BMC Cancer

RESEARCH Open Access

Enumeration, classification and clinical application of circulating tumor cells in advanced gallbladder adenocarcinoma

Chun Liu[1], Cheng Yan[1], Weichang Zhang[1], Yuxin Sun[1], Youjun Lin[1] and Wenwu Cai[1*]

Abstract

Background The relationship between circulating tumor cells (CTCs) and patients with advanced gallbladder adenocarcinoma (aGA) has been rarely studied. This article was to demonstrate the enumeration, classification, and clinical application of CTCs in patients with aGA.

Materials and methods Peripheral blood samples were collected and CTCs were detected using the CanPatrol® technique. T test, χ^2 test, Wilcoxon rank sum test or Kruskal-Wallis test, log-rank test and Cox regression analysis were performed to conduct statistical analysis.

Results CTCs were detected at pre-treatment in 75.00% (27/36) of the patients. Both CTCs positive rate and CTCs enumeration at pre-treatment were significantly associated with clinicopathological parameters including Ca199 level ($P=0.014$, $P<0.001$ respectively), tumor differentiation ($P=0.007$, $P=0.002$ respectively), lymph infiltration ($P=0.010$, $P=0.025$ respectively), vascular infiltration ($P=0.007$, $P<0.001$ respectively), and distant metastasis ($P=0.015$, $P=0.002$ respectively). CTCs-positive patients had a significantly shorter OS (HR 0.335, 95% CI 0.165–0.678, $P=0.0023$) and PFS (HR 0.364, 95% CI 0.179–0.739, $P=0.0024$) than CTCs-negative patients. Mesenchymal CTCs enumeration was closely related to the chemotherapy response, and CTCs programmed cell death ligand-1 (PD-L1) was highly correlated with the immunotherapy response. Positive CTCs at pre-treatment was closely related to the poor OS (HR 0.089, 95% CI 0.020–0.399, $P=0.002$) as well as distant metastasis (HR 0.159, 95% CI 0.041–0.610, $P=0.007$), untreated with chemotherapy (HR 4.510, 95% CI 1.403–14.499, $P=0.011$) and untreated with immunotherapy (HR 6.845, 95% CI 1.894–24.738, $P=0.003$).

Conclusion Pretreatment-positive CTCs was closely related to the poor prognosis in patients with aGA. Monitoring the subtype and phenotype of CTCs may be one of the means to assess tumor treatment response.

Keywords Circulating tumor cells (CTCs), Gallbladder adenocarcinoma, Tumor treatment response, Chemotherapy, Immunotherapy, CanPatrol® technique

*Correspondence:
Wenwu Cai
caiwenwu1986@csu.edu.cn
[1]Department of General Surgery, Second Xiangya Hospital, Central South University, Number 139, Renmin Road, Changsha, Hunan 410011, P.R. China

Introduction

Gallbladder carcinoma (GBC), of which gallbladder adenocarcinoma is the predominant pathological type, is the most common type of malignant tumor of the biliary tract and the sixth most common type of malignant tumor of the digestive tract [1–2]. Early diagnosis and curative resection, which is the most effective and preferred treatment method, may enable patients to have an improved long-term survival [3–4]. However, owing to insidious and atypical early clinical symptoms, most patients are diagnosed at an advanced stage, and only 15–47% of patients have a chance to undergo resection [5–6]. The prognosis is poor. A previous study has reported the median survival time was only 6 months, and the overall 5-year survival rate was not more than 5% [7]. Most patients eventually succumb to metastatic disease [8].

Circulating tumor cells (CTCs), first reported by Ashworth in 1869 [9], are defined as tumor cells that have been detached from the primary tumor and extravasated into the blood circulation. They can be detected and captured in peripheral blood circulation, and were believed to be regarded as a "liquid biopsy" of the tumor instead of traditional surgical tumor biopsy in providing a convenient and effective way to identify the tumor nature, monitor progression and metastasis, and evaluate treatment response [10–11]. Enumeration of CTCs in peripheral blood has been proven to be an independent prognostic indicator in patients with tumors [12]. However, the extremely rare number and mixed presence of numerous other hematological cells make the detection and enumeration of CTCs a technical challenge [13–14]. In our previous study, we had successfully used the CanPatrol®, a CONFORMITE EUROPEENNE (CE)-certified CTCs enrichment technique, to detect and classify the CTCs in 32 patients with advanced gastric carcinoma [15].

The limited reported data about the subtype and phenotype characteristics of CTCs and their clinical application precipitated the present study, which aimed to investigate the correlations of CTCs enumeration at pretreatment with clinicopathological parameters and prognosis, and the dynamic change in CTCs and correlation of classification with tumor treatment response during chemotherapy and immunotherapy in patients with aGA.

Materials and methods

Patient samples

Between January 2018 and January 2023, consecutive patients with histologically confirmed aGA and Eastern Cooperative Oncology Group Performance Status (ECOG-PS) ≤ 1, who underwent standard strategical treatment according to the 8th AJCC gallbladder cancer guidelines [16] were included in this retrospective study. The exclusion criteria were as follows: (1) history of malignancy other than gallbladder adenocarcinoma; (2) pregnancy or lactation; (3) unavailability of follow-up data. The requirement for written informed consent was waived owing to the retrospective study design. This study was approved by the Research Ethics Committee of Second Xiangya Hospital (approval No. 179 in 2017) and was performed in accordance with the Declaration of Helsinki.

Diagnosis and treatment regimens

The diagnosis and staging of aGA were achieved based on the findings of contrast-enhanced Computed Tomography (CT) and/or Magnetic Resonance Imaging (MRI) and pathology. The selection of treatment regimens regarding standardized staging, therapeutic benefits, and risks was discussed by a multidisciplinary tumor board comprising surgeons, hepatologists, oncologists, radiologists, and pathologists. Patients and clinicians made a final decision based on consensus. Biliary drainage (percutaneous transhepatic cholangial drainage, PTCD or biliary stents) was performed when the patient experienced jaundice.

Patients received at least one of the following treatment regimens: (1) gemcitabine: 1000 mg/m^2 administered intravenously (IV) on days 1 and 8, every 3 weeks; (2) gemcitabine plus S-1: gemcitabine 1000 mg/m^2 IV on days 1 and 8, plus S-1: 60 mg/m^2 taking orally twice per day till 2 weeks, every 3 weeks; (3) gemcitabine plus oxaliplatin: gemcitabine 1000 mg/m^2 IV on day 1, followed by oxaliplatin 100 mg/m^2 IV on day 2, every 2 weeks; and (4) pembrolizumab: 200 mg IV, every 3 weeks. Treatment regimens was continued until the patient declined further doses or until limiting toxicity or disease progression occurred. The drug dosages or treatment cycles were adjusted when intolerant toxicity or disease progression occurred. The adjustment of treatment regimens was not related to the presence or absence of CTCs.

