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Clinical Cancer Research

# Evolutionary Expression of HER2 Conferred by Chromosome Aneuploidy on Circulating Gastric Cancer Cells Contributes to Developing Targeted and Chemotherapeutic Resistance

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## Abstract

**Purpose:** Previous human epidermal growth factor receptor-2 (HER2)-derived resistance studies were based on *ex vivo* models, which could not mirror evolutionary expression of HER2 during therapy. To investigate dynamic expression of HER2 and its contribution to developing therapeutic resistance conferred by chromosome aneuploidy, both the HER2 phenotype and chromosome 8 (Chr 8) aneuploidy on circulating tumor cells (CTC) were coexamined in advanced gastric cancer (AGC) patients.

**Experimental Design:** A total of 115 AGC patients, including 56 of histopathologic HER2$^+$ (hHER2$^+$) subjects who received first-line HER2-targeted therapy plus chemotherapy, and 59 of hHER2$^-$ patients who received chemotherapy alone, were prospectively enrolled. Both HER2 phenotype and Chr8 aneuploidy of CTCs in patients were coexamined by HER2-iFISH during therapy.

**Results:** A fluctuated positive HER2 phenotype on CTCs (cHER2$^+$) was revealed, showing cHER2$^+$ at different time intervals during treatment. Acquisition of the cHER2$^+$ phenotype in 91.0% of hHER2$^+$ and 76.2% hHER2$^-$ patients was demonstrated to correlate with development of resistance to trastuzumab-targeted therapy for hHER2$^+$ patients and chemotherapy alone for hHER2$^-$ patients. Aneuploid Chr8 was demonstrated to participate in the acquisition of the cHER2$^+$ phenotype, which provides a growth advantage to HER2$^+$ CTCs against therapeutic pressure, leading to the development of therapeutic resistance.

**Conclusions:** Compared with low positivity of conventional histopathologic hHER2 examination routinely performed once, significant higher positivity of cHER2$^+$ on CTCs was observed. Continuously examining cHER2 shows unique advantages with respect to monitoring therapeutic resistance in real time in carcinoma patients. Moreover, contribution of chromosome aneuploidy to the phenotypic evolution of HER2 expression on CTCs may help elucidate underlying mechanisms of developing therapeutic resistance. *Clin Cancer Res; 1–11. ©2018 AACR.*

## Introduction

Gastric cancer has high incidence and mortality (1–3). Approximately 10% to 30% of patients with advanced gastric cancer (AGC) who are histopathologic human epidermal growth factor receptor-2 (HER2)-positive (hHER2$^+$) receive HER2-targeted trastuzumab. Among targeted therapy-treated patients, a decline in efficacy from 60% (the first-line therapy) down to 37% (the second-line treatment) was reported (4–6). Recent phase III clinical trials (TyTAN and GATSBY) to study the efficacy of the second-line HER2-targeted therapy showed that administration of additional lapatinib or trastuzumab emtansine to the second-line chemotherapy was not as efficacious as the first-line trastuzumab therapy for prolonging progression-free survival (PFS) and overall survival (OS) to those hHER2$^+$ gastric cancer patients (7, 8). Regarding those remaining 70% of hHER2$^-$ gastric cancer patients, they cannot benefit from HER2-targeted therapy due to inexistence of HER2 to drive malignant development and tumor progression (5, 9). Nevertheless, recent studies indicated conversion from hHER2$^-$ to hHER2$^+$ following metastasis or recurrence in 5.7% of hHER2$^-$ gastric cancer patients, suggesting the existence of variable driving mechanisms for gastric cancer development (10).

Human malignant carcinomas, including gastric cancer, are highly heterogeneous, and their molecular characteristics evolve to create therapeutic resistance during cancer development (11–14). However, how malignant evolution (such as HER2 expression) occurs and subsequently contributes to developing therapeutic resistance in both hHER2$^+$ and hHER2$^-$ gastric cancer patients are not clear. Thus, it is necessary to develop better strategies to continuously examine dynamically expressed

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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## Translational Relevance

Previous human epidermal growth factor receptor-2 (HER2)-derived resistance studies were based on *ex vivo* models, which could not mirror evolutionary HER2 expression during treatments. Our findings in the present study demonstrate the dynamic HER2 phenotype on circulating tumor cells (CTC; cHER2) in gastric cancer patients during therapy in both histopathologic HER2$^+$ (hHER2$^+$) and hHER2$^-$ patients. Compared with conventional one-time hHER2 examination showing low positivity on primary tumor, more than 70% of patients in both hHER2$^+$ and hHER2$^-$ cohorts dynamically acquired cHER2$^+$ phenotype on CTCs. Acquisition of cHER2$^+$ correlated with resistance to the targeted and/or chemotherapy. Obtained results revealed unique advantages of continuous examination of cHER2 status on CTCs with respect to monitoring resistance in real time. In addition, aneuploid chromosome 8 was confirmed to participate in dynamic acquisition of cHER2$^+$ and further to provide a growth advantage for cHER2$^+$ CTCs against therapeutic pressure. Obtained results help elucidate the mechanisms of developing resistance.

HER2 with respect to monitoring the development of resistance in real time during therapy.

Circulating tumor cells (CTC) in peripheral blood offer a "liquid biopsy" option for noninvasive monitoring therapeutic responses of various cancers, such as gastric cancer (15–18). CTCs are involved in every "checkpoint" from dissemination to distant tumor formation. They could mirror genotypic and phenotypic characteristics of malignant lesions and therefore could be applied to monitor the genotypic and phenotypic evolution of carcinomas (19, 20). Taking advantage of the well-established integrated subtraction enrichment and immunostaining-fluorescence *in situ* hybridization (SE-iFISH) strategy (21–25), we previously performed studies on both AGC patients and a metastatic patient-derived xenograft (mPDX) model of gastric neuroendocrine cancer and showed diverse chromosomal aneuploidies of CTCs might possess either intrinsic or acquired resistance to cisplatin (DDP)- or paclitaxel (PTX)-based chemotherapy (23, 25). Moreover, an additional study indicated that phenotypic HER2 expression on CTCs was highly heterogeneous (25).

To further investigate how HER2 expression dynamically evolves and contributes to developing therapeutic resistance, we longitudinally examined both dynamic variation of HER2 phenotype and chromosome aneuploidy in enriched CTCs by HER2-iFISH in 115 prospectively enrolled AGC patients, including 56 hHER2$^+$ and 59 hHER2$^-$ subjects. In addition, contribution of chromosomal aneuploidy to dynamic HER2 expression was also investigated.

## Materials and Methods

### Patient enrollment and sample collection

The classification and clinicopathologic characteristics of the 115 enrolled patients are shown in Supplementary Fig. S1 and Supplementary Table S1, respectively.

