Comparison of CellSearch and Circulating Tumor Cells (CTC)-Biopsy Systems in Detecting Peripheral Blood Circulating Tumor Cells in Patients with Gastric Cancer

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BCEF 1 Dawei Ning
BC 2 Kai Cui
D 2 Min Liu
BCEF 3 Yang Ou
B 4 Zhendan Wang
B 5 Benkui Zou
B 6 Yangyang Shen
B 7 Xinyang Lu
AG 8 Sheng Li
AB 9 Pang Li

1 Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences, Jinan, Shandong, P.R. China
2 Department of Hepatobiliary Surgery, Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences, Jinan, Shandong, P.R. China
3 Department of General Surgery, The 5th People's Hospital of Jinan, Jinan, Shandong, P.R. China
4 Department of Thoracic Surgery, Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences, Jinan, Shandong, P.R. China
5 Department of Urology, Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences, Jinan, Shandong, P.R. China
6 Department of Anesthetics, Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences, Jinan, Shandong, P.R. China
7 Department of Gastrointestinal Surgery, Affiliated Hospital of Jining Medical University, Jining Medical University, Jining, Shandong, P.R. China
8 Shandong First Medical University and Shandong Academy of Medical Sciences, Jinan, Shandong, P.R. China
9 Department of Cardiovascular Surgery, Guangzhou Red Cross Hospital, Medical college, Jinan University, Guangzhou, Guangdong, P.R. China

Corresponding Authors: Pang Li, e-mail: Slashpang82@163.com, Sheng Li, e-mail: e-mail: lisheng@sdfmu.edu.cn
Source of support: Funding for this study was provided by the Academic Promotion Program of Shandong First Medical University (2019QL004), the National Key R&D Plan (2016YFC010600) and the Major Science and Technology Innovation Project of Shandong province (2017CXGC1204)

Background: The purpose of this study was to compare circulating tumor cells (CTCs)/circulating tumor microemboli (CTM) detection rates of the CellSearch and CTC-Biopsy systems in patients with gastric cancer (GC). We also investigated potential correlations between clinicopathological characteristics and prognosis in patients with GC.

Material/Methods: This prospective study was conducted at the Shandong Institute of Cancer Prevention and Control in China. Fifty-nine patients with GC and 22 healthy volunteers were recruited and their peripheral blood samples were examined by the CTC-Biopsy system and CellSearch system for CTC.

Results: The rate of detection of CTCs/CTM was significantly higher with the CTC-Biopsy system than with the CellSearch system (59.32% vs. 27.12%, P<0.001). The Kappa value was 0.179, indicating poor consistency. CTCs detected with the CellSearch system in patients with stage III/IV GC was significantly correlated with neutrophil count (P=0.020), neutrophil/lymphocyte ratio (N/L ratio) (P=0.009), CA19-9 (P=0.049), tumor size (P=0.026), and the extent of vascular invasion (P=0.007). CTCs detected with the CTC-Biopsy system correlated with tumor differentiation (P=0.010). CTM in patients with stage I/II GC and stage II/IV GC correlated with CEA (P=0.004) and tumor differentiation (P=0.030), respectively. A CTC count >3 detected with the CellSearch system, and not the CTC-Biopsy system, correlated with reduced progression-free survival and overall survival.

Conclusions: The CTC-Biopsy system was superior to the CellSearch system for detecting CTCs in GC patients. CTM were detected with the CTC-Biopsy system but not with the CellSearch system. CTCs detected with the CellSearch system correlated with various clinicopathological factors and long-term survival outcomes.

MeSH Keywords: Neoplastic Cells, Circulating • Prognosis • Stomach Neoplasms

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/926565

📄 3142   📊 9   📈 4   📚 30

Background

Gastric cancer (GC) is the fourth most common cancer worldwide and the third most common cause of tumor-related deaths [1]. GC is an aggressive tumor, as almost 50% of patients suffer from tumor recurrence or metastasis after curative resection. The 5-year survival rate is less than 30% [2,3].

Circulating tumor cells (CTCs) are the cells shed from a primary tumor into the peripheral blood [4]. With the help of adhesion molecules, CTCs can adhere to form circulating tumor microemboli (CTM) [5]. CTCs and CTM may be responsible for the development of distant metastases in patients with malignant tumors [6,7]. In a study of patients with advanced GC, the number of CTCs detected in the peripheral blood after 6 weeks of chemotherapy was associated with the rate of disease control ($P<0.013$), treatment efficacy ($P<0.016$), short-term progression-free survival (PFS), and overall survival (OS) [8]. Other studies have shown that CTCs may be an independent predictor of short-term PFS and a potential prognostic marker in advanced GC [9,10].

To detect CTCs, it is important to first separate tumor cells from blood cells. One of the most widely used method for separation and detection of CTCs is the CellSearch system [11,12]. Another technique, which is based on the principle of filtration and isolation of tumor cells according to cell size, allows for the separation of CTCs and CTM from whole blood using a membrane filter [13]. Our research group has developed the novel CTC-Biopsy detection system based on this filtration principle. The CTC-Biopsy system can differentiate peripheral blood tumor cells from normal cells based on tumor cell diameter and capacity of deformation. A filtration membrane was used to increase the yield of CTCs from the whole blood sample.

In this study, we compared the CTC detection rates of the CellSearch and CTC-Biopsy systems. We also analyzed the relationships among CTC, clinicopathological features, and prognosis in patients with GC.

Material and Methods

Patient selection

In this prospective study, patients with GC who were admitted to Shandong Cancer Hospital and Institute from May 2014 to December 2014 and met the inclusion criteria were recruited for the study.

The inclusion criteria were age >18 years, pathological diagnosis of GC, treatment-naïve status, and good performance status allowing for subsequent treatment and blood collection.

Patients with benign stomach lesions or other malignancies were excluded from the study, as were patients receiving antitumor treatment.

During the same period, 22 healthy volunteers (age >18 years) were included as the negative controls, whose routine blood parameters, liver biochemistry, kidney function tests, tumor markers, and radiological imaging within the previous 3 months were normal. The study protocol was approved by the Ethics Committee of Shandong Cancer Prevention and Control Research Institute. Individuals who met the inclusion criteria signed written informed consent for enrollment in the study.

Sample and data collection

Peripheral blood was collected from the patients, and the relevant clinical data were recorded. The initial 1 mL of collected blood was discarded to prevent epithelial cell contamination and the associated interference with the study results. About 7.5 mL of blood was collected into a CellSave blood preservation tube, which had an anticoagulant effect. Samples were processed by the CellSearch system and stored at room temperature. The blood samples were tested within 96 h.

