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HER2 phenotyping of CTCs by peptide-functionalized nanoparticles as the diagnostic biomarkers of anti-HER2 treatment in breast cancer

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Abstract

Background: The efficacy of anti-human epidermal growth factor receptor 2 (HER2/*neu*) treatment is impacted by tissue-based evaluation bias due to tumor heterogeneity and dynamic changes of HER2 in breast cancer. Circulating tumor cell (CTC)-based HER2 phenotyping provides an integral and real-time assessment, benefiting accurate HER2 diagnosis for improved therapeutics.

Methods: The study was exploratory. Fifty-four breast cancer patients, including 26 histopathologic HER2/*neu*-positive (*Histo-HER2/neu*⁺) and 28 HER2/*neu*-negative (*Histo-HER2/neu*⁻), were enrolled for blood draws before a new line of treatment. We used peptide-functionalized magnetic nanoparticles to isolate CTCs from 2.0 mL whole blood and determined HER2 phenotypes of the enriched CTCs by immunocytochemistry (ICC). We investigated the correlation of the enumeration and

HER2 phenotyping on baseline CTCs with the diagnosis, the prognosis, and the efficacy of the trastuzumab in combination with chemotherapeutic agents of breast cancer. We also explored the dynamic change of HER2 phenotypes on CTCs after the combination therapy in a cohort of *Histo-HER2/neu*⁺ individuals (n = 14).

Results: We achieved high-efficient detection of the CTC-positive cases (≥ 3 CTCs) (38/54, 70.4%), from whom 71.1% (27/38) had a concordant HER2 status on CTCs and tumor tissues at baseline. Pretherapeutic CTC enumeration showed a significant correlation with the histopathologic diagnosis (e.g. estrogen receptor/progesterone receptor (ER/PR) status or proliferation of cancer cells) and the progression-free survival/overall survival (PFS/OS) of breast cancer. However, it was not a practicable biomarker to predict the efficacy of the trastuzumab-based combination therapy. In contrast, we demonstrated a significantly higher possibility of good overall responses in the patients with < 3 CTCs ($P = 0.006$) or with HER2 overexpression in CTCs (*CTC-HER2*⁺) at baseline, as compared to the individuals without HER2 overexpression in CTCs (*CTC-HER2*⁻) ($P = 0.028$).

Conclusions: We demonstrate the significance of the peptide-functionalized magnetic nanoparticle in noninvasive detection and HER2 phenotyping of CTCs, and highlight its diagnostic and prognostic values for the patients about to start the anti-HER2 treatment.

Keywords: Circulating tumor cells, Magnetic isolation, HER2 phenotyping, Prognosis, Breast cancer

Background

Breast cancer is responsible for a leading cause of cancer-associated deaths for global females [1, 2]. Human epidermal growth factor receptor 2 (*HER2/neu*) plays a vital role in tumorigenesis, invasion, and metastasis of breast cancer, leading to a poor prognosis and shorter survival [3, 4]. Anti-HER2 monoclonal antibodies (e.g. trastuzumab) alone or in combination with other therapies have been recommended as the standard first-line regimen for the histopathologic *HER2/neu*-positive (*Histo-HER2/neu*⁺) patients [5-

7]. However, a cohort of patients fails to achieve good outcomes from trastuzumab (20% – 50%) [8, 9]. It might be because of the spatiotemporal HER2 heterogeneity in primary/metastatic tumor sites [10-12]. Tissue biopsies by immunohistochemistry (IHC) and/or fluorescence in situ hybridization (FISH) have been the clinical “gold standard” to determine the *Histo-HER2/neu*⁺ candidates for anti-HER2 therapy [13, 14]. Nevertheless, conventional sampling of tumor tissues through surgery or puncture is constrained by complicated manipulation and harmful invasion, therefore not feasible in the repeated, real-time biopsies for a comprehensive tumor portrait (e.g. HER2) [15, 16]. Moreover, the HER2/*neu* analysis by tissue biopsies might produce uninterpretable results due to insufficient tumor cells or strong background [8]. Therefore, it is necessary to achieve more sensitive and real-time biomarkers for the HER2 diagnosis of breast cancer.

A noninvasive blood-based detection using the circulating tumor cells (CTCs) that have similar genetic and epigenetic properties to the primary/metastatic tumor cells is blooming to be a surrogate to the histopathologic assessments [17]. Multiple investigations have been implemented to explore the clinical significances of CTCs in the early diagnosis, prognostication, and monitoring of solid tumors [18-22]. CTC enumeration has been a valid and independent staging and prognostic biomarker for breast cancer but not for predicting the survival of the HER2-overexpressed patients during anti-HER2 treatment [23-25]. Exploring the gene/protein portraits of breast cancer CTCs should be complementary to the personalized diagnoses and treatments. HER2/*neu* overexpression or amplification on CTCs has been investigated in breast cancer over the last decade. The discordance of HER2/*neu* status on tumor tissues and CTCs was present (38% – 86%) [26-29]. Besides, Jordan et al. demonstrated a spontaneous interconversion of HER2 phenotypes on CTCs *ex vivo* [30]. Hence, HER2 reassessments on CTCs might overcome the spatio-temporal heterogeneity of HER2 expression in breast cancer. Nonetheless, up to date, there is no widely accepted consensus about the criteria of HER2 phenotyping of enriched CTCs, primarily because various tests and definitions of HER2 status were adopted by different trials in a small sample size. On the other hand, as the only U. S. Food and Drug Administration (FDA)-

approved detection system of epithelial CTCs in solid tumors, *CellSearch* was extensively used in various studies, whereas it is inevitably weak in the limited rate of CTC detection from breast cancer patients (22% – 70%) [31-34], leading to a portion of patients that are unqualified in the HER2 analysis by *CellSearch*. Therefore, it remains necessary to explore the clinical potentials of HER2 phenotyping on CTCs in breast cancer on the basis of a more sensitive detection of CTCs and HER2 proteins.

Previously, our group has developed an innovative EpCAM-targeted peptide (Pep10)-functionalized magnetic nanoparticle (Pep10@MNPs) that can be used to efficiently capture the rare epithelial CTCs in 2.0 mL whole blood (WB) from patients with various solid tumors [35-37]. In the present study, the Pep10@MNPs and classical four-color immunocytochemistry (ICC) were applied to detect EpCAM-overexpressed CTCs and to assess HER2 status of the enriched CTCs. We achieved the analysis of the blood samples from 54 breast cancer patients at baseline and from 15 cases of the *Histo-HER2/neu*⁺ individuals with the second blood draws after anti-HER2 therapy in combination with chemotherapy. Thereafter, we consolidated the diagnostic and prognostic values of CTC enumeration with the determined cutoff that was published in our previous report [36]. Furthermore, the evaluation of HER2 overexpression on CTCs was achieved based on the fluorescent quantification of HER2 immunostaining. We emphatically analyzed the relationship of the HER2 phenotypes of baseline CTCs or the evolutionary HER2 status in CTCs with the efficacy of the HER2-based combination treatment, which may further help understand the prognostication values of HER2 status of the enriched CTCs in guiding anti-HER2 treatment.

Methods

Study design

We were approved by the institutional review board at the Medical Ethical Committee of Peking Cancer Hospital (No. 2013KT29) to design and carry out the exploratory trial. The participants including breast cancer patients and healthy donors were enrolled by our collaborators from Peking Cancer Hospital. The criteria for the HER2 phenotyping

on CTCs were determined by peptides-functionalized magnetic nanoparticle (Pep10@MNPs) and immunocytochemistry. We explored the relationship of the HER2 phenotyping on baseline CTCs with the clinical characteristics, the progression-free survival/overall survival (PFS/OS), and the efficacy of the treatment with trastuzumab in combination with the chemotherapeutic agents of breast cancer patients. With the consent from patients, second blood draws were completed during the trastuzumab-based combination therapies and used to investigate the dynamic change of CTC enumeration and HER2 phenotype in predicting the real-time responses. The clinical characteristics at baseline, the clinical responses from the Response Evaluation Criteria in Solid Tumors (version 1.1) and the data of the PFS/OS were informed after all the tasks of the CTC detection were finished.

