Clinical prognostic value of circulating tumor cells in the treatment of pancreatic cancer with gemcitabine chemotherapy

XIAOGUANG WANG[*], LINGYU HU[*], XIAODAN YANG[*], FEI CHEN, HAOKAI XU, HAITAO YU, ZHENGWEI SONG, JIANGUO FEI and ZHENGXIANG ZHONG

Department of Surgery, The Second Affiliated Hospital of Jiaxing University, Jiaxing, Zhejiang 314000, P.R. China

Abstract. Pancreatic cancer (PC) is a highly malignant tumor type with a high early metastasis rate and no obvious symptoms. Gemcitabine is a first-line chemotherapeutic drug for PC. Since there is no distinct method to determine the efficacy of chemotherapy with gemcitabine in patients with PC, the purpose of the present study was to determine whether positivity for circulating tumor cells (CTCs) in patients with advanced PC is associated with response to gemcitabine chemotherapy and to explore whether CTCs may be used as a predictor of prognosis of patients with advanced PC undergoing chemotherapy. First, immunomagnetic microspheres (magnetic beads; MIL) were prepared to detect CTCs. The patients' clinical characteristics and survival data, as well as efficacy and adverse effects of chemotherapy, were prospectively obtained and their association with CTCs was analyzed. The results indicated that CTC-positive patients with advanced PC had a higher probability of developing resistance to gemcitabine chemotherapy than CTC-negative patients. Survival in the CTC-negative group was significantly higher than in the CTC-positive group (χ^2=14.58, P<0.001). CTC-positive patients with advanced PC also had shorter progression-free survival (PFS) after chemotherapy with gemcitabine (P=0.01). In conclusion, CTC-positive patients with PC are more likely to develop gemcitabine resistance, have poor PFS and low incidence of thrombocytopenia. CTCs are expected to become a prognostic indicator for chemotherapy response in patients with PC.

Correspondence to: Dr Zhengxiang Zhong, Department of Surgery, The Second Affiliated Hospital of Jiaxing University, 397 Huancheng North Road, Jiaxing, Zhejiang 314000, P.R. China
E-mail: jxeygdkzzx@126.com

[*]Contributed equally

Introduction

Pancreatic cancer (PC) is a digestive system malignancy and the most common pathologic type is pancreatic ductal adeno-carcinoma (PDAC). In 2018, an estimated 458,918 patients were newly diagnosed with PDAC worldwide, accounting for ~2.5% of the total new cases in all cancer types, and ~432,242 PDAC-associated mortalities occurred, accounting for ~4.5% of the total cancer-associated deaths (1,2). Furthermore, PC is often diagnosed at an advanced stage and the only treatment available is palliative care, such as chemo-therapy and radiotherapy (3). PC is not sensitive to palliative treatment during the late stage in the majority of cases (3). Complete surgical resection is the only effective method for the treatment of PDAC (4). However, only 15-20% of patients with PDAC may have the opportunity to undergo curative surgical tumor resection, while the remaining patients can only receive adjuvant therapies. In this case, adjuvant therapies refer to chemotherapy or radiation therapy alone, as extensive metastasis and advanced tumor stage means surgery is no longer an option (5).

Gemcitabine is a first-line chemotherapy drug approved by the Food and Drug Administration for PDAC (6,7). The development of gemcitabine resistance during chemotherapy affects the prognosis of advanced PDAC and has become an increasingly common phenomenon (8). Although PDAC is not sensitive to chemotherapy, gemcitabine may still alleviate the symptoms of patients with PDAC and prolong their survival time (9). However, it has been reported that PDAC is gradually becoming resistant to gemcitabine and its efficacy declines (10,11). As patients with advanced PDAC cannot be treated with surgery, they may only be treated with gemcitabine chemotherapy; however, a considerable number of patients develop resistance. Therefore, it is important to predict whether patients will develop gemcitabine resistance through certain methods.

Circulating tumor cells (CTCs) may be used as a predictor of advanced metastasis of malignant tumors (12). Previous studies have indicated that the amount of CTCs in the blood may predict the prognosis of breast cancer and PC (13-15). PC subtypes with CTCs are more aggressive and meta-static (16,17). In addition, an increase in the number of CTCs in the portal vein blood may reduce the survival of patients with PDAC (18,19). CTCs have been determined to be an

independent prognosticfactor of PDAC; however, the relationship between CTCs and gemcitabine resistance has been rarely studied (20). In the present study, it was hypothesized that CTCs may be used as an independent prognostic factor for patients with PDAC and to be related to gemcitabine resistance in patients with advanced PC. The immunomagnetic microsphere used for sorting CTC in this study were EpCAM (12). The purpose of the present study was to test the above hypothesis by detecting changes in CTCs during PDAC treatment.

Materials and methods

Patients and clinical samples. The clinicopathological factors and blood samples in the present study were obtained from 87 patients with advanced PDAC who underwent chemotherapy with gemcitabine between June 2013 and June 2017 at the Second Affiliated Hospital of Jiaxing University (Jiaxing, China). The patient inclusion criteria were as follows: i) Accurate pathological diagnosis of primary PDAC; ii) complete clinicopathological and follow-up data; iii) lack of opportunity of surgery and tumor-node-metastasis (TNM) stage of III or IV. The patient exclusion criteria were as follows: i) The patient had undergone surgery and chemotherapy prior to admission; ii) the diagnosis was not clear; iii) presence of more than two primary tumors.

All patients were tested with serum tumor markers prior to chemotherapy, such as carcinoembryonic antigen (CEA) and carbohydrate antigen 199 (CA199). Patients were classified and staged based on the TNM classification for PDAC established by the Union for International Cancer Control (21). The clinicopathological data are provided in Table I. The present study was approved by the Ethics Committee of the Second Affiliated Hospital of Jiaxing University (Jiaxing, China) and performed in accordance with the Declaration of Helsinki. Informed consent was obtained from each participant. All patients had advanced PC and no chance of surgery. Endoscopic ultrasound-guided fine-needle aspiration was used for pathological examination and 5 patients had squamous cell carcinoma. CT and MRI were used to examine vascular invasion.

Chemotherapy and clinical evaluation. All 87 patients were treated with gemcitabine as a first-line chemotherapy drug. Each patient was treated with gemcitabine 1,000 mg/m^2 administered as an intravenous drip for 30 min. This medication was given on the 1st, 8th and 15th days of the course of treatment with a rest period for 14 days and every course of treatment lasted 28 days. This was repeated until progression or adverse reactions of chemotherapy became intolerable. All 87 patients received chemotherapy for >8 weeks. As the evaluation standard for adverse reactions, the grading standard (CTCAE3.0) established by the National Cancer Center of the United States was used (22). Progression-free survival (PFS) was defined as the time from the date of the diagnosis to the date of progression or death. Overall survival (OS) was defined as the time from the date of the diagnosis to the date of death or the last follow-up examination.