To reduce the damage caused by venous blood sampling, 5 mL of peripheral blood was processed with the CTC-Biopsy system. Blood samples were collected in an EDTA anticoagulant blood tube (BD Vacutainer batch no: 367863) and submitted for analysis by the CTC-Biopsy system within 24 h. The peripheral blood of 22 healthy volunteers was collected and processed as described above.

CTC detection with the CellSearch system

The CellSearch system was operated in strict accordance with the manufacturer's instructions [14]. CTCs were defined as round or oval epithelial cells, expressing cell surface antigen epithelial cell adhesion molecule (EpCAM), cytokeratin (CK), and DAPI (4',6-2-amino-2-benzene indole), without the expression of leukocyte common antigen (CD45): EpCAM+/CK+/DAPI+/CD45–. The results were analyzed independently by 2 experienced technicians. If there was any inconsistency, double-blind reanalysis was carried out by the assessment technician. Test results were considered positive if 1 or more CTCs were detected.

CTC detection with the CTC-Biopsy system

CTCs were separated by a newly designed CTC-Biopsy system (Wuhan YZY Medical Science and Technology Co., Ltd., Wuhan, China). To maintain the cell morphology for analysis, 5 mL of blood sample was diluted to 8 mL with 0.2% paraformaldehyde and transferred to a centrifuge tube. The mixture was

aerated and absorbed by the Pasteur tube and fixed at room temperature for 10 min. After fixation, the blood sample was filtered through a filter membrane with an aperture of 8 μm. Five kilopascals were applied to the filters. The cells remaining on the filter membrane were stained with Wright-Giemsa stain and dried at room temperature. After the test, the filter membrane was taken out, adhered to a slide, and dried at 50°C to 60°C for 30 min. The slide was then sealed and read.

criteria for CTC diagnosis

The criteria for judging malignant abnormal cells (CTCs) were as follows:
1) Nuclear atypia (irregular nuclear morphology such as nodular and lobulated);
2) High ratio of nucleus to cytoplasm (> 0.8);
3) Cell diameter (long end) >15 μm;
4) Dark and uneven staining of the nucleus;
5) Nuclear membrane appeared thickened, with depressions and/or folds;
6) Nuclear chromatin migration (nuclear deviation), large nucleoli, or signs of abnormal nuclear division.

Based on the evaluation criteria proposed in previous studies, we considered samples that met ≥4 of the parameters described above as CTC [15–17]. A sample was also classified as CTC if it met criterion (6) and 2 additional criteria. Three or more cells were considered to be a CTC agglomerate, which we will refer to from here on as a CTM. The results were interpreted by 2 cytopathologists. All decisions were made after consensus was reached. If 1 or more CTCs were detected, the test was considered to have yielded positive results.

Statistical analysis

SPSS version 19.0 was used for statistical analysis. Consistency testing was used to compare the CellSearch and CTC-Biopsy systems in detecting peripheral blood CTCs in patients with GC. Continuous variables were expressed as mean±standard deviation, and the data conforming to a normal distribution with equal variance were tested by t test. Nonparametric tests were used for data without a normal distribution. Categorical variables were listed as frequencies and percentages. Chi-square or Fisher's exact test were used to compare categorical data. The Cox proportional risk regression model was used to analyze the factors affecting prognosis. Survival curves were constructed with the Kaplan-Meier method. Survival data were analyzed with the log-rank test. P values less than 0.05 were considered statistically significant.

Results

Demographic details

A total of 59 patients with GC and 22 healthy volunteers were included in this study. In the GC group, there were 49 men and 10 women, with a mean age of 57 years (Table 1).

CTC detection in healthy volunteers

CTCs were not detected in the peripheral blood of any of the healthy volunteers by either the CellSearch or the CTC-Biopsy system.

Comparison between the CellSearch and CTC-Biopsy systems

The CellSearch system did not detect CTM, and the CTC detection rate was 27.12% (16/59) (Table 2, Figure 1). The CTC/CTM detection rate was 59.32% (35/59) with the CTC-Biopsy system (Table 3, Figure 2). This difference was statistically significant (P<0.001) (Table 4). The Kappa coefficient value was 0.179, which confirmed that the consistency in results between systems was poor (Table 4).

Correlation between the presence of CTCs and clinicopathological factors of patients with GC

With the CellSearch system, a significant association was found between detectable CTCs and various clinicopathological factors such as neutrophil count, lymphocyte count, neutrophil/lymphocyte ratio, serum CA 19-9, distant metastasis, clinical stage, lymph node metastasis, tumor location, tumor size, and the extent of vascular invasion (P<0.05) (Table 5).

Although no statistically significant differences were found in patients with stage I/II GC (P>0.05), there was a significant correlation between detectable CTCs and various clinicopathological factors in patients with stage III/IV GC (P<0.05) (Table 5).

With the CTC-Biopsy system, there was no significant correlation between detectable CTC/CTM and clinicopathological factors (Table 6). However, subgroup analysis showed a significant correlation with neutrophil/lymphocyte ratio and serum CA19-9 in patients with stage I/II GC, and in patients with stage III/IV GC. Also, detectable CTCs were correlated with the degree of tumor differentiation (P<0.05) (Table 6).

Correlation between CTMs detected with the CTC-Biopsy system and clinicopathological factors

Among the 59 patients with GC, a CTM was detected by the CTC-Biopsy system in 15 patients. There was no significant

Table 1. Baseline characteristics of the study population.

Characteristics	n (%) (n=59)
Age, median (range), y	57.09±13.42 (28–81)
Platelet, median (range), 10^9/L	261.69±83.46 (119–437)
Neutrophil lymphocyte ratio, median (range)	2.73±1.48 (0.83–6.97)
Sex	
Male	49
Female	10
Serum CEA, median, ng/mL	27.92±134.61 (0.463–1000)
Grade of differentiation	
Well	4
Moderate	16
Poor	12
Other	27
T stage	
T1	9
T2	10
T3	26
T4	14
Lymph node metastasis	
Yes	35
No	15
Unknown	9
Venous invasion	
Yes	9
No	41
Unknown	9
Neural invasion	
Yes	13
No	37
Unknown	9
Location	
Antrum	28
Gastric angle	2
Cardia	16
Gastric body	10
Fundus	3

Table 2. Circulating tumor cells detected with the CellSearch system in patients with gastric cancer.

