Clinical significance of circulating tumor cells in predicting disease progression and chemotherapy resistance in patients with gestational choriocarcinoma

Weiling He\textsuperscript{1,2}, Minzhi Hou\textsuperscript{3,4}, Hui Zhang\textsuperscript{2}, Chao Zeng\textsuperscript{1,3}, Shanyang He\textsuperscript{5}, Xinlin Chen\textsuperscript{6}, Manman Xu\textsuperscript{5}, Cong Sun\textsuperscript{1}, Wenting Jiang\textsuperscript{1}, Han Wang\textsuperscript{1}, Hongwei Shen\textsuperscript{2}, Yang Zhang\textsuperscript{1}, Jing Liu\textsuperscript{6}, Shijun Sun\textsuperscript{9}, Neng Jiang\textsuperscript{1}, Yongmei Cui\textsuperscript{1}, Yu Sun\textsuperscript{1}, Yangshan Chen\textsuperscript{1}, Jessica Cao\textsuperscript{10}, Chunlin Wang\textsuperscript{11}, Mengzhen Li\textsuperscript{12}, Yi Zhang\textsuperscript{13}, Liantang Wang\textsuperscript{1}, Jianhong Wang\textsuperscript{14}, Millicent Lin\textsuperscript{15} and Zunfu Ke\textsuperscript{1}

\textsuperscript{1}Department of Pathology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, People's Republic of China
\textsuperscript{2}Institute of Precision Medicine, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, People's Republic of China
\textsuperscript{3}Department of Gynecology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, People's Republic of China
\textsuperscript{4}Department of Gynecology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, Guangdong, People's Republic of China
\textsuperscript{5}Department of Pathology, Guangdong Medical College, Dongguan, Guangdong, People's Republic of China
\textsuperscript{6}School of Basic Medical Science, Guangzhou University of Chinese Medicine, Guangzhou, Guangdong, People's Republic of China
\textsuperscript{7}Biomedical Engineering, The University of Texas at El Paso, El Paso, TX
\textsuperscript{8}Department of Anesthesiology, Guangdong Women and Children Hospital, Guangzhou, Guangdong, People's Republic of China
\textsuperscript{9}Molecular Diagnosis Center, The Affiliated Zhongshan Hospital, Sun Yat-Sen University, Zhongshan, Guangdong, People's Republic of China
\textsuperscript{10}Cumming School of Medicine, University of Calgary, Calgary, AB, Canada
\textsuperscript{11}Chapter Diagnostics, Menlo Park, CA
\textsuperscript{12}MyGene Diagnostics, Guangzhou International Biotech Island, Guangdong, People’s Republic of China
\textsuperscript{13}Biomedical Imaging Research Institute, Cedars-Sinai Medical Center, Los Angeles, CA
\textsuperscript{14}Precision Medicine Center, Shenzhen People’s Hospital, Shenzhen, Guangdong, People’s Republic of China
\textsuperscript{15}Department of Genetics, Harvard Medical School, Boston, MA

Gestational choriocarcinoma (GC) is a highly aggressive tumor. In our study, we systematically investigated EpCAM/CD147 expression characteristics in patients with GC and assessed the role of circulating tumor cells (CTCs) in predicting chemotherapy response and disease progression. GC tissues were positive for either epithelial cellular adhesion molecule (EpCAM) or CD147, and all samples exhibited strong human chorionic gonadotropin (HCG) expression. Among all the recruited

Key words: gestational choriocarcinoma, circulating tumor cell, β-HCG, progression, chemotherapy resistance

Abbreviations: CNS: central nervous system; CT: computed tomography; CTCs: circulating tumor cells; DAB: 3,3'-diaminobenzidine; DAPI: 4,6-diamidino-2-phenylindole-dihydrochloride; DMEM: Dulbecco’s modified Eagle’s medium; EGFR: epidermal growth factor receptor; MUC-1: mucin 1; EpCAM: epithelial cellular adhesion molecule; FIGO: International Federation of Gynecology and Obstetrics; GC: gestational choriocarcinoma; GTN: gestational trophoblastic neoplasia; H&E: hematoxylin–eosin; HCG: human chorionic gonadotropin; HER-2: human epidermal growth factor receptor 2; HR: hazard ratio; HRP: horse radish peroxidase; IHC: immunohistochemistry; MRI: magnetic resonance imaging; PD: progression of disease; PFS: progress-free survival; ROC: receiver operating characteristic; WBCs: white blood cells

Additional Supporting Information may be found in the online version of this article.