Cell culture

Six cell lines (human breast cancer cell lines SK-BR-3, MDA-MB-453, MCF-7, MDA-MB-468; human mammary epithelial cell line MCF-10A; human histiocytic lymphoma cell U937) were all purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in various basic media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco-BRL, Grand Island, NY, USA), including RPMI Medium 1640 (Gibco-BRL) for SK-BR-3 and U937, high-glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL) for MDA-MB-453, MCF-7 and MDA-MB-468, and mammary epithelial cell growth medium (MEGM) kit (Lonza Group Ltd., Basel, Switzerland)) for MCF-10A. The cells were maintained at 37°C in an atmosphere with 95% moisture and 5% CO₂.

Flow cytometry analysis

The cells were harvested by 0.25% (w/v) trypsin/2.5 g/L EDTA. 5% bull serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) was used to block the cells for 30 min at 37 °C. Cells were respectively incubated with phycoerythrin (PE)-conjugated antibodies for 60 min at 37 °C, including anti-EpCAM (BioLegend, San Diego, CA, USA), anti-HER2 (Sino Biological, Beijing, China) and IgG isotype controls (IgG2bk

for anti-EpCAM (Biolegend); IgG1 for anti-HER2 (Cell Signaling Technology, Danvers, MA, USA)). The BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA, USA) was applied to detect the EpCAM and HER2 immunostaining with PE channel (Ex./Em., 488/574 nm).

Western blot analysis

The harvested cells were lysed in 1% protease inhibitor-contained Pierce™ IP lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) on ice for 60 min, followed by high-speed centrifugation (15,000 rpm, 10 min, 4°C). Total proteins were quantified with BCA protein quantification kit (Solarbio, Beijing, China) and denatured by 5 x SDS-PAGE loading buffer (95 °C, 10 min) (CW BIO, Beijing, China). The proteins were separated in NUPAGE™ 10% Bis-tris polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) by electrophoresis. Proteins in gels were then transferred onto the polyvinylidene fluoride (PVDF) membranes, followed by blocking with 5% nonfat milk solution (w/v) (BD Biosciences) for 2.0 h at room temperature. Primary antibodies (anti-EpCAM (Cell Signaling Technology); anti-HER2 (Cell Signaling Technology, Danvers, MA, USA); anti-β-actin (EASYBIO, Beijing, China)) were then incubated with the membranes overnight at 4 °C. Horseradish peroxidase (HRP)-conjugated secondary antibodies were used to target the primary antibodies for 60 min at room temperature, including horse anti-mouse IgG (Cell Signaling Technology) and goat anti-rabbit IgG (Cell Signaling Technology). Accessible to the ChemiDoc™ MP imaging system (Bio-Rad, Hercules, CA, USA), the HRP-labeled proteins (EpCAM/HER2/β-actin) on PVDF membranes were visualized with an enhanced chemiluminescence kit (Thermo Fisher Scientific).

Isolation of breast cancer cells by Pep10@MNPs in simple cell models

The streptavidin-modified magnetic nanoparticles (SA-MNPs, maghemite $\gamma\text{-Fe}_2\text{O}_3$) (Ademtech, Gironde, PESSAC, France) and the biotin-labeled EpCAM-targeted peptides (Pep10) (Guoping Pharmaceutical Co., Anhui, China) were purchased. The Pep10@MNPs were constructed as previously described [38]. Briefly, with the volume

ratio (v/v) of 1:2, the SA-MNPs (5 mg/mL) were incubated with Pep10 (1 mg/mL) for 60 min at 37 °C, followed by three-time washing. As a control, the antibody@MNPs were prepared with anti-EpCAM (Abcam, Cambridge, MA, UK) using a similar protocol. The constructed nanoparticles were confirmed by dynamic light scattering (DLS) (Zetasizer Nano ZS90, UK) and transmission electron microscope (TEM) (Tecnai G2 20 S-TWIN, USA). In simple cell models, 1000 cells that were pre-stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) suspended in 1.0 mL PBS and incubated with the Pep10/anti-EpCAM@MNPs for 60 min at 37 °C. The Olympus IX73 fluorescent microscope (Olympus, Tokyo, Japan) was used to enumerate the added or captured cells. The capture efficiency (%) was determined as the number of the captured cancer cells/the number of the added cancer cells.

HER2 immunostaining on the breast cancer cells in cancer cell models

Cancer cell models were constructed with 1000 breast cancer cells in 1.0 mL PBS (simple, System 1) or in 1.0 mL PBS containing 10⁶ monocyte-like U937 cells (complex, System 2). The breast cancer cells were pre-stained with DAPI (nucleic acid dye)/DIO (plasma membrane dye). The U937 cells were only pre-stained with DAPI. Pep10@MNPs were incubated with System 1/2 for 60 min at 37 °C, followed by magnetic enrichment. After the fixation with 2% paraformaldehyde (PFA) for 30 min, the captured cells were blocked with 5% BSA solution. HER2 immunostaining of the captured cells was then achieved with Alexa Fluor 647-labeled anti-HER2 for 60 min (BioLegend, San Diego, CA, USA). The Olympus IX73 fluorescent microscope (Olympus, Tokyo, Japan) was used to quantify the mean fluorescent intensities of HER2 immunostaining from 50 captured cells.

Patients and clinical blood samples

The eligible breast cancer patients (N = 54) for baseline blood draws were newly diagnosed locally advanced or recurrent/metastatic individuals and were about to accept new-line treatments (chemotherapy alone or in combination with anti-HER2 or other targeted therapies). The patients with secondary malignancies within 5 years, or already

in systematic therapies within 7 days, or painful to blood draw were all excluded. As a negative control, 10 healthy donors were enrolled who were evaluated as no medical history of any serious disease and any surgery within 6 months. The corresponding clinicopathological and radiological evidence of the 54 enrolled patients was documented in **Table 1**. Most of the patients (~85%) had recurrent breast tumors, and almost all of patients (~98%) were metastatic. The differentiation, estrogen receptor (ER), progesterone receptor (PR), HER2/*neu* and Ki67 labeling index (Ki67 LI) on tumor tissues were all assessed by the expert pathologists. Twenty-six individuals (26/54, 48.2%) were diagnosed as the poorly-differentiated (G2~3, G3) and 29 individuals (29/54, 53.7%) were determined as the histopathologic ER/PR-positive (*Histo-ER/PR*⁺). All of the patients had histopathologic HER2/*neu* status, including 26 HER2/*neu*-overexpressed/amplified individuals (*Histo-HER2/neu*⁺) and 28 individuals without HER2/*neu* overexpression/amplification (*Histo-HER2/neu*⁻). Besides, on account of the cutoff (14%) of Ki67 LI [39, 40], a cohort of patients (44/54, 81.5%) with high level ($\geq 14\%$) of Ki67 LI could be identified. 21 (26) of the *Histo-HER2/neu*⁺ patients were treated with the combination therapies with trastuzumab and chemotherapeutic agents, among whom 14 cases provided second blood draws after therapies. The periods from baseline to follow-up in the 14 patients ranged from 7.4 to 18.6 weeks. The responses at the follow-up visit and the best overall responses were both assessed by Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 guidelines [41].

Totally, we collected 54 baseline blood samples and 14 follow-up blood samples from breast cancer patients, and 10 blood samples from healthy donors. For each blood sample, whole blood was drawn into an anticoagulation-contained plastic vacuum blood collection tube (NANOPEP BIOTECH CO., Beijing, China), and delivered to the lab for Pep10@MNPs-based detection of CTCs within 24 h.

Capture and HER2 immunostaining of CTCs in clinical blood samples

Briefly, 10 μ L Pep10@MNPs were incubated with 2.0 mL whole blood for 60 min at 37 °C. The magnetically isolated CTCs were then fixed with 2% PFA for 30 min and

blocked with 5% BSA solution for 30 min. We achieved the immunostaining of cytokeratins (CKs)/leukocyte common antigen (CD45)/HER2 on CTCs and/or white blood cells (WBCs) with primary antibodies for 60 min, including Alexa Fluor 488-linked anti-CKs (Abcam, Cambridge, MA, UK), PE-DyLight 594-conjugated anti-CD45 (Abcam), and Alexa Fluor 647-linked anti-HER2 (BioLegend, San Diego, CA, USA). The cell nuclei were stained with DAPI (1 $\mu\text{g/mL}$) for 2 min. In the end, the Olympus IX73 fluorescent microscope (Olympus, Tokyo, Japan) were applied to visualize the CTCs and WBCs. The fluorescent quantification of the CKs/CD45/HER2 immunostaining was applied to enumerate CTCs and HER2 phenotyping.

