IJC
International Journal of Cancer

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

Weiling He[1,2], Minzhi Hou[3,4], Hui Zhang[2], Chao Zeng[1,5], Shanyang He[3], Xinlin Chen[6], Manman Xu[3], Cong Sun[1], Wenting Jiang[1], Han Wang[1], Hongwei Shen[3], Yang Zhang[7], Jing Liu[8], Shijun Sun[9], Neng Jiang[1], Yongmei Cui[1], Yu Sun[1], Yangshan Chen[1], Jessica Cao[10], Chunlin Wang[11], Mengzhen Li[12], Yi Zhang[13], Liantang Wang[1], Jianhong Wang[14], Millicent Lin[15] and Zunfu Ke[1]

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

Conflict of interest: The authors declare no potential conflicts of interest.

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

Grant sponsor: Guangzhou and Guangdong/Guangzhou Science and Technology Planning Program; **Grant numbers:** 2014J4100132, 2012B031800115, 2013B02180021, 2015A, 2015ykzd07, 16ykjc08, 201704020094, 2016A020215055, 2015A020214010, 2013B02180021, 2012B031800115, 2014J4100132; **Grant sponsor:** Natural Science Foundation of Guangdong Province; **Grant numbers:** 2011B031800025, S2012010008378, 2015A030313036 and; **Grant sponsor:** Guangdong Natural Science Foundation; **Grant numbers:** 2015A030313036, S2012010008378, 2011B031800025; **Grant sponsor:** National Natural Science Foundation of China; **Grant numbers:** 30900650, 81372501, 81572260 and 81773299

DOI: 10.1002/ijc.31742

History: Received 23 Dec 2017; Accepted 20 Jun 2018; Online 2 Aug 2018

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

patients (n = 115), 103 had at least 1 CTC in a 7.5-mL peripheral blood sample, and the percentage of patients with ≥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 ≥4 pretreatment CTCs, while only 7 of the 51 non-progressive patients had ≥4 pretreatment CTCs ($p < 0.001$). In multivariate analysis, CTCs (≥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 ≥4 CTCs were more likely to be resistant to chemotherapy than those with <4 CTCs ($P < 0.001$). These findings demonstrates 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 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 β-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,[1] with an incidence of approximately 0.002% among live deliveries.[2,3] Characterized by its reliance on hematogenous dissemination for metastasis, GC generally spreads to the lungs, liver, central nervous system (CNS) and vagina.[4] This feature is also partially responsible for the high malignancy rate and resistance to chemotherapy of GC,[5,6] 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 β-HCG is currently one of the most effective markers for the clinical diagnosis of GC.[7,8] However, critical clinical information, such as the full extent of metastasis and resistance to conventional chemotherapy, which manifests in some cases,[9,10] cannot be fully revealed and assessed by measuring the β-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.[10–12] 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[13–15] and feasibility for molecular and functional research[16,17] 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)],[18] label-free devices,[19] microfluidic[20] and cytology-based ISET platforms[21] and the application of nanomaterials,[22] the CTC capture techniques have been rapidly developed,[23] 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.[24] 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 chemotherapeutic 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 tumorous 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.[25] To assess the treatment effects and progression statuses of the patients, the following tests were performed: serum β-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 β-HCG levels or new metastases were referred to as progression of disease (PD). A persistent rise or plateau of β-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, Germany, SAB4502541, 1:100) antibodies overnight at 4 °C, followed by biotinylated IgG (Vector Labs, USA, PK6101) at 1:200 dilution. Streptavidin-HRP and DAB detection kit (Ventana Medical Systems, Switzerland) were used to visualize the staining reaction, and Mayer's hematoxylin was used for subsequent counterstaining. The staining results were estimated semiquantitatively. The staining intensity was scored as follows: 0, colorless; 1, buff; 2, brownish yellow and 3, dark brown. The percentage of positive cells was scored as follows: 0, no positive cells; 1, 20% or fewer positive cells; 2, 21–75% 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% CO_2 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.[26] 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, Germany, rabbit 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 10^3 JEG-3 cells in 10^6 WBCs obtained from the blood of a healthy donor and was utilized as a positive control.[27] 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.[28] 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,[19,29] and studies have shown that both EpCAM[30] and CD147[31] 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. 1*a*; 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. 1*b*). 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. 2*a* and 2*b*). A significant association between CTC levels and the number of metastases was observed ($r = 0.603$, $p < 0.001$, Figs. 2*c* and 2*d*). No correlation between CTC counts and serum β-HCG levels was found ($r = 0.026$, $p = 0.208$, Figs. 2*e* and 2*f*).