Pathological stage	n	CTC detection rate (%)
Total	59	16/59 (27.12)
Stage I	9	0/9 (0.00)
Stage II	10	1/10 (10.00)
Stage III	27	6/27 (22.22)
Stage IV	14	9/14 (64.29)

correlation between CTM detection and clinicopathological factors (P>0.05) (**Table 7**). A subgroup analysis of patients with stage I/II GC showed that CTM was correlated with serum CEA levels (P<0.05) (**Table 7**). In patients with stage III/IV GC, the CTM detected with the CTC-Biopsy system was correlated with the degree of tumor differentiation (P<0.05) (**Table 7**).

Factors affecting 4-year progression-free survival in patients with GC who underwent surgical/interventional/chemoradiotherapy treatment

The results of the univariate analysis showed that patients with GC with a CTC count >3, as measured with the CellSearch system, had significantly lower progression-free survival (PFS) than patients with fewer CTCs. No such association with PFS was observed for elevated CTC counts measured with the CTC-Biopsy system (**Table 8**). The results of univariate analysis also showed that lower PFS was significantly associated with advanced pathological stage, distant metastasis, lymph node metastasis, large tumor size, and vascular infiltration (**Table 8**).

The multivariate analysis showed that, with a CTC count > 3, advanced pathological stage and distant metastasis were independent factors associated with low PFS in patients with GC, as determined by the CellSearch system (**Table 8**).

Factors affecting 4-year OS in patients with GC who underwent surgical/interventional/chemoradiotherapy treatment

The results of univariate analysis showed that patients with GC with a CTC count >3, as measured with the CellSearch system, had significantly lower OS than patients with fewer CTCs. The same association was not found with the CTC-Biopsy system (**Table 9**). Additional factors associated with decreased OS in the univariate analysis were advanced pathological stage, distant metastasis, lymph node metastasis, large tumor size, and vascular infiltration (**Table 9**). In the multivariate analysis, distant metastasis and a CTC count >3, as measured with the CellSearch system, were independent predictors of lower OS in patients with GC (**Table 9**).
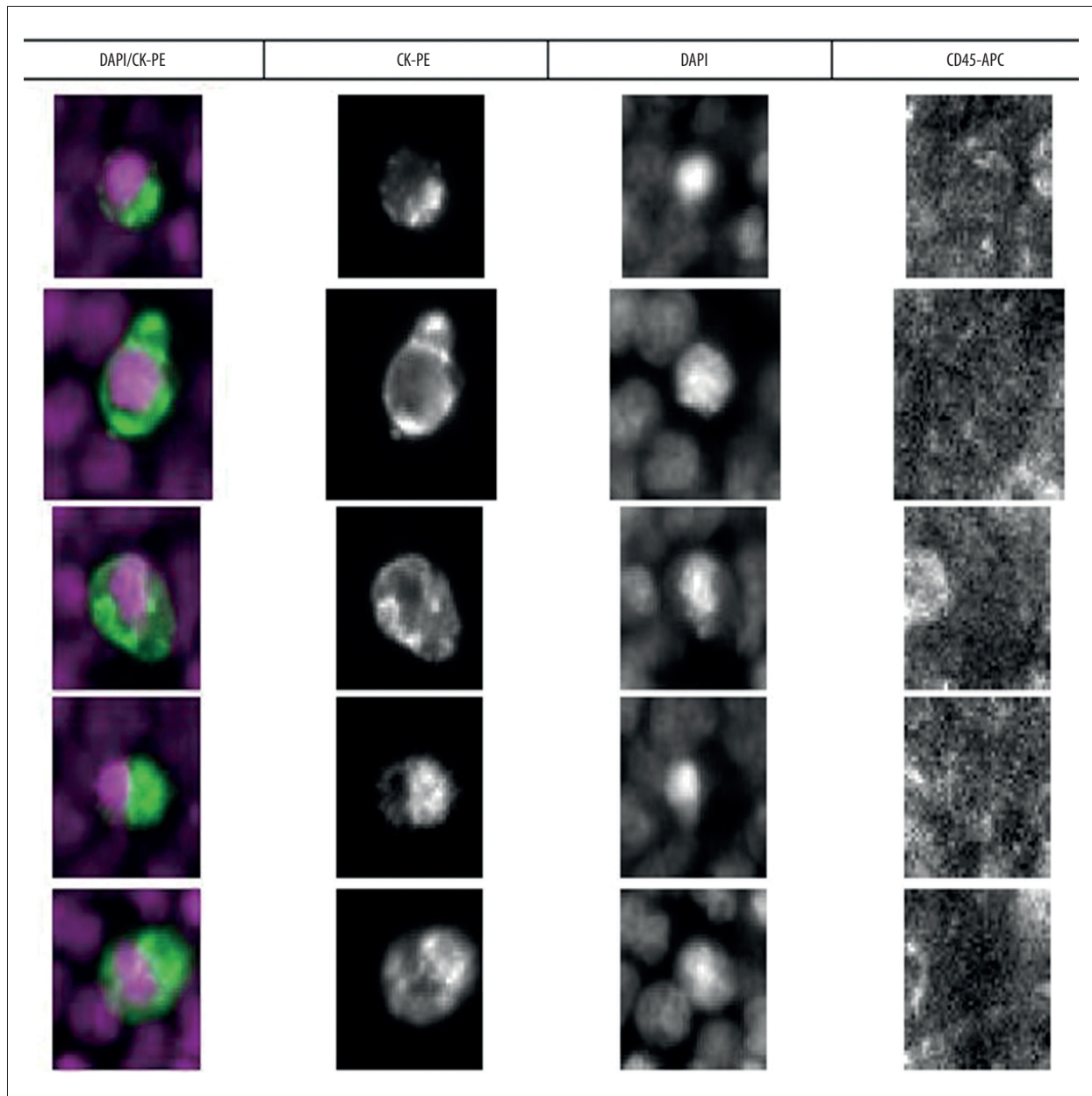
Figure 1. Circulating tumor cells (CTCs) were detected with the CellSearch system. In this system, CTCs were defined as circular or elliptical epithelial cells with specific cell surface antigen characteristics (EpCAM+/CK+/DAPI+/CD45–).

Table 3. Circulating tumor cells detected with the CTC-Biopsy system in patients with gastric cancer.