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W.H., M.H., H.Z., S.H. and W.J. contributed equally to this work

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Correspondence to: Zunfu Ke, Department of Pathology, The First Affiliated Hospital, Sun Yat-sen University, No. 58, ZhongShan Second Road, Guangzhou, Guangdong 510080, People’s Republic of China, Tel.: 86-20-87331780; Fax: 86-20-87331780, E-mail: kezunfu@mail.sysu.edu.cn

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patients ($n = 115$), 103 had at least 1 CTC in a 7.5-ml peripheral blood sample, and the percentage of patients with $\geq 4$ CTCs in a particular FIGO stage group increased with a higher FIGO stage ($p < 0.001$). Furthermore, the pretreatment CTC count was related to tumor size ($r = 0.225$, $p = 0.015$) and the number of metastases ($r = 0.603$, $p < 0.001$). A progression analysis showed that among the 115 included patients who qualified for further examination, 52 of the 64 patients defined as progressive had $\geq 4$ pretreatment CTCs, while only 7 of the 51 non-progressive patients had $\geq 4$ pretreatment CTCs ($p < 0.001$). In multivariate analysis, CTCs ($\geq 4$) remained the strongest predictor of PFS when other prognostic markers, FIGO score and FIGO stage were included. Moreover, based on the chemotherapy response, patients with $\geq 4$ CTCs were more likely to be resistant to chemotherapy than those with $< 4$ CTCs ($p < 0.001$). These findings demonstrate the feasibility of CTC detection in cases of GC by adopting EpCAM/CD147 antibodies together as capturing antibodies. The CTC count is a promising indicator in the evaluation of biological activities and the chemotherapy response in GC patients.

What’s new?
Gestational choriocarcinoma tumor cells tend to spread to distant organs by hematogenous dissemination. This study shows that circulating tumor cells (CTCs) in patients with gestational choriocarcinomas can be readily captured by targeting the highly expressed membrane antigens EpCAM and CD147. Elevated CTC levels, defined as $\geq 4$ or more CTCs per 7.5 ml of peripheral blood, were found to predict chemotherapy resistance and to more effectively predict disease progression where compared with traditional $\beta$-human chorionic gonadotropin. The findings suggest that CTC enumeration could be used to stratify gestational choriocarcinoma patients for personalized clinical intervention.

Introduction
Gestational choriocarcinoma (GC) is an aggressive, malignant and rare form of gestational trophoblastic neoplasia (GTN). GC occurs in the uterus after a wide range of pregnancies, including molar pregnancy, ectopic pregnancy, stillbirth/miscarriage and preterm or term delivery, with an incidence of approximately 0.002% among live deliveries. Characterized by its reliance on hematogenous dissemination for metastasis, GC generally spreads to the lungs, liver, central nervous system (CNS) and vagina. This feature is also partially responsible for the high malignancy rate and resistance to chemotherapy of GC, for which the detection of metastasis would be highly valuable for evaluating disease progression.

Due to the human chorionic gonadotropin (HCG)-generating function of GC, serum $\beta$-HCG is currently one of the most effective markers for the clinical diagnosis of GC. However, critical clinical information, such as the full extent of metastasis and resistance to conventional chemotherapy, which manifests in some cases, cannot be fully revealed and assessed by measuring the $\beta$-HCG level; thus, additional cytological evidence is required to assist in the diagnosis. Therefore, further exploration of effective clinical indicators of GC to assist with evaluating the disease status and chemotherapy response is of great significance and given this importance, directly monitoring tumor cells would be a promising approach to guiding clinical judgment. Currently, cell-level investigations on GC confront challenges for several reasons, such as the lack of tissue specimens, ideal cell lines or animal models. Therefore, a new method to capture the target cell for further biological investigation of GC is in urgent demand.

Circulating tumor cells (CTCs) originating from the primary tumor tissue and later disseminating to the peripheral blood circulation are the source of hematogenous cancer metastasis. Capturing and enumerating CTCs has great prognostic value and feasibility for molecular and functional research on various solid tumors, serving as a method of “liquid biopsy”. With the emergence of multimarker sets [epithelial cellular adhesion molecule (EpCAM), human epidermal growth factor receptor 2 (HER-2), epidermal growth factor receptor (EGFR) and mucin 1 (MUC-1)], label-free devices, and microfluidic and cytology-based ISET platforms and the application of nanomaterials, the CTC capture techniques have been rapidly developed, guaranteeing a better capture efficiency and accuracy. A few recent investigations showed that CTC clusters have a potentially high metastasis capacity, offering new insights into tumor metastasis. Such improvements led to our interest in investigating CTC characteristics in GC due to the tendency of GC to undergo hematogenous dissemination, as mentioned above.

The aims of our study were to verify the feasibility of CTC enumeration in GC patients, to reveal the correlation of the CTC count with a patient’s disease status and chemotherapy responsiveness and to further prospectively investigate the value of CTC detection and assess its role in evaluating chemotherapy response and disease progression.