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

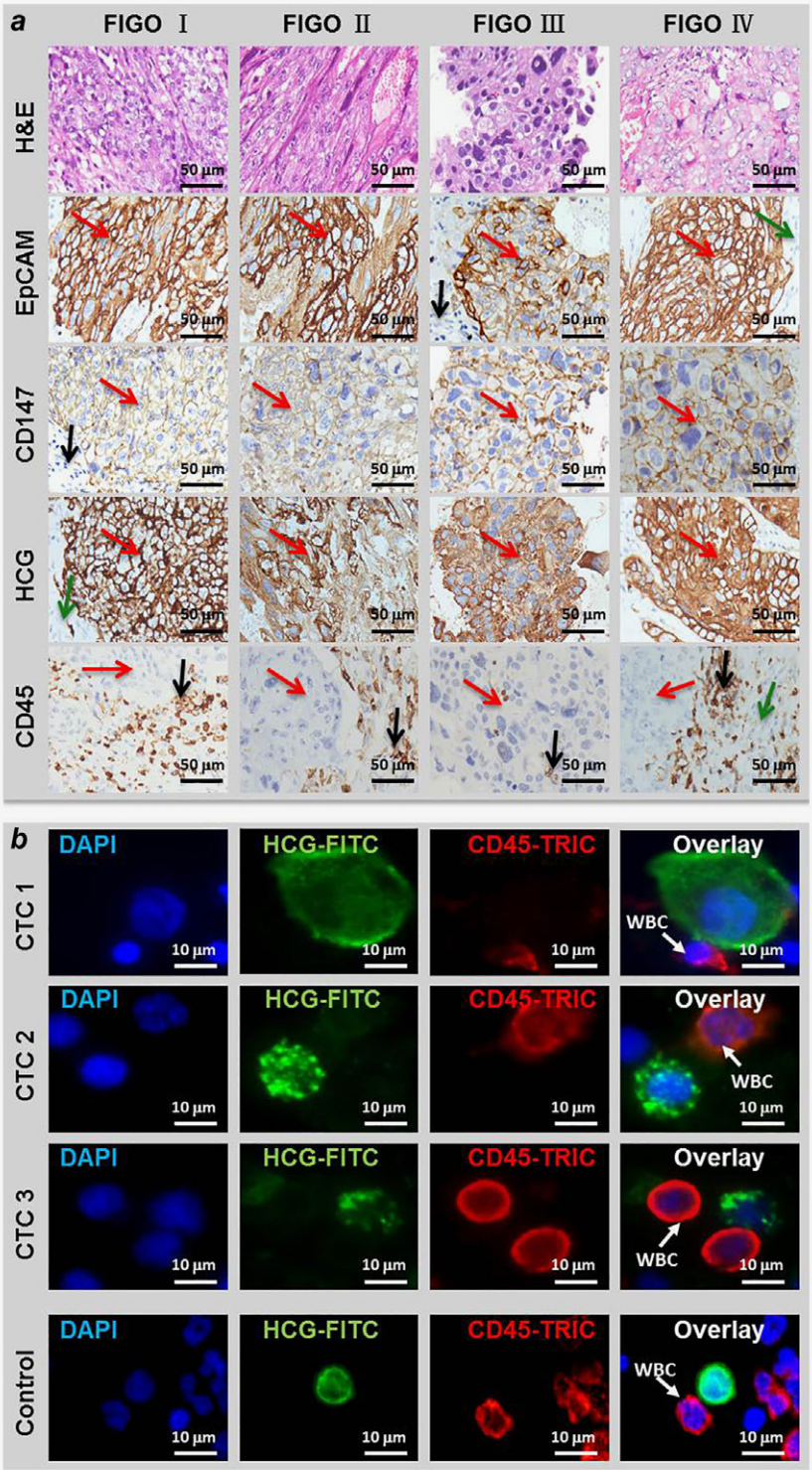

Figure 1. Immunohistochemical analysis of GC tissues and representative images of the immunofluorescence staining of CTCs. (*a*) Strong expression of EpCAM was detected both on the cell membrane and partially in the cytoplasm of GC cells, and CD147 was identified through intense membranous staining. 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]