All the subjects were enrolled at Peking University Cancer Hospital from January 2015 to February 2017. All patients were $\geq$18 years of age and had locally advanced, recurrent, and/or metastatic adenocarcinoma at either stomach or gastroesophageal junction, which were histopathologically confirmed. Enrolled nontreated patients with Karnofsky performance status $\geq$60, and adequate organ function as well as evaluable disease were eligible for the study. Enrolled patients were classified into two cohorts: hHER2$^+$ patients treated with the first-line trastuzumab-targeted therapy plus PTX- or DDP-based chemotherapy, and hHER2$^-$ patients treated with the first-line PTX or DDP-based chemotherapy alone. Therapeutic procedures were similar to those previously described (16, 25).

hHER2$^+$ patients histopathologically exhibited either 3+ of immunohistochemistry staining, or 2+ HER2 expression with positive HER2 gene amplification. hHER2$^-$ patients are those with 0, 1+, or 2+ expression with no HER2 gene amplification (4).

Six weeks following therapy initiated, evaluation of clinical response was performed with CT according to the RECIST version 1.1 criteria (26). With respect to gastric cancer, because primary tumor was unmeasurable according to RECIST, unidimensional measurement of lymph nodes or distant metastases was applied to evaluate clinical response with medical imaging. Responses were categorized as partial responses (PR, a minimum 30% decrease in diameters of target lesions), progressive disease (PD, a minimum 20% increase in diameters of target lesions), or stable disease (SD, neither sufficient shrinkage to be qualified as a PR nor a sufficient increase to be qualified as PD).

Six milliliters of peripheral blood was collected prior to therapy in 115 assessable patients. For patients whose CTCs were longitudinally investigated (44 of hHER2$^+$ and 42 of hHER2$^-$ subjects; Supplementary Fig. S1), blood samples were collected at the beginning of each treatment cycle.

Informed consent forms were signed and obtained from all subjects prior to participation. The study, approved by the Ethics Committee of Peking University Cancer Hospital, was performed according to the Declaration of Helsinki.

### Subtraction enrichment

Subtraction enrichment (SE) of CTCs was performed according to the manufacturer's instructions (Cytelligen) and the previously published protocols (21, 22). Briefly, 6 mL of the peripheral blood of patients with AGC was collected into a tube containing ACD anticoagulant (Becton Dickinson). Samples were stored at room temperature for no more than 48 hours prior to processing. Briefly, blood samples were centrifuged at 200 $\times$ *g* for 15 minutes at room temperature. Sedimented blood cells were gently mixed with 3.5 mL of hCTC buffer, followed by loading on the nonhematopoietic cell separation matrix in a 50-mL tube, and subsequent centrifugation at 450 $\times$ *g* for 5 minutes. Solution containing white blood cells (WBC) and tumor cells, but without red blood cells was collected into a 50-mL tube and subjected to incubation with 300 μL of immunomagnetic beads conjugated to a cocktail of antileukocyte mAbs, at room temperature for 30 minutes with gentle shaking. WBCs bound to immunobeads were depleted using a magnetic separator. Solutions free of magnetic beads were collected into a 15-mL tube, followed by adding hCTC buffer to 14 mL. Samples were spun at 500 $\times$ *g* for 4 minutes at room temperature. Supernatants were aspirated down to 50 μL. Sedimented cells in 50 μL solution were gently resuspended, followed by mixing with the special fixative developed by

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Cytelligen, and then applied to the coated and formatted CTC slides. Samples were dried overnight at 37°C for subsequent iFISH analyses.

### HER2-iFISH

HER2-iFISH was performed similarly to that previously published with minor modification (22), and according to the manufacturer's protocol (Cytelligen). Briefly, dried monolayer cells on the coated and formatted CTC slides (Cytelligen) were rinsed and incubated with PBS at room temperature for 3 minutes, followed by hybridization with Vysis chromosome 8 centromere probe (CEP8) SpectrumOrange (Abbott Laboratories) for 4 hours using an S500 StatSpin Thermo Brite Slide Hybridization/Denaturation System (Abbott Molecular). Samples were subsequently incubated with Alexa Fluor (AF) 488 (green) and 594 (red), respectively, conjugated to the mAbs recognizing HER2 and CD45 at room temperature for 20 minutes in the dark. After washing, samples were mounted with mounting media containing DAPI for nucleus staining (Vector Laboratories) and subjected to automated CTC image scanning and analyses.

### Automated CTC scanning and image analysis performed by Metafer-iFISH

Fully automated scanning, image acquiring and analyses of positive iFISHed CTCs on the coated and formatted slides (Cytelligen) were performed using an automated Metafer-iFISH scanning and image analyzing system (Carl Zeiss, MetaSystems, and Cytelligen; ref. 22). Automated X–Y scanning with cross Z-sectioning of all cells performed at 1-μm steps of depth was performed in four fluorescent color channels (DAPI, HER2, CEP8, and CD45). Positive target cells ($HER2^+$ CTCs) are defined as $DAPI^+$, $CD45^-$, and $HER2^+$ with diploid or aneuploid Chr8.

Automated CTC classification and statistical analyses were performed upon cell size, cell cluster, quantified immunostaining intensity of HER2 expression, and chromosome ploidy.

The precise copy number of chromosome 8 in each single CTCs was assessed for all the enrolled subjects.

### Detection of HER2 expression on cell lines

Breast cancer cell lines including MCF-7, BT20, MDA-MB-453, and SK-BR-3, each with different amount of HER2 expression, were obtained from Cell Resource Center, Chinese Academy of Medical Science and Peking Union Medical College (Beijing, China), and cultured as previously described (25). To quantify HER2 expression intensity performed by HER2-iFISH, approximately 500 cells were spiked into the blood collected from healthy donors. Specimens were subjected to SE to enrich tumor cells, followed by HER2-iFISH.

### Statistical analysis

All statistical analyses were performed with SPSS 21.0 software (IBM Corp., New York, USA). The threshold of $HER2^+$ CTCs in $hHER2^+$ and $hHER2^-$ cohorts was established based on nonparametric receiver operating characteristic (ROC) analysis, and optimal cutoff values were determined to maximize the area under the curve (AUC) of ROC. PFS was defined as the time from initial blood collection to the date that clinical progression was confirmed or was censored at the last follow-up. OS was defined as the time from initial blood collection to the date that death occurred or was censored at the last follow-up. Kaplan–Meier survival plots for PFS or OS were generated based on $HER2^+$ CTCs, and the survival curves were compared using log-rank tests. Cox proportional hazards regression was used to determine the univariate and multivariate hazard ratios of PFS and OS. $HER2^+$ CTCs and standard clinical factors, including gender, age, primary tumor site, liver metastasis, peritoneal metastases, bone metastasis, and Lauren classification, were subjected to univariate analysis for PFS and OS. Significant variables from univariate analyses were included in multivariate analyses. Correlations of variations of aneuploid CTCs and $HER2^+$ CTCs were performed using the Spearman test. $P < 0.05$ was considered statistically significant.