Statistical analysis

Based on the software GraphPad Prism (Version 8.0; La Jolla, CA, USA), we assessed the differences with P values from the unpaired Student's t -test (significant levels; *, < 0.05; **, < 0.01; ***, < 0.001; ****, < 0.0001) and analyzed the linear correlations with Pearson's coefficient r (the closer to 1, the stronger correlation). Receiver operating characteristic (ROC) analysis and Kaplan-Meier plots were achieved by GraphPad Prism as well. On the other hand, with the IBM SPSS Statistics 21 software (Version 21.0; Armonk, NY, USA), the contingency table analysis by Fisher's exact test or Chi-square test was useful to figure out the relationships among various diagnostic parameters ($P < 0.05$ as a significant correlation). Chi-square test was used when the sample size was large ($n \geq 40$) and the minimum of the observed frequencies ($T_{\min}$) was larger than five; otherwise, the Fisher's exact test was applied [32].

Results

Isolation and identification of breast cancer cells

We first functionalized the streptavidin-modified magnetic nanoparticles (SA-MNPs) with peptides (Pep10) or anti-EpCAM (Pep10/anti-EpCAM@MNPs). Dynamic light scattering (DLS) showed a slight increase of the hydrodynamic diameters and an obvious decrease of the zeta potentials both for Pep10@MNPs and anti-EpCAM@MNPs compared to SA-MNPs (Fig. 1a), in accordance with the previous

report [35]. Transmission electron microscope (TEM) images showed no obvious changes in the morphology and dispersity of the nanoparticles after peptide or antibody functionalization (Supplementary Fig. S1a-c), and meanwhile indicated a slight increase of the diameters of the functionalized nanoparticles (Supplementary Fig. S1d-f).

We then evaluated the expression level of EpCAM in the four human breast cancer cell lines (SK-BR-3, MCF-7, MDA-MB-453, MDA-MB-468) and the normal basal mammary epithelial cell MCF-10A. Flow cytometry (FCM) analysis revealed a significant positive level of EpCAM in the four breast cancer cell lines compared to MCF-10A (**** $P < 0.0001$) (Fig. 1b; Supplementary Fig. S2a), as expected as the EpCAM overexpression in breast cancer [42, 43]. Pep10@MNPs were then used to capture DAPI pre-stained cells in System 1 for the selected cell lines, i.e. 1000 cells were resuspended in 1.0 mL PBS. The captured cells were enumerated by a fluorescence microscope (Supplementary Fig. S3). We found a capture efficiency of 70% – 90% for the four breast cancer cell lines compared with the capture efficiency of ~30% for the MCF10A cells, similar to the one by anti-EpCAM@MNPs (Fig. 1c). Considering the compelling advantages of peptides (e.g. high yield, good stability, low cost, etc.) [35, 44], Pep10@MNPs serve as a competitive strategy for cancer cell isolation.

We therefore applied Pep10@MNPs to capture CTCs in 2.0 mL whole blood (WB) from breast cancer patients. On the basis of the four-color immunocytochemical (ICC) staining of nuclei/CKs/CD45/HER2, the captured cells that exhibited Nuclei⁺/CK⁺/CD45⁻ were identified as CTCs, distinguished from the contaminative WBCs that exhibited Nuclei⁺/CK⁻/CD45⁺ or the other blood components (Fig. 1d) [22].

HER2 phenotyping of breast cancer cells by immunocytochemistry (ICC)

We evaluated the expression level of HER2 in the four breast cancer cell lines. Both FCM and western blot assays showed that the expression level of HER2 was the strongest in SK-BR-3, moderate in MDA-MB-453, low in MCF-7, and negative in MDA-MB-468 cells using the MCF-10A as control (Fig. 2a; Supplementary Fig. S2a,

b), correlated with the reported expression level of HER2 in these cell lines [42]. We therefore chose these cells with high expression of EpCAM and different expression levels of HER2 to establish a model system for cell isolation and HER2 phenotyping.

We achieved the HER2 testing of the captured cells by ICC in model systems. The fluorescent intensities from HER2 immunostaining were 10-fold higher ($****P < 0.0001$) in the HER2-overexpressed SK-BR-3 cells compared to the other cell lines. MDA-MB-453 that had the moderate expression of HER2 exhibited higher fluorescent intensities ($****P < 0.0001$) from HER2 immunostaining than MCF-7 and MDA-MB-468. MCF-7 and MDA-MB-468 that had relatively low expression of HER2 could also be significantly differentiated ($***P < 0.001$) by HER2 immunostaining (Fig. 2b, c). Correlation analysis demonstrated good correlation (Pearson's coefficient $r = 0.9996$; $P = 0.0004$, $***$, two-tailed) between ICC-based and FCM-based HER2 phenotyping (Fig. 2d). These results indicated the excellent capability of ICC staining after Pep10@MNPs isolation for HER2 phenotyping of breast cancer cells. For each breast cancer cell line, we then mixed 1000 cells with 10^6 human monocyte-like cells U937 to better mimic the complicated blood system that had abundant WBCs (System 2), and found no obvious changes in the ICC-based HER2 phenotyping (Supplementary Fig. S4). Taken together, results above consolidated the feasibility of ICC-based HER2 phenotyping of the captured CTCs.

Diagnostic and prognostic significance of baseline CTC enumeration in breast cancer patients

Fifty-four histopathologically confirmed breast cancer patients were enrolled for analysis of CTCs with baseline blood draws. CTCs could be detected in 81.5% (44/54) of the enrolled patients at baseline. We found that the patients had a broad range of CTC enumeration (0 – 683 CTCs per 2.0 mL WB; mean $\pm$ standard deviation (S. D.), 47.2 ± 111.5) (Fig. 3a), whereas the epithelial cells were rare from healthy donors (mean $\pm$ S. D., 0.3 ± 0.7), consistent with the findings from Cristofanilli et al. [18, 22]. The receiver operator characteristics (ROC) curve in Supplementary Fig. S5 told us the cutoff of CTCs (≥ 3 CTCs/2.0 mL WB) at baseline could be used to detect breast cancer (area

under the curve (AUC), 0.880; 95% confidence interval (95% CI) of AUC, 0.797 to 0.963; $P = 0.0002$; sensitivity, 70.4%; 95% CI of sensitivity, 57.2% to 80.9%; specificity, 100%; 95% CI of specificity, 72.3% to 100%) (Supplementary Table S1). We then compared the capability of different cut-offs for CTC enumeration in correlating with the histopathologic diagnoses of breast cancer. Results showed that baseline CTC enumeration did not exhibit significant correlations with the grading, distant metastasis and HER2/*neu* status of breast cancer patients (Fisher's exact test or Chi-square test, $P > 0.05$, two-tailed) (Supplementary Table S2), coincident with the assessments in other reports [18, 22, 31]. Whereas, we found patients without the estrogen receptor/progesterone receptor (ER/PR)-overexpressed tumors were more possibly detected with a high CTC level (≥ 3 CTCs/2.0 mL WB) (Fisher's exact test, $P = 0.012$, two-tailed) (Supplementary Table S2). In addition, among the HER2⁽²⁺⁾ patients by immunohistochemistry (IHC) ($n = 18$), *neu*⁺ and *neu*⁻ individuals determined by fluorescent in situ hybridization (FISH) exhibited a significant difference in the cases detected with ≥ 4 CTCs (Fisher's exact test, $P = 0.044$, two-tailed) (Supplementary Table S2). With aspect to the histopathologic Ki67 labeling index (Ki67 LI), 79.6% of the patients determined with high Ki67 LI ($\geq 14\%$) on tumor tissues were detected with ≥ 3 CTCs at baseline, significantly higher than the cases with low Ki67 LI (Fisher's exact test, $P = 0.003$, two-tailed) (Supplementary Table S2). Thereafter, we explored the CTC enumeration in predicting progression-free survival/overall survival (PFS/OS) of breast cancer patients. As shown in Supplementary Fig. S6, the patients who had ≥ 3 CTCs at enrollment exhibited a significantly worse PFS/OS as compared to that of the cases with < 3 CTCs (median PFS, 8.7 vs 19.4 months; hazard ratio (HR) of progression = 2.56; 95% CI of HR, 1.13 to 5.79) (log-rank test, P of PFS = 0.048, P of OS = 0.040), in accordance with the previous work by Liu et al. [33]. Taken together, the detection of the CTC-positive cases (≥ 3 CTCs in 2.0 mL WB) showed promising diagnostic and prognostic values in breast cancer.

