Supplementary Data:

Methods

Patient demographics

The demographic and clinical characteristics of 180 enrolled GC patients were listed in Table S1. Forty-seven patients had a short interval from the time of antecedent pregnancy to the time of onset of symptoms (median, 2.61±0.97 months; range, <4 months), and 133 patients had a long interval (median, 46.15±11.80 months; range, ≥4 months). The meanβ-HCG level before treatment was 28502.15 ± 9817.31 IU/L (range, 15 to 268043). Forty-nine (27.22%) stage I, 36(20%) stage II, 60 (33.33%) stage III, and 35 (19.44%) stage IV GCs were diagnosed in 180 patients based on the 2009 FIGO staging system.[1] According to the new FIGO/WHO prognosis scoring system, 75 of the 180 evaluable patients were low risk(score≤6), and 105 were high risk (score>6). The distribution of total distant organ metastasis among the patients was characterized as the following organs: lungs (n=60), lungs/spleen/kidneys (n=5), lungs/gastrointestinal tract (n=7), lungs/liver (n=18), lungs/brain (n=1), liver (n=2) and brain (n=2). By the time of analysis, the average follow-up time for all patients was 57.18±19.78 months (range, 17 to 94 months).

Chemotherapy Regimen

FAEV (5-fluorouracil/FUDR, actinomycin-D, vincristine, etoposide) and FAV (5-fluorouracil/FUDR, actinomycin-D, vincristine) were used as the first-line treatment in 106 and 74 GC patients, respectively. After 2-3 additional courses of consolidation chemotherapy, patients is discontinued the treatment. The salvage chemotherapies included EMA/CO (etoposide, methotrexate, actinomycin-D/cyclophosphamide, vincristine), EMA/ EP (etoposide, methotrexate, actinomycin-D /etoposide, cisplatin), TE/TP (paclitaxel, etoposide/paclitaxel, cisplatin) and other cisplatin-based chemotherapies.

We defined complete remission (CR) as consecutively normal serumβ-HCG levels for at least 4 weeks. Either tumor lesions (tumor volume or number of metastasis) were reduced by 30 % or the serum β-HCG decreased by more than 50 % were defined as partial remission (PR). Progression of disease (PD) refers to conditions when there were either consistent or rising serum β-HCG levels or new metastases. Patients were found to have new lesions or rising serum β-HCG level after CR, were defined as recurrence.

Follow-up

Patients were followed-up according to the recommendations of the European Society for Medical Oncology,[2]and the items including a physical examination, regular serumβ-HCG test, ultrasonography, chest X ray or CT and MRI. Progression-free survival (PFS) and overall survival (OS) were defined from the date inclusion to the date of the last follow-up or cancer progression, and from the date of inclusion to the date of last follow-up or death resulting from cancer. PFS and OS were defined by an independent physician who was blinded to the study.

Immunohistochemistry Staining

Tissue sections (4 μm thick) from 180 GC patients were prepared using a rotary microtome (Leica, Wetzlar, Germany) on samples obtained from the formalin-fixed paraffin-embedded tissue archive. Before immunohistochemistry (IHC) staining, for each paraffin-embedded tissue (representing an individual GC patient), the diagnosis of GC was histologically confirmed by 2 experienced pathologists in a double-blind manner using hematoxylin-eosin (H&E) staining. All the tissue sections (4 μm thick) 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 minutes at 37℃ to accomplish

antigen retrieval, followed by blocking endogenous peroxidase with 0.5% H_2O_2 in distilled water for 8 minutes. The sections were incubated with anti-β-HCG (Abcam, 2092, USA, 1:120), anti-CD147 (Abcam, ab108317, 1:250), anti-EpCAM (Abcam, ab71916, 1:100) or anti-CD45 (Sigma, SAB4502541, 1:100) antibodies overnight at 4℃, followed by 30 minutes of post-primary blocking. 3, 3′-diaminobenzidine was used to visualize the staining reaction, and Mayer's hematoxylin was used for subsequent counterstaining. The staining results were estimated semi-quantitatively. 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% to 75% positive cells; 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.