## Results

### Pretherapeutic cHER2 phenotype on CTCs is distinct from HER2 expression in primary lesions

Classification and relevant clinicopathologic characteristics of all the enrolled 115 patients are described in Supplementary Fig. S1 and Supplementary Table S1. Quantification of HER2 expression intensity performed by HER2-iFISH on breast cancer cell lines is depicted in Supplementary Fig. S2A and S2B.

Pretherapeutic cHER2 status on CTCs in the cohorts of $hHER2^+$ and $hHER2^-$ patients is shown in Fig. 1. $cHER2^+$ CTCs in both cohorts had a similar positivity rate (Fig. 1A). Although the $cHER2^+$ phenotype on CTCs did not match hHER2 status in primary lesions, $cHER2^+$ expression was correlated with a homogeneous histologic HER2 expression pattern in $hHER2^+$ patients. Illustrated in Fig. 1B and C, correlation of the homogeneous expression pattern of $hHER2^+$ with $cHER2^+$ CTCs was observed. Among $hHER2^-$ subjects, pretherapeutic $cHER2^+$ CTCs could be detected in patients whose histologic HER2 expression score was 0 (Fig. 1D), indicating that CTCs were able to have a pretherapeutic positive cHER2 phenotype, despite negative hHER2 expression in primary lesions ($hHER2^-$).

None of other clinicopathologic characteristics, including gender, age, primary tumor site, Lauren classification, and metastatic sites, significantly correlated with pretherapeutic cHER2 expression on CTCs.

### Dynamic acquisition of $cHER2^+$ phenotype on CTCs during therapy

Among 115 subjects recruited in this follow-up clinical study, 29 participants did not return following initial examination, remaining 86 patients, including 44 of $hHER2^+$ and 42 of $hHER2^-$ patients were eligible to subject to longitudinal study (Supplementary Fig. S1). cHER2 phenotype on CTCs was longitudinally monitored among 86 patients during therapy. Revealed in Fig. 2A, the amount of $cHER2^+$ CTCs fluctuated during the therapeutic process in both $hHER2^+$ and $hHER2^-$ cohorts. Pretherapeutic $cHER2^-$ CTCs were able to acquire $cHER2^+$ phenotype during therapy, showing 91.0% (40 of 44) of $hHER2^+$ as well as 76.2% (32 of 42) of $hHER2^-$ patients exhibited $cHER2^+$ phenotype at different time intervals following treatment initiation (Fig. 2B).

However, hHER2 status of the primary lesions is relatively stable during therapy. Figure 2C and D (data obtained from 2 patients) shows that the pretherapeutic $cHER2^-$ patient could be converted to posttherapeutic $cHER2^+$, whereas hHER2 status of the primary tumor in both the patients remained negative,

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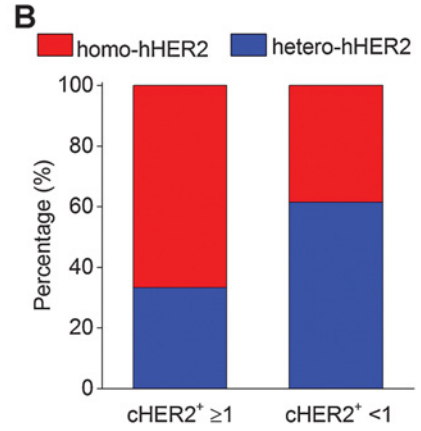
A

The positivity of HER2⁺ CTCs prior to therapy in AGC patients

| Patients | No. | HER2⁺ CTCs (≥1) | |
|---|---|---|---|
| | | N | n% |
| hHER2⁺ | 56 | 19 | 33.9% |
| hHER2⁻ | 59 | 25 | 42.4% |
| Overall | 115 | 44 | 38.3% |



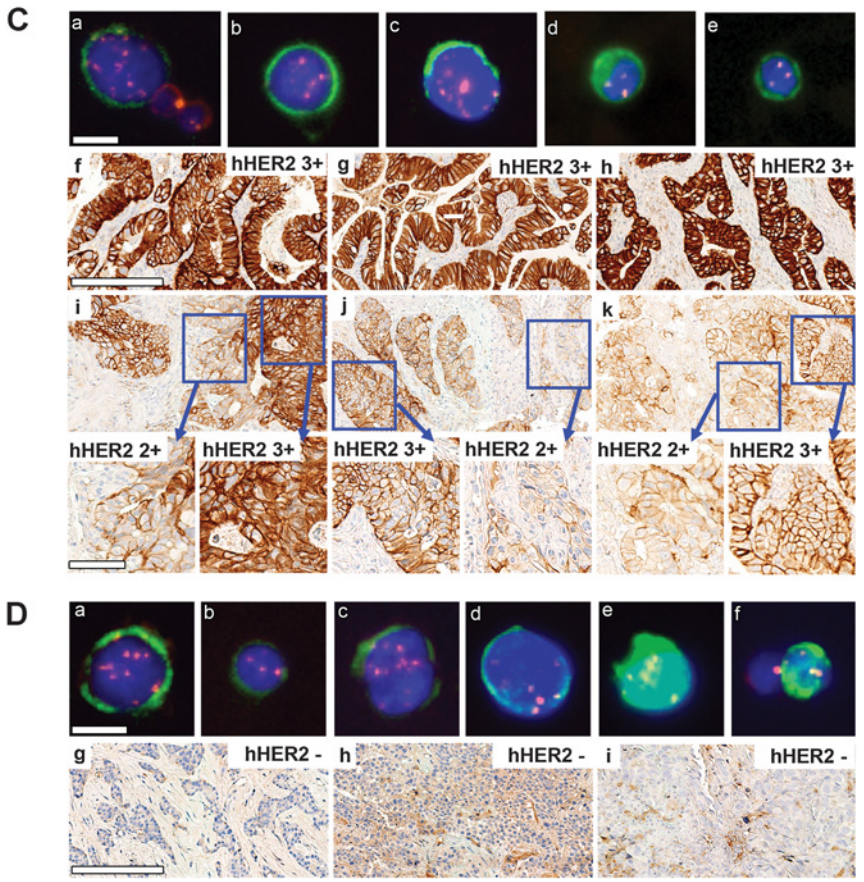







**Figure 1.**

Correlation of pretherapeutic HER2 expression on CTCs and in primary tumors. **A,** The positivity of cHER2$^+$ CTCs in 115 enrolled patients with AGC. **B,** Percentage of hHER2$^+$ patients with homogeneous or heterogeneous hHER2 expression in primary lesions. Homo-hHER2: hHER2$^+$ patients with homogeneous hHER2 expression; hetero-hHER2: hHER2$^+$ patients with heterogeneous hHER2 expression; cHER2$^+$: positive HER2 expression on CTCs. **C,** hHER2$^+$ cohorts: **a–e,** cHER2$^+$ CTCs identified by HER2-iFISH; **f–k,** hHER2$^+$ signal in primary lesions revealed by immunohistochemistry staining. Bars in **a, f**, and **i**, 5, 200, and 60 μm, respectively. **D,** hHER2$^-$ cohorts: **a–f**, cHER2$^+$ CTCs identified by HER2-iFISH; **g–i**, hHER2$^-$ images of primary lesions revealed by immunohistochemistry staining. Bars in **a** and **g**, 5 and 200 μm, respectively.

suggesting the existence of evolutionary expression of cHER2 on CTC during therapy, which was irrelevant to the hHER2 status of the primary lesions.