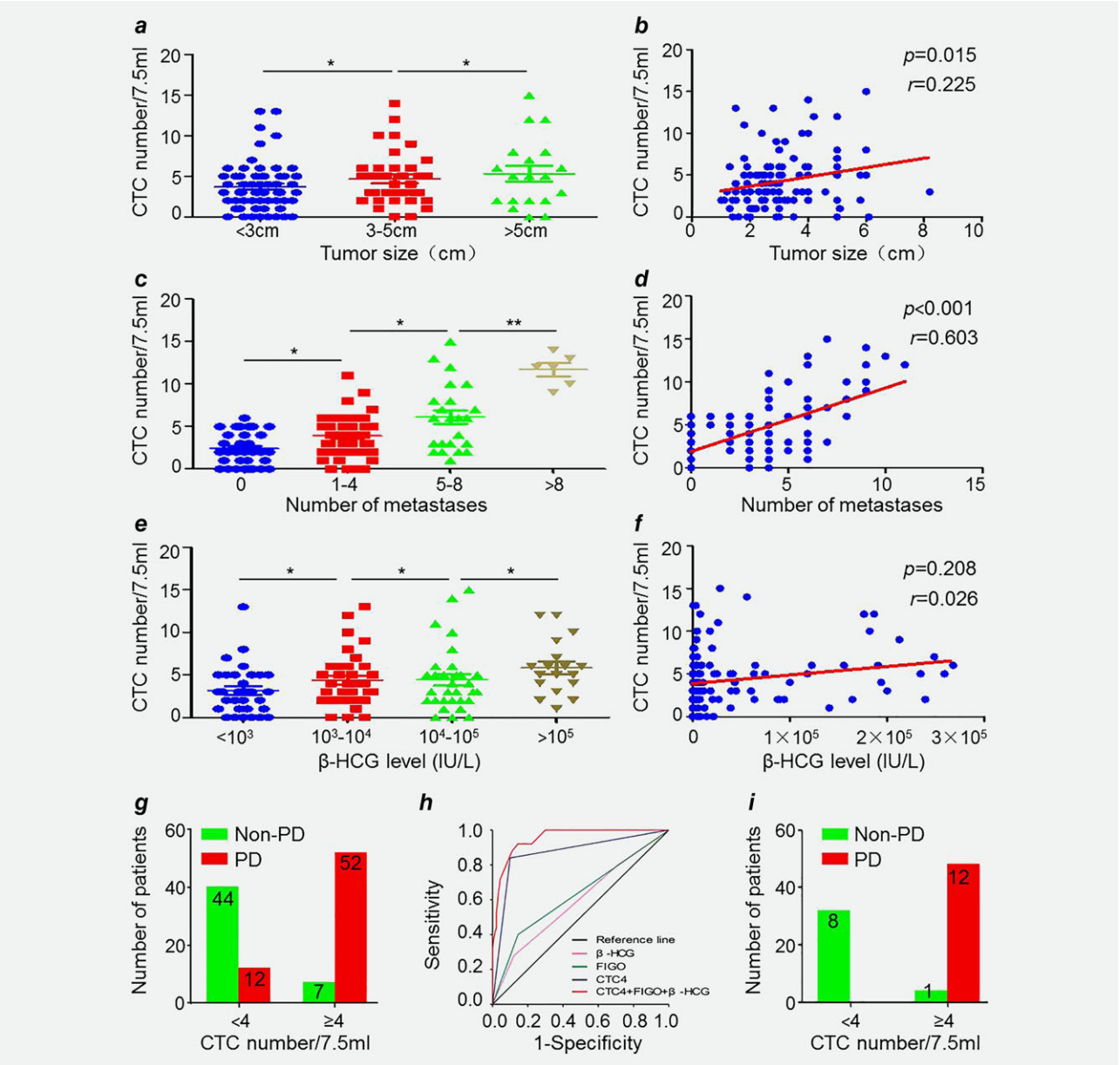

Figure 2. Relationship between CTC and existing markers and tumor progressions. (*a* and *b*) There is a positive correlation between CTC levels (range 0–15) and primary tumor sizes ($p = 0.015$, $r = 0.225$). (*c* and *d*) There is a positive correlation between CTC levels and number of metastases ($p < 0.001$, $r = 0.603$). (*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: 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 ($p < 0.001$). *, p›0·05; **, $p < 0·05$. [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. 2*g* and Table 3). Using ROC analysis, we found that ≥4 CTCs cutoff value (area

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

	Pretreatment CTC number		
	<4 (n = 56)	4 (n = 59)	p (Fisher's exact)
Age (29.0, 22–55), years			0.752
<40	45	46	
40	11	13	
Antecedent pregnancy			0.150
Mole	34	36	
Abortion	10	17	
Term and ectopic pregnancy	12	6	
Interval months from index pregnancy			0.364
<4	17	11	
4–6	11	10	
7–12	11	12	
>12	17	26	
Pretreatment β-HCG level (IU/L)			0.068
$<10^3$	19	12	
10^3–10^4	17	18	
10^4–10^5	16	15	
$>10^5$	4	14	
Largest tumor mass (cm)			0.473
<3	32	27	
3–5	16	21	
>5	8	11	
Site of metastases			0.014
Lung	23	37	
Spleen, kidney	0	3	
Gastrointestinal	0	3	
Liver, brain	1	13	
Number of metastases			0.003
0	22	10	
1–4	26	28	
5–8	8	12	
>8	0	9	
Previous failed chemotherapy			0.007
No	38	32	
Monotherapy	13	8	
Combined therapy	5	19	
FIGO score			<0.001
6	30	13	
>6	26	46	
FIGO stage			<0.001
I	22	10	
II	13	10	
III	20	19	
IV	1	20	

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: 60, $p = 0.153$) (Fig. 2*h*). For 21 GC patients with continuous low β-HCG level, in PD subgroup, the positive rate of ≥4 CTCs was 100%, and the positive rate of ≥4 CTCs was only 11.1% in non-PD subgroup ($p < 0.001$, Fig. 2*i*).

When assessing the absolute changes in target lung lesions, we found that 42 of 45 (93.33%) patients with ≥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. 3*a*). Additionally, 42 of 45 (93.33%) patients with ≥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. 3*b*). 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. 3*c*). 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 ≥4 CTCs. Among those patients who were not resistant to the chemotherapy (n = 92), 39 patients (42.39%) had ≥4 CTCs, while the other 53 patients (57.61%) had <4 CTCs. The patients with ≥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, β-HCG has served as an ideal tumor marker for GC diagnosis and disease status