CTCs detecting

According to a previously reported method [15], 5 mL blood sample was drawn from the peripheral blood vessel at pre-treatment, 1 month and 3 months post-treatment and kept at room temperature until processing within 72 h. After enrichment by nanomembrane filtration, all samples were analyzed and CTCs were detected using the CanPatrol® technique. The enumeration and classification of CTCs were successfully performed using a multiplex RNA in situ hybridization (RNA-ISH) assay based on branched DNA (bDNA) signal amplification. Epithelial biomarkers (EpCAM and CK8/18/19, R&B systems, Minneapolis, MN, USA), mesenchymal biomarkers (Vimentin and Twist, R&B Systems, Minneapolis, MN, USA), programmed cell death ligand-1 (PD-L1) (Abcam, Cambridge, MA, USA), and leukocyte biomarkers

(CD45, BD Biosciences, New Jersey, USA) were used to identify and characterize CTCs subsets. Five-color fluorescently labeled probes were added and incubated with the cells. The cell nuclei were stained with DAPI (Sigma, Aldrich, Delaware, USA). Cells were analyzed using an automatic Axio Imager Z2 fluorescence microscope (Carl Zeiss Meditec AG, Jena, Germany). Epithelial and mesenchymal biomarkers presented with red and green fluorescence, respectively, whereas PD-L1 and CD45 present with violet and bright blue fluorescence, respectively [15, 17]. If both epithelial and mesenchymal biomarkers were detected, the cells were defined as hybrid CTCs. The presence of one CTC per 5 mL of peripheral blood was considered positive according to a previous study [18]. The results were stored in a database, and following the closure of the observation period, CTCs enumeration in combination with other clinical data were imported into SPSS for statistical analysis.

PD-L1 expression and microsatellite instability (MSI) status determination

Expression of PD-L1 in tumor tissues was detected using immunohistochemistry (IHC). Anti-human PD-L1 mAb was purchased from Abcam. The DAB kit was supplied by Beijing Suolaibao Technology Co., Ltd, Beijing, China. The paraffin-imbedded tumor tissues were used. After dewaxing, EDTA solution was used to repair the antigen, and the tumor tissues were blocked with an endogenous peroxidase blocker for 15 min. The antibody was incubated at room temperature according to the recommended concentration of the antibody instructions. Finally, a DAB kit was used for color analysis, and hematoxylin was used for re-staining. The results were statistically analyzed based on the clinical response. Five high-power visual fields (×200 times) were randomly selected from the tumor cells and tumor stroma with yellow to brown granules in the cytoplasm or cell membrane, respectively. The scoring criteria for the staining intensity of positive cells were as follows: no staining, 0 points; light yellow, light brown, and brown scored as 1, 2, and 3, respectively. The scores of positive cell density were as follows: the number of positive cells ≤ 70% was 1 point, 71–80% was 2points, 81–90% was 3 points, and > 90% was 4 points. Multiplication of cell staining intensity and density scores ≥ 6 scores as positive expression.

Expression of MLH1 in tumor tissues was detected using IHC as described above. Each slice was randomly selected from five high-power visual fields with a high positivity rate. A total of 100 cells were counted in each visual field. The positive score criteria were 0 for non-positive cells, 1 for positive cells (≤ 10%), 2 for positive cells (10–50%), 3 for positive cells (50–80%), and 4 for positive cells (> 80%). The grading standards for the dyeing strength were 0 for colorless, 1 for light yellow, 2 for light brown, and 3 for brown. The final score was multiplied by the two scores. The final score was 0 for protein deletion and (≥ 2) for protein-positive expression.

Expression of PD-L1 in CTCs was detected using immunofluorescence (IFC) method. The proportion of PD-L1-positive cells among total CTCs in peripheral blood of 5 ml from patients with aGA was counted. The positive score criteria were 0 for non-positive cells, 1 for positive cells (< 20%), 2 for positive cells (20–50%), and 3 for positive cells (> 50%). The final score was (≥ 2) for CTCs-PD-L1-positive expression.

Data collection and follow-up

In addition to CTCs enumeration and classification, the following data were recorded: gender, age, Ca199 level, CEA level, total bilirubin, tumor differentiation, lymph and vascular infiltration, distant metastasis, AJCC-stage (V8) [16]-TNM stage, treatment regimens, and gallbladder stone. Continuous variables were dichotomized at the following thresholds: Ca199 ≥ 200 kU/L, total bilirubin ≥ 50 umol/L and CEA ≥ 5 mg/mL.

After the initiation of the treatment regimens, patients were observed during follow-up every 1 month in the first 6 months of the year and then every 3 months. Clinical status, Ca199 level, CEA level and CT and/or MRI were routinely performed to assess the disease status and evaluate the tumor treatment response, which was classified according to the Response Evaluation Criteria in Solid Tumors criteria [19], including complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). The follow-up period was until January 2024. The primary outcome was overall survival (OS), defined as the time interval between the date of treatment regimens and date of death. The secondary outcome was progression-free survival (PFS), defined as the time interval between the date of treatment regimens and date of disease progression.

Statistical analysis

The statistical software (SPSS version 27, International Business Machines Corporation, Armonk, NY, USA) was used to perform statistical analysis. Data were shown as mean ± standard deviation (SD), median with range, or number with percentage. Continuous variables were compared using t-test. Categorical variables were compared using the χ^2 test. Non-parametric correlation analysis was conducted using the Wilcoxon rank sum test for two samples or Kruskal-Wallis test for more than two samples.

OS and PFS were calculated using the Kaplan-Meier method and the Log-rank test for differences in curve pairs. Graphs were prepared using GraphPad Prism 8.0.2. Univariate and multivariate backward Cox regression analyses were performed and the hazard ratio (HR) with

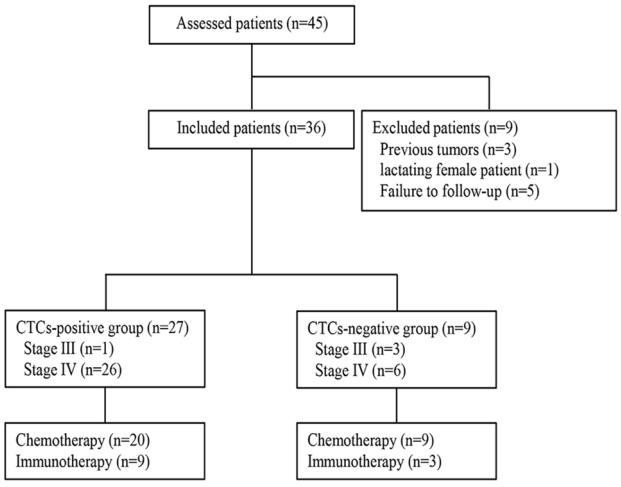

Fig. 1 A flowchart of the study population. CTCs, circulating tumor cells

a 95% confidence interval (CI) was calculated to confirm risk factors for shorter OS in patients with aGA. Statistical tests were two-sided, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characteristics of the study population

A flowchart of the patient selection process was presented in Fig. 1. During the study period, a total of 45 consecutive patients were assessed, of whom 9 (20.00%) patients were excluded for the following reasons: 1 patient had a history of breast carcinoma, 2 patients had a history of lung carcinoma in the last decade, 1 female patient was undergoing lactation, and 5 patients were lost to follow up due to being unable to get through or never visiting to the hospital after discharge.