Diagnostic and prognostic significance of the HER2 phenotyping of

baseline CTCs in breast cancer patients

The enrolled patients included 26 HER2/*neu*-overexpressed/amplified cases (*Histo*-HER2/*neu*⁺) and 28 HER2/*neu*-negative cases (*Histo*-HER2/*neu*⁻) from histopathologic validations (Table 1) [13, 14]. Based on the quantitative analysis of MFIs from HER2 immunostaining, the enriched CTCs could be classified into four subpopulations with various MFI ranges, including HER2⁽⁰⁾ CTCs (MFI 0 – 100 a.u.), HER2⁽¹⁺⁾ CTCs (MFI 100 – 400 a.u.), HER2⁽²⁺⁾ CTCs (MFI 400 – 600 a.u.) and HER2⁽³⁺⁾ CTCs (MFI > 600 a.u.) (Fig. 3b). As shown, the vast majority of CTCs from patients with various *Histo*-HER2/*neu* status had an overlapped HER2 immunostaining in the MFI ranges of HER2^(0/1+). Sixteen of the 23 *Histo*-HER2/*neu*⁻ cases (69.6%) and 10 of the 21 *Histo*-HER2/*neu*⁺ cases (47.6%) have the MFI values within the HER2^(0/1+) levels. Furthermore, of the *Histo*-HER2/*neu*⁻ patients, five cases (5/23, 21.7%) have the MFI up to HER2⁽²⁺⁾ and only two cases (2/23, 8.7%) have the MFI up to HER2⁽³⁺⁾. Whereas 5 (21) of the *Histo*-HER2/*neu*⁺ patients (23.8%) showed the MFI up to HER2⁽²⁺⁾ and 6 (21) (28.6%) reached the level of HER2⁽³⁺⁾. These resulted in additional statistical cutoff used to justify the positive staining of HER2 on CTCs.

ROC curves of HER2⁽²⁺⁾ CTCs and HER2⁽³⁺⁾ CTCs provided a reasonable ratio cutoff of CTCs (12%) used to differentiate the cases with various HER2 expression by IHC in the CTC-positive patients (Fig. 4a-c; Supplementary Fig. S7a, b). We therefore determined three levels of HER2 expression of the enriched CTCs, including negative expression of HER2 on CTCs (*CTC*-HER2⁻; only with HER2^(0/1+) CTCs, or with ≤ 12% of HER2⁽²⁺⁾ CTCs and no detectable HER2⁽³⁺⁾ CTCs), the score (2+) of HER2 expression on CTCs (*CTC*-HER2⁽²⁺⁾; with > 12% of HER2⁽²⁺⁾ CTCs and no detectable HER2⁽³⁺⁾ CTCs, or with ≤ 12% of HER2⁽³⁺⁾ CTCs) and the score (3+) of HER2 expression on CTCs (*CTC*-HER2⁽³⁺⁾; with > 12% of HER2⁽³⁺⁾ CTCs). As expected, there's a good correlation between the HER2 expression on CTCs and tumor tissues (Fisher's exact test, overall $P = 0.021$) (Fig. 4d-f; Supplementary Fig. S7c). None of the patients with negative expression of HER2 by IHC (*IHC*-HER2⁻) had the *CTC*-HER2⁽²⁺⁾ or *CTC*-HER2⁽³⁺⁾ phenotypes, whereas a cohort of the individuals with HER2 moderate/strong expression by IHC (*IHC*-HER2^(2+/3+)) (40% – 50%) were determined

as the *CTC-HER2*^(2+/3+) phenotypes (Supplementary Fig. S7c).

Herein, the *CTC-HER2*^(2+/3+) patients were described as the cases with HER2 overexpression in CTCs (*CTC-HER2*⁺). As shown in Table 2, in the CTC-positive patients at enrollment (n = 38), 10 (20) of the *Histo-HER2/neu*⁺ patients (50.0%) were identified as the *CTC-HER2*⁺ and 17 (18) of the *Histo-HER2/neu*⁻ patients (94.4%) were determined as the *CTC-HER2*⁻, suggesting an overall coincidence rate of 71.1% (27/38) and a good correlation of the CTC-based HER2 phenotyping with the histopathologic HER2/*neu* status (Kappa's test, $\kappa = 0.434$, $P = 0.004$ (two-tailed); Fisher's exact test, $P = 0.004$, two tailed). Statistically, detection of the *CTC-HER2*⁺ phenotype showed a most potential effect size in detecting the *Histo-HER2/neu*⁺ cases (AUC, 0.722, $P = 0.019$; sensitivity, 50.0%; 95% CI of sensitivity, 29.9% to 70.1%; specificity, 94.4%; 95% CI of specificity, 74.2% to 99.7%), superior to that of other CTC-derived parameters (the count/ratio of the *HER2*^(2+/3+) CTCs or the *HER2*⁽³⁺⁾ CTCs) (Supplementary Fig. S8; Table S3). Thereafter, we investigated the relationship of the HER2 phenotypes on baseline CTCs (*CTC-HER2*⁺ or *CTC-HER2*⁽³⁺⁾) with the histopathologic parameters of breast cancer, whereas no significant associations were achieved (Supplementary Table S4), in concordance with the conclusions by others [16, 37]. Nonetheless, we found several intriguing relationships of the CTC-based HER2 phenotyping with the grading, bone metastasis and lung metastasis, which are close to the significant level (Supplementary Table S4).

We also assessed the prognostic significance of the CTC-based HER2 phenotyping at enrollment in predicting the PFS/OS. The *CTC-HER2*⁺ patients showed a significantly worse PFS than the CTC-negative cases (log-rank $P = 0.024$; hazard ratio (HR) of progression = 3.19, 95% CI of HR (1.01 to 10.11)) (Supplementary Fig. S9). No significant difference of the PFS was achieved between the patients with various HER2 phenotypes of CTCs, but the *CTC-HER2*⁻ individuals had a shorter median PFS than the one of *CTC-HER2*⁺ individuals (7.5 vs 9.2 months) (Supplementary Fig. S9). In OS analysis, we found the patients with *CTC-HER2*⁻ phenotype exhibited a worst OS (log-rank test, overall $P = 0.015$; P of *CTC-HER2*⁻ vs *CTC-HER2*⁺ = 0.099; P of *CTC-HER2*⁻ vs CTC-negative = 0.014) (Supplementary Fig. S9).

Predictive significance of CTC-based HER2 phenotyping during anti-HER2 targeted therapy

Of the 26 *Histo*-HER2/*neu*⁺ patients, 20 individuals who started the therapies with trastuzumab in combination with chemotherapeutic agents were eligible to the analysis of the relationship of CTC enumeration and HER2 phenotyping with the efficacy of anti-HER2 therapy in breast cancer. Supplementary Table S5 recorded the enumeration of CTCs and HER2^(2+/3+) CTCs at baseline and the best overall responses of the 20 patients. 10 (20) of the individuals (50.0%) could acquire partial response (PR). One patient showed the non-complete response/non-progressive disease (Non-CR/non-PD) as the best state. The other patients had the stable disease (SD) (n = 8) or progressive disease (n = 1) as the best overall response. Hence, an objective response rate (ORR) of 50.0% and a disease control rate (DCR) of 95.0% were achieved in the small sample size in our trial.