Preparation of immunomagnetic microspheres. To perform modification of epithelial cell adhesion molecule (EpCAM)

by glycidylhexadecylamine (GHDC), 57.1 μg of EpCAM (cat. no. ab223582; Abcam), dissolved 100 μg of GHDC (cat. no. 30-CG-049; Huzhou Liyuan Medical Laboratory Co., Ltd.) and 3.0 ml PBS (pH 7.4) was reacted with magnetic stirring at 4°C overnight. The molecules to be retained in the dialysis bag with a molecular weight of 8,000-14,000 Da were used the next day for 12 h and the buffer (PBS) was changed every 2 h. The antibody derivative EpCAM-GHDC obtained by dialysis and lyophilization was weighed. GHDC, immunomagnetic microspheres and dialysis bags were purchased from Huzhou Liyuan Medical Laboratory Co., Ltd. The preparation procedure of EpCAM immunomagnetic microspheres is displayed in Fig. 1A. During the preparation of immunomagnetic microspheres, GHDC was able to interact with transferrin antibodies. Hexadecyl-quaternized (carboxymethyl) chitosans (cat. no. 30-CG-050; Huzhou Liyuan Medical Laboratory Co., Ltd.) increased the grafting quantities of magnetic microspheres and antibodies through reactive groups and exerted emulsification, dispersion and surface activation functions (23,24).

Characterization of EpCAM magnetic spheres. The size distribution and zeta potential of the EpCAM magnetic spheres were measured by a Zetasizer (Nano-ZS 90; Malvern Instruments, Ltd.). The molecular morphology of folic acid magnetic spheres was determined via atomic force microscopy (BioScope SPM; Bruker Corporation). The magnetic properties of EpCAM magnetic spheres were measured via a vibrating sample magnetometer (Model 7407; Lake Shore Cryotronics, Inc.) and the ultraviolet absorption spectrum of EpCAM magnetic spheres was measured using a UV-2501PC UV-Vis spectrophotometer (Shimadzu Corporation).

CTC detection. The CTC detection procedure for PC is displayed in Fig. 1B. EpCAM immunomagnetic microspheres, prepared according to Fig. 1A, were added to the peripheral blood of patients with PDAC and the captured CTCs were identified and counted by immunofluorescence. Peripheral blood (4 ml) from 87 patients with advanced PDAC was collected in anticoagulation tubes prior to any treatment, and subsequently, 14 ml of red blood cell lysate from the same patient was added within 2 h. The sample was mixed gently by pipetting, placed in a refrigerator at 4°C for 15 min and then centrifuged at 111 x g for 5 min at 4°C. The supernatant was discarded and to the cell pellet, 10 ml buffer [PBS 500 ml + EDTA 0.375 g + BSA (Thermo Fisher Scientific, Inc.) 2.5 g] was added using the Nextctc FS100 Nano microfluidic chip (Wuxi Nao Biomedical Co., Ltd.). Obtained CTCs were evenly poured onto the slide. Sections were fixed with 2% paraformaldehyde for 10 min at room temperature and 0.25% Triton X-100 for 10 min. Subsequently, sections were washed with PBS for 15 min (three times for 5 min), and incubated with 2% BSA for 30 min at room temperature to block nonspecific binding. The sample was then incubated for 20 min at room temperature with EpCAM antibody (1:200 dilution; cat. no. ab223582; Abcam), cytokeratin (CK)-18 antibody (1:200 dilution; cat. no. ab181597; Abcam) or CD45 antibody (1:200 dilution; cat. no. SAB4502541; Sigma-Aldrich; Merck KGaA). Subsequently, the samples were rinsed with PBS for 15 min (3 times for 5 min). The sections were incubated

Table I. Characteristics of patients with advanced pancreatic cancer (n=87).

Parameter	Value
Age, years	61.8 (45-86)
Sex (male/female)	50/37
Pathological type (adenocarcinoma/squamous cell carcinoma)	79/8
Serum CA199, U/l (positive/negative)	52/35
Serum CEA, U/l (positive/negative)	24/53
TNM stage (III/IV)	40/47
Tumor location (head and neck/tail)	65/22
Tumor size, cm	3.9 (2-7)

Values are expressed as the mean (range) or n. CEA, carcinoembryonic antigen; CA199, carbohydrate antigen 199.

with DyLight 488-conjugated donkey anti-mouse IgG (H+L) (1:200 dilution; cat. no. ab150105; Abcam) and anti-rabbit IgG (1:200 dilution; cat. no. ab150073; Abcam) for 2 h at room temperature. Subsequently, the sections were washed with PBS for 15 min, mounted with mounting medium and examined under a fluorescence microscope (Olympus BX51; Olympus Corporation). CTCs were characterized by lacking CD45 expression and expressing EpCAM. CK immunocytofluorescence staining was also assessed on detected CTCs.

PFS follow-up. The 87 patients with advanced PDAC were followed up. The deadline for follow-up was June 1, 2017. Patients who had regular follow-up visits were followed up by outpatient clinics, while those who were not able to be followed up on time were followed up by telephone. The PFS rate was defined as the proportion of patients with no disease progression from enrollment to follow-up. The patients were divided into CTC-positive ($\geq$1 cell) and CTC-negative groups according to their circulating immune cell test results.

Statistical analysis. SPSS statistical analysis software 22.0 (IBM Corp.) was used for statistical analysis. The χ^2 test and t-test were utilized to determine the association between the presence of CTCs with chemotherapy adverse reactions, chemotherapeutic efficacy of gemcitabine and different clinical characteristics (if the sample size was <40 or the minimum theoretical frequency was <1, Fisher's exact test was used). For the OS and PFS analysis, Kaplan-Meier curves were drawn and the log-rank test was applied to compare differences between CTC-positive and CTC-negative patients. Univariate analysis of the relationship between CTCs and clinicopathological characteristics was performed by the χ^2 test/t-test. Multivariate analysis was conducted with multivariate logistic regression using the Cox proportional hazards model for analysis. P<0.05 was considered to indicate statistical significance.