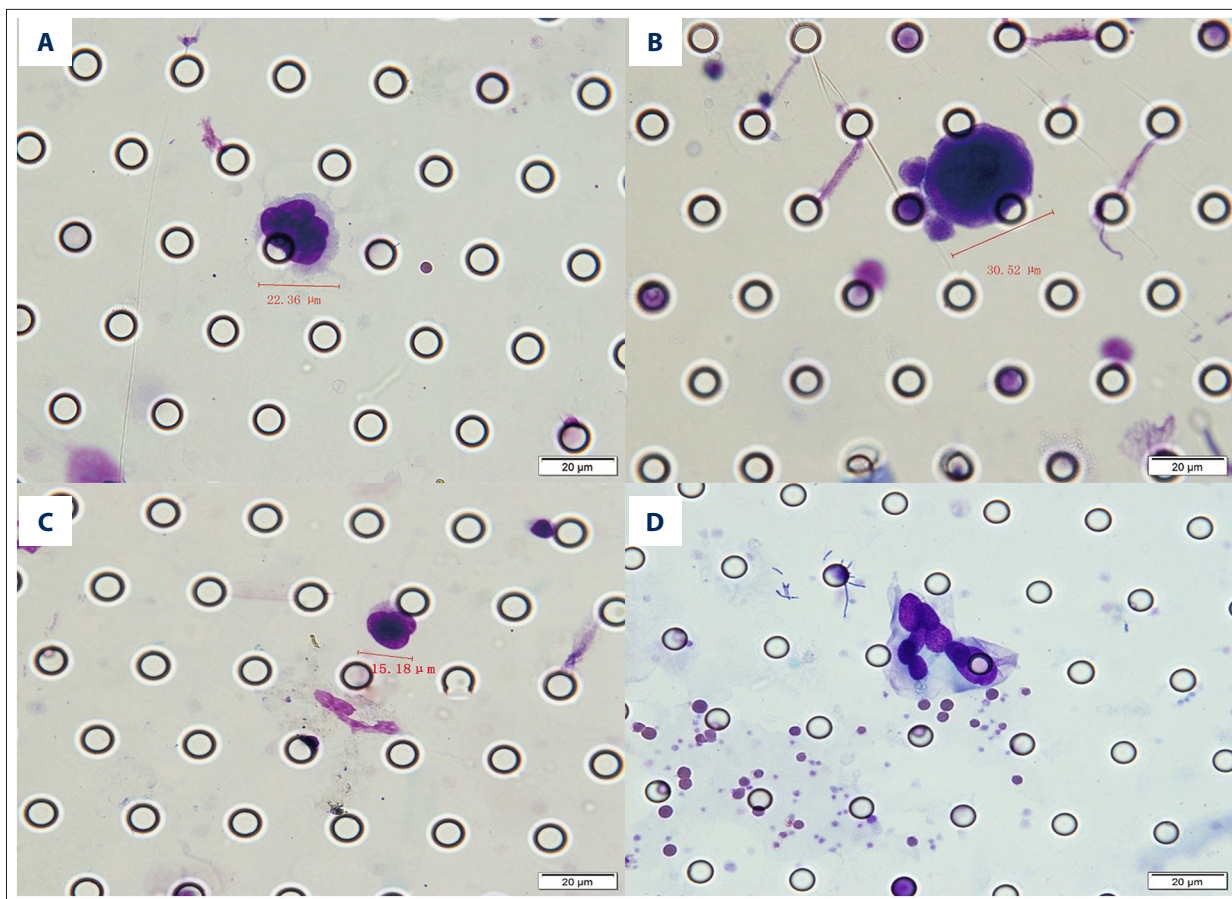| Pathological stage	n	CTC+CTM detection rate (%)	CTM detection rate (%)	CTC detection rate (%)
Total	59	35/59 (59.32)	15/59 (25.42)	28/59 (47.46)
Stage I	9	4/9 (44.44)	2/9 (22.22)	3/9 (33.33)
Stage II	10	4/10 (40.00)	0/10 (0.00)	4/10 (40.00)
Stage III	26	17/26 (65.38)	10/26 (38.46)	13/26 (50.00)
Stage IV	14	10/14 (71.43)	3/14 (21.43)	8/14 (57.14)

Figure 2. Circulating tumor cells (CTC)/circulating tumor microemboli (CTM) detected with the CTC-Biopsy system in patients with gastric cancer. Wright-Giemsa staining was performed for cytomorphological analysis. (**A**) CTC: nuclear-cytoplasmic ratio >0.8, irregular nuclear shape. (**B**) CTC: cell diameter >15 μm; abnormally large nucleoli. (**C**) CTC: thickened and wrinkled, with jagged nuclear membranes and side-shifted chromatin. (**D**) CTM: aggregation of tumor cells (≥3). All cells were analyzed under 40× magnification. Scale bar: 20 μm.

Table 4. Comparison of CellSearch and CTC-Biopsy systems in terms of circulating tumor cells detection rate.

CellSearch	CTC-Biopsy		Total	P-value	Kappa value
	Positive	**Negative**			
Positive	12	4	16	<0.001	0.138*
Negative	24	19	43		

* Kappa <0.40 indicates poor consistency.

Impact of CTC count, as detected with the CellSearch system, on survival in patients with GC

The 4-year PFS of patients in the CTC ≤3 group was significantly higher than that of patients in the CTC >3 group (*P*<0.001) (**Figure 3**). Similarly, the 4-year OS of patients in the CTC ≤3 group was significantly higher than that of patients in the CTC >3 group (*P*<0.001) (**Figure 4**).

Discussion

The presence of CTCs has been used as a "liquid pathology" index for tumor metastasis and to assess the risk of tumor recurrence. CTCs have also been used for the real-time detection of tumor response and for targeted therapies [12]. However, the concentration of CTCs in human peripheral blood is very low and varies significantly among individuals. Advanced technology is required to detect CTCs [13]. In this study, we used the CellSearch system, which is based on cell-surface immune

Table 5. Correlation between circulating tumor cells detected with the CellSearch system and clinicopathological factors.