### Acquisition of cHER2$^+$ phenotype on CTCs correlates to developing therapeutic resistance and poor prognosis

Following subjected to therapy, 33 of 44 (75%) hHER2$^+$ patients had a PD. In the hHER2$^-$ cohort of 42 patients, 23 of 42 subjects (55%) developed PD following therapy. The longitudinal variation of cHER2 phenotype in both cohorts of PD patients is depicted in Fig. 3A and B, showing that 63.6% (21 of 33) of hHER2$^+$ PD patients and 73.9% (17 of 23) of hHER2$^-$ PD patients acquired the cHER2$^+$ phenotype when therapeutic resistance was developed, suggesting that acquisition of the cHER2$^+$ phenotype during therapy might be involved in developing therapeutic resistance.

The correlation of cHER2$^+$ CTCs with prognosis in the hHER2$^+$ and hHER2$^-$ cohorts was examined. ROC curves and AUC area data are demonstrated in Supplementary Fig. S3, showing that the cutoff values of ≥2 cHER2$^+$ CTCs for hHER2$^+$ cohort, and ≥3 for hHER2$^-$ cohort, which maximizes the AUC area were, respectively, selected as the high-risk thresholds for the analyses in Fig. 3C–F. Figure 3C–F depicts Kaplan–Meier plots with respect to predicting PFS and OS by pretherapeutic

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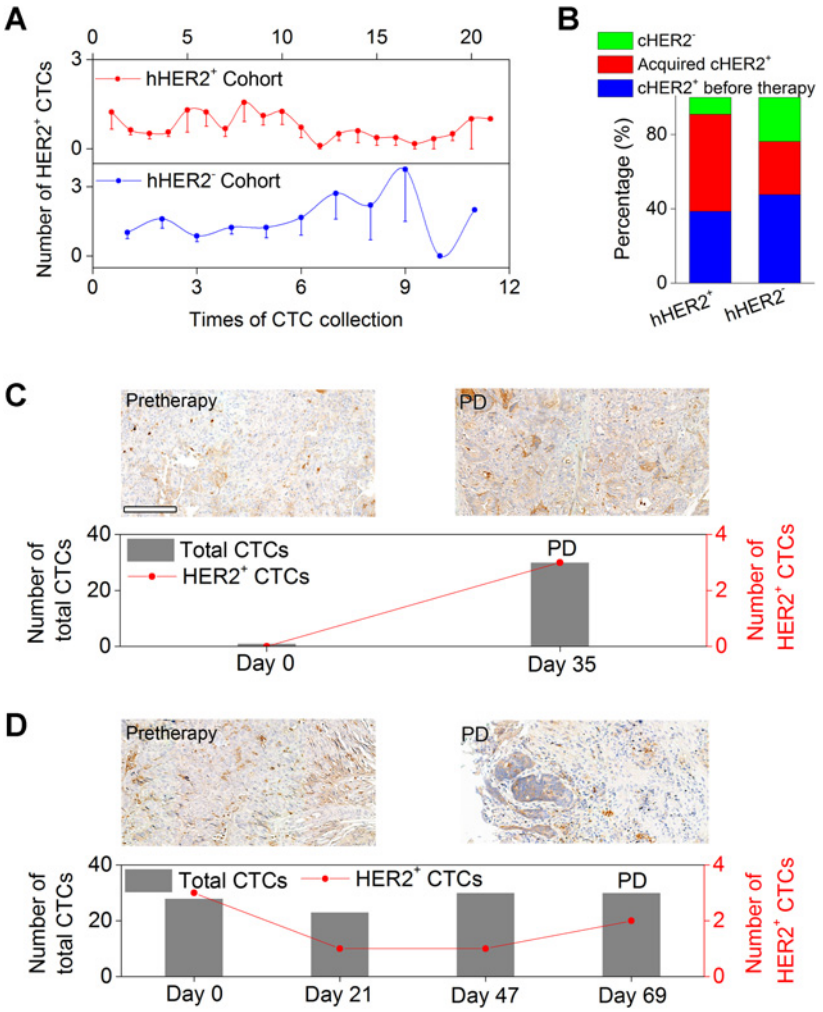


**Figure 2.**

Evolutionary expression of cHER2 on CTCs during therapy. **A,** Quantitative variation of cHER2$^+$ CTCs during therapy in the hHER2$^+$ and hHER2$^-$ cohorts. Error bars, SEM. **B,** Percentage of patients with pretherapeutic cHER2$^+$, acquired posttherapeutic cHER2$^+$, or cHER2$^-$ phenotypes in hHER2$^+$ and hHER2$^-$ patients, respectively. **C** and **D,** Two hHER2$^-$ patients with variable cHER2 phenotypes during therapy showed nonchanged stable hHER2$^-$ status in the duration between pretherapy and PD (progressive disease). Bar, 200 μm.

cHER2$^+$ CTCs in both hHER2$^+$ and hHER2$^-$ cohorts, showing that acquisition of the cHER2$^+$ phenotype correlated with reduced therapeutic efficacy, developing therapeutic resistance, and poor PFS as well as OS in both cohorts of patients. This observation was further confirmed by Cox proportional hazards regression, indicating that both hHER2$^+$ and hHER2$^-$ patients with the cHER2$^+$ CTCs had a high risk for inferior PFS and OS as illustrated in Supplementary Tables S2 and S3.

### Aneuploid Chr8 contributes to acquisition of the cHER2$^+$ phenotype on CTCs

As shown in Fig. 4A, various heterogeneous polysomic Chr8 were observed in cHER2$^+$ CTCs. Analysis of the ratio of chromosome aneuploidy and HER2 status is depicted in Fig. 4B and C. In the hHER2$^+$ cohort, 81.2% of cHER2$^+$ CTCs were multiploid, and the percentages of chr8 haploidy, diploidy, triploidy, and tetraploidy in cHER2$^+$ CTC are 0.3%, 2.9%, 5.7%, and 6.0%, respectively. Similarly, in the hHER2$^-$ cohort, 85.5% cHER2$^+$ CTCs are chr8 multiploid, and the percentages of chr8 diploidy, triploidy, and tetraploidy are 2.6%, 4.0%, and 5.9%, respectively. Overall, more than 90% of cHER2$^+$ CTCs had aneuploid Chr8, and most of them were multiploid (≥5 copy number of Chr8). Supplementary Fig. S4 and Fig. 4D demonstrate that quantitative variation of either aneuploid (Supplementary Fig. S4) or multiploid (Fig. 4D) CTCs in the duration from pretherapy to PD, significantly correlated with quantitative variation of number of cHER2$^+$ CTCs, indicating that aneuploid Chr8, particularly multiploid Chr8, was involved in the dynamic acquisition of the posttherapeutic cHER2 phenotype in AGC patients.

