible for their massive activation following chemotherapy.

**TABLE 4**

CTC counts and clinicopathological features in eight metastatic breast cancer patients

The following abbreviations are used: MBC, metastatic breast cancer; CTC, circulating tumor cell; ER, estrogen receptor; PR, progesterone receptor; IDC, invasive ductal carcinoma. CTC number from eight MBC patients was quantified by CellSearch®. The CTCs were isolated from peripheral blood from these eight patients using the magnetic beads coupled with anti-EpCAM antibody for positive selection and beads with anti-CD45 and anti-CD15 antibodies for negative selection to isolate CTCs. PTCs were isolated from primary tumor biopsies of the same patients. Isolated CTC and PTCs were used for further experiments.

| Case no. | Age | Primary tumor size | Pathological classification | ER | PR | Metastatic sites | Grade | CTC counts* |
|----------|-----|-------------------|-----------------------------|----|----|-----------------|-------|-------------|
| 1        | 60  | 5.6               | IDC                         | +  | +  | Lung, bone      | III   | 336         |
| 2        | 44  | 5.0               | IDC                         | -  | -  | Brain           | II    | 218         |
| 3        | 45  | 3.6               | IDC                         | -  | -  | Liver, bone     | III   | 1134        |
| 4        | 64  | 3.8               | IDC                         | +  | -  | Lung, bone      | III   | 257         |
| 5        | 41  | 3.4               | IDC                         | +  | -  | Liver           | III   | 346         |
| 6        | 40  | 4.2               | IDC                         | -  | -  | Liver           | III   | 806         |
| 7        | 56  | 5.5               | IDC                         | -  | -  | Liver, bone     | III   | 354         |
| 8        | 44  | 4.0               | IDC                         | +  | -  | Liver           | III   | 406         |

* Quantification was by CellSearch® system per 7.5 ml of blood.
To further evaluate whether partial pre-activation of DNA damage checkpoints in suspended breast cancer cells is responsible for efficient post-chemotherapeutic DNA repair, we used specific inhibitors for Chk1 and Chk2. Chir-124 (a Chk1 inhibitor) and Chk2 inhibitor II efficiently inhibited the activity of Chk1 at 250 nM and Chk2 at 10 μM in suspended MCF-7 cells, respectively, and DBH at 3 μM inhibits both Chk1 and Chk2 activity (Fig. 5C). Checkpoint inhibitors added 2 h prior to the chemotherapeutic drugs only slightly enhanced DSB in the adherent cells (Fig. 5D) probably due to their low basal activity of checkpoints prior to chemotherapy. However, addition of Chir-124 or Chk2 inhibitor II to suspended MCF-7 cells at 2 h prior to chemotherapy inhibited Chk1 or Chk2 activity (Fig. 5C) and dramatically enhanced DSB at 1 h after cisplatin withdrawal, which was aggravated at 2 h after drug withdrawal (Fig. 5D). Moreover, inhibiting the activity of both Chk1 and Chk2 with DBH at 3 μM (Fig. 5C) further enhanced the severity of cisplatin-induced DNA damage in the suspended cells as compared with inhibition of either checkpoint (Fig. 5D). These data suggest that pre-treatment of suspended cells with checkpoint inhibitors prior to chemotherapy enhances DNA damage by inhibiting checkpoint pre-activation before chemotherapy.

We next investigated whether inhibition of DNA damage checkpoints may reverse chemoresistence of breast cancer cells induced by ECM detachment. Annexin V staining showed that pre-treatment of suspended MCF-7 cells with either Chk1 or Chk2 inhibitor prior to chemotherapy resulted in significantly more apoptosis at 1 and 2 h after withdrawal of cisplatin (p < 0.05 for Chk1 inhibition and p < 0.01 for Chk2 inhibition), and blocking both checkpoints before chemotherapy synergistically increased cell death (p < 0.001, Fig. 5E). More importantly, pre-treatment of suspended cells with Chk1 or Chk2 inhibitor reduced the IC_{50} value for cisplatin by 66.7 and 77.8%, respectively, and the IC_{50} value for epirubicin by 52 and 68%, respectively, which were further reduced by inhibiting both checkpoints (Fig. 5F). Therefore, blocking the partial pre-activation of DNA damage checkpoints in cancer cells that lack ECM support may enhance their sensitivity to chemotherapy by reducing the efficiency of DNA repair.

To study the clinical implication of the above findings, we inhibited Chk1 and Chk2 activity prior to treatment with cisplatin in the CTCs isolated from breast cancer patients (Table 4). Similar to the breast cancer lines in suspension culture, pre-treating CTCs with either or both of the checkpoint inhibitors prior to chemotherapy enhanced DSB (Fig. 5G) and apoptosis after cisplatin withdrawal (Fig. 5H), but it did not change those without chemotherapy. The finding suggested that upon detachment the ROS-induced activation of DNA damage response could be responsible for the protective effect of suspended cells following genotoxic agent exposure. Therefore, partial pre-activation of DNA damage checkpoints in CTCs may contribute to efficient DNA damage repair and chemotherapeutic resistance.