Factors	Positive (n=16)	Negative (n=43)	P	Stage I–II (n=1)	P	Stage III–IV (n=15)	P
Age			0.937*		1**		0.935*
≥60	8	21		1		8	
<60	8	22		0		7	
Sex			0.378*		1**		0.414*
Male	12	37		1		11	
Female	4	6		0		4	
Lymphocyte (×10^9/L)	1.30±0.38	1.74±0.56	**0.004#**	1.29	0.148#	1.29±0.39	0.085#
Neutrophils (×10^9/L)	4.62±1.85	3.71±1.41	**0.046#**	5.82	0.327#	4.54±1.88	**0.020#**
Platelet (×10^9/L)	269.00±83.03	258.98±84.43	0.685#	181.00	0.351#	274.87±82.44	0.527#
N/L	3.86±1.75	2.35±1.26	**0.001#**	4.51	0.080#	3.82±1.81	**0.009#**
D-dimer	3.54±6.33	1.03±1.78	0.138#	0.90	0.918#	3.71±6.51	0.0549#
Serum CEA			0.770*		0.730**		0.904*
<3.4	8	26		1		7	
≥3.4	7	15		0		7	
Other	1	2		0		1	
Serum CA199			**0.024***		0.838*		**0.049***
<39	9	36		1		8	
≥39	6	3		0		6	
Other	1	4		0		1	
Lymph node metastasis			**0.014***		1**		0.519*
No	1	17		1		0	
Yes	15	26		0		15	
Tumor location			0.026*		0.080*		0.254*
Upper third	6	17		0		6	
Middle third	6	4		1		5	
Lower third	4	22		0		4	
Tumor size, cm			**0.006***		0.784*		**0.026***
<5	3	26		1		2	
≥5	9	15		0		9	
Other	4	2		0		4	
Vascular invasion			**0.002***		0.892*		**0.007***
No	6	35		1		5	
Yes	4	6		0		4	
Other	6	2		0		6	

Table 5 continued. Correlation between circulating tumor cells detected with the CellSearch system and clinicopathological factors.

Factors	Positive (n=16)	Negative (n=43)	P	Stage I–II (n=1)	P	Stage III–IV (n=15)	P
Differentiation level			0.650**		0.404**		0.404**
Low	10	22		0		10	
Low medium	2	8		1		1	
Medium	2	8		0		2	
High	2	2		0		0	
Other	0	3		0		2	

* χ^2 test; ** Fisher accurate inspection; # Student's t-test.

Table 6. Correlation between circulating tumor cells detected with the CTC-Biopsy system and clinicopathological factors.

Factors	Positive (n=35)	Negative (n=24)	P	Stage I–II (n=8)	P	Stage III–IV (n=27)	P
Age			0.914*		0.552*		0.427*
≥60	17	12		4		13	
<60	18	12		4		14	
Sex			0.451*		0.202*		0.177*
Male	28	21		8		20	
Female	7	3		0		7	
Lymphocyte (×10⁹/L)	1.58±0.50	1.67±0.58	0.51#	1.75±0.36	0.192#	1.53±0.53	0.411#
Neutrophils (×10⁹/L)	3.97±1.66	3.94±1.49	0.94#	4.96±1.97	0.084#	3.67±1.47	0.327#
Platelet (×10⁹/L)	266.00±70.65	128.63±26.17	0.64#	273.38±84.76	0.487#	263.81±67.61	0.988#
N/L	2.82±1.53	2.82±1.53	0.72#	3.01±1.67	**0.045#**	2.77±1.51	0.278#
D-dimer	2.01±4.58	1.32±2.15	0.451#	0.74±0.49	0.459#	2.40±5.19	0.297#
Serum CEA			0.964*		0.355*		0.569*
<3.4	20	14		5		15	
≥3.4	13	9		2		11	
Other	2	1		1		1	
Serum CA199			0.882*		**0.050****		0.367*
<39	26	19		5		21	
≥39	6	3		1		5	
Other	3	2		2		1	
Lymph node metastasis			0.498*		0.737*		1**
No	9	9		7		2	
Yes	26	15		1		25	
Tumor location			0.319*		0.581*		0.093*
Upper third	22	11		3		9	
Middle third	8	2		1		7	
Lower third	15	11		4		11	

This work is licensed under Creative Common Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0)

e926565-8

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Table 6 continued. Correlation between circulating tumor cells detected with the CTC-Biopsy system and clinicopathological factors.

Factors	Positive (n=35)	Negative (n=24)	P	Stage I–II (n=8)	P	Stage III–IV (n=27)	P
Tumor size, cm			0.301*		0.396*		0.807*
<5	15	14		6		9	
≥5	15	9		1		14	
Other	5	1		1		4	
Vascular invasion			0.388*		0.243*		0.685*
No	22	19		7		15	
Yes	7	3		0		7	
Other	6	2		1		5	
Differentiation level			0.196*		0.753*		**0.010***
Low	22	10		2		20	
Low medium	7	3		2		5	
Medium	3	7		2		1	
High	1	1		1		1	
Other	2	3		1		0	

* χ^2 test; ** Fisher accurate inspection; # Student's t-test.

Table 7. Correlation between circulating tumor microemboli detected with the CTC-Biopsy system and clinicopathological factors.

Factors	Positive (n=15)	Negative (n=44)	P	Stage I–II (n=2)	P	Stage III–IV (n=13)	P
Age			0.330*		1**		0.427*
≥60	9	20		1		8	
<60	6	24		1		5	
Sex			0.445*		1**		0.237*
Male	11	38		2		9	
Female	4	6		0		4	
Lymphocyte (×10⁹/L)	1.50±0.52	1.66±0.54	0.307#	1.89±0.04	0.950#	1.43±0.54	0.711#
Neutrophils (×10⁹/L)	3.70±1.12	4.04±1.71	0.476#	3.65±1.13	0.635#	3.71±1.17	0.716#
Platelet (×10⁹/L)	281.87±60.93	254.82±89.42	0.282#	345.50±95.46	0.109#	272.08±52.85	0.671#
N/L	2.88±1.55	2.72±1.57	0.727#	1.94±0.64	0.654#	3.03±1.61	0.864#
D-dimer	0.87±1.15	2.04±4.33	0.306#	0.55±0.07	0.703#	0.92±1.23	0.260#
Serum CEA			0.903*		**0.004***		0.324*
<3.4	8	26		0		8	
≥3.4	6	16		1		5	
Other	1	2		1		0	
Serum CA199			0.820*		0.154**		0.287*
<39	11	34		1		10	
≥39	3	6		0		3	
Other	1	4		1		0	

Table 7. Correlation between circulating tumor microemboli detected with the CTC-Biopsy system and clinicopathological factors.