Figure 5A displays quantity variation of diverse cHER2$^+$ CTCs with aneuploid Chr8 in patients monitored from pretherapy to PD. Compared with tri- and tetraploid cHER2$^+$ CTCs, only multiploid (≥5 copies) cHER2$^+$ cells increased following therapeutic resistance developed. Quantitative variations of triploid and tetraploid cHER2$^+$ CTCs were irrelevant to prognosis (Supplementary Tables S4 and S5), whereas elevated specific subtype of multiploid cHER2$^+$ CTCs in the duration from pretherapy to the first-time evaluation of clinical response, significantly correlated with both decreased PFS (Fig. 5B; Supplementary Table S6) and inferior OS depicted in Kaplan–Meier plots (Fig. 5C). Multiploid cHER2$^+$ CTCs do not show significant correlation with OS when subjected to Cox proportional hazards regression analysis (Supplementary Table S6). The results indicate that multiploid Chr8 help improve adaption of cHER2$^+$ CTCs in the therapeutic environment, and further to facilitate developing resistance to the targeted and chemotherapy.

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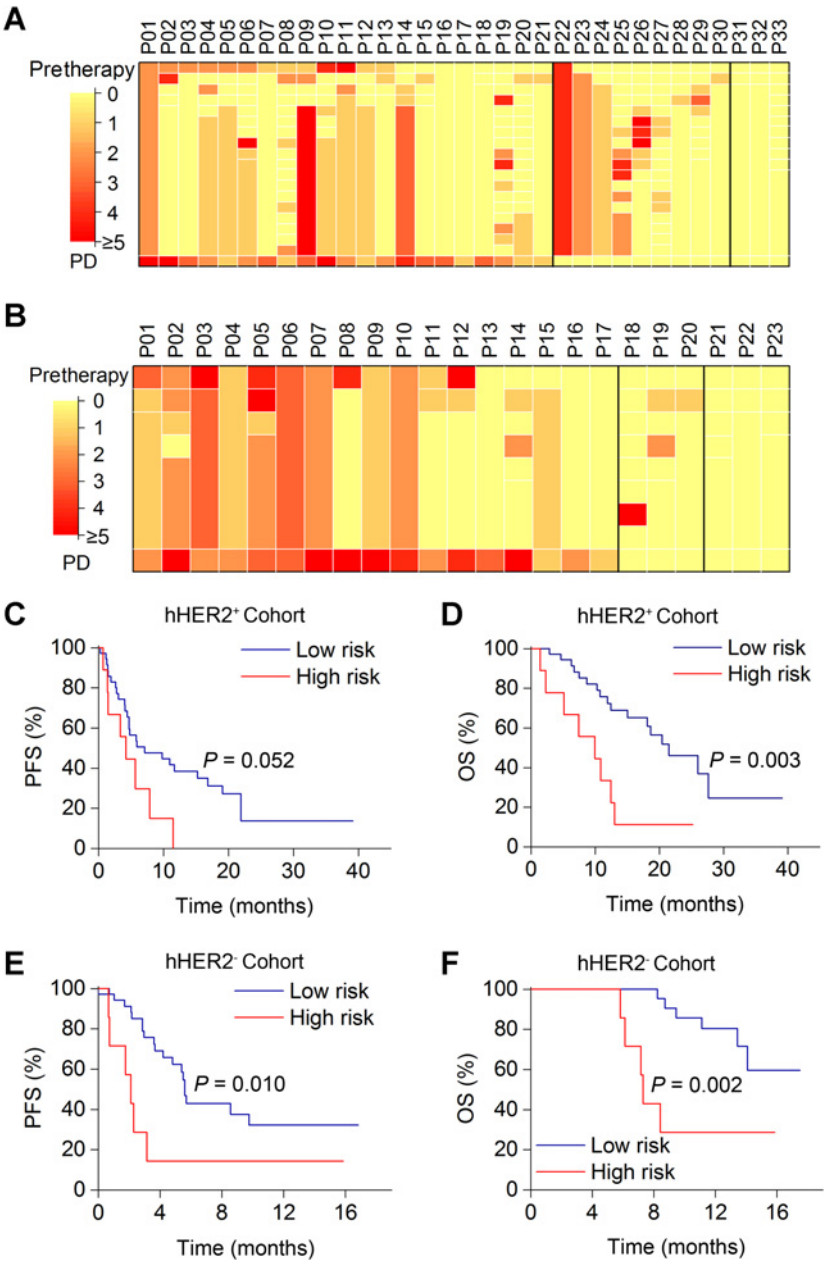


**Figure 3.**

Acquisition of the cHER2$^+$ phenotype correlates with therapeutic resistance and inferior prognosis in patients developing PD. **A** and **B,** Heat maps of variation of cHER2$^+$ CTCs in response to therapy in patients who developed PD following therapy, including (**A**) 33 of hHER2$^+$ PD patients and (**B**) 23 of hHER2$^-$ PD patients. X-axis represents each patient, and Y-axis represents the number of cHER2$^+$ CTCs in each examination in the duration from pretherapy to PD. **C** and **D,** Kaplan–Meier plots to predict PFS (**C**) and OS (**D**) with pretherapeutic cHER2$^+$ CTCs in the hHER2$^+$ cohort. Low risk, patients with pretherapeutic cHER2$^+$ CTCs < 2; high risk, patients with pretherapeutic cHER2$^+$ CTCs ≥2. **E** and **F,** Kaplan–Meier plots to predict PFS (**E**) and OS (**F**) with pretherapeutic cHER2$^+$ CTCs in the hHER2$^-$ cohort. Low risk, patients with pretherapeutic cHER2$^+$ CTCs < 3; high risk, patients with pretherapeutic cHER2$^+$ CTCs ≥ 3.

**Trastuzumab reduces quantity of cHER2$^+$ and cHER2$^-$ CTCs**

Two of hHER2$^-$ gastric cancer patients were subjected to the trastuzumab-targeted therapy when the cHER2$^+$ phenotype was developed.