Results

Characterization and performance evaluation of immunomagnetic microspheres. In order to examine the performance of the constructed CTC capture system, a series of functional tests were used to analyze the characteristics of the magnetic beads constructed. The UV spectrum proved that the EpCAM antibody had a broad absorption peak at 278 nm (Fig. 2A). For EpCAM-magnetic beads (MIL), an absorption peak was present at 281 nm. This indicated that EpCAM was indeed attached to the surface of the magnetic spheres. The UV spectrum of EpCAM-MIL had diffraction peaks at 31.5, 36.7, 42.9, 53.2, 58.2 and 61.5°, respectively, which corresponded to the (219), (312), (401), (420), (509) and (439) crystal plane structures of Fe_3O_4, respectively (Fig. 2B). The above results indicated that the magnetic beads were composed of Fe_3O_4 and that EpCAM was successfully attached to the surface of the magnetic beads. The immunomagnetic beads exhibited the crystal characteristics of magnetic nanoparticles. The magnetization curve indicated that the Fe_3O_4-MIL and EpCAM-MIL had no hysteresis at room temperature, but were superparamagnetic, and the magnetizing curve was displayed at 302 Kelvin. In addition, these results also proved that the saturation magnetization of the MIL was 27.75 Am^2/kg and the synthetic saturated magnetization of EpCAM-MIL was 10.03 Am^2/kg, indicating the saturation magnetization of the magnetic beads on the surface of the antibody and protein coating was immunomagnetic (Fig. 2C). The atomic force microscopy image of EpCAM-MIL illustrated that the size of the EpCAM-MIL was spherical and no agglomeration was present, which indicated that the microspheres had good stability and shape (Fig. 2D). As presented in Fig. 2E, the particle size test results of EpCAM-MIL suggested that the size of the spheres was ~400 nm and the average particle size was 323.9 nm, which indicated liposome-like vesicle properties. At the same time, the zeta potential analysis results of EpCAM-MIL suggested that the zeta potential was +23.9 mV (Fig. 2F). In summary, the magnetic beads prepared in the present study have smaller particle size and higher stability than those described in a in previous study (25).

Detection results of CTCs and efficacy of chemotherapy. The present study included 87 patients with PDAC treated between June 2013 and June 2017. As indicated in Table I, the age ranged between 45 and 86 years (mean age, 61.8 years), the cohort contained 50 males and 37 females, 22 tumors were located in the tail of the pancreas and 65 cases were located in the head and neck of the pancreas. Among them, 49 patients (56%, 49/87) had one or more CTCs/4 ml blood detected, with CTC numbers ranging from 2 to 298 (mean ± standard deviation, 109.2±71.6) (Fig. 3A). The Kaplan-Meier plots for PFS and OS for patients with PC were drawn and it was indicated that CTC positivity was associated with poor PFS compared with CTC negativity (P=0.01). However, the OS rate of CTC-positive patients was not significantly different from that of CTC-negative patients (P=0.091; Fig. 3B and C).

Relationship between CTCs and clinicopathological characteristics of patients with advanced PC. The relationship between CTCs and the clinicopathological characteristics of 87 patients with advanced PDAC was analyzed by the χ^2 test
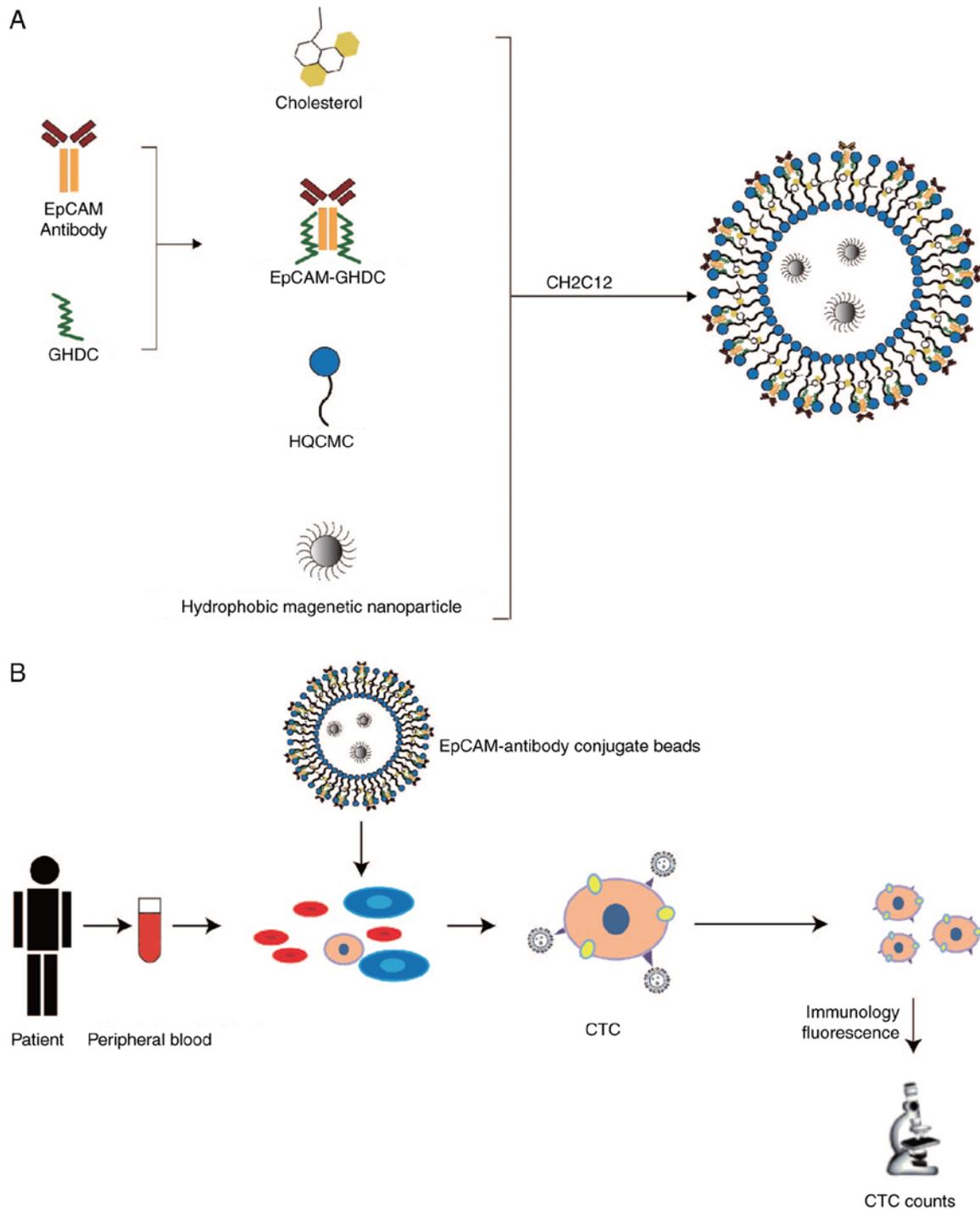
Figure 1. Preparation of immunomagnetic microspheres and flow chart for the detection of CTCs in patients with PC. (A) Flow diagram of EpCAM-MIL particle preparation. (B) Detection of CTCs in patients with PC by the MILs. CTC, circulating tumor cell; EpCAM, epithelial cell adhesion molecule; MIL, magnetic microspheres; GHDC, glycidylhexadecylamine; PC, pancreatic cancer; HQCMC, hexadecyl-quaternized (carboxymethyl) chitosans.