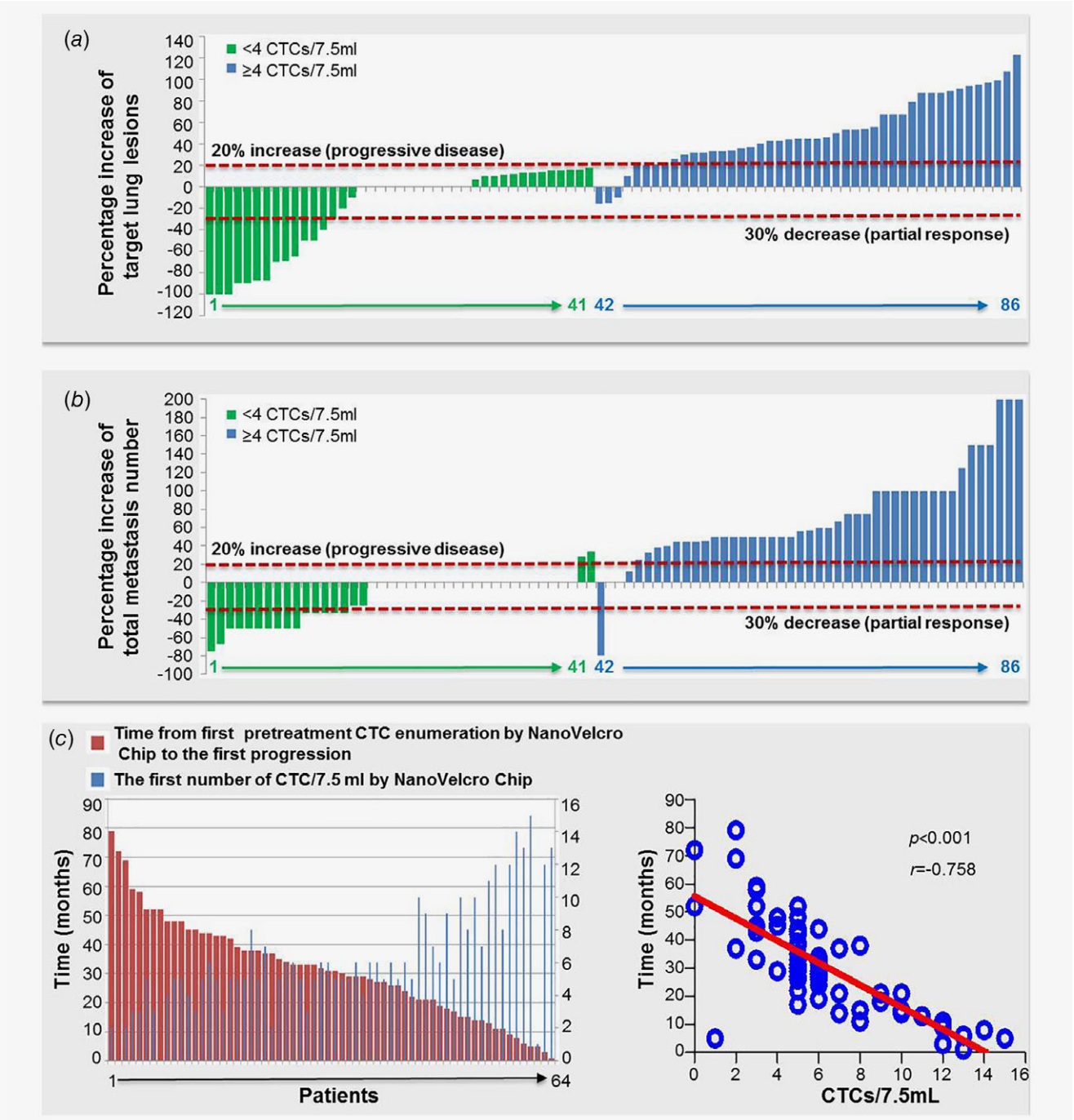

Figure 3. Relationship between pretreatment CTC levels and the percentage increments of primary tumor volume, metastasis number and progression time. (*a* and *b*) The trend of percentage increments of lung-targeted lesions and the number of metastases in the GC patients grouped according to pretreatment CTC count. Each bar represents an individual case. Those with CTC counts ≥4 are presented on the right side of the waterfall chart, whereas those with CTC counts <4 are presented on the left side. © The left side of the chart represents the distribution of the time from the first pretreatment CTC enumeration to the first progression as well as the pretreatment CTC number. Each bar indicates an individual case ($n = 64$). The result shows that these two factors are negatively related ($r = -0.758$, $p < 0.001$). [Color figure can be viewed at wileyonlinelibrary.com]

evaluation.[37] However, a growing body of evidence concerning false-positive tests raises new challenges for the future clinical application of β-HCG, creating the demand for a new indicator for GC patients.[7] 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