The remaining 36 (80.00%) patients were included in this study (Table 1). Based on the CTCs detection status at pre-treatment, 27 (75.00%) patients were assigned to the CTCs-positive group. Among them, median age was 64.78 ± 11.15 years, 20 (74.07%) patients were female, and gallbladder stones were observed in 24 (88.89%) patients. Ca199 level was more than 200 kU/L in 21 (77.78%) patients, CEA level was more than 5 mg/mL in 16 (59.26%) patients, and total bilirubin level was more than 50 umol/L in 10 (37.04%) patients. Tumor differentiation was moderate in 7 (25.93%) patients, and poor in 20 (74.07%) patients. Lymph infiltration was observed in 25 (92.59%) patients, vascular infiltration in 22 (81.48%) patients, and distant metastasis in 23 (85.19%) patients. Tumor stage was stage III in 1 (3.70%) patient, and stage IV in 26 (96.30%) patients. 20 (74.07%) patients received chemotherapy, and 9 (33.33%) patients received immunotherapy. The remaining 9 (25.00%) patients were assigned to the CTCs-negative group. Among them, median age was 60.44 ± 8.26 years, 7 (77.78%) patients were female, and gallbladder stones were observed in 7 (77.78%) patients. Ca199 level was more than 200 kU/L in 3 (33.33%) patients, CEA level was more than 5 mg/mL in 3 (33.33%) patients, and total bilirubin level was more than 50 umol/L in 1 (11.11%) patient. Tumor differentiation was well in 3 (33.33%) patients, moderate in 2 (22.22%) patients, and poor in 4 (44.45%) patients. Lymph infiltration was observed in 5 (55.56%) patients, vascular infiltration in 3 (33.33%) patients, and distant metastasis in 4 (44.44%) patients. Tumor stage was stage III in 3 (33.33%) patients, and stage IV in 6 (66.67%) patients. 9 (100.00%) patients received chemotherapy, and 3 (33.33%) patients received immunotherapy. At the end of the follow-up period, 3 patients in the CTCs-positive group and 2

Table 1 The baseline characteristics of the patients between subgroups

	CTCs-positive group ($n=27$)	CTCs-negative group ($n=9$)	*P* value
Age (years)	64.78 ± 11.15	60.44 ± 8.26	0.293
Gender, female	20 (74.07%)	7 (77.78%)	0.824
Ca199, ≥ 200 kU/L	21 (77.78%)	3 (33.33%)	0.014
CEA, ≥ 5 mg/mL	16 (59.26%)	3 (33.33%)	0.177
Total bilirubin, ≥ 50 umol/L	10 (37.04%)	1 (11.11%)	0.144
Tumor differentiation			0.007
Well	0	3 (33.33%)	
Moderate	7 (25.93%)	2 (22.22%)	
Poor	20 (74.07%)	4 (44.45%)	
Lymph infiltration			0.010
Absence	2 (7.41%)	4 (44.44%)	
Presence	25 (92.59%)	5 (55.56%)	
Vascular infiltration			0.007
Absence	5 (18.52%)	6 (66.67%)	
Presence	22 (81.48%)	3 (33.33%)	
Distant metastasis			0.015
Absence	4 (14.81%)	5 (55.56%)	
Presence	23 (85.19%)	4 (44.44%)	
TNM stage			0.014
III	1 (3.70%)	3 (33.33%)	
IV	26 (96.30%)	6 (66.67%)	
Chemotherapy			0.089
Untreated	7 (25.93%)	0	
Treated	GEMZ: 8 (29.63%) GS: 6 (22.22%) GEMOX: 6 (22.22%)	GEMZ: 3 (33.33%) GS: 3 (33.33%) GEMOX: 3 (33.33%)	
Immunotherapy			1.000
Untreated	18 (66.67%)	6 (66.67%)	
Treated	9 (33.33%)	3 (33.33%)	
Gallbladder stone			0.404
Absence	3 (11.11%)	2 (22.22%)	
Presence	24 (88.89%)	7 (77.78%)	

CTCs, Circulating tumor cells; Ca199, Carbohydrate antigen 199; CEA, Carcinoembryonic antigen; GEMZ, GS, GEMOX, chemotherapy regimens; GEMZ, Gemcitabine; GS, Gemcitabine and S-1; GEMOX, Gemcitabine and Oxaliplatin

patients in the CTCs-negative group were still alive, and the remaining patients eventually died.

Detection status of CTCs at pre-treatment and correlations with clinicopathology parameters and prognosis

In the present study, the overall positivity rate for CTCs at pre-treatment was 75.00% (27/36). The median total CTCs enumeration was 6.5 (range from 0 to 48), epithelial CTCs enumeration was 1.5 (range from 0 to 16), mesenchymal CTCs enumeration was 0 (range from 0 to 32), and hybrid CTCs enumeration was 1.0 (range from 0 to 9) per 5 mL of peripheral blood in patients with aGA (Fig. 2A-B).

Both CTCs positive rate and CTCs enumeration at pre-treatment were significantly associated with the Ca199 level ($P = 0.014$, $P < 0.001$ respectively), tumor differentiation ($P = 0.007$, $P = 0.002$ respectively), lymph infiltration ($P = 0.010$, $P = 0.025$ respectively), vascular infiltration ($P = 0.007$, $P < 0.001$ respectively), and distant metastasis ($P = 0.015$, $P = 0.002$ respectively) in patients with aGA as presented in Table 2; Fig. 2C-G.

Patients in the CTCs-positive group had a significantly shorter OS (HR 0.335, 95% CI 0.165–0.678, $P = 0.0023$, Fig. 3A) and PFS (HR 0.364, 95% CI 0.179–0.739, $P = 0.0024$, Fig. 3B) than those in the CTCs-negative group.