The 20 *Histo*-HER2/*neu*⁺ patients were determined into three groups on the basis of CTC enumeration and HER2 phenotyping at baseline, including the CTC-negative (n = 5), the *CTC*-HER2⁻ (n = 6) and the *CTC*-HER2⁺ (n = 9). We first showed the CTC enumeration at baseline was not a valid biomarker to predict the responses of the combination therapy (Fisher's exact $P = 0.051$, two tailed) (Fig. 5a). With the contribution of CTC-based HER2 phenotyping at baseline, the CTC-negative patients (n = 5) were detected with 4 cases with PR and one case with Non-CR/non-PD, and meanwhile the *CTC*-HER2⁺ patients (n = 9) showed 6 cases with PR, , whereas all of the *CTC*-HER2⁻ individuals (n = 6) reached the SD/PD (Fig. 5b; Supplementary Table S5), indicating an increased ORR (71.4%) if the cases with negative expression of HER2 on CTCs were excluded. We statistically demonstrated a significant difference of the best overall responses in the three cohorts of patients (Fisher's exact test, overall $P = 0.004$, two tailed) (Fig. 5b). Furthermore, the *CTC*-HER2⁺ phenotype at baseline was assessed with a most potential effect size in predicting the responses of PR/Non-CR/non-PD (AUC = 0.833, $P = 0.034$; sensitivity, 66.7%; 95% CI of sensitivity, 35.4% to 87.9%; specificity, 100%; 95% CI of specificity, 61.0% to 100%), superior to other CTC-derived parameters (e.g. the count/ratio of the HER2^(2+/3+) CTCs, the count/ratio of

the HER2⁽³⁺⁾ CTCs, the count of total CTCs) (Fig. 5c; Supplementary Table S6).

Thereafter, based on the analysis of the second blood draws of the 14 individuals of the patients with trastuzumab-based combination therapies, we investigated the significance of the dynamic change of CTC count and HER2 phenotype from baseline to the follow-up visit (Supplementary Table S5). At second blood draws, 8 (14) of the patients had the PR or Non-CR/non-PD, 5 (14) of the cases showed the status of SD and one patient already progressed. As shown in Fig. 6a, b, we vividly demonstrated the dynamic change of total CTC count from baseline to the follow-up visit, whereas no significant correlation with the real-time responses was found. By contrast, we statistically showed a significant association of the evolutionary HER2 phenotypes on CTCs with the follow-up efficacy of combination therapies (Fisher's exact test, overall $P = 0.007$) (Fig. 6c, d). The patients with the depletion of CTC-HER2⁻ phenotype were all assessed with the responses of SD/PD at second blood draws, contributing to the significant differences from the patients with depletion of CTC-HER2⁺ phenotype (Fisher's exact test, $P = 0.015$) or with consistently CTC-negative (Fisher's exact test, $P = 0.048$). Taken together, the HER2 phenotyping of the baseline CTCs or its dynamic change were valuable for predicting the outcomes of the trastuzumab-based combination therapies in breast cancer.

Discussion

The FDA-approved *CellSearch* system is extensively utilized to detect CTCs in 7.5 mL whole blood (WB). However, a limited portion of patients with primary breast tumors (22% – 24%) [31, 32] or metastatic breast tumors (45% – 70%) [18, 33, 34] could be detected with CTCs by *CellSearch*, which might be because of the limited anti-EpCAM molecules on the surface of 10 nm ferrofluid. With the advantages of peptides and larger magnetic nanoparticles, our team developed an innovative EpCAM-targeted technique (i.e. Pep10@MNPs) to enrich CTCs in 2.0 mL WB, which was validated with a high rate of CTC detection (70% – 97%) in solid tumors (breast cancer, lung cancer, etc.) [20, 36, 45-47]. In the present study, 81.5% of the enrolled breast cancer patients were

detected with CTCs by Pep10@MNPs (mainly 1 – 300 CTCs/2.0 mL WB), competitive to the one of *CellSearch* (mainly 1 – 500 CTCs/7.5 mL blood) [17, 48]. Moreover, our data showed that the rate of the cases with more than 3 CTCs that were determined with a worse prognosis by Liu et al. could reach 70.4% [46]. By contrast, by *CellSearch*, Cristofanilli and Pestrin et al. showed a lower recovery rate of the metastatic breast cancer (MBC) patients with ≥ 2 CTCs (34.5% or 60.6%) [22, 49]. Therefore, the efficient capture of CTCs by Pep10@MNPs in 2.0 mL WB might allow us to achieve the analysis of CTCs for the undetectable cases by *CellSearch*.

The cancer staging guidelines (version 8) of breast cancer by the American Joint Committee on Cancer (AJCC) recommended CTC enumeration as a valid staging indicator of breast cancer [23]. In this trial, our data demonstrated the high level of CTCs (≥ 3 CTCs) by Pep10@MNPs was significantly correlated with the negative expression of ER/PR on tumor tissues, different from the studies by *CellSearch* to the best of our knowledge [18, 22, 33]. Nonetheless, in breast cancer, the basal-like subtype (ER/PR⁻) are more aggressive and showed a worse prognosis as compared to the luminal A/B subtypes (ER/PR⁺), suggesting the rationality of the finding [50]. Furthermore, the expression level of Ki67 antigen (Ki67 LI) represents the cell proliferation, correlated with the malignancy and therapeutic outcomes of various cancers [51-55]. The histopathologic Ki67 LI has been valuable biomarker used to determine the luminal A/B subtypes of breast cancer [39, 40]. Two teams (Lucci's and Sandri's) previously investigated the relationship between CTC count and Ki67 LI of breast tumors, but both found no significant correlation [27, 56]. Differently, our data demonstrated a significant association ($P = 0.003$) with the cutoff of ≥ 3 CTCs in 2.0 mL WB. The various dichotomous cutoff of the Ki67 LI and CTC enrichment by *CellSearch* from Sandri's group might be the reasons for the different result [57]. As demonstrated in some *CellSearch*-based reports, we did not find significant correlations of CTC count with grading, sites of metastasis and HER2 expression on tumor tissues as well [18, 22, 31, 33, 57]. Intriguingly, enumerating CTCs had a strong correlation with the *neu* amplification in our trial, suggesting the potential to aid the decision making of the IHC-based HER2⁽²⁺⁾ patients. This finding is consistent with the

one by Lucci's group, whereas both are yet limited by the sample size [56]. Moreover, as expected, a strong correlation of the CTC enumeration (≥ 3 CTCs) with the PFS/OS of breast cancer patients was achieved, in concordance with the other *CellSearch/TumorFisher*-based publications [18, 22, 25, 36]. Taken together, the prevalence of epithelial CTC by Pep10@MNPs could serve as a valid diagnostic and prognostic marker of breast cancer. Nevertheless, these findings should be supported by a larger cohort of breast cancer patients in the future.

HER2/*neu* is predominant in the classification and treatment of breast cancer. Niikura et al. demonstrated that a cohort of breast cancer patients (6% – 64%) showed a loss of HER2/*neu*⁺ status at primary/metastatic tumors after preoperative systematic therapy and some patients (2% – 15%) exhibited an acquisition of HER2/*neu*⁺ status at metastases, which resulted in a shorter overall survival than the one of concordant HER2/*neu* status [12]. However, based on the invasive and biased tissue biopsies, it is difficult to overcome the spatio-temporal molecular heterogeneity of breast cancer. The noninvasive blood-based analysis of CTCs that derive from the primary/metastatic tumors might be ideal biomarkers of breast cancer for integral and dynamic HER2 portrait. Up to date, researchers have reported the encouraging significances of the CTC-based HER2 reassessment in the diagnosis and prognostication of breast cancer in a small sample size, where the *CellSearch* and immunocytochemistry (ICC) are the most extensive methods to detect the HER2 expression on CTCs [26-28, 31, 33]. Our trial was the first one to employ the Pep10@MNPs and ICC to achieve another sensitive HER2 phenotyping on CTCs, mainly benefiting from the efficient capture of CTCs in 2.0 mL WB. The *CellSearch* and ICC-based criteria of HER2 overexpression of the enriched CTCs (CTC-HER2⁺) were quite different by various groups. Some investigators determined the positive level of HER2 on CTCs with at least 2 CTCs and at least one HER2-strongly/moderately stained (HER2^(2+/3+)) CTCs in the primary/metastatic breast cancer patients [31, 58]. In other previous studies, the metastatic breast cancer patients with at least 5 CTCs and at least one HER2⁽³⁺⁾ CTCs were determined as the CTC-HER2⁺ cases [16, 28]. Besides, some trials explored the contribution of the ratio of HER2-positive CTCs in a reasonable definition of the