and presented in Table II. Univariate analyses demonstrated that CTCs were closely associated with vascular invasion (P<0.001), TNM stage (P=0.005) and liver metastasis (P=0.005). However, there was no significant difference between CTCs and other clinical parameters, including age, sex, symptoms, tumor size, tumor location, pathological type, lymph node metastasis, neurological invasion, CA199 and CEA. Multivariate regression analysis indicated that vascular invasion (P<0.001) and liver metastasis (P=0.002) were independent predictors of CTCs.

Relationship between chemotherapy effect and CTCs. According to the analysis, 77.5% of the CTC-positive patients were resistant to gemcitabine, while 47.4% of CTC-negative patients developed resistance to gemcitabine. The detailed data for the association between peripheral blood CTC and chemotherapeutic efficacy of gemcitabine are displayed in Fig. 4, which indicated that the efficacy of gemcitabine was also affected by CTCs (χ^2=8.501, P=0.004). The sensitivity and specificity of CTC detection for gemcitabine resistance was calculated as follows: Sensitivity, 67.86% and
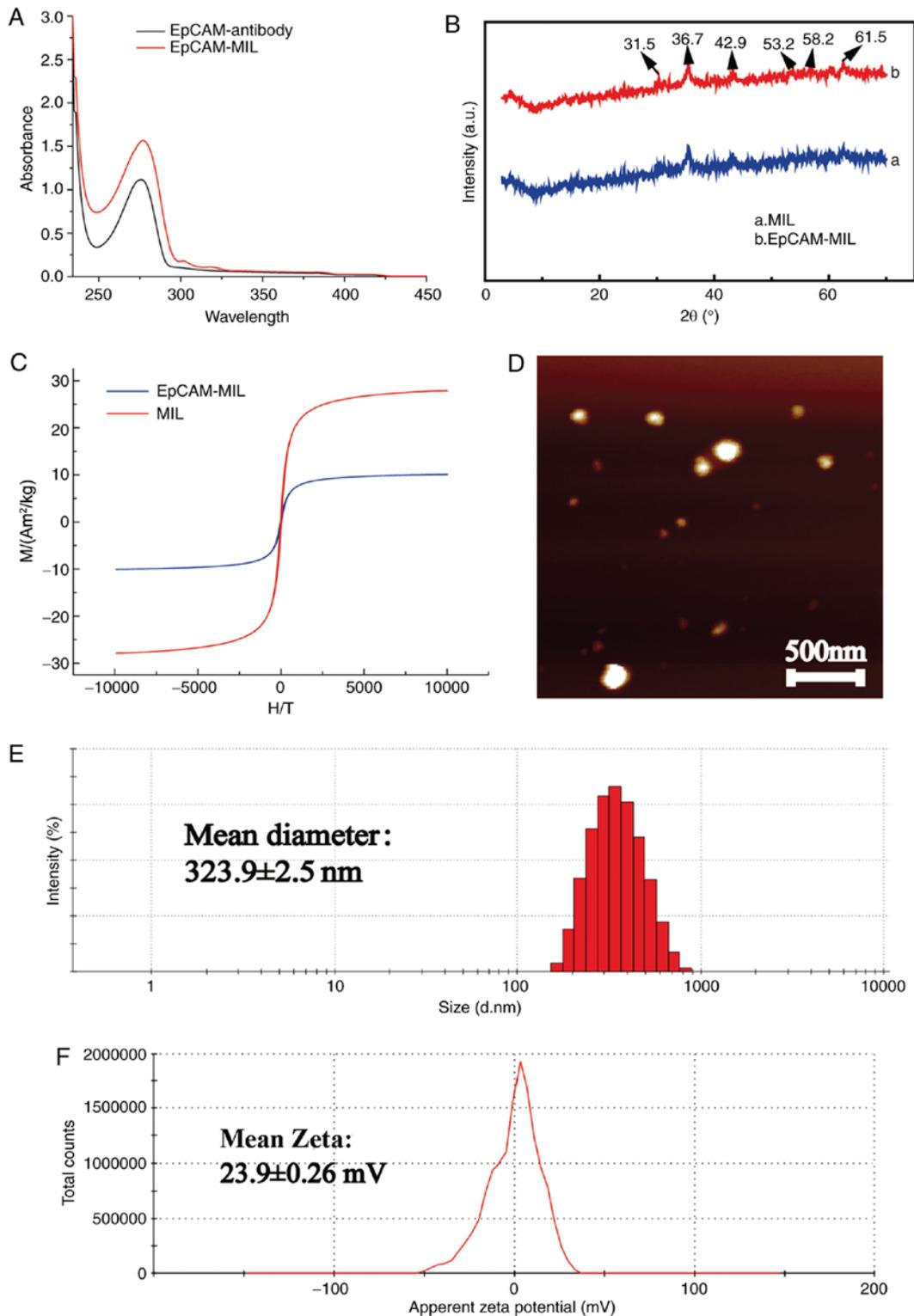
Figure 2. Material characterization and performance evaluation of immunomagnetic microspheres. (A) Ultraviolet absorption spectrum of EpCAM antibody and EpCAM-MIL. (B) X-ray diffraction spectrum of MIL and EpCAM-MIL. (C) Magnetization curves of Fe3O4 MIL and EpCAM-MIL. M, ratio of saturation magnetization to the density of the material; H/T, the saturated magnetization of the magnetic beads. (D) Atomic force microscopy image of EpCAM-MIL (scale bar, 500 nm). (E) Particle-size-distribution based intensity of EpCAM-MIL. (F) Zeta potential of EpCAM-MIL. EpCAM, epithelial cell adhesion molecule; MIL, magnetic microspheres; d, diameter.

specificity, 64.52%. However, there was no significant association between the chemotherapy effect and other clinical parameters, including age, sex, symptoms, tumor size, tumor location, CA-199, CEA, liver metastasis and TNM stage (Table III).