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[1]
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[1]
Pretreatment β-HCG level (IU/L)				
‹10^3	31	1.0		
10^3–10^4	35	1.4	0.4–4.3	
10^4–10^5	31	1.3	0.4–4.1	
›10^5	18	3.0	0.9–9.6	0.215[1]
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[1]
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[1]
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[1]
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
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
CTC count				
‹4	56	1.0		
4	59	51.1	2.6–1003.4	0.010

(Continues)

Table 2. (Continued)

For multivariate analyses, stepwise method was used to select the variables with statistical significance.
[1]Overall *p*-value.
Abbreviation: CTC, circulating tumor cell.

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,[38,39] perhaps reflecting the high affinity for blood vessels exhibited by trophoblastic cells and the tendency of GC to metastasize through the hematogenous route.[40] 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.[13,15] However, there was no correlation found between the CTC level and serum β-HCG concentration. This finding conflicts with the findings of some studies.[41,42] 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*.[43,44] The β-HCG is mainly synthesized by syncytiotrophoblast. Since only a small part of circulating CTCs are cluster "syncytial cells" to generate β-HCG, and 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.[45] Therefore, the CTC count, as an indirect indicator of the anatomical metastasis status,[46] may assist the stratification for FIGO staging at the time of GC diagnosis. Furthermore, the

Table 3. The relationship of CTC with GC progression and drug resistance of the first chemotherapy cycle

	Progressive status		Treatment outcome	
	Nonprogressive (n = 51)	Progressive (n = 64)	Nonresistance (n = 92)	Resistance (n = 23)
Pretreatment CTC number		$p < 0.001$		$p < 0.001$
<4	44	12	52	4
4	7	52	40	19

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.[47] 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 the number of metastases. Given the cost and radiation injury imposed on patients during serial imaging,[48] 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[49] 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.[50] 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.[10–12] 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.[51] 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.[51] 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.

References

1. Seckl MJ, Fisher RA, Salerno G, et al. Choriocarcinoma and partial hydatidiform moles. *Lancet* 2000;356:36–9.
2. Seckl MJ, Sebire NJ, Fisher RA, et al. Gestational trophoblastic disease: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2013;24(Suppl 6): vi39–50.
3. Schmid P, Nagai Y, Agarwal R, et al. Prognostic markers and long-term outcome of placental-site trophoblastic tumours: a retrospective observational study. *Lancet* 2009;374:48–55.
4. Savage P. Clinical observations on chemotherapy curable malignancies: unique genetic events, frozen development and enduring apoptotic potential. *BMC cancer* 2015;15:11.
5. Al-Husaini H, Soudy H, Darwish A, et al. Gestational trophoblastic neoplasia: treatment outcomes from a single institutional experience. *Clin Transl Oncol* 2015;17:409–15.
6. Baergen RN. Gestational choriocarcinoma. *Gen Diagn Pathol* 1997;143:127–41.
7. Ng TY, Wong LC. Diagnosis and management of gestational trophoblastic neoplasia. *Best Pract Res Clin Obstet Gynaecol* 2003;17:893–903.
8. McDonald TW, Ruffolo EH. Modern management of gestational trophoblastic disease. *Obstet Gynecol Surv* 1983;38:67–83.
9. Alifrangis C, Agarwal R, Short D, et al. EMA/CO for high-risk gestational trophoblastic neoplasia: good outcomes with induction low-dose etoposide-cisplatin and genetic analysis. *J Clin Oncol* 2013;31:280–6.
10. Shih IM. Gestational trophoblastic neoplasia—pathogenesis and potential therapeutic targets. *Lancet Oncol* 2007;8:642–50.
11. Kobayashi Y, Shimizu T, Naoe H, et al. Establishment of a choriocarcinoma model from immortalized normal extravillous trophoblast cells transduced with HRASV12. *Am J Pathol* 2011;179:1471–82.
12. Grummer R, Donner A, Winterhager E. Characteristic growth of human choriocarcinoma xenografts in nude mice. *Placenta* 1999;20:547–3.
13. Bidard FC, Peeters DJ, Fehm T, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. *Lancet Oncol* 2014;15: 406–14.
14. Okubo K, Uenosono Y, Arigami T, et al. Clinical impact of circulating tumor cells and therapy response in pancreatic cancer. *Eur J Surg Oncol* 2017;43:1050–5.
15. Krebs MG, Sloane R, Priest L, et al. Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer. *J Clin Oncol* 2011;29:1556–63.
16. Lohr JG, Adalsteinsson VA, Cibulskis K, et al. Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer. *Nat Biotechnol* 2014;32:479–NaN.
17. Krebs MG, Metcalf RL, Carter L, et al. Molecular analysis of circulating tumour cells-biology and biomarkers. *Nat Rev Clin Oncol* 2014;11:129–44.
18. Ghazani AA, Castro CM, Gorbatov R, et al. Sensitive and direct detection of circulating