Dynamic change of CTCs and correlations of subtype and phenotype with tumor treatment response

Compared to the pre-treatment, the median total CTCs enumeration was 7.5 (range from 0 to 50) ($P = 1.000$), epithelial CTCs enumeration was 0 (range from 0 to 12) ($P = 0.636$), mesenchymal CTCs enumeration was 4.0 (range from 0 to 37) ($P = 0.099$), and hybrid CTCs enumeration was 0.5 (range from 0 to 10) ($P = 0.813$) at 1 month post-treatment, and median total CTCs enumeration 10.0 (range from 0 to 51) ($P = 0.637$), epithelial CTCs enumeration 0 (range from 0 to 8) ($P = 0.346$), mesenchymal CTCs enumeration 7.5 (range from 0 to 42) ($P = 0.099$), and hybrid CTCs enumeration 0.5 (range from 0 to 9) ($P = 0.813$) at 3 months post-treatment per 5 mL of peripheral blood in patients with aGA.

The results in patient No.17 showed that when patients were in PD during chemotherapy, mesenchymal CTCs enumeration increased rapidly, while total CTCs enumeration, serum Ca199 concentration, and imaging findings were all obviously unaltered (Fig. 4A). In addition, the results in patient No.24 showed that even when the expression of PD-L1 negative in tumor tissues, the MSI-H patient with aGA still presented good immune responses during immunotherapy when PD-L1 was positively detected in CTCs (Fig. 4B).

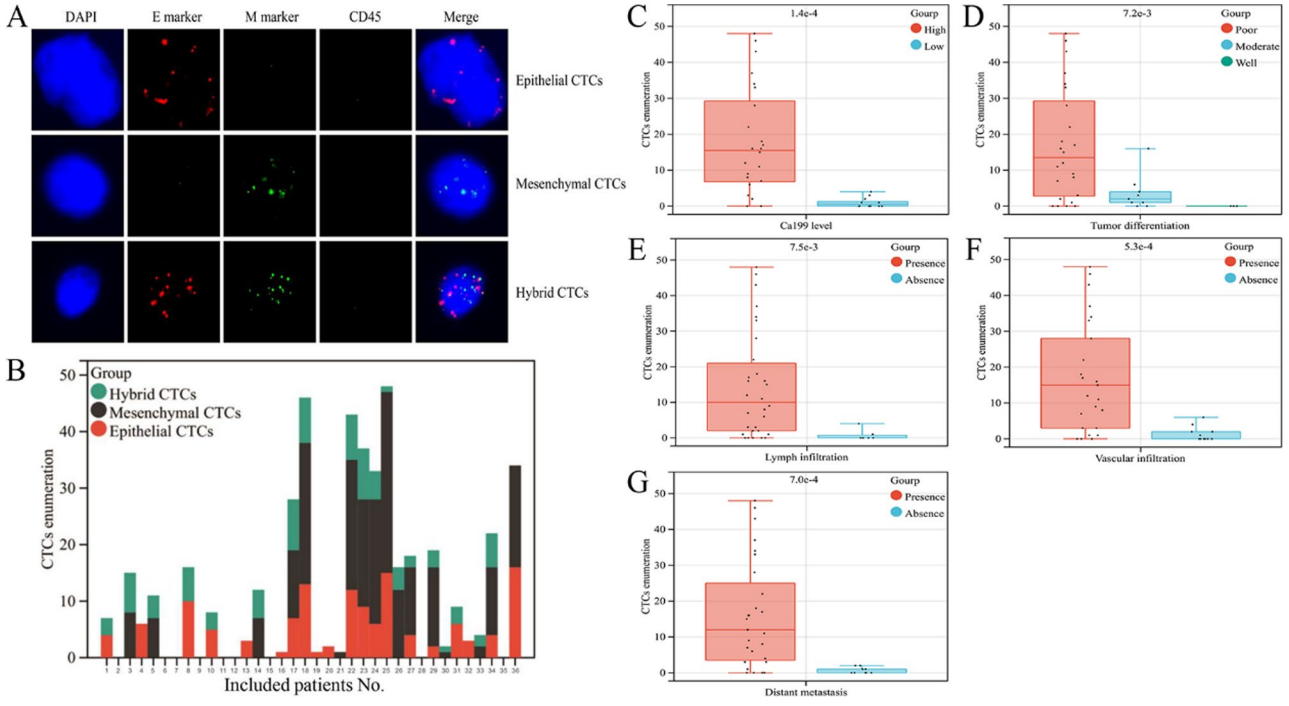

Fig. 2 (**A**) CTCs identified from patients with aGA. Immunocytochemistry method based on EpCAM/cytokeratin (E markers, red), Vimentin/Twist (M markers, green), PD-L1 (violet) as well as DAPI nuclear staining; (**B**) CTCs enumeration and classification results obtained from 36 patients with aGA; Boxplots for CTCs enumeration at pre-treatment of patients with aGA (**C**) with Ca199 level; (**D**) tumor differentiation; (**E**) lymph infiltration; (**F**) vascular infiltration; and (**G**) distant metastasis. CTCs, circulating tumor cells; aGA, advanced gallbladder adenocarcinoma; PD-L1, programmed cell death ligand-1

Table 2 The correlations of CTCs at pre-treatment with clinicopathological parameters

	CTCs positive rate *N* (%)	P^a value	CTCs enumeration median (range)	P^b value
Age (years)		0.439		0.737
≥ 65	16 (44.44%)		6.5 (0–48)	
<65	11 (30.56%)		5.5 (0–37)	
Gender		0.824		1.000
Female	20 (55.56%)		6 (0–48)	
Male	7 (19.44%)		9 (0–43)	
Ca199 (kU/L)		0.014		< 0.001
≥200	21 (58.33%)		15.5 (0–48)	
<200	6 (16.67%)		0.5 (0–4)	
CEA (mg/mL)		0.177		< 0.001
≥5	16 (44.44%)		16 (0–48)	
<5	11 (30.56%)		1 (0–22)	
TB (umol/L)		0.144		0.030
≥50	10 (27.78%)		15 (0–48)	
<50	17 (47.22%)		2 (0–37)	
TD		0.007		0.002*
Well	0		0	
Moderate	7 (19.44%)		2 (0–16)	
Poor	20 (55.56%)		13.5 (0–48)	
Lymph infiltration		0.010		0.025
Absence	2 (5.56%)		0 (0–4)	
Presence	25 (69.44%)		10 (0–48)	
Vascular infiltration		0.007		< 0.001
Absence	5 (13.89%)		0 (0–6)	
Presence	22 (61.11%)		15 (0–48)	
Distant metastasis		0.015		0.002
Absence	4 (11.11%)		0 (0–2)	
Presence	23 (63.89%)		12 (0–48)	
TNM stage		0.014		0.112
III	1 (2.78%)		2 (0–6)	
IV	26 (72.22%)		8.5 (0–48)	
Gallbladder stone		0.404		0.054
Absence	3 (8.33%)		1 (0–6)	
Presence	24 (66.67%)		9 (0–48)	