Relationship between chemotherapy adverse reactions and CTCs. All 87 patients with advanced PDAC were able to tolerate adverse reactions to gemcitabine chemotherapy and no chemotherapy-related death occurred. The major adverse reactions were digestive tract reactions, myelosuppression
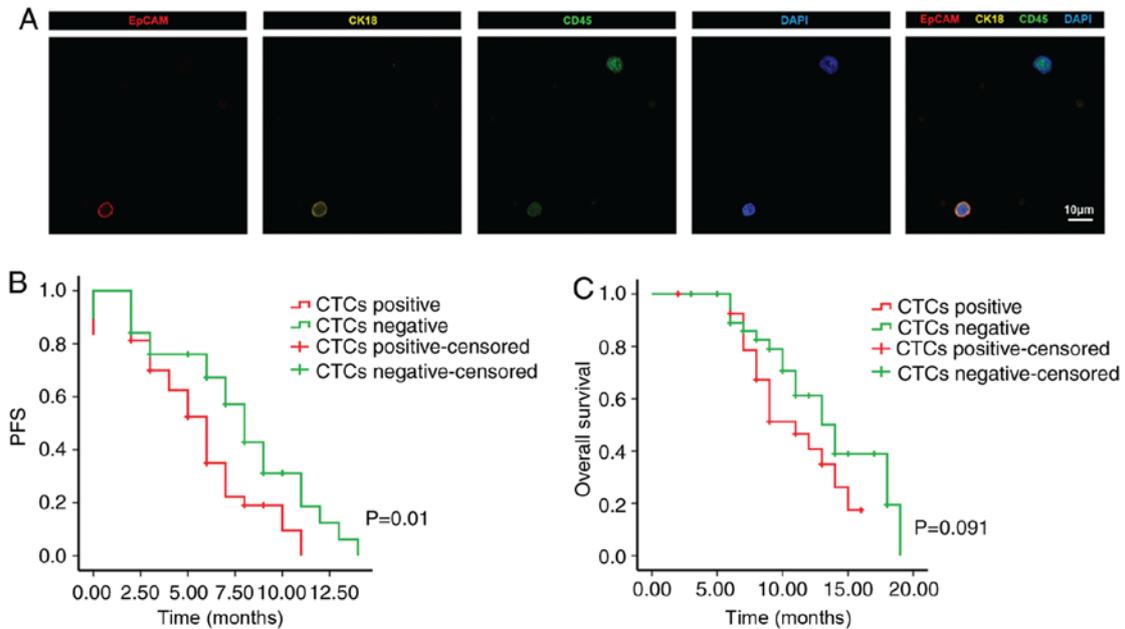
Figure 3. Influence of CTCs on the efficacy of chemotherapy. (A) Immunofluorescence observation of CTCs in clinical blood samples captured by EpCAM immunomagnetic particles (scale bar, 10 μm). (B and C) Kaplan-Meier plots of (B) PFS and (C) overall survival in patients with pancreatic cancer. The survival time of patients who died within 1 month was recorded as 0, thus, the red and green lines initially overlapped. CTC, circulating tumor cell; EpCAM, epithelial cell adhesion molecule; PFS, progression-free survival; m, months; CK18, cytokeratin 19.
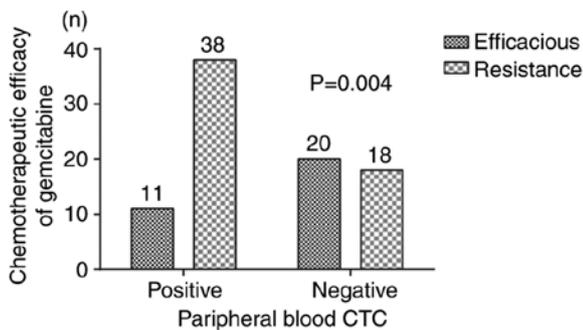
Figure 4. Association between chemotherapeutic efficacy of gemcitabine and CTCs in patients with advanced pancreatic cancer. CTC, circulating tumor cell.

and flu-like symptoms. The incidence of thrombocytopenia in the CTC-negative and -positive groups was 57.8 and 18.4%, respectively, and that in the CTC-negative group was significantly higher than that in the-positive group (χ^2=14.58, P<0.001), but other adverse reactions, including digestive tract reactions, myelosuppression, anemia, liver damage and flu-like symptoms were not associated with CTCs (Table IV).

Discussion

In the present study, it was confirmed that patients with advanced PDAC with positive CTCs have poor prognosis and short survival. Furthermore, CTC-positive patients with advanced PDAC had a higher ratio of resistance to gemcitabine and lower efficacy of chemotherapy. The use of CTC count statistics and related research is of great significance for the dynamic monitoring of PDAC clinical samples (26,27).

PDAC is the fourth leading cause of death worldwide. The lack of early symptoms and screening usually results in late diagnosis and poor prognosis. CTCs have been a promising novel biomarker in solid tumors. Over the past two decades, >100 articles have been published on this topic. Most of the studies evaluated the use of CTCs as a prognostic marker and its association with the survival of patients with PDAC (28). Patients with advanced PDAC may exhibit multiple complications associated with distant metastasis (29,30). The present study indicated that the positive rate of peripheral blood CTCs in 87 patients with advanced PDAC was 56%. Han *et al* (17) combined nine articles in a meta-analysis, revealing a CTC-positive rate of 43% in 623 patients with PDAC. The meta-analysis suggested that CTC-positive patients with pancreatic cancer exhibited worse levels of PFS and OS, compared with CTC-negative patients (17). Of note, the CTC data of patients with metastatic PDAC using the CellSearch® system indicated that the detection rate of CTCs is ~50% (18-20). The higher CTC-positive rate in the present study was likely due to the patients having advanced PDAC and the limited sample size. This still indicated that the self-assembled lipid beads used had a good CTC capture ratio.