CTC-HER2⁺, such as 30% or 50% as the cutoff [29, 33, 49]. By contrast, in the *CTC*-positive cases (≥ 3 *CTCs*/2.0 mL WB), we determined the individuals with $> 12\%$ *HER2*⁽²⁺⁾ *CTCs* or with at least one *HER2*⁽³⁺⁾ *CTCs*, whose rationality was supported by the *HER2* testing by IHC. The *HER2* phenotyping of the enriched *CTCs* by Pep10@MNPs, could achieve a competitive overall accuracy ($\sim 71\%$) in identifying the patients with various histopathologic *HER2/neu* status, as compared to the reported concordance rates (38% – 86%) (Supplementary Table S7). Half of the patients with *HER2/neu*-overexpressed/amplified tumor tissues were determined with the negative expression of *HER2* on *CTCs*. Various sensitivities from ICC and IHC might be one of the intrinsic reasons. The heterogeneity of *HER2* profile in primary/metastatic breast tumors and dynamic change of *HER2* on *CTCs* should also be included in the reasons for this discordance [11, 12, 30]. With respect to the underlying diagnostic values of the *HER2* reassessments on *CTCs*, our findings demonstrated the potential of the *CTC*-based *HER2* phenotyping in detecting the poor differentiation or bone/lung metastasis of breast cancer was close to the significant levels, superior to the *CTC* enumeration. Similarly, Brandt's group found the prevalence of *HER2*-positive *CTCs* in the poorly-differentiated patients ($P = 0.033$) [59]. Cristofanilli's team demonstrated an increased likelihood of *HER2*-positive *CTCs* in the patients with bone metastasis, whereas they found no correlation with the other metastatic sites [60]. Hence, the significances described in the trials above were controversial at some extent. As compared to the *CTC* enumeration at baseline, *HER2* phenotyping on *CTCs* achieved a more sensitive prediction of PFS/OS of breast cancer, where the *CTC-HER2⁻* phenotype indicated an increased risk of progression and death. However, the findings about the independent prognostication of the *CTC*-based *HER2* phenotyping also remained controversial in breast cancer. Munzone et al. demonstrated the shortest PFS of the patients with *HER2/neu* overexpression/amplification on *CTCs*, conversely to the reports by Wallwiener and Zhang et al [27, 29, 37]. Moreover, no obvious difference of OS in the patients with various *CTC*-based *HER2* phenotypes was observed by Munzone et al. [27].

Clinically, breast cancer patients should be recommended to accept anti-*HER2*

monoclonal antibodies alone or in combination after the HER2/*neu*-positive primary/metastatic breast tumors are determined. As demonstrated by Jordan et al, the HER2-negative CTCs were insensitive to anti-HER2 monoclonal antibodies and easily resistant to cytotoxic chemotherapy [30]. Hence, the CTC-HER2⁻ cases at baseline might suggest an undesirable outcome of trastuzumab-based combination therapies. Our investigation preliminarily provided proofs to the assumption in a small sample size (n = 20), where the objective response rate reached 71.4% when the exclusion of the baseline CTC-HER2⁻ individuals were done in the analysis. As a comparison, the potential of CTC enumeration in predicting the outcomes of the combination therapies was also investigated in our trial and showed no association, consistent with the implications from a larger retrospective trial by Cristofanilli's group [25]. Therefore, beyond enumerating CTC, the HER2 phenotyping on CTCs before therapies may serve as a more useful predictor for the personalized administration of anti-HER2 therapy in breast cancer. Second, in a small sample size with second blood draws (n = 14), we showed the dynamic change of CTC enumeration and CTC-HER2 phenotypes after the combination therapy. As expected, the depletion of epithelial CTCs was far from a practical predictive biomarker of the treatment outcomes [61]. A few groups investigated the clinical significances of evolutionary HER2 status on CTCs. Agelaki et al. observed, after the treatment with lapatinib, the decrease of HER2-overexpressed CTC was significantly correlated with the stabilization of breast tumors [62]. Wang et al. demonstrated the patients with the loss of high-risk HER2 status on CTCs showed a significantly elongated PFS [16]. However, these trials only recruited the patients with HER2/*neu*-negative breast tumors. We indicated the depletion of CTC-HER2⁺ or consistently CTC-negative in the *Histo*-HER2/*neu*⁺ patients predicted a common response of PR at the follow-up visit after the trastuzumab-based combination therapies.

To the end, a few limitations should also be concerned in the present study. First of all, the aforesaid clinical implications of CTC detection and HER2 phenotyping in breast cancer are based on small sample size, so it needs to enroll more MBC patients to consolidate the diagnostic and prognostic values we demonstrated in this trial. In the future, a long follow-up with regular blood draws should be considered as well before

patients are assessed as progressive disease. If so, more rigorous thoughts about the dynamic change of CTC-HER2 phenotype could be uncovered for breast cancer, important for the decision making through personalized longitudinal evaluations.

Conclusions

The present study shows the opportunities of CTC enumeration and HER2 phenotyping on CTCs as novel aided biomarkers for the diagnosis and prognostication of breast cancer. Pep10@MNPs can achieve a sensitive detection of breast cancer (70.4%) with the cutoff (≥ 3 CTCs/2.0 mL whole blood). CTC enumeration by Pep10@MNPs carries the critical relevance to the histopathologic ER/PR or Ki67 LI status and the risk of progression/death, whereas not a valid predictor in the trastuzumab-based combination therapies. Interestingly, the heterogeneity of HER2 profiles on CTCs and tumor tissues at enrollment suggests a translational potential in predicting the efficacy of the trastuzumab-based combination treatments in the histopathologic HER2/*neu*-overexpressed/amplified breast cancer patients. Nonetheless, our findings need the validation in a larger sample size, ideally with the prospective or retrospective clinical trials.

List of abbreviations

HER2/*neu*: Human epidermal growth factor receptor 2; CTCs: Circulating tumor cells; AJCC: American Joint Committee on Cancer; *Histo*-HER2/*neu*^{+/-}: Histopathologic HER2/*neu*-positive/negative; CTC-HER2^{+/-}: CTC-based HER2-positive/negative; IHC: Immunohistochemistry; FISH: Fluorescence in situ hybridization; ICC: Immunocytochemistry; ER/PR: Estrogen receptor/progesterone receptor; LI: Labeling index; RECIST: Response Evaluation Criteria in Solid Tumors; PR: Partial response; Non-CR/non-PD: Non-complete response/non-progressive disease; SD: Stable disease; ORR: Objective response rate; DCR: Disease control rate; PFS: Progression-free survival; OS: Overall survival; HR: Hazard ratio; FDA: U. S. Food and Drug Administration; WB: Whole blood; CK: Cytokeratin; CD45: Leukocyte common antigen; EpCAM: Epithelial cell adhesion molecule; SA-MNPs: Streptavidin-modified

magnetic nanoparticles; Pep10@MNPs: Peptide (Pep10) functionalized magnetic nanoparticles; anti-EpCAM@MNPs: Anti-EpCAM antibody modified magnetic nanoparticles; DLS: Dynamic light scattering; TEM: Transmission electron microscope; FCM: Flow cytometry; MFIs: Mean fluorescent intensities; ROC: Receiver operating characteristic; AUC: Area under the curve; S. D.: Standard deviation; CI: Confidence interval; MBC: Metastatic Breast Cancer.

Supplementary data

Supplementary data to this article can be found online at

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Authors' contributions

MW, YL, HL, LZ, PL and YY designed the study. MW, LZ, PL and YY was the main contributors to the investigation, data curation, formal analysis of the work. YL, BS, XL, HL coordinated the enrollment of participants and provided the corresponding clinical information. CW, LZ and YY supervised, administrated, and supported the project. MW wrote the original draft. All authors reviewed and edited the original draft, and approved the final manuscript.

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Availability of data and material

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

This project was under the approval of the institutional review board at the Medical Ethical Committee of Peking Cancer Hospital (No. 2013KT29) from November 2018 to December 2019. All the participants signed the informed consent forms before enrolled in the trial.

Consent for publication

Not applicable.

Competing interests

There are no conflicts of interest to declare.