Patient 1 (Fig. 6A) was diagnosed with poorly differentiated nongastroesophageal junction (non-EGJ) adenocarcinoma with multiple hepatocellular metastases. The Lauren's histologic type was intestinal, with hHER2$^-$ status (immunohistologic staining 2+ for HER2 expression/no HER2 gene amplification). However, the patient was pretherapeutic cHER2$^+$ on CTCs prior to the first-line therapy. Accordingly, the subject was treated with XELOX (capecitabine + oxaliplatin) plus trastuzumab.

Patient 2 (Fig. 6B) was diagnosed with poorly differentiated non-EGJ adenocarcinoma with ovarian metastases. Histopathologic examination showed hHER2$^-$ status (immunohistologic staining 1+ for HER2 expression). The patient was subjected to palliative gastric and uterine surgical resection, followed by the first-line chemotherapy alone, including PTX plus S-1, and peritoneal perfusion with PTX and DDP. Following PD developed, positive HER2 expression on CTCs (cHER2$^+$ phenotype) was identified. Trastuzumab plus PTX were subsequently administrated to this postchemotherapeutic cHER2$^+$ patient.

Figure 6A and B shows that combined targeted (trastuzumab) and chemotherapy could maintain or reduce the quantity of cHER2$^+$ or total (both cHER2$^+$ and cHER2$^-$) CTCs. The patient with the pretherapeutic cHER2$^+$ phenotype (PFS: 4.7 m; OS: 11.4 m), and the other with postchemotherapeutic acquisition of cHER2$^+$ (PFS: 1.8 m; OS: 6.7 m) did not show obvious extended PFS and OS when trastuzumab was administrated to these 2 patients.

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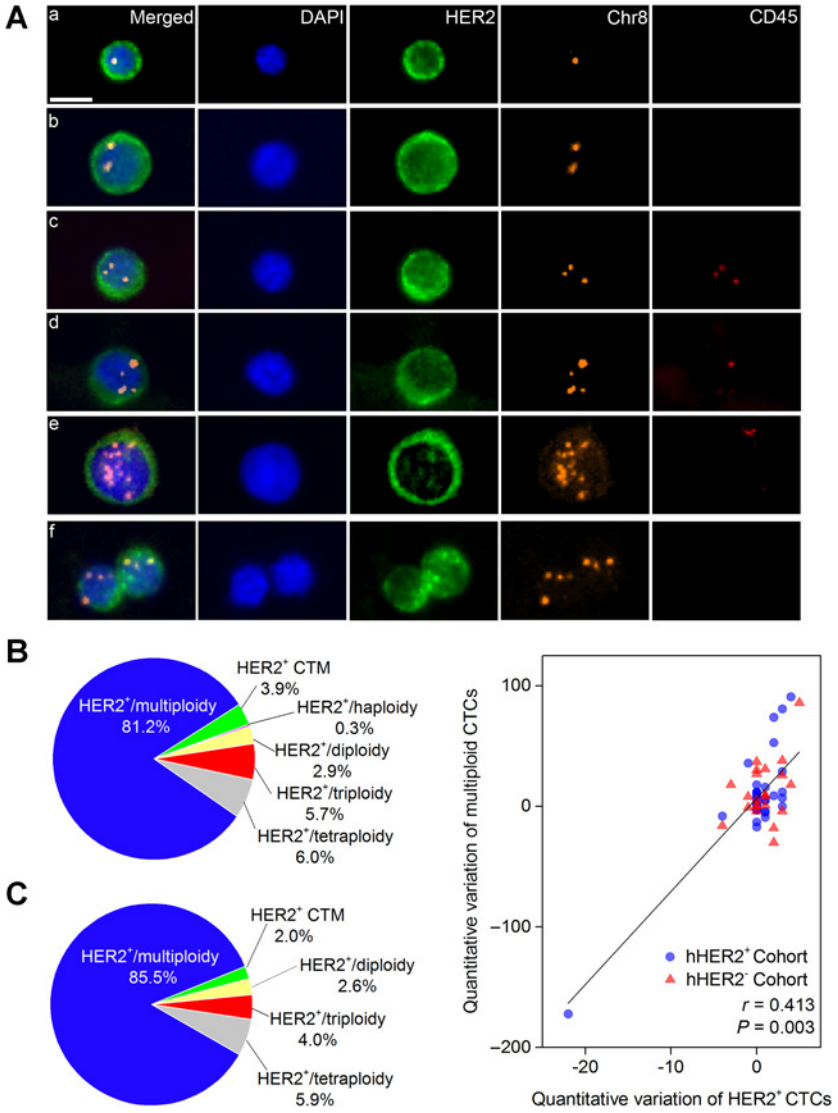


**Figure 4.**

Aneuploid Chr8 contributes to variation of the cHER2 phenotype on CTCs. cHER2$^+$ CTCs are identified by HER2-iFISH. **A,** Diverse Chr8 aneuploidy in cHER2$^+$ CTCs and CTM. **a–e**, cHER2$^+$ CTCs with haploid, diploid, triploid, tetraploid, and multiploid Chr8, respectively. **f,** cHER2$^+$ CTM. Bar, 5 μm. **B** and **C,** Distribution of aneuploid Chr8 in cHER2$^+$ CTCs in hHER2$^+$ (**B**) and hHER2$^-$ (**C**) cohorts. **D,** Quantitative variation of multiploid CTCs is in accordance with quantitative variation of cHER2$^+$ CTCs in the duration from pretherapy to PD. CTM, circulating tumor microemboli.

## Discussion

HER2 is an important therapeutic target for gastric cancer and is involved in tumorigenesis and malignant progression (4, 5, 9). Understanding biological mechanisms of HER2-related tumor development and therapeutic resistance will help improve therapeutic efficacy. To date, HER2-targeted therapeutic resistance studies have focused on compensatory activation of either alternative tyrosine kinase receptors (EGFR, HER3, FGFR2, and MET) or relevant signaling pathways (Src and Notch; refs. 27, 28), or performing relevant studies on homogeneous *ex vivo* models. However, reported studies were unable to mirror heterogeneous evolution of carcinomas following developing therapeutic resistance and tumor progression.

HER2 protein is encoded by the *ERBB2* gene. Detection of the amplified *ERBB2* gene is conventionally applied to guide selection of patients suitable for HER2-targeted therapy. However, processes from DNA to protein along the intracellular biochain are regulated at checkpoints of transcription and translation (29), resulting in potential negative expression of HER2 protein in carcinoma cells with the amplified *ERBB2* gene (30). Examination of phenotypic expression of HER2 protein on CTCs, therefore, provides a direct convincing evidence showing the neoplasm cells applicable to be recognized and targeted by the therapeutic antibody trastuzumab.