Materials and Methods
Patient characteristics
GC patients ($n = 115$) from multicenter between January 2009 and January 2013 were recruited. All of the patients had...
different index pregnancy statuses. Patients were included in the study according to the following criteria. First, the patients were without any other tumor disease, had normal renal and liver function and could undergo chemotherapy. Eligible patients were at least 18 years of age and had histological and immunohistochemical proof of GC confirmed by two pathologists. Second, all the patients could be followed up through the present or until the end point (death) (range 8–94 months, median 67 months). Those with a simultaneous pregnancy or who became pregnant during the treatment were excluded. Additionally, patients lost to follow-up were excluded. Because of the effect of recent chemotherapy on CTC count, patients who had received single- or multiagent chemotherapy within the previous 3 months were excluded at the time of the first CTC test. Our project was approved by the Medical Ethics Committee of each independent center, and all enrolled patients signed a consent form.

International Federation of Gynecology and Obstetrics (FIGO) staging and scoring was classified using the published systems for gynecological cancers in the twenty-sixth volume of the FIGO Annual Report. To assess the treatment effects and progression statuses of the patients, the following tests were performed: serum \( \beta \)-HCG level (no less frequently than every 1–2 weeks) and a computed tomography (CT) scan of the chest if the chest X-ray results were negative (although pulmonary micrometastases observed only on the CT scan were not used in staging), and a CT scan or magnetic resonance imaging (MRI) of the abdomen, brain and pelvis. Conditions in which there were either consistent or increasing serum \( \beta \)-HCG levels or new metastases were referred to as progression of disease (PD). A persistent rise or plateau of \( \beta \)-HCG levels during chemotherapy indicated resistant disease.

Immunohistochemistry staining

Tissue sections (4 μm) from 115 GC patients were prepared using a rotary microtome (Leica, Wetzlar, Germany) obtained from the formalin-fixed paraffin-embedded tissue archive. All the tissue sections were deparaffinized in xylene and then rehydrated in graded (100%–90%–80%–75%) alcohol solutions. Next, the sections were subjected to a trypsin solution (0.1%) for 2 min at 37 °C to accomplish antigen retrieval. The sections were incubated with anti-HCG (Abcam, UK, 2092, 1:120), anti-EpCAM (Abcam, UK, ab71916, 1:100), anti-CD147 (Abcam, UK, ab108317, 1:250) or anti-CD45 (Sigma, 1:120), anti-EpCAM (Abcam, UK, ab71916, 1:100), anti-

positive cells and 3, more than 75% positive cells. We obtained the staining index by multiplying the staining intensity score by the positive tumor cell score. Based on the heterogeneity of the measure, we defined a staining index of 1–2 as weak, 3–4 as moderate and 6–9 as strong staining.

Cell culture

The GC cell line JEG-3 (the third passage of monoclonal cell line originated from Bewo cell) was obtained from the Guangzhou Cellcook Biotech (Guangzhou, China). JEG-3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Waltham, USA) supplemented with 10% bovine serum, penicillin (100 U/mL) and streptomycin (100 U/mL) at 37 °C for 24 hr in a 5% CO₂ atmosphere.

CTC enrichment using the NanoVelcro system

Blood samples (7.5 mL of venous blood) were collected in EDTA tubes and processed within 24 hr before the first line chemotherapy. CTC enrichment was performed using the NanoVelcro system as described in our previous article. A combination of anti-EpCAM and anti-CD147 antibodies was used to modify the surface of the NanoVelcro chip to effectively capture CTCs in peripheral blood samples from the GC patients (Fig. S1).

Captured CTCs were fixed with PBS containing 2.0% formaldehyde, washed and blocked with 1% donkey serum in PBS. Then, a commonly used three-color immunocytochemistry method was utilized to discriminate CTCs from white blood cells (WBCs); the method included a TRITC-conjugated anti-CD45 antibody (CD45, a marker for WBCs) (Sigma, USA, mouse antibody, 1:50), a FITC-conjugated anti-HCG antibody (HCG, a protein marker for GC cells) (Abcam, USA, mouse antibody, 1:150) and 4,2-diamidino-2-phenylindole-dihydrochloride (DAPI) (Sigma, Germany) for nuclear staining. A manual blood sample was prepared by spiking \( 10^7 \) JEG-3 cells in \( 10^6 \) WBCs obtained from the blood of a healthy donor and was utilized as a positive control. WBCs in each blood sample were used as an internal negative control.

Statistical analysis

To obtain the most appropriate CTC cutoff for distinguishing PFS, all the enrolled GC patients were randomly split into the training and validation cohorts according to the methods used in a previous study. In the training phase, a range of baseline CTC values for 59 enrolled patients was tested to establish an optimal cutoff level. In the validation phase, the optimal cutoff level was then evaluated with new data collected from an independent cohort of 56 enrolled GC patients.