CTCs, Circulating tumor cells; Ca199, Carbohydrate antigen 199; CEA, Carcinoembryonic antigen; TB, Total bilirubin; TD, Tumor differentiation

P^a value from the χ2 test; P^b value from the Wilcoxon rank sum test, and $^*P^b$ value from the Kruskal-Wallis test

Univariate and multivariate analysis

The predictors of poor OS for patients with aGA in the univariate analysis were the positive CTCs at pre-treatment ($P = 0.005$), Ca199 ≥ 200 kU/L ($P = 0.003$), CEA ≥ 5 mg/mL ($P = 0.004$), total bilirubin ≥ 50 umol/L ($P = 0.036$), with distant metastasis ($P = 0.004$), untreated with chemotherapy ($P < 0.001$) and untreated with immunotherapy ($P = 0.009$).

Therefore, these variables were included in the multivariate analysis, and the results showed that positive CTCs at pre-treatment remained closely related to the poor prognosis (HR 0.089, 95% CI 0.020–0.399, $P = 0.002$). In addition, distant metastasis (HR 0.159, 95% CI 0.041–0.610, $P = 0.007$), untreated with chemotherapy (HR 4.510, 95% CI 1.403–14.499, $P = 0.011$), and untreated with immunotherapy (HR 6.845, 95% CI 1.894–24.738, $P = 0.003$) also had a significant and adverse prognostic impact. The results were presented in Table 3.

Discussion

In the present study, a strong association between both the CTCs positivity rate and CTCs enumeration at pre-treatment and serum Ca199 concentration, poor tumor differentiation, lymph and vascular infiltration, and distant metastasis was confirmed in patients with aGA. In addition, a close relationship between positive CTCs at pre-treatment and shorter median OS and PFS was observed in these patients. The results showed that the presence of CTCs at pre-treatment had a significantly adverse prognostic impact on patients with aGA.

Only 3 previous studies have reported the relationship between CTCs and GBC. Wang et al. reported that higher CTCs enumeration was closely related to worse chemotherapy treatment response, and poor PFS and OS in patients with advanced GBC, implying that CTCs measurement may serve as a novel marker to formulate individualized treatment and optimize the prognostication [20]. Awasthi et al. reported that levels of CTCs significantly correlated with clinicopathological parameters and the detection and quantification of CTCs may be considered as a non-invasive biomarker for the diagnosis of GBC in correlation with radiological studies [18]. Zhang et al. designed a new ultrasensitive electrochemical cytosensor to recognize peripheral blood CTCs in patients with gallbladder cancer, and achieved the diagnosis of chemotherapeutic resistance by monitoring phenotypic changes in CTCs [21].

The overall positive frequency of CTCs in the peripheral blood was 75.00% in the present study, which was slightly lower than that reported in previous studies (Wang et al., almost all patients, and Awasthi et al., 92.59%). The following possible reasons may explain this result: (1) In China, owing to poor economic conditions and an imperfect medical insurance system, most patients with aGA do not intent to be hospitalized and ultimately give up treatment. Therefore, only 36 patients with advanced disease were included in the study. The smaller sample size may have had an impact on the positive detection rate, (2) in the previous two studies, CTCs were both detected and identified by flow cytometry technology. The obvious drawbacks of flow cytometry technology to detect CTCs are its low sensitivity and high false-positive rate [22]. Therefore, it is a big issue worth our consideration, that whether there was a very high

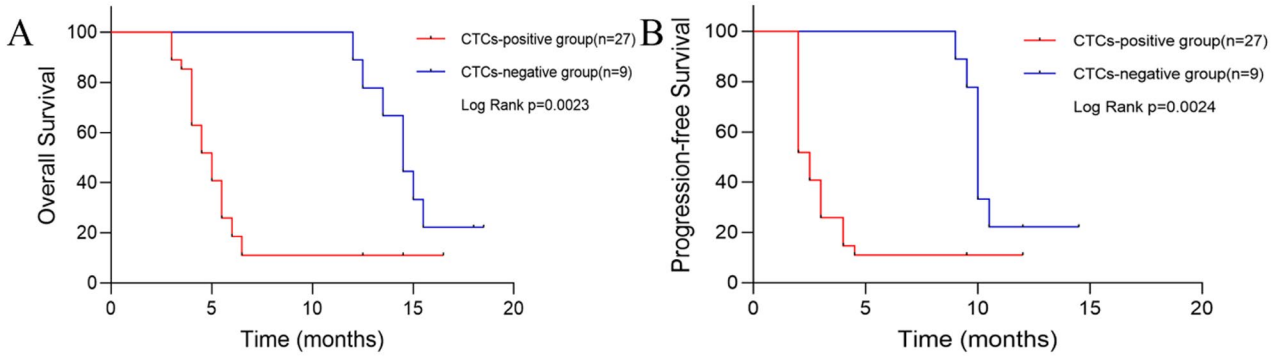

Fig. 3 Survival curve according to the CTCs status in patients with aGA. (**A**) OS curve for the CTCs-positive and CTCs-negative patients; (**B**) PFS curve for the CTCs-positive and CTCs-negative patients. CTCs, circulating tumor cells; aGA, advanced gallbladder adenocarcinoma; OS, overall survival; PFS, progression-free survival

false-positive rate in the two previous studies, and (3) a sample size of only 5 mL may be another explanation for the lower positive rate. Previous studies had demonstrated that larger volumes of peripheral blood could improve the sensitivity of CTCs [23–24]. However, it is seldom used in clinical practice, because of not only the high expenses, but also the heavy workload [25]. Nonetheless, larger sample volumes of peripheral blood should be collected and detected by the CanPatrol® technique for potential improvement of risk classification by CTCs in future studies on patients with aGA, which may affect both the specificity and sensitivity of results reported by the present study.

A previous studies reported that Epithelial – Mesenchymal transformation (EMT) is a process by which epithelial cells transdifferentiate into mesenchymal cells [26]. It can endow tumor cells with the traits of migration and invasion and induce cancer stem cell properties [27–28]. Besides, it also plays an important role in drug resistance [29]. Classification of CTCs according to its EMT phenotype is helpful for identifying the most aggressive CTCs subset and providing data for clinical application. In the present study, CTCs were successfully divided into three subsets using the CanPatrol® technique, and an interesting finding in patient No.17 was that when in PD during chemotherapy, the mesenchymal CTCs enumeration changed earlier than total CTCs enumeration, serum Ca199 concentration and image findings, which indicated that the mesenchymal CTCs perhaps could be a more aggressive subset and monitoring its dynamic change could be a more sensitive method for evaluating the disease status and chemotherapy response in patients with aGA. Surely, this was just an isolated case, and more future researches are needed to confirm this result.