In the current study, we longitudinally monitored phenotypic HER2 expression on CTCs in AGC patients subjected to the first-line therapy. Obtained results demonstrated evolutionary expression of cHER2 on CTCs in patients during targeted and/or chemotherapeutic treatment, despite relatively stable status of hHER2$^-$ in primary lesions. Phenotypic cHER2 expression on CTCs fluctuated during therapy was from spontaneous interconversion of the HER2 phenotype between cHER2$^+$ and cHER2$^-$ CTCs, which is in accordance with the observation reported by others on breast cancer studies (31). Published studies demonstrated that, compared with cHER2$^+$ CTCs, the cHER2$^-$ category of cells have a higher potential in terms of phenotypic conversion, resulting in more than half of hHER2$^+$ and hHER2$^-$ patients acquired cHER2$^+$ on CTCs, which exceeds hHER2 positivity of 10% to 30% examined by a

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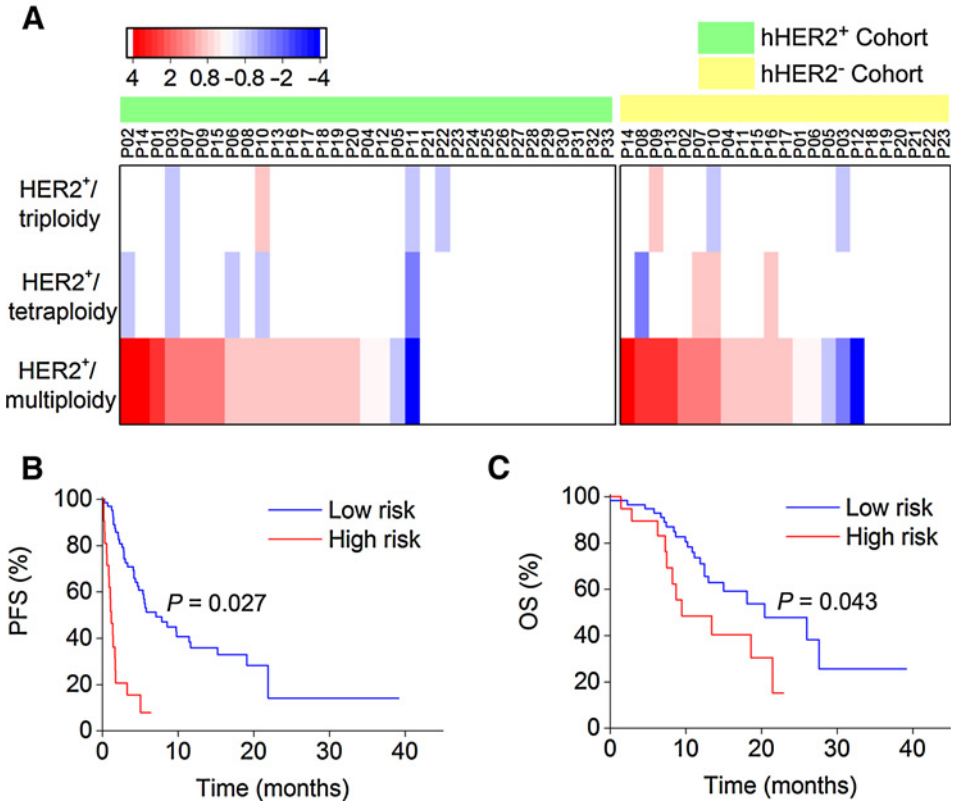


**Figure 5.**
Increased multiploid cHER2$^+$ CTCs correlate with poor prognosis. **A,** Heat map of variations of multiploid cHER2$^+$ CTCs in the duration between pretherapy and PD in 33 of hHER2$^+$ patients and 23 of hHER2$^-$ patients developing PD. White, absence of aneuploid cHER2$^+$ CTCs; red, increased aneuploid cHER2$^+$ CTCs; blue, decreased aneuploid cHER2$^+$ CTCs. **B** and **C,** Kaplan–Meier plots to predict PFS (**B**) and OS (**C**) with quantitative variations in multiploid cHER2$^+$ CTCs. Low risk, patients with stable or decreased multiploid cHER2$^+$ CTCs following therapy initiated; high risk, patients with increased multiploid cHER2$^+$ CTCs following therapy.

conventional histopathologic approach routinely performed only once on primary lesions (2, 31).

In the present study, we demonstrated that phenotypic acquisition of cHER2 in CTCs is associated with developing therapeutic resistance and poor prognosis. A majority of evaluable AGC patients in the hHER2$^+$ or hHER2$^-$ cohorts acquired a cHER2$^+$ phenotype when therapeutic resistance developed. Pretherapeutic emergence of cHER2$^+$ CTCs in both cohorts of patients led to poor PFS and OS. Thus, acquisition of posttherapeutic cHER2$^+$ phenotype is involved in clonal evolution against therapeutic pressure, and further promotes development of therapeutic resistance in AGC patients.

Previous studies performed by others indicated that abnormalities of genomic structure or gene expression may induce phenotypic variation (32–34). The present study investigated whether abnormal chromosome copy number correlated with HER2 phenotypic variation on CTCs, which may subsequently facilitate development of therapeutic resistance. *In situ* coexamination of Chr8 aneuploidy and cHER2 phenotyping on CTCs performed with HER2-iFISH revealed that aneuploid Chr8 (multiploidy of Chr8 in particular) contributed to acquisition of the posttherapeutic cHER2$^+$ phenotype on CTCs, which is in accordance with the published observations, showing correlation between protein expression and chromosomal copy-number variations in budding yeast (35). The impact of chromosome number variation on the noncoding protein contributes to compensatory posttranslational mechanisms which reduce protein expression when their encoding genes are in excess (36, 37). Similarly, it is reasonable to speculate that aneuploid Chr8 with extra copies of chromosome in CTCs may have an impact on noncoding HER2 protein in a similar way. Moreover, it was observed that unique aneuploidy was able to facilitate tumorigenesis via generating phenotypic variants and to provide a growth advantage under selective pressure (35, 38–40). Indeed, in the present study we demonstrated that multiploid Chr8 contributed to phenotypic conversion from cHER2$^-$ to cHER2$^+$ on CTCs, which is speculated to improve the cellular adaption of cHER2$^+$ CTCs against therapeutic pressure, and in turn to facilitate development of resistance to therapy.

The existence of a cHER2$^+$ phenotype on CTCs in hHER2$^-$ patients suggests that those patients might benefit from HER2-targeted therapy. Indeed, 2 hHER2$^-$/cHER2$^+$ AGC patients who prospectively received trastuzumab in this study showed reduced quantity of CTCs, including both cHER2$^+$ and cHER2$^-$ neoplasm cells, although prognosis was not significantly improved for these 2 patients. A previously published study on breast cancer patients may provide one of possible explanations, showing acquisition of cHER2$^+$ phenotype was accompanied by activation of multiple receptor tyrosine kinases and progrowth signaling pathways (31). Accordingly, a prospective interventional study with a large cohort of enrolled patients could help prove whether additional targeted therapeutic strategies, such as pan-HER2 inhibitors or multiple-targeted therapies, may help improve cHER2$^+$ carcinoma patients' prognosis.