Pancreatic adenocarcinoma has a moderate response to gemcitabine-based chemotherapy, which is the most widely used monotherapy for PDAC. Tadros *et al* (31) discovered a marked increase in gemcitabine resistance in patients with pancreatic cancer following the orlistat-induced inhibition of fatty acid biosynthesis. Using the Cancer Genome Atlas dataset, Tadros *et al* indicated that fatty acid biosynthetic pathway manipulation may help overcome the stress and regulation of gemcitabine in PDAC (31). Furthermore, Shukla *et al* (32) declared that targeting HIF-1 cells or *de novo* pyrimidine biosynthesis, combined with gemcitabine, may significantly

Table II. Association between CTC status and clinicopathological characteristics of patients with pancreatic cancer.

| Clinical characteristic | Peripheral blood CTCs | | Univariate analysis P-value | Multivariate analysis | |
	Positive (n=49)	Negative (n=38)		OR (95% CI)	P-value
Sex			0.612		
Male	27	23			
Female	22	15			
Age, years	61.29±9.00	62.61±8.57	0.491		
Symptoms			0.069		
Present	25	12			
Absent	24	26			
Tumor location			0.489		
Head and neck	38	27			
Tail	11	11			
Tumor size, cm	4.00 (3.00,5.00)	3.00 (3.00,4.00)	0.203		
Pathological type			1.000[a]		
Adenocarcinoma	44	35			
Squamous cell carcinoma	5	3			
Lymph node metastasis			0.163		
Present	16	18			
Absent	33	20			
Vascular invasion			<0.001	Reference 57.321 (7.138-460.297)	<0.001
Present	37	9			
Absent	12	29			
Neurological invasion			0.093		
Present	32	18			
Absent	17	20			
TNM stage			0.005	Reference 2.202 (0.411-11.804)	0.357
III	16	24			
IV	33	14			
Liver metastasis			0.005	Reference 27.285 (3.380-220.272)	0.002
Present	29	11			
Absent	20	27			
CA199			0.450		
Normal	18	17			
Elevated	31	21			
CEA			0.463		
Normal	37	26			
Elevated	12	12			

Hypothesis testing was carried out on the whole model, that is, testing whether the relationship between the dependent variable and the independent variable can be expressed by the established regression equation. It has been tested whether the single regression coefficient is 0, that is, whether the influence of a single independent variable on the dependent variable exists. The variables with statistically significant differences in univariate analysis were taken as independent variables and included in the Logistic regression model. The stepwise forward regression method was adopted for analysis. The inclusion standard was 0.05 and the exclusion standard was 0.1. [a]Fisher's exact test. Values are expressed as the mean ± standard deviation, median (range) or n. CTC, circulating tumor cell; CEA, carcinoembryonic antigen; CA199, carbohydrate antigen 199; OR, odds ratio.

reduce the tumor burden and decrease the expression of transketolase and cytidine triphosphate synthase 1. In addition, Mehla and Singh (33) revealed that a glycolytic subtype indicates poor survival in patients with PDAC, whereas the holesterogenic subtype correlates with more favorable outcomes, potentially due to a higher energy expenditure.

The detection of CTCs may be of important clinical value for the prognosis of PC. The purpose of the present study

Table III. Association between efficacy of chemotherapy and CTCs.

| Clinical characteristic | Chemotherapeutic efficacy of gemcitabine | | t or χ^2 | P-value |
	Efficacious (n=31)	Resistance (n=56)		
Sex			0.287	0.592
Male	19	31		
Female	12	25		
Age, years	65.83±10.94	68.64±10.88	-1.007	0.318
Symptoms			0.007	0.934
Present	13	24		
Absent	18	32		
Tumor location			0.187	0.666
Head and neck	24	41		
Tail	7	15		
Tumor size, cm	3.45±1.36	4.31±1.78	-1.158	0.251
CA-199, U/l			0.001	0.974
≥37	21	31		
<37	10	25		
CEA, U/l			0.05	0.822
≥5	9	15		
<5	22	41		
Liver metastasis			2.653	0.103
Present	17	23		
Absent	11	32		
TNM stage			1.024	0.312
III	12	28		
IV	19	28		
CTC status			8.501	0.004
Positive	11	38		
Negative	20	18		

Values are expressed as the mean ± standard deviation or n. CTC, circulating tumor cell; CEA, carcinoembryonic antigen; CA199, carbohydrate antigen 199.

Table IV. Association between adverse reactions to chemotherapy and CTCs.

| Chemotherapy adverse reaction | CTC status | | t or χ^2 | P-value |
	Positive (n=49)	Negative (n=38)		
Nausea, emesis	27	17	0.920	0.338
Diarrhea	5	3	0.000	1.000[a]
Leukocytopenia	23	17	0.042	0.838
Thrombocytopenia	9	22	14.580	<0.001
Anemia	9	6	0.100	0.752
Hepatic function damage	8	7	0.066	0.798
Rash	4	3	0.000	1.000[a]
Flu-like symptoms	27	18	0.513	0.474

[a]Fisher's exact test. CTC, circulating tumor cell.

was to evaluate the role of CTCs in recurrence, metastasis and treatment efficacy by detecting the differences in CTCs between patients with PDAC. Most previous studies have explored the association between CTC detection and PC

diagnosis. Both Earl *et al* (34) and Liu *et al* (35) reported that CTCs are a promising marker for the management of patients with PDAC; however, the correlation between CTCs and gemcitabine resistance in patients with PDAC has remained largely elusive. The present study not only confirmed that CTCs are a prognostic marker in patients with advanced PC undergoing chemotherapy, but also that CTC-positive patients with PC are more likely to develop gemcitabine resistance. In the present study, all 87 patients with advanced PDAC received gemcitabine monotherapy. Among them, 56 patients were resistant to gemcitabine and the drug resistance rate was 64%. The resistance rate of gemcitabine in CTC-positive patients with PDAC was as high as 77.6% (28/39). Previous studies have indicated that the resistance rate of patients with PDAC to gemcitabine is gradually increasing, and the efficacy of gemcitabine is also reduced by >20%, compared to the results established ~10 years prior (5,6). The present clinical study confirmed that positivity for CTCs prior to chemotherapy in patients with PC indicates drug resistance, but the mechanisms have remained elusive. Based on the combination of results of previous studies, it may be hypothesized that epithelial to mesenchymal transition (EMT) may be associated with changes in CTCs and gemcitabine resistance (16,36). This is a process associated with the separation of cancer cells from the primary tumor, which may lead to CTCs that metastasize, contributing to cancer progression. Thus, the number of CTCs can be used as an indicator for cancer progression and its degree of malignancy. The further the cancer has progressed, the worse the prognosis, and the poorer the efficacy of gemcitabine. Although the expression of EpCAM was detected and the relevant literature was reviewed, it was determined that the mechanism of action underlying CTCs in pancreatic cancer may be associated with EMT. In the present study, the method used was not able to detect the expression of E-cadherin and vimentin due to the use of peripheral blood primary cells of patients for CTC detection. Furthermore, peripheral blood samples cannot easily be stored for long periods of time, thus, relevant substances in the blood are lost over time. This is also the difficulty of CTC detection at present. Previous methods have also failed to do this, for example, Xie *et al* (37) used an *in vivo* CellCollector® method to detect the number of CTC in patients. In the future, more convenient and sensitive testing methods will be applied (37). Previous studies have indicated that gemcitabine combined with nab-paclitaxel chemotherapy may optimize the chemotherapy effect of PDAC and prolong the survival time (38,39). Therefore, improving the sensitivity of PDAC cells to gemcitabine and combined chemotherapy may improve the chemotherapy effect and prolong the survival time of patients. In the present study, the median PFS was 8.0 months in CTC-positive patients compared to 7.0 months in CTC-negative patients. However, OS did not differ significantly between CTC-positive and CTC-negative patients with PDAC. In general, the median survival time of PDAC is low, but a minority of patients with PDAC have undergone complete surgical resection, so their survival time is particularly long (40). In those patients eligible for surgery, the cancer was at an early stage without metastasis, and the associated prognosis was improved. Furthermore, the present study determined that patients with advanced PDAC

with CTCs were less likely to develop thrombocytopenia after receiving gemcitabine, but the reason for this remains elusive.