**Chk1/Chk2 Inhibitors Suppress Chemoresistance of CTCs in Xenografted Mice**—To examine the effect of DNA damage checkpoint inhibition on chemotherapy in vivo, MDA-MB-231 breast cancer cells that stably expressed green fluorescence protein (GFP) were inoculated into the mammary fat pads of nude mice. Tumor growth, CTC apoptosis, and distal metastasis after cisplatin administration were evaluated when xenografts reached 1.5 cm in diameter. Intravenous administration of cisplatin at a dosage of 5 mg/kg weekly for 4 consecutive weeks with or without injection of Chir-124 at 10 mg/kg or Chk2 inhibitor II at 1 mg/kg 2 h prior to chemotherapy did not significantly influence the xenograft tumor growth (data not shown). Circulating tumor cells in the blood of mice were retrieved using cardiac puncture and followed by purification procedure. Although checkpoint inhibitor alone did not influence the apoptosis of CTCs, administering either checkpoint inhibitor followed by cisplatin injection 2 h later significantly increased the percentage of apoptotic CTCs (p < 0.01, Fig. 6, A and B).

For the mice xenografted with MDA-MB-231 cells that stably expressed luciferase, luminescence imaging for the whole mice (Fig. 6, C and E) or for the harvested lungs and livers (Fig. 6, D, F, and G) demonstrated that sequential injection of either DNA damage checkpoint inhibitor and cisplatin dramatically suppressed lung and liver metastasis, whereas chemotherapy or checkpoint inhibition alone did not influence the metastasis (Fig. 6, C–G). In agreement, immunohistochemical staining using anti-human PCNA antibody confirmed that chemotherapy together with DNA damage checkpoint inhibition suppressed lung and liver metastasis of the mice bearing MDA-MB-231 xenografts as compared with chemotherapy or checkpoint inhibition alone (Fig. 6H). Collectively, these data suggest that DNA damage checkpoint inhibition applied prior to chemotherapy enhances the anti-metastatic effect of DNA-damaging chemotherapy probably by sensitizing CTCs to the drugs. All these interesting findings are schematically summarized in Fig. 7.

**Discussion**

Tumor cells circulating in the bloodstream of cancer patients are considered as the seeds of tumor relapse or distant metastasis because of their strong capacity of tumorigenesis and invasiveness (4, 6, 33). Thus, the efficacy of systemic anti-cancer...
therapies in preventing cancer recurrence relies heavily on their ability to eradicate CTCs. Unfortunately, the biology of CTCs is so different from their parental PTCs that the chemotherapeutic sensitivity of PTCs cannot represent that of CTCs (4, 19). Here, we showed that CTCs isolated from advanced breast cancer patients are far more resistant to the DNA-damaging and

**FIGURE 6.** Checkpoint inhibitors sensitize the response of cisplatin-induced CTC apoptosis in breast cancer xenografts. A and B, nude mice xenografted in the mammary fat pads with MDA-MB-231 cells stably expressing enhanced GFP (n = 8/group). Intravenous injection of Chk1 inhibitor (Chir-124) or Chk2 inhibitor II (Chk2 inhibitor II) 0 and 6 h after intravenously injection of cisplatin were performed weekly when xenografts were palpable. Mice were subjected to a total of four cycles of therapy. M30-positive (A) or TUNEL (B)-positive cells of CTC expressing enhanced GFP were quantified by confocal scanning microscope. C and E, nude mice xenografted in the mammary fat pad with MDA-MB-231 cells expressing luciferase. Mice were subjected to the above-mentioned therapy (n = 8/group). C, whole body luminal imaging; D, representative luminal images. The photon intensities in the chest and upper abdomen (E), lungs (F), and livers (G) were shown in the tumor-bearing mice. Anti-human PCNA immunohistochemical staining for lungs (upper) and livers (lower) (×200) were shown in tumor-bearing mice (H). Bars correspond to mean ± S.D. **, p < 0.01 as compared with cisplatin. ###, p < 0.001 versus Chir-124. $$, p < 0.01, or $$$, p < 0.001 versus Chk2 inhibitor II.
pro-apoptotic effects of chemotherapy than the ECM-attached PTCs. More importantly, apoptosis of CTCs, but not that of PTCs, correlates well with systemic chemotherapeutic response and disease progression of the patients upon chemotherapy, which extends previous knowledge that CTC enumeration is a serologic marker to predict prognosis. Therefore, monitoring the primary tumor alone is not sufficient to predict chemotherapeutic sensitivity of breast cancers, whereas the chemoresistance of CTCs to chemotherapy seems to be a better indicator of patients’ prognosis.