The statistical tests in our study were performed using SPSS 16.0 for Windows (SPSS Inc., USA) and GraphPad Prism 5.0 (GraphPad Software, USA). The association between CTC numbers and the clinical parameters was assessed using Fisher’s exact test. The Spearman test was used to compute the concordance rate of the CTC level with progression time, primary...
tumor size, number of metastases and serum β-HCG level. Time-dependent receiver operating characteristic (ROC) analysis was applied to compare the predictive accuracy of CTC with the clinicopathological parameters. The Kaplan–Meier method was applied to analyze survival differences between groups. Multivariable Cox regression was applied to the selected significant variables for PFS using stepwise methods (forwardstepwise selection [Wald] method). All the tests were two-sided, and a difference with \( p < 0.05 \) was defined as statistically significant.

Results

Characteristics of participants

Table S1 lists the clinical characteristics of 115 consecutive patients enrolled into the study. Forty-five patients suffered previous failed chemotherapeutic treatments prior to the diagnosis of GC, 24 of whom had previous multiagent therapy failures. Twenty-eight patients had a short interval between the first time of GC diagnosis and the end of follow-up (<4 months), and 87 patients had a long interval (≥4 months). Thirty-two stage I, 23 stage II, 39 stage III and 21 stage IV GCs were diagnosed in 115 patients based on the 2009 FIGO staging system. According to the new 2009 FIGO prognosis scoring system, 43 of the 115 evaluable patients were low risk, and 72 were high risk.

EpCAM and CD147 expression characteristics in GC tissues and CTC enrichment

The membrane markers EpCAM and CD147 are recognized as suitable markers for CTC detection and enumeration, and studies have shown that both EpCAM and CD147 are detectable in gestational trophoblastic diseases. To ensure that our NanoVelcro system is suitable for GC study, the expression characteristics of EpCAM and CD147 were investigated in paraffin-embedded GC tissues by immunohistochemical analysis. EpCAM was expressed mostly on the cell membrane and partially in the cytoplasm of the GC cells, and CD147 staining was mainly observed on the cell membrane (Fig. 1a; Table S2). Among the 115 GC patients, positive expression of EpCAM was detected in 110 patients, and only 5 patient samples were negative for EpCAM expression. For CD147, 112 patients were found to be positive for expression, whereas only 3 were negative for staining. No patients were negative for both EpCAM and CD147 (Table S3). As a unique marker of trophoblastic disease, strong expression of HCG was observed in all the GC tissues, with cytoplasmic staining or membrane staining. However, all the GC cells exhibited negative staining for CD45, the WBC-specific marker (Fig. 1a).

Blood samples (7.5 mL) were drawn from each patient and applied to the NanoVelcro system. After capturing suspected CTCs, we performed immunofluorescence staining to confirm the accuracy of the CTC capture. HCG was found to be expressed only in CTCs, which were negative for CD45 (Fig. 1b). This finding is consistent with the IHC results for the GC tissues.

CTC cutoff definition and relationship of CTC counts to existing markers

Prior to obtaining the optimum CTC count cutoff, a series of CTC thresholds from 1 to 15 were systematically evaluated for their estimate of PFS by the Kaplan–Meier method and log-rank test in a training set of 59 patients. After comparing the hazard ratios (HRs) and differences by multiple-threshold testing, a cutoff of 4 CTCs per 7.5 mL was found to offer optimal PFS prediction (Table S4). Thus, a cutoff of 4 CTCs was used thereafter to distinguish between high- and low-risk patients.

The reliability of our CTC cutoff was further verified in a validation cohort. To ensure the uniformity and quality of the random distribution, we compared the difference in patient counts above the cutoff value between the training and validation sets using Fisher’s exact test (\( p = 0.636; \) Table S5). Furthermore, the mean PFS of the two independent data sets showed no significant difference (\( p = 0.338; \) Table S6). As shown in Table S7, the cutoff of 4 CTCs per 7.5 mL for PFS was fully supported by the validation set.

Patients with larger primary GC tumors tended to have more CTCs in their peripheral blood (\( r = 0.225, p = 0.015, \) Figs. 2a and 2b). A significant association between CTC levels and the number of metastases was observed (\( r = 0.603, p < 0.001, \) Figs. 2c and 2d). No correlation between CTC counts and serum β-HCG levels was found (\( r = 0.208, \) Figs. 2e and 2f).