Statistical Analysis

Previous studies have stratified cancer patients into high- and low-risk subgroups based on "favorable" and "unfavorable" CTC counts.[3, 4] Assuming a power of ≥90% and a two-sided α of 0.05, a sample size of 153 would meet the statistical requirements for detecting the difference between a median PFS of 43 months for the "favorable" CTC group and a median PFS of 21 months for the "unfavorable" CTC group. Because we were not sure of the proportion of patients randomly allocated to each group, we increased the sample size to 180 to allow for favorable-to-unfavorable CTC group ratio as low as 0.5 or as high as 2.3.[5]

To obtain the most appropriate CTC cutoff for distinguishing prognosis, all the enrolled GC patients were randomly split into the training and validation cohorts according to the methods used in a previous study.[6] In the training phase, a range of baseline CTC values for 90 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 90 enrolled GC patients.

References

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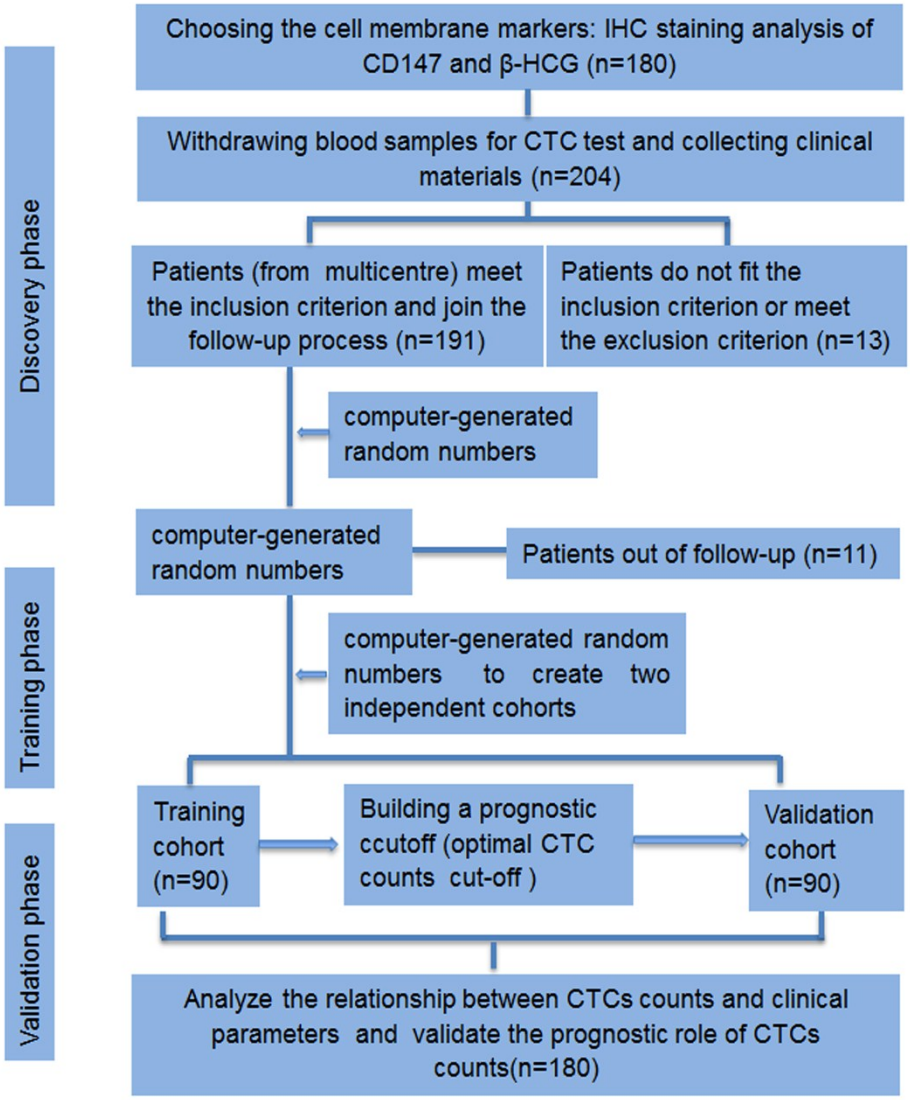
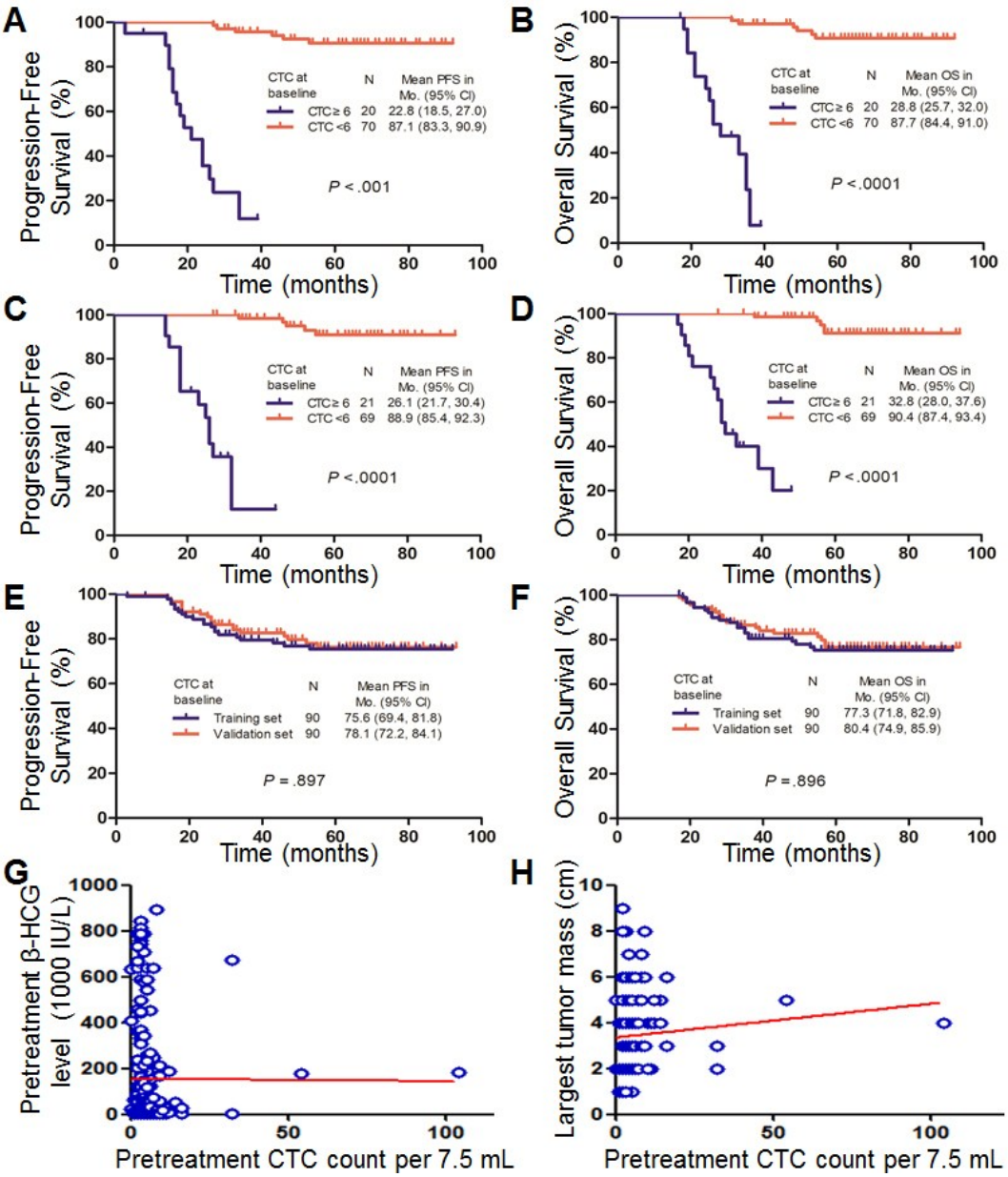
Supplementary Figures and figure legends



Supplementary Fig. S1. Consort diagram of study design. The inclusion criterion: 1). With age older than 18 years; 2). With histological and immunohistochemical proof of GC confirmed by two pathologists; 3). No concurrent treatment with experimental drugs; 4). Signed informed consent. The exclusion criterion: 1). Receiving any chemotherapy with in precious 3 months at the time of first CTC test; 2). Out of the follow-up; 3). With any other malignant tumors before or after the diagnosis of GC; 4). Receiving any other treatments such as molecular targeted therapy or experimental drugs; 6). Patients with pregnancy; 7). Patients with PSTT (placental site trophoblastic tumor) or ETT (epithelial trophoblastic tumor); 8). Death caused by other reasons not related to GC.