Two possible mechanisms may underlie the different chemosensitivity between CTCs and PTCs. CTCs may be derived from heterogenic primary tumor cells that are intrinsically resistant to chemotherapy, or they are induced to acquire resistance due to lack of ECM support. It has been reported that CTCs of cancers (34) express some biomarkers of cancer stem cells and are highly tumorigenic (6). Breast cancer stem cells are resistant to chemotherapy by overexpressing an ATP-binding cassette half-transporter ABCG2, which expels chemotherapeutic drugs from the cells (26, 29). Nevertheless, ABCG2 expression was not elevated in CTCs, and the ability of CTCs to expel dyes and chemotherapeutic drugs was not enhanced. Therefore, CTCs of breast cancer are resistant to chemotherapy through mechanisms different from CSCs.

Our study demonstrated that re-establishing ECM support for CTCs by adherent culturing sensitizes them to chemotherapy, whereas depriving ECM support for primary breast cancer cells and a breast cancer line by suspension culture recapitulates chemoresistance of CTCs. Detachment from the extracellular matrix is a major environmental change for CTCs that may lead to their biological difference from PTCs. Under normal circumstances, epithelial cells undergo apoptosis upon detachment from ECM, a phenomenon noted as anoikis (22). In contrast, many cancer cells are resistant to anoikis because of their property of anchorage-independent growth (22). While surviving the process, cancer cells without ECM support undergo oxidative stress and produce increased amounts of ROS (35). Excessive ROS production is toxic to cancer cells, which is an anti-cancer mechanism of anthracyclines by inducing massive and irreversible DNA damage (36). However, our data showed that CTCs produce increased amounts of ROS that induces mild DNA damage and partially pre-activates DNA damage checkpoints. This is responsible for the efficient DNA damage repair upon chemotherapy because inhibiting ROS production dramatically reduces the efficiency of post-chemotherapy DNA damage repair. Therefore, the amount of ROS production in CTCs because of detachment from ECM is not lethal to the cells, but it generates enough DNA breaks to facilitate efficient DNA damage repair and prepares the cells for more severe DNA insults. Different from CTCs, breast CSCs had lower levels of ROS as compared with non-CSCs (37), confirming that ROS-induced efficient DNA damage repair upon chemotherapy is not a mechanism of drug resistance in CSCs.

It has been documented that enhanced capability of DNA damage repair in cancer cells not only results in genomic instability and tumor progression (38) but also leads to tumor resistance to DNA-damaging therapies (39, 40). Because DNA-damaging agents, including various anthracyclines and platinum, are commonly used chemotherapeutic drugs in breast cancer, the enhanced ability of DNA damage repair in CTCs poses a significant obstacle for these drugs to efficiently eradicate CTCs and disseminated tumor cells. By rapidly repairing DNA damages, CTCs could survive the lethal assaults of chemotherapy and remain the source of tumor recurrence.

Our findings further suggest that the efficient DNA damage repair in CTCs following chemotherapy is due to partial pre-activation of DNA checkpoints. In the process of DNA damage repair, various DNA damage checkpoints are activated, and DNA repair proteins are recruited to the chromatin near the DSB lesions (41). Checkpoint activation prevents further progression of the cell cycle, which enables DNA repairing via homologous recombination or nonhomologous end joining (42). If the genotoxic insult exceeds the repairing capacity, the damaged cells undergo cell death via apoptosis or mitotic catastrophe (43). Therefore, adequate and timely activation of cell cycle checkpoints is the key to efficient DNA damage repair and survival of cancer cells. Our present data indicate that ATM/Chk2 and ATR/Chk1, which are two major kinase signaling pathways involved in the canonical DNA damage response network, are pre-activated in the suspended breast cancer cells and the CTCs that lack ECM attachment prior to chemotherapy. Furthermore, activation of these kinases results in cell cycle arrest by controlling the G_{1}/S and G_{2}/M checkpoints. This is of vital importance to the chemoresistance of these cells as inhibiting the activation of Chk2 and Chk1 synergistically leads to severe DNA damage and massive cell death following chemotherapy. Supporting our findings, previous studies have confirmed that

DNA Checkpoint Activation and CTC Chemoresistance
activation of checkpoint kinases in the process of DNA damage response represents an important mechanism limiting chemotherapeutic efficacy (43, 44). However, several Chk1/Chk2 inhibitors, including XL-844, AZD7762, and PF00477736, have been shown to potentiate the effects of DNA-damaging therapies by abrogating DNA damage-induced cell cycle arrest and have entered clinical trials for cancer therapy in combination with chemotherapeutic drugs (45). Our present findings went one step forward by showing that Chk1/Chk2 inhibitors sensitize the chemoresistant CTCs to conventional chemotherapy in vitro, and upon combining with chemotherapy, the checkpoint inhibitors reduce the number of CTCs and inhibit lung and liver metastasis in the immunocompromised mice with breast cancer xenografts. Therefore, inhibitors for DNA checkpoints, in combination with conventional chemotherapies that damage DNA, may have the potential to efficiently eliminate CTCs and thus control cancer metastasis. However, numerous clinical trials have failed to demonstrate that Chk1/Chk2 inhibitors enhance the overall efficacy of genotoxic agents against a wide range of tumor types (46–48). Checkpoint inhibitors combined with chemotherapy would be of great value in a selected subgroup with increased CTC burden, but to date, the value of this strategy remains to be demonstrated.

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