Immune checkpoint blockade, a new molecular targeted therapy, has attracted attention in patients with melanoma [30–31], lung cancer [32–33], gastric cancer [34–35], and other malignant tumors [36–37]. However, the clinical effect of PD-1/PD-L1 immune checkpoint inhibitors on GBC is limited compared to other malignancies [38–39], and overall survival is still not significantly improved. Therefore, the identification of predictive biomarkers and screening of the target population are urgently needed in patients with aGA. In general, patients with MSI-H tumors and PD-L1 positivity in the tumor tissues are considered more suitable for immunotherapy. In the present study, the results showed another interesting finding in patient No.24 was that even PD-L1 was negative in tumor tissues, when PD-L1 was detected positively in CTCs, the MSI-H patient with aGA still presented good immune responses during immunotherapy, which indicated that the expression of CTCs PD-L1 may overcome tumor heterogeneity and perhaps could be more representative to evaluate the true status of the body and immunotherapy response in patients with aGA. As we mentioned above, this was also an isolated case, and more future researches are needed to confirm this result.

The results showed that positive CTCs at pre-treatment was closely related to the poor prognosis in patients with aGA. As we mentioned above, CTCs were more easily detected in patients with higher Ca199 level, poorer tumor differentiation, lymph infiltration, vascular infiltration, and distant metastasis. These patients usually had a poorer general condition, which would definitely lead to a worse prognosis. However, the small sample size of this study made propensity score matching (PSM), which was intended to use to adjust the baseline conditions of the two groups, to be difficult to implement. In the future, a larger sample study is needed to confirm this result. In addition to positive CTCs at pre-treatment, the results showed that untreated with chemotherapy or immunotherapy also had a significantly adverse prognostic impact on patients with aGA. Therefore, when these patients are willing to receive chemotherapy and/or immunotherapy, CTCs PD-L1 was positively detected, and their physical

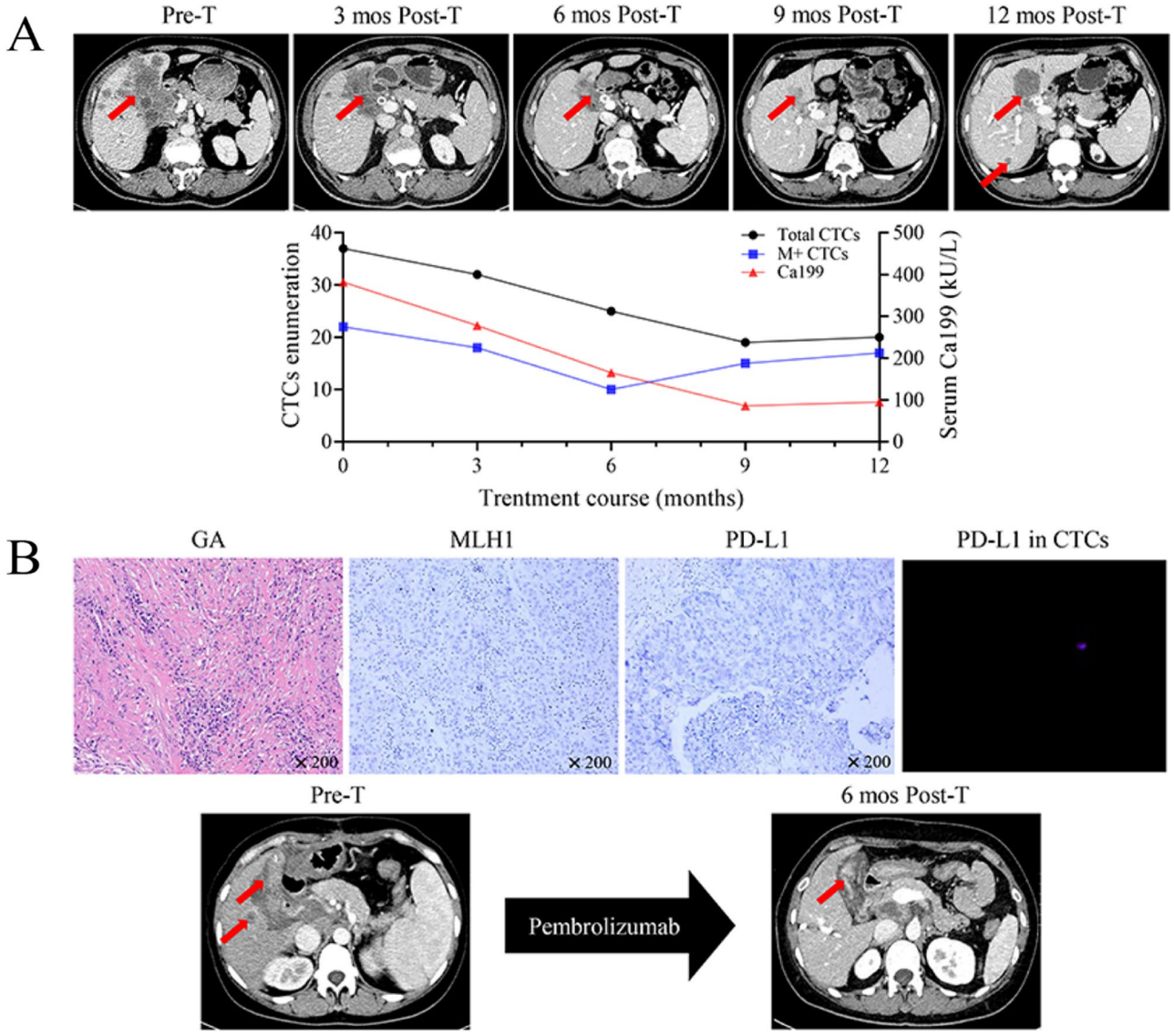

Fig. 4 (**A**) Numerical analysis of a patient with aGA received GEMOX showing total CTCs enumeration, M + CTCs enumeration, serum Ca199 concentration and CT scan in identifying disease progression; (**B**) Relationship performance between PD-L1 and treatment response to pembrolizumab in an MSI-H patient with aGA with PD-L1- in tumor tissue and PD-L1 + in CTCs. The red arrows indicate tumor locations. aGA, advanced gallbladder adenocarcinoma; GEMOX, Gemcitabine and Oxaliplatin; CTCs, circulating tumor cells; M + CTCs, mesenchymal CTCs; Ca199, Carbohydrate antigen 199; CT, computed tomography; PD-L1, programmed cell death ligand-1; MSI, microsatellite instability; Pre-T, pre-treatment; mos, months; Post-T, post-treatment

conditions allow them to tolerate the side effects of therapeutic drugs, chemotherapy and/or immunotherapy are strongly recommended.