Relationship between pretreatment CTC counts and clinical characteristics of GC

Among the 115 recruited patients, the pretreatment CTC number ranged from 0 to 15/7.5 mL of peripheral blood, and 103 of the patients had at least 1 CTC/7.5 mL, including 59 patients with ≥4 CTCs per 7.5 mL of peripheral blood. The percentage of patients with ≥4 CTCs increased in accordance with the FIGO stage (\( p < 0.001 \)). The FIGO score (\( p < 0.001 \)), sites of metastasis (\( p = 0.0140.001 \)), and number of metastases (\( p = 0.003 \)) as well as previous failed chemotherapy (\( p = 0.007 \)) were also found to be significantly correlated with the pretreatment CTC count (<4 vs. ≥4). There was no correlation between the CTC count (<4 vs. ≥4) and age (\( p = 0.752 \)), antecedent pregnancy (\( p = 0.150 \)), interval in months from the index pregnancy (\( p = 0.364 \)), pre-treatment β-HCG level (\( p = 0.068 \)) or largest tumor mass (\( p = 0.473, \) Table 1). The percentage of patients with ≥4 CTCs increased gradually along with GC progression as reflected by the FIGO stage. In particular, for patients in FIGO stage IV, the positive rate for ≥4 CTCs reached 95.24%. However, the positive rate for ≥4 CTCs in FIGO stage I patients is 31.25% (Table 1).

Univariate analyses revealed that the clinical factors significantly associated with a poor prognosis were age, interval months from index pregnancy, largest tumor mass, site of metastases, number of metastases, previous failed chemotherapy, FIGO score and FIGO stage. A multivariate analysis showed that CTC count (≥4 CTCs), FIGO score and FIGO
Figure 1. Immunohistochemical analysis of GC tissues and representative images of the immunofluorescence staining of CTCs. (a) Strong expression of EpCAM was detected both on the cell membrane and partially in the cytoplasm of GC cells, and CD147 was identified through intense membranous staining. HCG was positively detected in GC cells with strong cytoplasmic staining or membrane staining. CD45 was negatively detected in GC cells, but was positively expressed on the infiltrating lymphocytes. Red, green and black arrows represent GC cells, smooth muscle cell and infiltrating lymphocytes, respectively. (b) GC CTCs enriched by NanoVelcro system were positive for HCG (coupled with FITC, green) and negative for CD45 (coupled with TRITC red). Blood spiked manually with JEG-3 cells was used as the positive control for HCG. [Color figure can be viewed at wileyonlinelibrary.com]
stage were independent predictor factors for PFS, consistent with the univariate analysis (Table 2).

**Correlation of CTC count with tumor progression and chemotherapy resistance**

Accumulating studies have reported that the CTC count is associated with tumor progression. 13–15,32 To analyze the relationship between the CTC count and tumor progression, the disease status of the patients was evaluated according to the RECIST standards. Fifty-two of 64 (81.25%) patients with progressive disease had ≥4 pretreatment CTCs, whereas only 7 of 51 (13.73%) patients with non-progressive disease had ≥4 pretreatment CTCs (p < 0.001, Fig. 2g and Table 3). Using ROC analysis, we found that ≥4 CTCs cutoff value (area

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**Figure 2. Relationship between CTC and existing markers and tumor progressions.** (a and b) There is a positive correlation between CTC levels (range 0–15) and primary tumor sizes (p = 0.015, r = 0.225). (c and d) There is a positive correlation between CTC levels and number of metastases (p < 0.001, r = 0.603). (e and f) There is no correlation between CTC levels and serum β-HCG level (p = 0.208, r = 0.026). (g) The number (52/64, 81.25%) of PD patients who had ≥4 pretreatment CTCs was significantly higher than that (7/51, 13.73%) in non-PD patients (p < 0.001). (h) ROC analysis showed that ≥4 CTCs (area under curve: 0.870; sensitivity: 0.84, specificity: 0.90, p < 0.001) was better to predict GC progression than traditional β-HCG (area under curve: 0.594; sensitivity: 0.52, specificity: 0.60, p = 0.153). (i) In 21 patients with continuous low β-HCG level, the positive rate of ≥4 CTCs (100%, 12/12) in PD subgroup was significantly higher that (11.1%, 1/9) in non-PD subgroup (p < 0.001). [Color figure can be viewed at wileyonlinelibrary.com]
under curve: 0.870; sensitivity: 0.84, specificity: 0.90, \( p < 0.001 \) was better to predict GC progression than traditional \( \beta \)-HCG (area under curve: 0.594; sensitivity: 0.52, specificity: 0.60, \( p = 0.153 \)) (Fig. 2h). For 21 GC patients with continuous low \( \beta \)-HCG level, in PD subgroup, the positive rate of \( \geq 4 \) CTCs was 100%, and the positive rate of \( < 4 \) CTCs was only 11.1% in non-PD subgroup (\( p < 0.001 \), Fig. 2i).

When assessing the absolute changes in target lung lesions, we found that 42 of 45 (93.33%) patients with \( \geq 4 \) CTCs experienced tumor volume growth, and 28 of 41 (68.29%) patients with \( < 4 \) CTCs had spontaneous tumor shrinkage or no tumor volume growth (Fig. 3a). Additionally, 42 of 45 (93.33%) patients with \( \geq 4 \) pretreatment CTCs exhibited an increase in the number of total metastases, whereas only 2 of 41 (4.88%) patients with \( < 4 \) pretreatment CTCs exhibited such an increase (Fig. 3b). The progression time (from first CTC detection to first progression) was negatively correlated with the pretreatment CTC numbers (\( r = -0.758 \), \( p < 0.001 \); Fig. 3c). This result indicated that patients with more pretreatment CTCs were more likely to suffer shorter times of non-progression.