tumor cells by multimarker mu-nuclear magnetic resonance. *Neoplasia* 2012;14:388.
19. Chen WQ, Weng SN, Zhang F, et al. Nanoroughened surfaces for efficient capture of circulating tumor cells without using capture antibodies. *ACS Nano* 2013;7:566–75.
20. Nagrath S, Sequist LV, Maheswaran S, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature* 2007;450:1235–NaN.
21. Poruk KE, Blackford AL, Weiss MJ, et al. circulating tumor cells expressing markers of tumor-initiating cells predict poor survival and cancer recurrence in patients with pancreatic ductal adenocarcinoma. *Clin Cancer Res* 2017;23:2681–90.
22. Yoon HJ, Kozminsky M, Nagrath S. Emerging role of nanomaterials in circulating tumor cell isolation and analysis. *ACS Nano* 2014;8:1995–2017.
23. Sarioglu AF, Aceto N, Kojic N, et al. A microfluidic device for label-free, physical capture of circulating tumor cell clusters. *Nat Methods* 2015; 12:685.
24. Hong Y, Fang F, Zhang Q. Circulating tumor cell clusters: what we know and what we expect (Review). *Int J Oncol* 2016;49:2206–16.
25. FIGO (International Federation of Gynecology and Obstetrics). 26th Annual Report on the Results of Treatment in Gynecological Cancer. *Int J Gynaecol Obstet* 2006;95(Suppl 1): S1–257.
26. Wang S, Liu K, Liu J, et al. Highly efficient capture of circulating tumor cells by using nanostructured silicon substrates with integrated chaotic micromixers. *Angewandte Chemie (International ed in English)* 2011;50:3084–8.
27. Traboulsi W, Sergent F, Boufettal H, et al. Antagonism of EG-VEGF receptors as targeted therapy for choriocarcinoma progression in vitro and in vivo. *Clin Cancer Res* 2017;23: 7130–40.
28. Altman DG, Vergouwe Y, Royston P, et al. Prognosis and prognostic research: validating a prognostic model. *BMJ (Clinical research ed)* 2009;338:b605.
29. Liu S, Tian Z, Zhang L, et al. Combined cell surface carbonic anhydrase 9 and CD147 antigens enable high-efficiency capture of circulating tumor cells in clear cell renal cell carcinoma patients. *Oncotarget* 2016;7:59877–91.
30. Schonberger S, Okpanyi V, Calaminus G, et al. EPCAM-A novel molecular target for the treatment of pediatric and adult germ cell tumors. *Genes Chromosomes Canc* 2013;52:24–32.
31. Singh M, Kindelberger D, Nagymanyoki Z, et al. Vascular endothelial growth factors and their receptors and regulators in gestational trophoblastic diseases and normal placenta. *J Reprod Med* 2012;57:197–203.
32. Olmos D, Baird RD, Yap TA, et al. baseline circulating tumor cell counts significantly enhance a prognostic score for patients participating in phase I oncology trials. *Clin Cancer Res* 2011;17: 5188–96.
33. Chen H, Cao B, Chen H, et al. Combination of antibody-coated, physical-based microfluidic chip with wave-shaped arrays for isolating circulating tumor cells. *Biomed Microdev* 2017;19:66.