Supplementary Fig. S2. **Kaplan-Meier curves of progression-free survival (PFS) and overall survival (OS) according to a cutoff value of 6 CTCs in 7.5 mL of blood at baseline in the training (A, B) and validation (C, D) sets.** The differences in PFS and OS between the training and validation sets (**E, F**). CTC levels were not related to serum β-HCG concentration (**G**) (r=-0.004, P=0.954) or largest tumor mass (**H**) (r=0.087, P=0.246).

Supplementary Table S1. Relationship between pretreatment CTC count and clinicopathological characteristics of GC patients.

	Pretreatment CTC count		
	<6 (n=139)	≥6 (n=41)	*P*
Age at baseline			0.058
<40	114	28	
≥40	25	13	
FIGO score			<0.001
≤6	69	6	
>6	70	35	
Antecedent pregnancy			0.840
Mole	81	26	
Abortion	35	9	
Term and ectopic pregnancy	23	6	
Interval months from index pregnancy			0.478
<4	40	7	
4-6	21	6	
7-12	24	9	
>12	54	19	
Pre-treatment β-HCG level (IU/L)			0.037
$<10^3$	48	5	
10^3-10^4	38	15	
10^4-10^5	36	12	
$>10^5$	17	9	
Largest tumor mass(cm)			
<3	63	13	0.281
3-5	62	22	
>5	14	6	
Site of metastases			<0.001
Lungs	83	8	
Spleen, kidneys	2	3	
Gastrointestinal tract	2	5	
Liver, brain	3	20	
Number of metastases			<0.001
0	49	0	
1-4	67	15	
5-8	22	15	
>8	1	11	
Previous failed chemotherapy			0.039
No	81	15	
Monotherapy	29	11	
Combined therapy	29	15	
Surgery			0.019
No	83	16	

Yes	56	25	
FIGO Stage			<0.001
I	49	0	
II	32	4	
III	53	7	
IV	5	30	

Supplementary Table S2. Establishment of cutoff CTC count in training set*.

CTC cutoff	Group	No. in each group	36-Month PFS (%)	Survival difference	HR	*P*
1	0	2	100	24	20.9	0.614
	≥ 1	88	76			
2	0-1	6	100	22	22.9	0.377
	≥ 2	84	78			
3	0-2	20	100	27	7.4	0.051
	≥ 3	70	73			
4	0-3	47	100	45	18.3	<0.001
	≥ 4	43	55			
5	0-4	58	98	57	28.2	<0.001
	≥ 5	32	41			
6	0-5	70	96	84	50.4	<0.001
	≥ 6	20	12			
7	0-6	76	92	84	39.2	<0.001
	≥ 7	14	8			
8	0-7	78	89	80	25.5	<0.001
	≥ 8	12	9			
9	0-8	80	87	76	17.8	<0.001
	≥ 9	10	11			
10	0-9	82	86	73	18.6	<0.001
	≥ 10	8	13			
11	0-10	84	84	67	17.3	<0.001
	≥ 11	6	17			
12	0-11	84	84	67	17.3	<0.001
	≥ 12	6	17			
13	0-12	85	83	63	13.6	<0.001
	≥ 13	5	20			
14	0-13	85	83	63	13.6	<0.001
	≥ 14	5	20			
15	0-14	87	82	82	63.8	<0.001
	≥ 15	3	0			
16	0-15	87	82	82	63.8	1.000
	≥ 16	3	0			
17	0-16	88	81	81	1.0	1.000
	≥ 17	2	0			
32	0-31	88	81	81	1.0	1.000
	≥ 32	2	0			
54	0-53	89	80	80	1.0	1.000

CTC cutoff	Group	No. in each group	36-Month PFS (%)	Survival difference	HR	*P*
	≥ 54	1	0			

Abbreviations: CTC, circulating tumor cell; HR, hazard ratio; PFS, progression-free survival.

*Groups were split into below and above different CTC cutoffs in rows.