Regarding chemotherapy resistance, 23 patients appeared to be resistant to the drug regimen; of the 23 patients, only 4 (17.39%) had \( < 4 \) pretreatment CTCs, while the remaining patients (82.61%) had \( \geq 4 \) CTCs. Among those patients who were not resistant to the chemotherapy (\( n = 92 \)), 39 patients (42.39%) had \( \geq 4 \) CTCs, while the other 53 patients (57.61%) had \( < 4 \) CTCs. The patients with \( \geq 4 \) CTCs were more likely to be resistant to chemotherapy than those with \( < 4 \) CTCs (\( p = 0.001 \)) (Table 3).

### Discussion

EpCAM is primarily used as a biomarker to target and capture CTCs.\(^{33,34}\) In addition to EpCAM,\(^ {30}\) CD147 has been reported to be highly expressed on the surface of GC cells.\(^ {31}\) We also found that none of the GC tissues were negative for both EpCAM and CD147, regardless of grade. Thus, a combined antibody panel targeting EpCAM and CD147, introduced in the development of our NanoVelcro Chip, ensured the efficient capture of CTCs. Additionally, based on the biological activity specific to chorion-originated tissues,\(^ {7,35}\) we innovatively used anti-HCG antibodies to assist in marking enriched CTCs. Immunostaining of a manually prepared blood sample that included Jeg-3 cells confirmed the high specificity of anti-HCG antibodies for identifying CTCs from WBCs. Further tests of clinical samples have verified that CTCs isolated from the peripheral blood of GC patients using the NanoVelcro system are trophoblastic in origin based on their HCG immunostaining characteristics.

GC is a heterogeneous disease consisting of abundant phenotypically and functionally distinct cell subpopulations, some of which have varying capacities to develop drug resistance and form metastases.\(^ {36}\) Traditionally, \( \beta \)-HCG has served as an ideal tumor marker for GC diagnosis and disease status.

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**Table 1. Relationship between pretreatment CTC number and clinical characteristic of choriocarcinoma patients**

| Pretreatment CTC number | \( n = 56 \) | \( n = 59 \) | \( p \) (Fisher’s exact) |
|-------------------------|-------------|-------------|--------------------------|
| Age (29.0, 22–55), years | \( < 40 \) | \( \geq 40 \) | 0.752 |
| \( < 40 \)            | 45          | 46          |              |
| \( \geq 40 \)         | 11          | 13          |              |
| Antecedent pregnancy   | \( 34 \)    | \( 36 \)    | 0.150        |
| Mole                   | \( 10 \)    | \( 17 \)    |              |
| Abortion               | \( 12 \)    | \( 6 \)     |              |
| Interval months from index pregnancy | \( < 4 \) | \( 4–6 \) | \( 7–12 \) | \( \geq 12 \) | 0.364 |
| \( < 4 \)             | 17          | 11          |              |
| \( 4–6 \)             | 11          | 10          |              |
| \( 7–12 \)            | 11          | 12          |              |
| \( \geq 12 \)         | 17          | 26          |              |
| Pretreatment \( \beta \)-HCG level (IU/L) | \( \leq 10^3 \) | \( 10^3–10^4 \) | \( 10^4–10^5 \) | \( > 10^5 \) | 0.068 |
| \( \leq 10^3 \)       | 19          | 12          |              |
| \( 10^3–10^4 \)       | 17          | 18          |              |
| \( 10^4–10^5 \)       | 16          | 15          |              |
| \( > 10^5 \)          | 4           | 14          |              |
| Largest tumor mass (cm) | \( \leq 3 \) | \( 3–5 \) | \( > 5 \) | 0.473 |
| \( \leq 3 \)          | 32          | 27          |              |
| \( 3–5 \)             | 16          | 21          |              |
| \( > 5 \)             | 8           | 11          |              |
| Site of metastases     | \( \leq 2 \) | \( 2–4 \) | \( 5–8 \) | \( > 8 \) | 0.014 |
| Lung                   | 23          | 37          |              |
| Spleen, kidney         | 0           | 3           |              |
| Gastrointestinal       | 0           | 3           |              |
| Liver, brain           | 1           | 13          |              |
| Number of metastases   | \( \leq 2 \) | \( 2–4 \) | \( 5–8 \) | \( > 8 \) | 0.003 |
| \( \leq 2 \)          | 22          | 10          |              |
| \( 2–4 \)             | 26          | 28          |              |
| \( 5–8 \)             | 8           | 12          |              |
| \( > 8 \)             | 0           | 9           |              |
| Previous failed chemotherapy | \( \leq 2 \) | \( > 2 \) | 0.007 |
| No                     | 38          | 32          |              |
| Monotherapy            | 13          | 8           |              |
| Combined therapy       | 5           | 19          |              |
| FIGO score             | \( \leq 6 \) | \( > 6 \) | \( \leq 6 \) | \( > 6 \) | \( \leq 0.001 \) |
| \( \leq 6 \)          | 30          | 13          |              |
| \( > 6 \)             | 26          | 46          |              |
| FIGO stage             | \( \leq III \) | \( IV \) | \( \leq III \) | \( IV \) | \( \leq 0.001 \) |
| I                      | 22          | 10          |              |
| II                     | 13          | 10          |              |
| III                    | 20          | 19          |              |
| IV                     | 1           | 20          |              |
evaluation. However, a growing body of evidence concerning false-positive tests raises new challenges for the future clinical application of $\beta$-HCG, creating the demand for a new indicator for GC patients. Since GC mainly spreads by hematogenous dissemination, we directed our attention to CTCs, which are a biomarker with increasing clinical value. In the present study, we mainly focused on the role of CTCs in evaluating clinically curative effects and disease status; to our
knowledge, our study is the first such investigation in GC patients to date.