The present study has certain limitations that are worth mentioning. Due to the limited number of patients included, the results of related studies should also be considered. The present study is a retrospective study and the results obtained require to be verified by larger prospective studies. In addition, the patients of the present study were not monitored for CTCs after treatment due to cost considerations. In subsequent studies, a comparative study evaluating CTCs prior to and after treatment may be performed.

In conclusion, CTC-positive patients with PC are more likely to develop gemcitabine resistance, and these patients have poor PFS and low incidence of thrombocytopenia. Thus, CTCs may be considered as a prognostic marker for chemotherapy in patients with advanced PC.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

ZZ was responsible for project design, obtaining funding and resources, and conceptualization. XW was also responsible for project conceptualization. FC was also responsible for obtaining resources. XW, LH and XY were responsible for drafting the manuscript. LH conceived and designed the CTC extraction experiment, analyzed the experimental data, wrote the results and discussion in the manuscript, and generated Figures 1 and 3, and Table II. In addition, LH made a significant contrution to manuscript revision. XY made substantial contributions to acquisition of data. HX was responsible for data curation, statistical analysis and experimentation. HY was responsible for carrying out the experiments. HX, FC and JF were responsible for performing the experiments. ZS made substantial contributions to analysis and interpretation of data, and producing figures and tables of analysis results. All authors confirm the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Jiaxing University (Jiaxing, China). Informed consent was obtained from each study participant.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018.
2. Mizrahi JD, Surana R, Valle JW and Shroff RT: Pancreatic cancer. Lancet 395: 2008-2020, 2020.
3. Rothenberg ML, Moore MJ, Cripps MC, Andersen JS, Portenoy RK, Burris HA III, Green MR, Tarassoff PG, Brown TD, Casper ES, *et al*: A phase II trial of gemcitabine in patients with 5-FU-refractory pancreas cancer. Ann Oncol 7: 347-353, 1996.
4. Li D, Xie K, Wolff R and Abbruzzese JL: Pancreatic cancer. Lancet 363: 1049-1057, 2004.
5. Mao Y, Xi L, Li Q, Wang S, Cai Z, Zhang X and Yu C: Combination of PI3K/Akt pathway inhibition and Plk1 depletion can enhance chemosensitivity to gemcitabine in pancreatic carcinoma. Transl Oncol 11: 852-863, 2018.
6. Di Marco M, Di Cicilia R, Macchini M, Nobili E, Vecchiarelli S, Brandi G and Biasco G: Metastatic pancreatic cancer: Is gemcitabine still the best standard treatment? (review). Oncol Rep 23: 1183-1192, 2010.
7. Loos M, Kleeff J, Friess H and Büchler MW: Surgical treatment of pancreatic cancer. Ann N Y Acad Sci 1138: 169-180, 2008.
8. Bidard FC, Pierga JY, Soria JC and Thiery JP: Translating metastasis-related biomarkers to the clinic-progress and pitfalls. Nat Rev Clin Oncol 10: 169-179, 2013.
9. Thalgott M, Heck MM, Eiber M, Souvatzoglou M, Hatzichristodoulou G, Kehl V, Krause BJ, Rack B, Retz M, Gschwend JE, *et al*: Circulating tumor cells versus objective response assessment predicting survival in metastatic castration-resistant prostate cancer patients treated with docetaxel chemotherapy. J Cancer Res Clin Oncol 141: 1457-1464, 2015.
10. Giuliano M, Giordano A, Jackson S, De Giorgi U, Mego M, Cohen EN, Gao H, Anfossi S, Handy BC, Ueno NT, *et al*: Circulating tumor cells as early predictors of metastatic spread in breast cancer patients with limited metastatic dissemination. Breast Cancer Res 16: 440, 2014.
11. Iinuma H, Watanabe T, Mimori K, Adachi M, Hayashi N, Tamura J, Matsuda K, Fukushima R, Okinaga K, Sasako M and Mori M: Clinical significance of circulating tumor cells, including cancer stem-like cells, in peripheral blood for recurrence and prognosis in patients with Dukes' stage B and C colorectal cancer. J Clin Oncol 29: 1547-1555, 2011.
12. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, Isakoff SJ, Ciciliano JC, Wells MN, Shah AM, *et al*: Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science 339: 580-584, 2013.
13. Barriere G, Riouallon A, Renaudie J, Tartary M and Rigaud M: Mesenchymal characterization: Alternative to simple CTC detection in two clinical trials. Anticancer Res 32: 3363-3369, 2012.
14. Arnoletti JP, Zhu X, Almodovar AJ, Veldhuis PP, Sause R, Griffith E, Corpus G, Chang JC, Fanaian N and Litherland SA: Portal venous blood circulation supports immunosuppressive environment and pancreatic cancer circulating tumor cell activation. Pancreas 46: 116-123, 2017.
15. Tien YW, Kuo HC, Ho BI, Chang MC, Chang YT, Cheng MF, Chen HL, Liang TY, Wang CF, Huang CY, *et al*: A high circulating tumor cell count in portal vein predicts liver metastasis from periampullary or pancreatic cancer: A high portal venous CTC count predicts liver metastases. Medicine (Baltimore) 95: e3407, 2016.
16. Effenberger KE, Schroeder C, Hanssen A, Wolter S, Eulenburg C, Tachezy M, Gebauer F, Izbicki JR, Pantel K and Bockhorn M: Improved risk stratification by circulating tumor cell counts in pancreatic cancer. Clin Cancer Res 24: 2844-2850, 2018.
17. Han L, Chen W and Zhao Q: Prognostic value of circulating tumor cells in patients with pancreatic cancer: A meta-analysis. Tumour Biol 35: 2473-2480, 2014.
18. Kurihara T, Itoi T, Sofuni A, Itokawa F, Tsuchiya T, Tsuji S, Ishii K, Ikeuchi N, Tsuchida A, Kasuya K, *et al*: Detection of circulating tumor cells in patients with pancreatic cancer: A preliminary result. J Hepatobiliary Pancreat Surg 15: 189-195, 2008.
19. Dotan E, Alpaugh RK, Ruth K, Negin BP, Denlinger CS, Hall MJ, Astsaturov I, McAleer C, Fittipaldi P, Thrash-Bingham C, *et al*: Prognostic significance of MUC-1 in circulating tumor cells in patients with metastatic pancreatic adenocarcinoma. Pancreas 45: 1131-1135, 2016.
20. Ko AH, Scott J, Tempero MA and Park JW: Detection and significance of circulating tumor cells (CTC) in patients with metastatic pancreatic cancer (PC) receiving systemic therapy. J Clin Oncol 25 (Suppl 18): S4596, 2007.
21. Allen PJ, Kuk D, Castillo CF, Basturk O, Wolfgang CL, Cameron JL, Lillemoe KD, Ferrone CR, Morales-Oyarvide V, He J, *et al*: Multi-institutional validation study of the American joint commission on cancer (8th edition) changes for T and N staging in patients with pancreatic adenocarcinoma. Ann Surg 265: 185-191, 2017.
22. Trotti A, Colevas AD, Setser A, Rusch V, Jaques D, Budach V, Langer C, Murphy B, Cumberlin R, Coleman CN and Rubin P: CTCAE v3.0: Development of a comprehensive grading system for the adverse effects of cancer treatment. Semin Radiat Oncol 13: 176-181, 2003.
23. Liang X, Li X, Chang J, Duan Y and Li Z: Properties and evaluation of quaternized chitosan/lipid cation polymeric liposomes for cancer-targeted gene delivery. Langmuir 29: 8683-8693, 2013.
24. Liang X, Tian H, Luo H, Wang H and Chang J: Novel quaternized chitosan and polymeric micelles with cross-linked ionic cores for prolonged release of minocycline. J Biomater Sci Polym Ed 20: 115-131, 2009.
25. Chen J, Chen L, Du S, Wu J, Quan M, Yin H, Wu Y, Ye X, Liang X and Jiang H: High sensitive detection of circulating tumor cell by multimarker lipid magnetic nanoparticles and clinical verifications. J Nanobiotechnology 17: 116, 2019.
26. Timme-Bronsert S, Bronsert P, Werner M, Kulemann B and Höppner J: Circulating tumor cells in pancreatic cancer: Results of morphological and molecular analyses and comparisons with the primary tumor. Pathologe 39 (Suppl 2): S311-S314, 2018 (In German).
27. Zhao XH, Wang ZR, Chen CL, Di L, Bi ZF, Li ZH and Liu YM: Molecular detection of epithelial-mesenchymal transition markers in circulating tumor cells from pancreatic cancer patients: Potential role in clinical practice. World J Gastroenterol 25: 138-150, 2019.
28. Martini V, Timme-Bronsert S, Fichtner-Feigl S, Hoeppner J and Kulemann B: Circulating tumor cells in pancreatic cancer: Current perspectives. Cancers (Basel) 11: 1659, 2019.
29. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2019. CA Cancer J Clin 69: 7-34, 2019.
30. Ducreux M, Cuhna AS, Caramella C, Hollebecque A, Burtin P, Goéré D, Seufferlein T, Haustermans K, Van Laethem JL, Conroy T, *et al*: Cancer of the pancreas: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 26 (Suppl 5): v56-v68, 2015.
31. Tadros S, Shukla SK, King RJ, Gunda V, Vernucci E, Abrego J, Chaika NV, Yu F, Lazenby AJ, Berim L, *et al*: De novo lipid synthesis facilitates gemcitabine resistance through endoplasmic reticulum stress in pancreatic cancer. Cancer Res 77: 5503-5517, 2017.
32. Shukla SK, Purohit V, Mehla K, Gunda V, Chaika NV, Vernucci E, King RJ, Abrego J, Goode GD, Dasgupta A, *et al*: MUC1 and HIF-1alpha signaling crosstalk induces anabolic glucose metabolism to impart gemcitabine resistance to pancreatic cancer. Cancer Cell 32: 71-87.e7, 2017.
33. Mehla K and Singh PK: Metabolic subtyping for novel personalized therapies against pancreatic cancer. Clin Cancer Res 26: 6-8, 2020.
34. Earl J, Garcia-Nieto S, Martinez-Avila JC, Montans J, Sanjuanbenito A, Rodríguez-Garrote M, Lisa E, Mendía E, Lobo E, Malats N, *et al*: Circulating tumor cells (CTC) and KRAS mutant circulating free DNA (cfDNA) detection in peripheral blood as biomarkers in patients diagnosed with exocrine pancreatic cancer. BMC Cancer 15: 797, 2015.
35. Liu H, Sun B, Wang S, Liu C, Lu Y, Li D and Liu X: Circulating tumor cells as a biomarker in pancreatic ductal adenocarcinoma. Cell Physiol Biochem 42: 373-382, 2017.