Factors	Positive (n=15)	Negative (n=44)	P	Stage I–II (n=2)	P	Stage III–IV (n=13)	P
Lymph node metastasis			0.094*		1**		1**
No	2	17		2		0	
Yes	13	27		0		13	
Tumor location			0.625*		0.638**		0.907*
Upper third	7	16		1		6	
Middle third	3	7		0		3	
Lower third	5	21		1		4	
Tumor size, cm			0.211*		0.605**		0.339*
<5	24	5		2		3	
≥5	15	9		0		9	
Other	5	1		0		1	
Vascular invasion			0.501*		0.501**		0.685*
No	9	32		2		7	
Yes	4	6		0		4	
Other	2	6		0		2	
Differentiation level			0.277*		0.346**		**0.030****
Low	11	21		0		11	
Low medium	3	7		1		2	
Medium	1	9		1		0	
High	0	2		0		0	
Other	0	5		0		0	

* χ^2 test; ** Fisher accurate inspection; # Student's t-test.

recognition, and the CTC-Biopsy system, which is based on cell morphology and size, to detect CTCs in the peripheral blood of patients with GC.

Detection techniques that are based on epithelial markers, such as with the CellSearch system, cannot capture cells that do not express EpCAM and cytokeratin on the cell membrane. Galatea et al. used breast cancer cell lines in their study and found that the filtration method had a higher recovery rate for tumor cells than did CellSearch [18]. Another study on pancreatic cancer found a 40% CTC detection rate with the CellSearch system, while the CTC detection rate achieved using a technology based on the filtration principle reached 93% [19]. In a study on esophageal squamous cell carcinoma, Li et al. reported that the CTC detection rate of the CTC-Biopsy system (32.8%) was significantly higher than that of the CellSearch system (1.6%) [20]. A similar study on renal cancer found that the CTC detection rates by the CellSearch system and technology based on the filtration principle were 19.4% and 36.1%, respectively [21]. In the present study, the CTC detection rate of the CTC-Biopsy system was significantly higher than that

of the CellSearch system in GC (59.32% *vs.* 27.12%, *P*<0.001). The CTC-Biopsy system isolated CTCs based on cell size, and its effectiveness was not affected by changes in the expression of epithelial markers. We believe that, in the present study, the low EpCAM and cytokeratin expression rates of CTC in patients with GC led to high rates of false-negative results from the use of the CellSearch technology. Meanwhile, a CTM was found in 16 GC patients with the CTC-Biopsy system but in no patients with the CellSearch system. This finding is consistent with reports published previously [22,23].

Recent studies have shown that CTCs are associated with advanced tumor stage and lymphatic vascular invasion [24,25]. In the present study, CTCs detected with the CellSearch system were positively correlated with the stage of disease (*P*=0.022). The CTC detection rate was highest in patients with stage IV disease (64.29%, 9/14) and lowest in those with stage I disease (0%, 0/9). There was also a positive correlation between CTCs and vascular invasion in patients with stage III/IV GC (*P*=0.007). This suggests that CTCs in GC are related to tumor invasiveness and tumor stage. Hence, the CTC count can be

CLINICAL RESEARCH

Table 8. Univariate and multivariate analysis of CellSearch-derived and other factors affecting progression-free survival in patients with gastric cancer.

Independent factors	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P
CTC (CellSearch) (Yes/No)	2.556	1.278–5.109	**0.008**	0.512	0.157–1.667	0.266
CTC (≤1/>1)	5.824	2.452–13.833	**0.000**	5.367	0.427–67.416	0.193
CTC (≤2/>2)	6.292	2.566–15.431	**0.000**	8.630	0.421–177.118	0.162
CTC (≤3/>3)	5.358	2.136–13.443	**0.000**	0.023	0.001–0.395	**0.009**
CTC (≤4/>4)	4.859	1.775–13.300	**0.002**	1.618	0.293–8.947	0.581
Age (≤60/>60)	1.527	0.790–2.951	0.208			
Sex (Male/Female)	1.449	0.634–3.314	0.380			
KPS (70–80/90–100)	0.685	0.367–1.279	0.235			
Platelet count	1.001	0.997–1.005	0.724			
N/L	1.116	0.905–1.377	0.305			
CEA (<3.4/≥3.4)	1.437	0.878–2.352	0.149			
CA199 (<39/≥39)	1.036	0.160–0.818	0.886			
Pathological stage (I–II/III–IV)	14.377	3.424–60.372	**0.000**	15.475	2.426–98.725	**0.004**
Distant metastasis (Yes/No)	11.478	4.451–29.595	**0.000**	10.310	2.796–38.019	**0.000**
Lymph node metastasis (Yes/No)	5.177	1.820–14.727	**0.002**	0.603	0.152–2.383	0.470
Tumor size (<5 cm/≥5 cm)	2.580	1.637–4.067	**0.000**	1.385	0.588–3.263	0.456
Vascular invasion(Yes/No)	1.912	1.266–2.889	**0.002**	0.917	0.420–2.003	0.829

Table 9. Univariate and multivariate analyses of factors affecting overall survival in patients with gastric cancer.