In total, 89.6% of the GC patients had detectable CTCs, and 54.4% of these patients had greater than 4 CTCs/7.5 mL. These levels are significantly higher than those observed with other tumor types, perhaps reflecting the high affinity for blood vessels exhibited by trophoblastic cells and the tendency of GC to metastasize through the hematogenous route. The number of CTCs is closely associated with the primary tumor size and the number of metastases, consistent with findings in non-small-cell lung cancer and breast cancer. However, there was no correlation found between the CTC level and serum β-HCG concentration. This finding conflicts with the findings of some studies. Indeed, GC invasion driven by β-HCG has been demonstrated by independent groups, each showing that β-HCG promotes the migration and invasion of GC cells in vitro. The β-HCG is mainly synthesized by syncytiotrophoblast. Since only a small part of circulating CTCs are syncytial cells to generate β-HCG, this may explain our result that no correlation between CTC counts and serum β-HCG levels. As such, these findings require further clinical validation or in vivo animal studies.

We observed that the percentage of patients with ≥4 CTCs increased gradually along with GC progression as reflected by the FIGO stage. In particular, for patients in FIGO stage IV, the positive rate for ≥4 CTCs reached 95.24%. The revised FIGO 2000 Classification of Gestational Trophoblastic Neoplasia includes the classical anatomical prognostic factors. Therefore, the CTC count, as an indirect indicator of the anatomical metastasis status, may assist the stratification for FIGO staging at the time of GC diagnosis. Furthermore, the

### Table 2. Univariate and multivariate analyses for PFS (n = 115)

| Risk factor                        | No. of patients | PFS | HR  | 95% CI  | p     |
|------------------------------------|-----------------|-----|-----|---------|-------|
| **Univariate analyses**            |                 |     |     |         |       |
| **Age (years)**                    |                 |     |     |         |       |
| <40                                | 91              | 1.0 |     |         |       |
| 40                                 | 24              | 2.9 | 1.3–6.5 | 0.009 |
| **Antecedent pregnancy**           |                 |     |     |         |       |
| Mole                               | 70              | 1.0 |     |         |       |
| Abortion                           | 27              | 0.5 | 0.2–1.6 |       |
| Term and ectopic pregnancy         | 18              | 0.5 | 0.2–1.9 | 0.382 |
| **Interval months from index pregnancy** |   |     |     |         |       |
| <4                                 | 28              | 1.0 |     |         |       |
| 4–6                                | 21              | 0.9 | 0.2–5.5 |       |
| 7–12                               | 23              | 1.9 | 0.4–8.6 |       |
| ≥12                                | 43              | 4.0 | 1.2–13.8 | 0.041 |
| **Pretreatment β-HCG level (IU/L)**|                 |     |     |         |       |
| <10³                              | 31              | 1.0 |     |         |       |
| 10³–10⁶                            | 35              | 1.4 | 0.4–4.3 |       |
| 10⁶–10⁹                            | 31              | 1.3 | 0.4–4.1 |       |
| ≥10⁹                              | 18              | 3.0 | 0.9–9.6 | 0.215 |
| **Largest tumor mass (cm)**        |                 |     |     |         |       |
| <3                                | 59              | 1.0 |     |         |       |
| 3–5                               | 37              | 2.5 | 0.9–6.4 |       |
| ≥5                                | 19              | 3.9 | 1.4–10.9 | 0.028 |
| **Site of metastases**             |                 |     |     |         |       |
| Lung                              | 60              | 1.0 |     |         |       |
| Spleen, kidney                    | 3               | 1.22| 0.60–2.47 |       |
| Gastrointestinal                  | 3               | 1.16| 0.54–2.50 |       |
| Liver, brain                      | 14              | 3.82| 1.58–9.23 | 0.040 |
| **Number of metastases**          |                 |     |     |         |       |
| 0                                 | 32              | 1.0 |     |         |       |
| 1–4                               | 54              | 3.16| 1.19–8.41 |       |
| 5–8                               | 20              | 3.90| 1.16–13.13 |       |
| ≥8                                | 9               | 5.81| 1.63–20.78 | <0.001 |
| **Previous failed chemotherapy**  |                 |     |     |         |       |
| No                                | 70              | 1.0 |     |         |       |
| Monotherapy                       | 21              | 3.0 | 0.9–9.9  |       |
| Combined therapy                  | 24              | 8.2 | 3.1–21.4 | <0.001 |
| **FIGO score**                    |                 |     |     |         |       |
| 6                                 | 43              | 1.0 |     |         |       |
| ≥6                                | 72              | 52.4| 2.0–1377.0 | 0.018 |
| **FIGO stage**                    |                 |     |     |         |       |
| I + II                            | 55              | 1.0 |     |         |       |
| III + IV                          | 60              | 79.7| 3.1–2040.4 | 0.008 |