36. El Amrani M, Corfiotti F, Corvaisier M, Vasseur R, Fulbert M, Skrzypczyk C, Deshorgues AC, Gnemmi V, Tulasne D, Lahdaoui F, *et al*: Gemcitabine-induced epithelial-mesenchymal transition-like changes sustain chemoresistance of pancreatic cancer cells of mesenchymal-like phenotype. Mol Carcinog 58: 1985-1997, 2019.
37. Xie N, Hu Z, Tian C, Xiao H, Liu L, Yang X, Li J, Wu H, Lu J, Gao J, *et al*: In vivo detection of CTC and CTC plakoglobin status helps predict prognosis in patients with metastatic breast cancer. Pathol Oncol Res 26: 2435-2442, 2020.
38. Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, *et al*: Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med 369: 1691-1703, 2013.
39. Von Hoff DD, Ramanathan RK, Borad MJ, Laheru DA, Smith LS, Wood TE, Korn RL, Desai N, Trieu V, Iglesias JL, *et al*: Gemcitabine plus nab-paclitaxel is an active regimen in patients with advanced pancreatic cancer: A phase I/II trial. J Clin Oncol 29: 4548-4554, 2011.
40. Kimura K, Amano R, Nakata B, Yamazoe S, Hirata K, Murata A, Miura K, Nishio K, Hirakawa T, Ohira M and Hirakawa K: Clinical and pathological features of five-year survivors after pancreatectomy for pancreatic adenocarcinoma. World J Surg Oncol 12: 360, 2014.