This study has two limitations that should be acknowledged. First, the sample size (only 36 patients) was relatively small because of the low incidence of GBC and poor willingness of patients with aGA to receive treatment. Second, restricted randomness is another limitation of this study. The results of a prospective randomized trial with multiple centers and a larger sample size will be more convincing in the future.

In conclusion, the detection of CTCs positivity at pretreatment was closely related to the poor prognosis in patients with aGA. The enumeration of mesenchymal CTCs was significantly associated with chemotherapy response. A close correlation was observed between immunophenotypes in CTCs and immunotherapy response. CTCs detection allowed noninvasive and continuous dynamic monitoring of clinical patients with aGA, suggesting its potential for individualized treatment and therapeutic evaluation in the future.

Table 3 The uni- and multivariate analysis of prognostic factors of OS for patients with aGA

	Level	*N*	Univariate analysis Odds ratio (95% CI)	*P* value	Multivariate analysis Odds ratio (95% CI)	*P* value
Age (years)			2.050 (0.992–4.236)	0.053		
	≥ 65	20				
	<65	16				
Gender			1.164 (0.515–2.632)	0.715		
	Male	9				
	Female	27				
CTCs			0.279 (0.114–0.687)	0.005	0.089 (0.020–0.399)	0.002
	≥ 1	27				
	<1	9				
Ca199 (kU/L)			4.362 (1.678–11.340)	0.003	1.168 (0.276–4.939)	0.833
	≥ 200	24				
	<200	12				
CEA (mg/mL)			3.195 (1.444–7.070)	0.004	1.464 (0.393–5.453)	0.570
	≥ 5	19				
	<5	17				
Total bilirubin (umol/L)			2.277 (1.055–4.914)	0.036	0.816 (0.289–2.299)	0.700
	≥ 50	11				
	<50	25				
Tumor differentiation				0.145		
	Poor	24	Ref			
	Well	3	1.378 (0.605–3.137)	0.445		
	Moderate	9	0.193 (0.024–1.564)	0.123		
Lymph infiltration			0.335 (0.109–1.030)	0.056		
	No	6				
	Yes	30				
Vascular infiltration			0.521 (0.235–1.154)	0.108		
	No	11				
	Yes	25				
Distant metastasis			0.237 (0.089–0.633)	0.004	0.159 (0.041–0.610)	0.007
	No	9				
	Yes	27				
TNM			0.708 (0.214–2.348)	0.573		
	III	4				
	IV	32				
Chemotherapy			11.408 (3.651–35.647)	< 0.001	4.510 (1.403–14.499)	0.011
	No	7				
	Yes	29				
Immunotherapy			2.991 (1.308–6.840)	0.009	6.845 (1.894–24.738)	0.003
	No	24				
	Yes	12				
Gallbladder stone			0.705 (0.246–2.025)	0.517		
	No	5				
	Yes	31				

OS, overall survival; aGA, advanced gallbladder adenocarcinoma; CTCs, Circulating tumor cells; Ca199, Carbohydrate antigen 199; CEA, Carcinoembryonic antigen

Abbreviations

CTCs	Circulating tumor cells
aGA	Advanced gallbladder adenocarcinoma
Ca199	Carbohydrate antigen 199
OS	Overall survival
PFS	Progression-free survival
PD-L1	Programmed cell death ligand-1
GBC	Gallbladder carcinoma
AJCC	American joint committee on cancer
CT	Computed tomography
MRI	Magnetic resonance imaging
PTCD	Percutaneous transhepatic cholangial drainage
IHC	Immunohistochemistry
IFC	Immunofluorescence
CEA	Carcinoembryonic antigen
CR	Complete response
PR	Partial response
SD	Stable disease
PD	Progressive disease
SD	Standard deviation

HR	Hazard ratio
CI	Confidence interval
EMT	Epithelial – Mesenchymal transformation
PD-1	Programmed cell death-1
GEMZ	Gemcitabine
GS	Gemcitabine and S-1
GEMOX	Gemcitabine and Oxaliplatin
TB	Total bilirubin
TD	Tumor differentiation

Acknowledgements
We are very grateful to Zijian Zhang for his assistance and suggestions in statistical analysis in this present study.

Author contributions
All the authors contributed to this study. W.C. designed the research. C.L. and Y.S. collected the clinical data. C.Y. and Y.L. collected the blood samples and detected the CTCs. C.L., C.Y. and W.Z. performed the statistical analysis and drafted the manuscript. W.C. and W.Z. reviewed and revised the final manuscript. The all authors have read and approved the published version of the manuscript.

Funding
This work was supported by the National Natural Science Foundation of China, Beijing (Grant No. 81703767, W.C) and the Hunan Natural Science Foundation of China, Changsha (Grant No. 2019JJ50891, W.C).

Data availability
The datasets used and/or analyzed during the current study are available within the article, or from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Between January 2018 and January 2023, a research project was implemented to study the capture efficiency of CanPatrol® technique for CTCs in patients with gallbladder adenocarcinoma. Now, the clinical data on patients with aGA were retrospectively reviewed to reveal the clinical significances of CTCs. The requirement for written informed consent was waived owing to the retrospective study design. This study was approved by the Research Ethics Committee of Second Xiangya Hospital (approval No. 179 in 2017) and was performed in accordance with the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 18 May 2024 / Accepted: 11 April 2025
Published online: 17 April 2025