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Figure Legends

Fig. 1 Efficient capture and identification of epithelial breast cancer cells by Pep10@MNPs and immunocytochemistry (ICC). **a** Hydrodynamic diameters (left) and zeta potentials (right) of streptavidin-conjugated magnetic nanoparticles (SA-MNPs) and Pep10/anti-EpCAM-functionalized MNPs (Pep10/anti-EpCAM@MNPs) analyzed by dynamic light scattering (DLS). Data are presented as means $\pm$ S. D. (n = 3). **** P < 0.0001, unpaired Student's t -test. **b** The mean fluorescent intensities (MFIs) of PE-labeled anti-EpCAM on a normal breast epithelial cell line (MCF10A) and four breast cancer cell lines (SK-BR-3, MCF-7, MDA-MB-453, MDA-MB-468) from flow cytometry (FCM) analysis. Data are presented as means $\pm$ S. D. (n = 3). **** P < 0.0001, unpaired Student's t -test. **c** Capture efficiency of different cell lines by Pep10@MNPs or anti-EpCAM@MNPs. Data are presented as means $\pm$ S. D. (n = 3). Simple cell model (1000 cells/1 mL PBS, as System 1) was established for each cell line. The cells were pre-stained with DAPI for fluorescent visualization (blue nuclei, Supplementary Fig. S3). The capture efficiency (%) is defined as the number of captured cancer cells as a percentage of the total number of added cancer cells. **d** Identification of enriched CTCs from breast cancer patients based on the fluorescent staining of nuclei/cytokeratin (CK)/CD45. CK, specific for epithelial CTCs. Leukocyte common antigen (CD45), specific for white blood cells (WBCs). The captured cells by Pep10@MNPs that exhibit clear nucleus (blue), CK⁺ (green), and CD45⁻ (no red) are identified as CTCs, different from the WBCs which express CD45 (red), but no CK (no green). Scale bar: 10 μ m.

Fig. 2 Sensitive HER2 expression assessment on the captured breast cancer cells by ICC. **a** The MFIs of PE-labeled anti-HER2 on four breast cancer cell lines (SK-BR-3, MDA-MB-453, MCF-7, MDA-MB-468) from FCM analysis. Data are presented as means $\pm$ S. D. ($n = 3$). $**P < 0.01$, $****P < 0.0001$, unpaired Student's *t*-test. **b** Fluorescent images of the isolated breast cancer cells. The cancer cells were pre-stained with DAPI (blue, nuclei) and DiO (green, membrane). The isolated cancer cells were stained with Alexa Fluor 647-conjugated anti-HER2 (pink, HER2). Scale bar: 50 μ m. **c** MFIs from HER2 immunostaining on the captured breast cancer cells by Pep10@MNPs in System 1 (1000 breast cancer cells/1 mL PBS). Data are presented as means $\pm$ S. D. (100 events). $****P < 0.0001$, $***P < 0.001$, unpaired Student's *t*-test. **d** Correlation analysis between the means of MFIs from HER2 immunostaining on the four breast cancer cell lines by ICC and by FCM. Pearson's correlation coefficient $r = 0.9996$ (a positive and strong correlation), with a significant *P* value (0.0004, $***$, two-tailed).

Fig. 3 CTC enumeration and quantitative analysis of HER2 expression on CTCs from breast cancer patients at baseline. **a** Total CTC count in 2.0 mL whole blood from 54 enrolled breast cancer patients (#1~54). 44 (54) of the enrolled patients (81.5%) were detected with ≥ 1 CTCs/2.0 mL whole blood. **b** Development of four levels of HER2 expression on CTCs based on fluorescent quantification (incl. HER2⁽⁰⁾ CTCs, HER2⁽¹⁺⁾ CTCs, HER2⁽²⁺⁾ CTCs, HER2⁽³⁺⁾ CTCs). Left, MFIs from HER2 immunostaining on enriched CTCs for each patient (#11~54) at baseline. Twenty-three *Histo-HER2/neu*⁻ cases (green, #11~33) and 21 *Histo-HER2/neu*⁺ cases (red, #34~54) who had baseline CTCs were included. The data are presented as means $\pm$ standard error of mean (SEM), together with boxes (min to max). *x* axis, the IDs of patients. HER2 expression on CTCs were quantitatively determined as four MFI ranges from HER2 immunostaining, including no staining/barely perceptible staining (HER2⁽⁰⁾; MFI 0 ~ 100 a.u.), faint staining (HER2⁽¹⁺⁾; MFI 100 ~ 400 a.u.), moderate staining (HER2⁽²⁺⁾; MFI 400 ~ 600 a.u.) and strong staining (HER2⁽³⁺⁾; MFI > 600 a.u.). Right, the representative fluorescent graphs of the captured CTCs with different levels of HER2 expression. Scale bar: 10 μ m.

Fig. 4 Comparison of the level of HER2 expression on CTCs and tumor tissues from breast cancer patients. **a, b and c** Count (left) and ratio (right) of CTCs in four cell subpopulations with various HER2 expression for each patient at baseline. Only the CTC-positive cases (≥ 3 CTCs/2.0 mL whole blood) were included. Patients with various HER2 phenotypes by immunohistochemistry (IHC) are presented, including the *IHC-HER2*⁻ (**a**; n = 13), the *IHC-HER2*⁽²⁺⁾ (**b**; n = 12) and the *IHC-HER2*⁽³⁺⁾ (**c**; n = 13). The enriched CTCs were classified into four subpopulations with various HER2 expression for each patient, i.e. HER2⁽⁰⁾ CTCs (black), HER2⁽¹⁺⁾ CTCs (gray), HER2⁽²⁺⁾ CTCs (yellow), and HER2⁽³⁺⁾ CTCs (red). **d, e, and f** Representative cases for displaying a good concordance between the HER2 expression level on CTCs and on tumor tissues at baseline. The patient #18 (**d**), #45 (**e**), and #53 (**f**) were determined with different HER2 expression on baseline CTCs (i.e. the *CTC-HER2*⁻, the *CTC-HER2*⁽²⁺⁾ and the *CTC-HER2*⁽³⁺⁾) (Left). The *CTC-HER2*⁻, including the cases detected with only HER2^(0/1+) CTCs, or with $\leq 12\%$ HER2⁽²⁺⁾ CTCs and no detectable HER2⁽³⁺⁾ CTCs. The *CTC-HER2*⁽²⁺⁾, including the cases with $> 12\%$ HER2⁽²⁺⁾ CTCs and no detectable HER2⁽³⁺⁾ CTCs, or with $\leq 12\%$ HER2⁽³⁺⁾ CTCs. The *CTC-HER2*⁽³⁺⁾, determined as the cases with $> 12\%$ HER2⁽³⁺⁾ CTCs. The corresponding tumor tissue-based HER2 diagnosis by IHC for the three cases are shown on the right. In the IHC images, the cell nuclei (blue) and HER2 proteins (brown) were visible due to immunochemical reactions.

Fig. 5 The CTC-HER2⁻ at baseline shows worse responses to trastuzumab-based combination therapy in the *Histo*-HER2/*neu*⁺ patients. **a** Ratio of the cases with various best overall responses to the combination treatment in the patients with various baseline CTC count, including the CTC-negative (< 3 CTCs; n = 5) and the CTC-positive (≥ 3 CTCs; n = 15). The best overall responses of the 20 individuals included the partial response (PR) (n = 10), the non-complete response/non-progressive disease (Non-CR/non-PD) (n = 1), the stable disease (SD) (n = 8), and the PD (n = 1). Fisher's exact $P = 0.051$ (> 0.05), not significant (ns). **b** Ratio of the cases with various best overall responses to the combination therapy in the patients with various HER2 phenotypes on baseline CTCs, including the CTC-negative (n = 5), the CTC-HER2⁻ (n = 6), and the CTC-HER2⁺ (n = 9). P values were analyzed by the Fisher's exact test (two-tailed), including $P = 0.006$ (CTC-negative vs CTC-HER2⁻) (< 0.01, **), $P = 0.028$ (CTC-HER2⁻ vs CTC-HER2⁺) (< 0.05, *) and overall $P = 0.004$ (**). **c** ROC curves of different CTC-derived factors at baseline used to differentiate the *Histo*-HER2/*neu*⁺ patients with various best overall responses to the anti-HER2 therapy (i.e. PR/Non-CR/non-PD, or SD/PD). Only the CTC-positive cases were analyzed (n = 15). The area under ROC curves (AUC values) are presented. Left, the CTC-HER2⁺ (red) (AUC1, 0.833), the count and ratio of HER2^(2+/3+) CTCs (count, dark green; ratio, blue) (AUC2, 0.843; AUC3, 0.741). Right, the count and ratio of HER2⁽³⁺⁾ CTCs (count, dark green; ratio, blue) (AUC1, 0.759; AUC2, 0.667), the total CTC count (black) (AUC3, 0.556). The dash line was the line of identity with AUC = 0.500. The detailed values of ROC analysis were contained in supporting information (Supplementary Table S6).