Supplementary Table S3. Establishment of cutoff CTC count in training set**

CTC cutoff	Group	No. in each group	36-Month OS (%)	Survival difference	HR	*P*
1	0	2	100	20	20.9	0.614
	≥ 1	88	80			
2	0-1	6	100	21	22.9	0.377
	≥ 2	84	79			
3	0-2	20	100	25	7.3	0.052
	≥ 3	70	75			
4	0-3	47	100	42	17.2	<0.001
	≥ 4	43	58			
5	0-4	58	98	54	22.2	<0.001
	≥ 5	32	44			
6	0-5	70	97	89	64.0	<0.001
	≥ 6	20	8			
7	0-6	76	93	85	39.0	<0.001
	≥ 7	14	8			
8	0-7	78	91	82	25.3	<0.001
	≥ 8	12	9			
9	0-8	80	89	78	17.7	<0.001
	≥ 9	10	11			
10	0-9	82	87	74	17.1	<0.001
	≥ 10	8	13			
11	0-10	84	85	68	19.8	<0.001
	≥ 11	6	17			
12	0-11	84	85	68	19.8	<0.001
	≥ 12	6	17			
13	0-12	85	84	64	15.0	<0.001
	≥ 13	5	20			
14	0-13	85	84	64	15.0	<0.001
	≥ 14	5	20			
15	0-14	87	83	83	59.1	<0.001
	≥ 15	3	0			
16	0-15	87	83	83	59.1	<0.001
	≥ 16	3	0			
32	0-31	88	82	82	49.8	<0.001
	≥ 32	2	0			
54	0-53	89	82	82	1.0	1.000
	≥ 54	1	0			

Abbreviations: CTC, circulating tumor cell; HR, hazard ratio; OS, overall survival.

*Groups were split into below and above different CTC cutoffs in rows.

Supplementary Table S4. Univariate analysis for prognostic factors in stage III and/or IV GC patients.

	No. of	PFS			OS		
Risk factor	patients	HR	95% CI	*P*	HR	95% CI	*P*
Stage III + IV (n = 95)							
CTC count							
<6	58	1.0		<0.001	1.0		<0.001
≥6	37	39.3	(13.0, 118.4)		47.7	(13.5, 168.3)	
FIGO score							
≤6	28	1.0		0.002	1.0		0.002
>6	67	24.7	(3.4, 180.2)		24.8	(3.4, 180.6)	
Number of metastases							
0	1	1.0		0.591	1.0		0.589
≥1	94	20.6	(0.0, 1.3E6)		20.6	(0.0, 1.2E6)	
Stage III (n = 60)							
CTC count							
<6	53	1.0		<0.001	1.0		<0.001
≥6	7	54.1	(9.8, 298.0)		90.4	(10.0, 821.9)	
FIGO score							
≤6	27	1.0		0.015	1.0		0.016
>6	33	12.4	(1.6, 95.1)		12.1	(1.5, 92.5)	
Number of metastases							
0	1	1.0		0.703	1.0		0.700
≥1	59	20.8	(0.0, 1E8)		20.8	(0.0, 1E8)	
Stage IV (n = 35)							
CTC count							
<6	5	1.0		0.024	1.0		0.041
≥6	30	5.5	(1.3, 24.7)		4.6	(1.1, 20.4)	
FIGO score							
≤6	1	1.0		0.602	1.0		0.478
>6	34	21.3	(0.0, 2.1E6)		21.9	(0.0, 1.1E5)	
Number of metastases							
0	0			-			-
≥1	35	-	-	-	-	-	-

Abbreviations: CTC, circulating tumor cell.

-: not available.

Supplementary Table S5. Univariate analyses for prognostic markers in GC patients (n = 106).

	PFS			OS		
Risk factor	HR	95% CI	*P*	HR	95% CI	*P*
CTC count after treatment						
<6	1.0			1.0		
≥6	44.5	5.8-342.5	<0.001	36.1	4.8-271.5	<0.001
CTC count at baseline and after treatment						
Both <6	1.0			1.0		
One ≥6	22.1	2.5-198.4	0.006	14.8	1.7-128.6	0.014
Both ≥6	526.7	46.7-5942.5	<0.001	1012.2	58.7-17441.6	<0.001

Abbreviations: CTC, circulating tumor cell.