34. Jackson JM, Witek MA, Kamande JW, et al. Materials and microfluidics: enabling the efficient isolation and analysis of circulating tumour cells. *Chem Soc Rev* 2017;46:4245–80.
35. Sisinni L, Landriscina M. The role of human chorionic gonadotropin as tumor marker: biochemical and clinical aspects. *Adv Exp Med Biol* 2015; 867:159–76.
36. Allias F, Bolze PA, Gaillot-Durand L, et al. reseau des maladies trophoblastiques g. [Gestational trophoblastic disease]. *Ann Pathol* 2014;34:434–7.
37. Essel KG, Bruegl A, Gershenson DM, et al. Salvage chemotherapy for gestational trophoblastic neoplasia: utility or futility? *Gynecol Oncol* 2017;146:74–80.
38. Lindsay CR, Faugeroux V, Michiels S, et al. A prospective examination of circulating tumor cell profiles in non-small-cell lung cancer molecular subgroups. *Ann Oncol* 2017;28:1523–31.
39. Khan MS, Kirkwood A, Tsigani T, et al. Circulating tumor cells as prognostic markers in neuroendocrine tumors. *J Clin Oncol* 2013;31:365–72.
40. Cong Q, Li GL, Jiang W, et al. Ectopic choriocarcinoma masquerading as a persisting pregnancy of unknown location: case report and review of the literature. *J. Clin Oncol* 2011;29:E845–NaN.
41. Cole LA, Khanlian SA, Riley JM, et al. Hyperglycosylated hCG in gestational implantation and in choriocarcinoma and testicular germ cell malignancy tumorigenesis. *J Reprod Med* 2006;51:919–28.
42. Cole LA, Butler SA. Hyperglycosylated human chorionic gonadotropin and human chorionic gonadotropin free beta-subunit: tumor markers and tumor promoters. *J Reprod Med* 2008;53: 499–512.
43. Zhang HJ, Siu MK, Yeung MC, et al. Overexpressed PAK4 promotes proliferation, migration and invasion of choriocarcinoma. *Carcinogenesis* 2011;32:765–1.
44. Hamada AL, Nakabayashi K, Sato A, et al. Transfection of antisense chorionic gonadotropin beta gene into choriocarcinoma cells suppresses the cell proliferation and induces apoptosis. *J Clin Endocr Metab* 2005;90:4873–9.
45. Committee FO. FIGO staging for gestational trophoblastic neoplasia 2000. FIGO Oncology Committee. *Int J Gynaecol Obstet* 2002;77:285–7.
46. Ignatiadis M, Lee M, Jeffrey SS. Circulating tumor cells and circulating tumor DNA: challenges and opportunities on the path to clinical utility. *Clin Cancer Res* 2015;21:4786–800.
47. Small W Jr, Lurain JR, Shetty RM, et al. Gestational trophoblastic disease metastatic to the brain. *Radiology* 1996;200: 277–80.
48. Jiao L, Ghorani E, Sebire NJ, et al. Intraplacental choriocarcinoma: systematic review and management guidance. *Gynecol Oncol* 2016;141:624–31.
49. Stenman UH, Alfthan H, Hotakainen K. Human chorionic gonadotropin in cancer. *Clin Biochem* 2004;37:549–61.
50. Alix-Panabieres C, Pantel K. Clinical Applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. *Cancer Discov* 2016;6: 479–91.
51. Jiang R, Lu YT, Ho H, et al. A comparison of isolated circulating tumor cells and tissue biopsies using whole-genome sequencing in prostate cancer. *Oncotarget* 2015;6:44781–93.