Evolutionary expression of HER2 on gastric cancer CTCs was demonstrated in the present study. Dynamic acquisition of the cHER2 phenotype is an important mechanism involved in developing therapeutic resistance, respectively to trastuzumab for hHER2$^+$ patients and chemotherapy for hHER2$^-$ patients. The results of this study suggest that, in contrast to relatively stable HER2 status in primary lesions prior to therapy, cHER2

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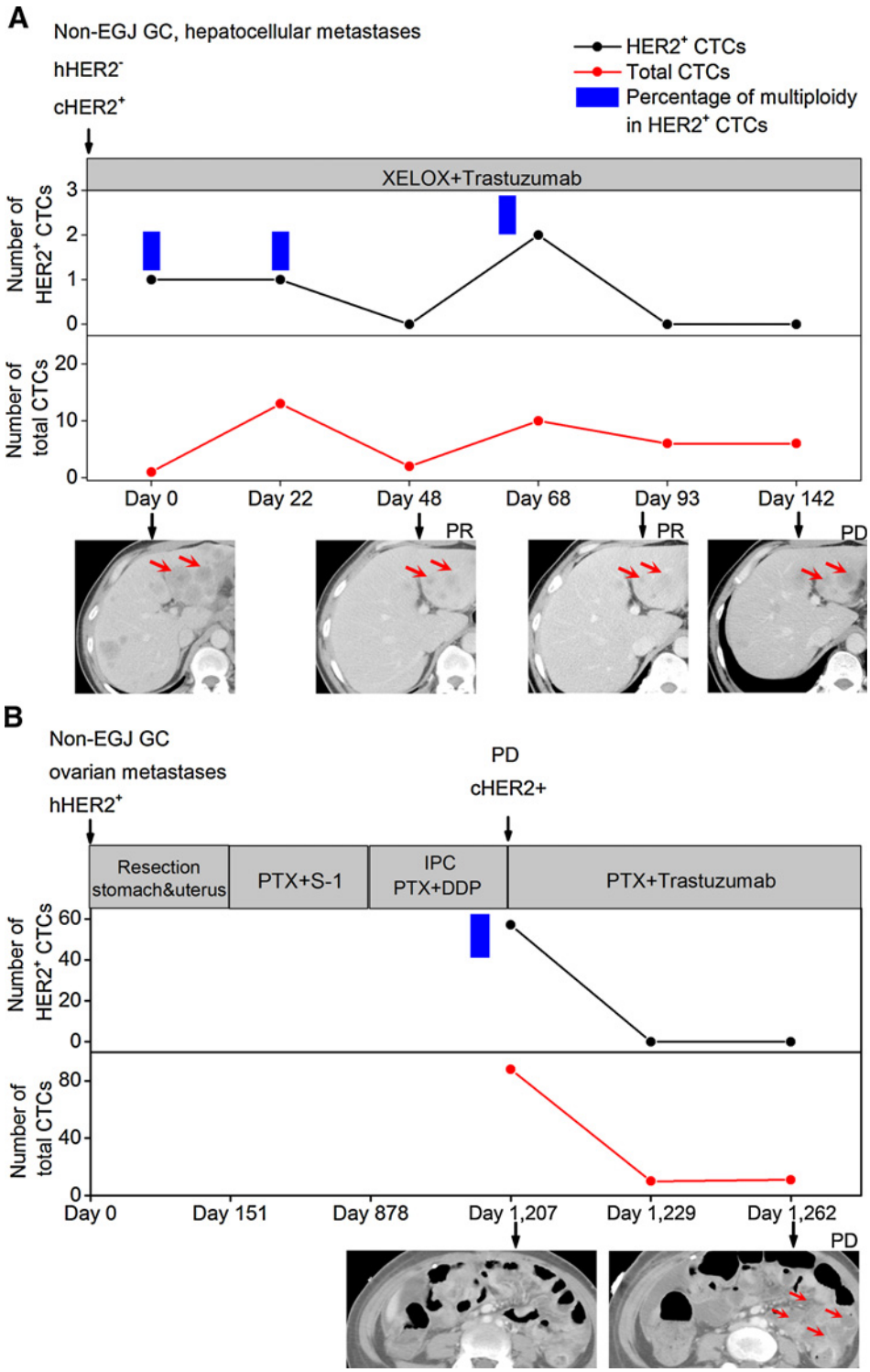


**Figure 6.**
Treatments of hHER2$^-$/cHER2$^+$ patients with trastuzumab reduces quantity of CTCs. Quantification of cHER2$^+$ and total CTCs (cHER2$^+$ and cHER2$^-$) and analysis of clinical response of patients to the therapy: **A,** Patient 1 with pretherapeutic cHER2$^+$ phenotype was subjected to the first-line XELOX plus trastuzumab. Red arrows indicate metastatic liver lesions. The lesion size is increased after PD. **B,** Patient 2 with postchemotherapeutic acquisition of the cHER2$^+$ phenotype was subjected to the combined PTX plus trastuzumab. XELOX, capecitabine plus oxaliplatin; PR, partial response; PD, progressive disease; PTX, paclitaxel; DDP, cisplatin; IPC, intraperitoneal chemotherapy. Red arrows indicate malignant ascites, which is developed after PD.

status on CTCs is more dynamic. Continuous examination of the cHER2 phenotype could help monitor resistance to therapy in AGC patients in real time. In addition, *in situ* codetection and characterization of HER2 protein expression and chromosomal aneuploidy of CTCs performed by HER2-iFISH illustrate that aneuploid Chr8 participates in dynamic acquisition of a post-therapeutic cHER2$^+$ phenotype, which may provide a growth advantage for cHER2$^+$ CTCs against therapeutic pressure and then lead to development of resistance to therapy. Published study from others indicated that gain of a single chromosome may induce genomic instability in carcinoma cells (41); it is speculated that extra copies of chromosome 8 in CTCs reported

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in this study might lead to dynamic variation of cHER2 phenotypes, which eventually facilitates development of therapeutic resistance in cancer patients.

The contribution of chromosome aneuploidy to evolutionary variation of cHER2 phenotypes on CTCs may provide additional perspectives with respect to understanding the mechanisms of developing therapeutic resistance in malignant carcinomas.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** Y. Li, X. Zhang, M. Li, L. Shen
**Development of methodology:** Y. Li, X. Zhang, D.D. Wang, S. Li, P.P. Lin
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** X. Zhang, D. Liu, J. Gong, Z. Peng, X. Wang, L. Shen
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** Y. Li, X. Zhang, X. Wang, M. Li, L. Shen
**Writing, review, and/or revision of the manuscript:** Y. Li, J. Gong, M. Li, L. Shen
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** Y. Li, X. Zhang, D. Liu, S. Li, Y. Li, L. Shen
**Study supervision:** Y. Li, X. Zhang, M. Li, L. Shen

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Clinical Cancer Research

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