Fig. 6 Dynamic changes of CTC count and *CTC-HER2* phenotype after the trastuzumab-based combination therapy. **a** Heat map of the CTC enumeration before and after the combination therapy in the 14 *Histo-HER2/neu*⁺ patients. Color scale from white to blue represent the CTC count (0 CTC, 1 CTC, 2 CTCs, ≥ 3 CTCs). *x* axis, the IDs of the 14 patients with clinical responses at the time point of CTC-based follow-up. **b** Comparison of patient responses to the combination therapy in three groups of patients with different changes of CTC count. The patients were classified into the cases with the change from CTC-positive to CTC-negative (i.e. from ≥ 3 CTCs to < 3 CTCs) (*n* = 11), or from CTC-negative to CTC-positive (*n* = 1), or with consistently CTC-negative (*n* = 2). *P* values were analyzed by the Fisher's exact test (two-tailed). Not significant (ns), *P* > 0.05. **c** Heat map of the *CTC-HER2* phenotype before and after the combination therapy for each monitored patient. Color blocks represented various *CTC-HER2* phenotypes (Cambridge blue, the CTC-negative; gray, the *CTC-HER2*⁻; pink, the *CTC-HER2*⁺). **d** Comparison of patient response to the combination therapy in the four groups of individuals with dynamic change of *CTC-HER2* phenotypes. The individuals were determined as the cases with depletion of *CTC-HER2*⁺ (*n* = 6), or with depletion of *CTC-HER2*⁻ (*n* = 5), or with acquisition of *CTC-HER2*⁺ (*n* = 1), or consistently with CTC-negative (*n* = 2). *P* values were analyzed by the Fisher's exact test (two-tailed). Significant level, **P* < 0.05, ***P* < 0.01, including *P* = 0.015 (depletion of *CTC-HER2*⁺ vs depletion of *CTC-HER2*⁻), *P* = 0.048 (depletion of *CTC-HER2*⁻ vs consistently with CTC-negative) and overall *P* = 0.007.

Table Legends

Table 1 Clinical characteristics of recruited breast cancer patients

Clinical factors	Patients (N = 54)	
	No.	%
Age, years		
Range	29~87	
Median	55	
Mean	53.4	
Standard deviation	11.4	
Grading		
G1	4	7.4
G2	23	42.6
G2~G3	3	5.6
G3	23	42.6
N/A *	1	1.9
Relapse		
Yes	46	85.2
No	8	14.8
Metastasis ^a		
No metastasis	1	1.9
Visceral metastasis	35	64.8
Nonvisceral metastasis	18	33.3
Histopathologic ER/PR status ^b		
<i>Histo</i> -ER/PR ⁺	29	53.7
<i>Histo</i> -ER/PR ⁻	11	20.4
N/A *	14	25.9
Histopathologic HER2/ <i>neu</i> status ^c		
<i>Histo</i> -HER2/ <i>neu</i> ⁺	26	48.1
<i>Histo</i> -HER2/ <i>neu</i> ⁻	28	51.9
Histopathologic Ki67 LI ^d		
High	44	81.5
Low	7	13.0
N/A *	3	5.5
Period before follow-up for CTCs, weeks		
Range	7.4~18.6	
Median	13.0	
Mean	12.6	
Standard deviation	4.0	

Annotations:

^a **Visceral metastasis**, including metastasis at sites of lung, liver, adrenal gland, brain, kidney, pancreas, peritoneal, pleural effusions, etc.; **Nonvisceral metastasis**, including metastasis at sites of the breast, lymph nodes, chest wall, bone, skin, abdomen, etc.;

^b **Histo-ER/PR⁺**, the estrogen receptor (ER)/progesterone receptor (PR)-positive phenotype on tumor tissues by immunohistochemistry (IHC), determined as the case with positive expression of either ER or PR; **Histo-ER/PR⁻**, the ER/PR-negative phenotype on tumor tissues by IHC, determined as the case with negative expression for both of ER and PR;

^c **Histo-HER2/neu⁺**, the histopathologic HER2/neu-positive subtype, assessed as the case with positive expression of HER2 by IHC (i.e. the *IHC-HER2⁽³⁺⁾*) or with *neu* amplification by FISH when the HER2 expression by IHC was equivocal (i.e. the *IHC-HER2⁽²⁺⁾* plus *neu⁺*); **Histo-HER2/neu⁻**, the histopathologic HER2/neu-negative subtype, assessed as the case with negative expression of HER2 by IHC (i.e. the *IHC-HER2⁻*) or with no *neu* amplification by FISH when the HER2 expression by IHC was equivocal (i.e. the *IHC-HER2⁽²⁺⁾* plus *neu⁻*);

^d **Ki67 LI**, the Ki67 labeling index, presented by the percentage of tumor cells that were positively expressed with Ki67 on tumor tissues. The cases whose tumor tissues were detected with $\geq 14\%$ Ki67 LI by IHC were classified as a high level of Ki67 expression;

* **N/A**, not available, used to show the missing/unvalidated histopathologic parameters at first diagnosis for some breast cancer patients;

Table 2 Comparison of the HER2 assessments on CTCs and tumor tissues from breast cancer patients at baseline

CTC-based	Tumor tissue-based	
	<i>Histo-HER2/neu</i> ^{-c} , no. (% [*])	<i>Histo-HER2/neu</i> ^{+d} , no. (% [*])
CTC-positive cases [†]	18	20
CTC-HER2 ^{-a}	17 (94.4)	10 (50.0)
CTC-HER2 ^{+b}	1 (5.6)	10 (50.0)

$\kappa = 0.434$ (Kappa's test, $P = 0.004$); Fisher's exact test, $P = 0.004$, two tailed.

Annotations:

[†] **CTC-positive cases**, the patients who were detected with ≥ 3 CTCs in 2.0 mL blood;

^a **CTC-HER2⁻**, the HER2-negative phenotype on CTCs for the CTC-positive cases, including two kinds of situations: (1) the case that was detected with only HER2^(0/1+) CTCs (i.e. HER2⁽⁰⁾ CTCs and/or HER2⁽¹⁺⁾ CTCs); (2) the case that was detected with $\leq 12\%$ HER2⁽²⁺⁾ CTCs in total CTCs and no detectable HER2⁽³⁺⁾ CTCs;

^b **CTC-HER2⁺**, the HER2-positive phenotype on CTCs for the CTC-positive cases, determined as the case that was detected with $> 12\%$ HER2⁽²⁺⁾ CTCs in total CTCs or with detectable HER2⁽³⁺⁾ CTCs;

^c ***Histo-HER2/neu*⁻**, the histopathologic HER2/*neu*-negative subtype, assessed as the case with negative expression of HER2 by IHC (i.e. the *IHC-HER2*^(0/1+)) or with no *neu* amplification by FISH when the HER2 expression by IHC was equivocal (i.e. the *IHC-HER2*⁽²⁺⁾ plus *neu*⁻);

^d ***Histo-HER2/neu*⁺**, the histopathologic HER2/*neu*-positive subtype, detected as the case with strong expression of HER2 by IHC (i.e. the *IHC-HER2*⁽³⁺⁾), or with amplification of *neu* by FISH when the HER2 expression by IHC was equivocal (i.e. the *IHC-HER2*⁽²⁺⁾ plus *neu*⁺);

^{*} %, the percentages of the patients with various CTC-HER2 phenotypes (CTC-HER2⁻ or CTC-HER2⁺) in two cohorts of the CTC-positive cases who were respectively diagnosed as the *Histo-HER2/neu*⁻ and *Histo-HER2/neu*⁺ subtype;

Figures

Figure 1

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Figure 2

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Figure 3

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Figure 4

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