References

1. Kanthan R, Senger JL, Ahmed S, Kanthan SC. Gallbladder Cancer in the 21st century. J Oncol. 2015: 967472.
2. Goldin RD, Roa JC. Gallbladder cancer: a morphological and molecular update. Histopathology. 2009;55:218–29.
3. Kakaei F, Beheshtirouy S, Nejatollahi SM, Zarrintan S, Mafi MR. Surgical treatment of gallbladder carcinoma: a critical review. Updates Surg. 2015;67:339–51.
4. Cziupka K, Partecke LI, Mirow L, et al. Outcomes and prognostic factors in gallbladder cancer: a single-centre experience. Langenbecks Arch Surg. 2012;397:899–907.
5. Lazcano-Ponce EC, Miquel JF, Muñoz N, et al. Epidemiology and molecular pathology of gallbladder cancer. CA Cancer J Clin. 2001;51:349–64.
6. Mekeel KL, Hemming AW. Surgical management of gallbladder carcinoma: a review. J Gastrointest Surg. 2007;11:1188–93.
7. Levy AD, Murakata LA, Rohrmann CA Jr. Gallbladder carcinoma: radiologic-pathologic correlation. Radiographics. 2001;21(questionnaire):295–314.
8. Butte JM, Matsuo K, Gönen M, et al. Gallbladder cancer: differences in presentation, surgical treatment, and survival in patients treated at centers in three countries. J Am Coll Surg. 2011;212:50–61.
9. Ashworth TR. A case of cancer in which cells similar to those in the tumors were seen in the blood after death. Med J Aust. 1869;14:146–9.
10. Pantel K, Alix-Panabieres C, Riethdorf S. Cancer micrometastases. Nat Rev Clin Oncol. 2009;6(6):339–51.
11. Kim MY, Oskarsson T, Acharyya S, et al. Tumor self-seeding by Circulating cancer cells. Cell. 2009;139(7):1315–26.
12. Racila E, Euhus D, Weiss AJ, et al. Detection and characterization of carcinoma cells in the blood. Proc Natl Acad Sci. 1998;95:4589–94.
13. Brown HK, Tellez-Gabriel M, Cartron P-F, Vallette FM, Heymann M-F, Heymann D. Characterization of Circulating tumor cells as a reflection of the tumor heterogeneity: myth or reality? Drug Discov Today. 2019;24(3):763–72.
14. Zieglschmid V, Hollmann C, Böcher O. Detection of disseminated tumor cells in peripheral blood. Crit Rev Clin Lab Sci. 2005;42(2):155–96.
15. Cheng BR, Tong GL, Wu X, et al. Enumeration and characterization of Circulating tumor cells and its application in advanced gastric Cancer. Onco Targets Ther. 2019;25:12: 7887–96.
16. Amin MB, Edge SB, Greene FL, et al. editors. AJCC Cancer staging manual. New York: Springer; 2017.
17. Wu SY, Liu SY, Liu ZM, et al. Classification of Circulating tumor cells by epithelial-mesenchymal transition markers. PLoS ONE. 2015;10(4):e0123976.
18. Awasthi NP, Kumari S, Neyaz A, et al. EpCAM-based flow cytometric detection of Circulating tumor cells in gallbladder carcinoma cases. Asian Pac J Cancer Prev. 2017;18(12):3429–37.
19. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European organization for research and treatment of cancer, National Cancer Institute of the united States, National Cancer Institute of Canada. J Natl Cancer Inst. 2000;92:205–16.
20. Wang YP, Yuan ZQ, Zhu SB, et al. The longitudinal change of Circulating tumor cell during chemotherapy and its correlation with disease features, treatment response and survival profile of advanced gallbladder carcinoma. Am J Transl Res. 2021;13(12):13590–8.
21. Zhang XZ, Li L, Zhang M, et al. Intelligent recognition of CTCs from gallbladder cancer by ultrasensitive electrochemical cytosensor and diagnosis of chemotherapeutic resistance. Biosens Bioelectron. 2023;228:115183.
22. Zhang KL, Gao XM, Qin LX. The biological characteristics and signifcance of Circulating tumor cells in patients with solid malignancies. Fudan Univ J Med Sci. 2016;43(1):94–8.
23. Fischer JC, Niederacher D, Topp SA, et al. Diagnostic leukapheresis enables reliable detection of Circulating tumor cells of nonmetastatic cancer patients. Proc Natl Acad Sci U S A. 2013;110:16580–5.
24. Rack B, Schindlbeck C, Jückstock J, et al. Circulating tumor cells predict survival in early average-to-high risk breast cancer patients. J Natl Cancer Inst. 2014;106. pii: dju066.
25. van Dalum G, Stam GJ, Scholten LFA, et al. Importance of Circulating tumor cells in newly diagnosed colorectal cancer. Int J Oncol. 2015;46:1361–8.
26. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and desease. Cell. 2009;139:871–90.
27. Fu XT, Dai Z, Song K, et al. Macrophage-secreted IL-8 induces epithelial-mesenchymal transition in hepatocellular carcinoma cells by activating the JAK2/STAT3/Snail pathway. Int J Oncol. 2015;46:587–96.
28. Micalizzi DS, Farabaugh SM, Ford HL. Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. J Mamary Gland Biol Neoplasia. 2010;15(2):117–34.
29. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008;133:704–15.
30. Edwards J, Tasker A, Pires Da Silva I, et al. Prevalence and cellular distribution of novel immune checkpoint targets across longitudinal specimens in treatment-naïve melanoma patients: implications for clinicaltrials. Clin Cancer Res. 2019;25(11):3247–58.
31. Nikolakis G, Brunner M, Boye H, et al. Enlarged mediastinal lymph nodes of a patient with malignant melanoma stage IV under pembrolizumab treatment. Hautarzt. 2019;70(6):443–6.
32. Tsunoda A, Morikawa K, Inoue T, et al. A prospective observational study to assess PD-L1 expression in small biopsy samples for non-small-cell lung cancer. BMC Cancer. 2019;19(1):546.
33. Oyanagi J, Koh Y, Sato K, et al. Predictive value of serum protein levels in patients with advanced non-small cell lung cancer treated with nivolumab. Lung Cancer. 2019;132:107–13.

34. Katz H, Biglow L, Alsharedi M. Immune checkpoint inhibitors in locally advanced, unresectable, and metastatic upper Gastrointestinal malignancies. J Gastrointest Cancer. 2019;118(1):77.
35. Ding N, Zou Z, Sha H, et al. iRGD synergizes with PD-1 knockout immunotherapy by enhancing lymphocyte infiltration in gastric cancer. Nat Comms. 2019;10(1):1336.
36. Sharma P, Allison JP. The future of immune checkpoint therapy. Science. 2015;348(6230):56–61.
37. Martin-Liberal J, Ochoa de Olza M, Hierro C, Gros A, Rodon J, Tabernero J. The expanding role of immunotherapy. Cancer Treat Rev. 2017;54:74–86.
38. Chen XF, Wu XF, Wu H, et al. Camrelizumab plus gemcitabine and oxaliplatin (GEMOX) in patients with advanced biliary tract cancer: a single-arm, open-label, phase II trial. J Immunother Cancer. 2020;8(2):e001240.
39. Oh DY, He AR, Qin S, et al. A phase 3 randomized, double-blind, placebo-controlled study of durvalumab in combination with gemcitabine plus cisplatin (GemCis) in patients (pts) with advanced biliary tract cancer (BTC): TOPAZ-1. J Clin Oncol. 2022;40(4suppl):378.

Publisher's note