For multivariate analyses, stepwise method was used to select the variables with statistical significance. Overall p-value.

Abbreviation: CTC, circulating tumor cell.

### Table 2. (Continued)

| Risk factor    | No. of patients | PFS | HR  | 95% CI  | p     |
|----------------|-----------------|-----|-----|---------|-------|
| **CTC count** |                 |     |     |         |       |
| <4             | 56              | 1.0 |     |         |       |
| 4              | 59              | 108.0| 4.0–2884.1 | 0.005 |
| **Multivariate analyses**       |                 |     |     |         |       |
| **FIGO score** |                 |     |     |         |       |
| 6              | 43              | 1.0 |     |         |       |
| ≥6             | 72              | 22.0| 1.1–434.0 | 0.042 |
| **FIGO stage** |                 |     |     |         |       |
| I + II         | 55              | 1.0 |     |         |       |
| III + IV       | 60              | 37.0| 1.9–718.8 | 0.017 |

For multivariate analyses, stepwise method was used to select the variables with statistical significance. Overall p-value.

Abbreviation: CTC, circulating tumor cell.

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Tumor Markers and Signatures
CTC count was positively correlated with the site of metastasis, further exhibiting a trend that the patients with CTC counts ≥4 confronted a significantly greater risk of distant multiple organ metastasis. Although the most common site for GC metastasis is the lungs, patients with cerebral metastases often present with severe neurological symptoms as a result of intracranial bleeding or increased intracranial pressure. Therefore, when a GC patient is referred with brain metastasis, a CTC count in the peripheral blood should be included in the diagnostic evaluation. Notably, most GC patients with ≥4 CTCs tended to have progression in both lung-targeted lesions and sites of metastases. Given the cost and radiation injury imposed on patients during serial imaging, dynamic CTC detection may be an important evaluation indicator to monitor disease status. In addition, during the period of chemotherapy monitoring, since a rise in β-HCG with pregnancy or other non-trophoblastic tumors will complicate the situation, it is important to prescribe a CTC detection. An increasing body of evidence demonstrates that, in the course of therapy, CTCs may offer more predictive assessment information than primary tumor samples, which do not reflect the real-time evolution of the tumor. We also found that for some GC patients with continuous low β-HCG level during chemotherapy process, the high positive rate of ≥4 CTCs indicated the high incidence of chemotherapeutic resistance. Thus, the dynamic detection of CTCs would not only indirectly reflect tumor progression status but also assist in guiding clinical treatment in GC patients.

Currently, lack of tissue specimens, ideal cell lines and animal models, make it challenging to investigate GC. Therefore, a new method to capture CTCs for further biological exploration of GC is in urgent demand. CTCs are postulated to be an alternative source of tissue samples for clinical and biomolecular studies. Researches showed similarities between CTCs and tumor tissues. Using high-quality WGS on single-CTCs, the shared genomic alterations between CTCs and tumor tissues was found, and most of the clonal mutations (about 86%) in CTCs could be traced back to either the primary or metastatic tumors. Thus, based the above facts, applying the CTC investigation technique to GC may pave a way for GC research. Our study show that CTC counts is associated with progression status and chemotherapy resistance. However, the bioinformation of CTCs in GC patients have not been investigated. In the future, we will focus on the bioinformation of CTCs and the circulating clusters cell in GC patients.

**Conclusion**

Our study is the first to provide a significant prospective application of CTC detection and enumeration in hematogenously spread GCs. CTC enumeration could be useful for assisting the stratification of high-risk GC patients for early clinical intervention and evaluating the effect of chemotherapy.

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