| Independent factors	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P
CTC (CellSearch) (Yes/No)	2.834	1.386–5.796	**0.004**	0.419	0.118–1.491	0.179
CTC (≤3/>3)	5.463	2.213–13.489	**0.000**	0.007	0.000–0.157	**0.002**
Age (≤60/>60)	1.563	0.776–3.151	0.211			
Sex (Male/Female)	1.109	0.456–2.696	0.820			
KPS (70–80/90–100)	0.679	0.349–1.322	0.255			
Platelet count	1.001	0.997–1.005	0.627			
N/L	1.165	0.946–1.434	0.150			
CEA (<3.4/≥3.4)	1.382	0.821–2.327	0.224			
CA199 (<39/≥39)	1.092	0.668–1.785	0.726			
Pathological stage (I–II/III–IV)	10.670	2.538–44.849	**0.001**	4.756	0.799–28.319	0.087
Distant metastasis (Yes/No)	7.165	3.199–16.050	**0.000**	8.623	2.296–32.382	**0.001**
Lymph node metastasis (Yes/No)	6.042	1.833–19.917	**0.003**	1.455	0.326–6.498	0.623
Tumor size (<5 cm/≥5 cm)	2.736	1.713–4.369	**0.000**	2.309	0.938–5.682	0.069
Vascular invasion (Yes/No)	1.917	1.246–2.951	**0.003**	0.480	0.197–1.171	0.107
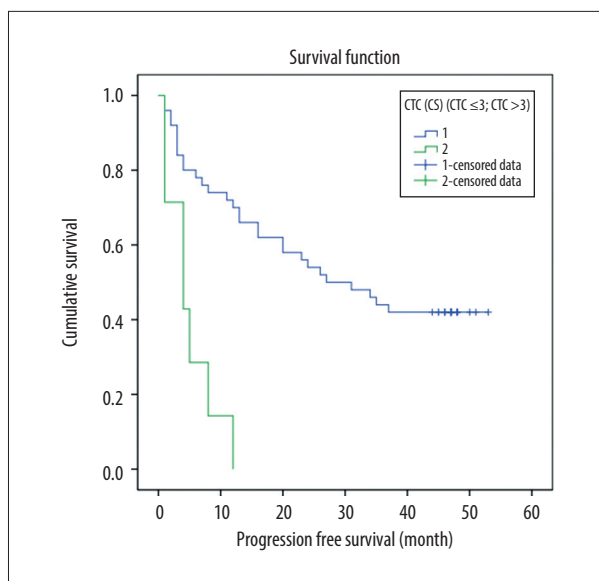
Figure 3. Correlation between circulating tumor cells (CTCs) detected with the CellSearch system and progression-free survival in 59 patients with gastric cancer (CTC ≤3 and CTC >3 group log-rank test, *P*<0.001).
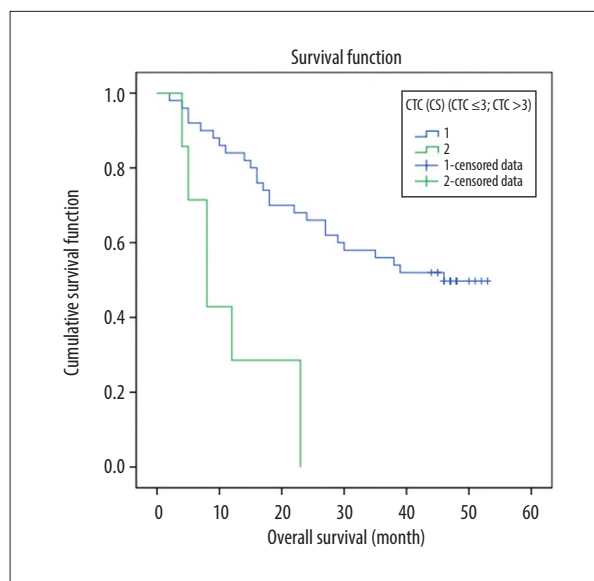

Figure 4. Correlation between circulating tumor cells (CTCs) detected with the CellSearch system and overall survival in 59 patients with gastric cancer (CTC ≤03 and CTC >3 group log-rank test, *P*<0.001).

used as an auxiliary indicator to judge tumor stage and the risk of recurrence.

CTMs are more invasive than CTCs [5]. Carlsson et al. found that the number of CTMs in patients with advanced lung cancer was much higher than that in patients with early lung cancer (0 to 184 *vs.* 0 to 2, respectively) [26]. One study showed that the rate of CTM detection correlated with serum CA125 levels in patients with GC [23]. Another study showed that high CTC counts were associated with poor tumor differentiation and high serum CEA levels (*P*=0.021 and 0.005, respectively) [27]. In this study, we found that, with use of the CTC-Biopsy system, CTM in patients with stage I/II GC was correlated with serum CEA (*P*<0.05), while CTCs (*P*=0.010) and CTMs (*P*=0.030) in patients with stage III/IV GC were correlated with the degree of tumor differentiation. CTM detection often indicates a poor prognosis. The rate of detection of CTM is different in patients at various stages of disease, and CTM detection may help in increasing the accuracy of clinical staging [22].

Neutrophils have been found to be associated with tumor growth, metastasis, and tumor angiogenesis. Previous studies have found that the number of neutrophils in GC tumor tissue is increased and associated with low OS rates in patients with GC [28]. A study of 1220 nonsurgical patients with advanced GC found that patients with an elevated neutrophil/lymphocyte ratio had poor OS and that the neutrophil/lymphocyte ratio was an independent prognostic factor [29]. In patients with GC undergoing radical resection and postoperative adjuvant chemotherapy, preoperative neutrophil/lymphocyte ratio values

were independent risk factors for OS and PFS [30]. In the present study, CTC count, as measured with the CellSearch system, was found to be correlated with neutrophils (*P*=0.020) and neutrophil/lymphocyte ratio (*P*=0.009) in patients with stage III/IV GC. The CTC detection rate obtained with the CTC-Biopsy system was correlated with neutrophil count in patients with stage I/II GC (*P*=0.045). We speculate that increases in neutrophil count and neutrophil/lymphocyte ratio promote tumor progression, leading to increased numbers of CTCs in peripheral blood. In the present study, the detection of >3 CTCs by the CellSearch system was an independent risk factor adversely affecting PFS and OS in patients with GC (*P*<0.05) (**Tables 8, 9**). However, there was no statistically significant correlation between the rate of CTC detection by the CTC-Biopsy system and oncologic outcomes such as PFS or OS (*P*>0.05).

This study had some limitations. First, because of the constraints of our study design, we could not compare the recovery rate and specificity of the 2 systems for detection of GC and WBC cell lines. Also, owing to technical limitations, we could not use immunocytofluorescence or immunocytochemistry to verify the results of the CTC-Biopsy in the GC group. Hence, we could not identify false-positive cases detected by the CTC-Biopsy system. In the future, we intend to perform a more comprehensive study with the use of immunocytofluorescence or immunocytochemistry to verify the nature of the cells detected by the CTC-Biopsy system and to validate cell lines. Second, there was a poor correlation between the CTCs detected with the CTC-Biopsy system and the clinicopathological or prognostic factors of patients. Moreover, the correlations

were different with the CellSearch system. We think that this may be related to the small number of patients in the experimental group and the short follow-up time. Therefore, to further clarify the significance of CTC detection in the prognostic evaluation of GC, additional large-sample, multi-center clinical trials are needed. Third, the identification criteria used by the CellSearch and CTC-Biopsy systems were not the same. Future experiments comparing these 2 systems with the same identification criteria are required to decrease the possibility of false-positive results.

Conclusions

In conclusion, the CTC-Biopsy system, which was designed based on the filtration principle, is more sensitive than the CellSearch system in the detection of CTCs. CTCs detected with the CellSearch system correlated with various clinicopathological factors and prognosis in GC patients. Future studies are required to validate the findings of this study.

Ethics statement

The study protocol was approved by the Ethics committee of Shandong Cancer Prevention and Control Research Institute (201405002).

Conflict of interest

Among the authors listed in this paper, Li Sheng proposed the theory of circulating tumor cell detection and designed and verified the feasibility of the detection method. The CTC- Biopsy device was produced by Wuhan YZY Medical Technology Co, Ltd. YZY provided related equipment, testing supplies, and technical support. Li Sheng's team carried out clinical trials and tests in Shandong Cancer Prevention and Control Institute. Li Sheng provided technical consulting services and academic promotion for the technical team of YZY. Li Sheng, Dawei Ning, Kai Cui, Min Liu, Yang Ou, Zhendan Wang, Benkui Zou, Yangyang Shen, Xinyang Lu, and Pang Li had no commercial interest in the CTC-Biopsy device. The terms of this arrangement have been reviewed and approved by the Shandong First Medical University and Shandong Academy of Medical Sciences in accordance with its policy on objectivity in research.

References:

1. Karimi P, Islami F, Anandasabapathy S et al: Gastric cancer: Descriptive epidemiology, risk factors, screening, and prevention. Cancer Epidemiol Biomarkers, 2014; 23:700–13

2. Duarte HO, Gomes J, Machado JC et al: Gastric cancer: Basic aspects. Helicobacter, 2018; Suppl. 1: e12523

3. Marrelli D, De Stefano A, de Manzoni G et al: Prediction of recurrence after radical surgery for gastric cancer: A scoring system obtained from a prospective multicenter study. Ann Surg, 2005; 241: 247–55

4. Liu Y, Gai J, Fu L et al: Effects of RSF-1 on proliferation and apoptosis of breast cancer cells. Oncol Lett, 2018; 16: 4279–84

5. Zhang ZY, Ge HY: Micrometastasis in gastric cancer. Cancer Lett, 2013; 336: 34–45

6. Duda DG, Ancukiewicz M, Isakoff SJ et al: Seeds and soil: Unraveling the role of local tumor stroma in distant metastasis. J Natl Cancer Inst, 2014; 106: dju187

7. 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

8. Danila DC, Heller G, Gignac GA et al: Circulating tumor cell number and prognosis in progressive castration-resistant prostate cancer. Clin Cancer Res, 2007; 13: 7053–58

9. Yang C, Chen F, Wang S et al: Circulating tumor cells in gastrointestinal cancers: Current status and future perspectives. Front Oncol, 2019; 9: 1427

10. Vona G, Béroud C, Benachi A et al: Enrichment, immunomorphological, and genetic characterization of fetal cells circulating in maternal blood. Am J Pathol, 2002; 160: 51–58

11. Cristofanilli M, Budd GT, Ellis MJ et al: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med, 2004; 351: 781–91

12. Palmirotta R, Lovero D, Cafforio P et al: Liquid biopsy of cancer: A multimodal diagnostic tool in clinical oncology. Ther Adv Med Oncol, 2018; 10: 1758835918794630

13. Hong B, Zu Y: Detecting circulating tumor cells: Current challenges and new trends. Theranostics, 2013; 3: 377–94

14. Huebner H, Fasching PA, Gumbrecht W et al: Filtration based assessment of CTCs and CellSearch® based assessment are both powerful predictors of prognosis for metastatic breast cancer patients. BMC Cancer, 2018; 18: 204

15. Hofman V, Long E, Ilie M et al: Morphological analysis of circulating tumour cells in patients undergoing surgery for non-small cell lung carcinoma using the isolation by size of epithelial tumour cell (ISET) method. Cytopathology, 2012; 23: 30–38

16. Vona G, Sabile A, Louha M et al: Isolation by size of epithelial tumor cells: A new method for the immunomorphological and molecular characterization of circulatingtumor cells. Am J Pathol, 2000; 156: 57–63

17. Vona G, Béroud C, Benachi A et al: Enrichment, immunomorphological, and genetic characterization of fetal cells circulating in maternal blood. Am J Pathol, 2002; 160: 51–58

18. Kallergi G, Politaki E, Alkahtani S et al: Evaluation of isolation methods for circulating tumor cells (CTCs). Cell Physiol Biochem, 2016; 40: 411–19

19. Khoja L, Backen A, Sloane R et al: A pilot study to explore circulating tumour cells in pancreatic cancer as a novel biomarker. Br J Cancer, 2012; 106: 508–16

20. Li H, Song P, Zou B et al: Circulating tumor cell analyses in patients with esophageal squamous cell carcinoma using epithelial marker-dependent and -independent approaches. Medicine, 2015; 94: e1565

21. Bai M, Zou B, Wang Z et al: Comparison of two detection systems for circulating tumor cells among patients with renal cell carcinoma. Int Urol Nephrol, 2018; 50: 1801–9

22. Abdallah EA, Braun AC, Flores BCTCP et al: The potential clinical implications of circulating tumor cells and circulating tumor microemboli in gastric cancer. Oncologist, 2019; 24: e854–63

23. Zheng X, Fan L, Zhou P et al: Detection of circulating tumor cells and circulating tumor microemboli in gastric cancer. Transl Oncol, 2017; 10: 431–41

24. Wang L, Zhou S, Zhang W et al: Circulating tumor cells as an independent prognostic factor in advanced colorectal cancer: A retrospective study in 121 patients. Int J Colorectal Dis, 2019; 34: 589–97

25. Pan L, Yan G, Chen W et al: Distribution of circulating tumor cell phenotype in early cervical cancer. Cancer Manag Res, 2019; 11: 5531–36

26. Carlsson A, Nair VS, Luttgen MS et al: Circulating tumor microemboli diagnostics for patients with non-small-cell lung cancer. J Thorac Oncol, 2014; 9: 1111–19

27. Liu Y, Ling Y, Qi Q et al: Prognostic value of circulating tumor cells in advanced gastric cancer patients receiving chemotherapy. Mol Clin Oncol, 2017; 6: 235–42

28. Zhao JJ, Pan K, Wang W et al: The prognostic value of tumor-infiltrating neutrophils in gastric adenocarcinoma after resection. PLoS One, 2012; 7: e33655

29. Jin H, Sun J, Zhu K et al: The prognostic value of neutrophil-lymphocyte ratio is superior to derived neutrophil-lymphocyte ratio in advanced gastric cancer treated with preoperative chemotherapy and sequential R0 resection: A 5-year follow-up. OncoTargets Ther, 2017; 10: 2655–64

30. Aliustaoglu M, Bilici A, Ustaalioglu BB et al: The effect of peripheral blood values on prognosis of patients with locally advanced gastric cancer before treatment. Med Oncol, 2010; 27: 1060–65