

Circulating Tumor Cells Count Predicts Survival in Colorectal Cancer Patients

Adriana Romiti¹, Salvatore Raffa¹, Roberta Di Rocco¹, Michela Roberto¹, Annalisa Milano¹, Angelo Zullo², Laura Leone¹, Danilo Ranieri¹, Francesca Mazzetta¹, Emanuela Medda³, Ida Sarcina¹, Viola Barucca¹, Chiara D'Antonio¹, Valeria Durante¹, Mario Ferri⁴, Maria Rosaria Torrisi¹, Paolo Marchetti¹

1) Clinical and Molecular Medicine Department, Sapienza University, Sant'Andrea Hospital;
2) Gastroenterology and Digestive Endoscopy, Nuovo Regina Margherita Hospital;
3) Genetic Epidemiology Unit, National Centre for Epidemiology, Surveillance and Health Promotion, Istituto Superiore di Sanità;
4) Surgical and Medical Department of Clinical Sciences, Biomedical Technologies and Translational Medicine, Sant'Andrea Hospital, Rome, Italy

ABSTRACT

Background & Aims: Data on the potential of circulating tumor cells (CTC) count in predicting overall survival (OS) in patients with colorectal cancer are timely and worthy of interest. This study aimed to evaluate the prognostic role of CTC count in both localized and metastatic colorectal cancer patients.

Methods: Consecutive patients with histological diagnosis of colorectal cancer were enrolled. CTC count was performed, by using a quantitative immunofluorescence method, at baseline (T0) and 1 month following start of chemotherapy (T1). A CTC count <2 was considered negative, whilst a CTC level ≥ 2 was positive. Overall survival was calculated accordingly.

Results: A total of 75 colorectal cancer patients were enrolled, including 54 stages I-III and 21 stage IV patients. Overall, 21 (28%) patients had a positive CTC count at baseline, and it was significantly associated with a worse prognosis as compared to a negative status (OS: 36.2 vs. 61.6 months; $P = 0.002$). CTC count remained positive after chemotherapy in 22.4% of the patients and it was an independent prognostic factor of OS ($P = 0.03$; Hazard Ratio: 3.55; 95% CI: 1.1-11.5).

Conclusions: This study found that the presence of CTCs is associated with a reduced survival in colorectal cancer patients. Further studies aimed at testing such a predictive value in early stage colorectal cancer are awaited.

Key words: circulating tumor cells – colorectal cancer – predictive value – survival.

Address for correspondence:

Adriana Romiti, MD
Clinical and Molecular Medicine Department
Sapienza University
Sant'Andrea Hospital
Via di Grottarossa 1035
00189 Rome - Italy
adriana.romiti@tin.it

INTRODUCTION

Colorectal cancer (CRC) is a common and deadly malignancy in both the US and Europe, with an estimated total of 258,000 and 136,000 new cases per year, respectively [1, 2]. However, overall survival (OS) of patients with advanced CRC has been improved by the combination of traditional anti-proliferative agents with newer targeted molecules, which inhibit signal transduction pathways [3, 4]. A current challenge concerns both the selection of patients with a poor prognosis to be directed towards the most effective treatment, and the identification of reliable predictive markers of response. Circulating tumor

cells (CTCs) have been recognized in patients carrying different cancer types [5, 6]. The CTC count was reported to correlate with disease stage, and to predict OS in patients with advanced CRC [7-9]. Moreover, low CTC levels before and during chemotherapy were associated with a better clinical response [10]. However, the prognostic role of CTCs in patients with CRC has not been definitely established so far. We therefore designed a cohort study aimed to evaluate the prognostic role of CTC count in CRC patients.

PATIENTS AND METHODS

Patients

Consecutive CRC patients observed from October 2007 to September 2009 in a single center were enrolled. Performance status (PS) was classified according to the Eastern Cooperative Oncology Group (ECOG) score. CTC count was performed on blood samples collected before chemotherapy (T0) and 1 month after start of therapy (T1). Patients provided their written informed consent to participate in this study. The study design was approved by the Ethical Committee of the Sant'Andrea Hospital (Prot. C.E. 1242/2013).

Received: 02.05.2014
Accepted: 05.08.2014

CTC detection

Briefly, 7.5 mL of peripheral blood was collected from each patient for CTC evaluation. Peripheral Blood Mononuclear Cells (PBMCs) were isolated using Ficoll–Paque (Miltenyi Biotec, Bergisch Gladbach, Germany) density centrifugation at 2,400 rpm for 30 min and re-suspended for magnetic labeling in 300 μ L of MACS[®] separation buffer (Miltenyi Biotec). According to the manufacturer's instructions, PBMCs were incubated for 30 minutes at 4°C with 100 μ L of CD326 (EpCAM) MicroBeads (Miltenyi Biotec). Then, MS separation columns (Miltenyi Biotec) had been equilibrated with 0.5 ml of MACS[®] separation buffer, and the micro-beads labeled cells were subjected to a magnetic field through the column passage. Thus, the column was removed from the magnetic separator and placed on a suitable collection tube for the recovery of sample enriched of CD326+ cells (Fig.1). The sample with CD326+ cells were incubated with anti-CD326 PE and anti-CD45 FITC monoclonal antibodies (1:10 in MACS[®] separation buffer) for 15 min at 4°C (Fig. 1C). Cells were then washed, centrifuged at 1,300 rpm for 6 min at 25°C and the pellet was re-suspended in 10 mL of cells solution and spotted on 8 wells diagnostic slides (Menzel-Glaser, Braunschweig, Germany), left to dry and fixed with acetone for 8 min at -20°C. Nuclei were stained with DAPI (1 ng/mL, Sigma Chemicals, St Louis, MO). After appropriate washing, cover slips were finally mounted with mowiol for observation. Cells were analyzed by conventional fluorescence or by scanning in a series of 0.5 mm sequential optical sections with an ApoTome

System (Zeiss, Oberkochen, Germany) connected with an Axiovert 200 inverted microscope (Zeiss). Image analysis was performed by the Axiovision software (Zeiss). Single optical sections were acquired by a CCD camera and image analysis was performed by the Axiovision software (Zeiss) [11]. The CTCs were identified as CD326+DAPI+CD45- cells. The CTC isolation technique was optimized by recovery experiments of serial dilutions (10^4 - 10^1 /7.5 mL in healthy volunteer's blood) of cancer cell lines from the colon (HT29, Caco2). A threshold to define unfavorable CTC levels was defined as ≥ 2 /7.5 mL peripheral blood, because the conventional unfavorable cut-off ≥ 3 CTCs could negatively affect the clinical utility of assay as a predictive marker in advanced stages CRC [12]. CTCs were not detected in 10 healthy volunteers, recruited as negative controls.

Statistical analysis

Data were compared by using the Chi-squared test and the Fisher's exact test, as appropriate. A logistic regression analysis was performed to assess the association between CTC count and clinical parameters. The analysis of OS, defined as the time from baseline blood collection to death, and progression-free survival (PFS), defined as the period from baseline blood collection until the detection of recurrent disease, were calculated with the Kaplan–Meier method and compared by using the log-rank test. Patients were censored at the last observation when disease progression or death did not occur. Multivariate Cox proportional-hazards regression was

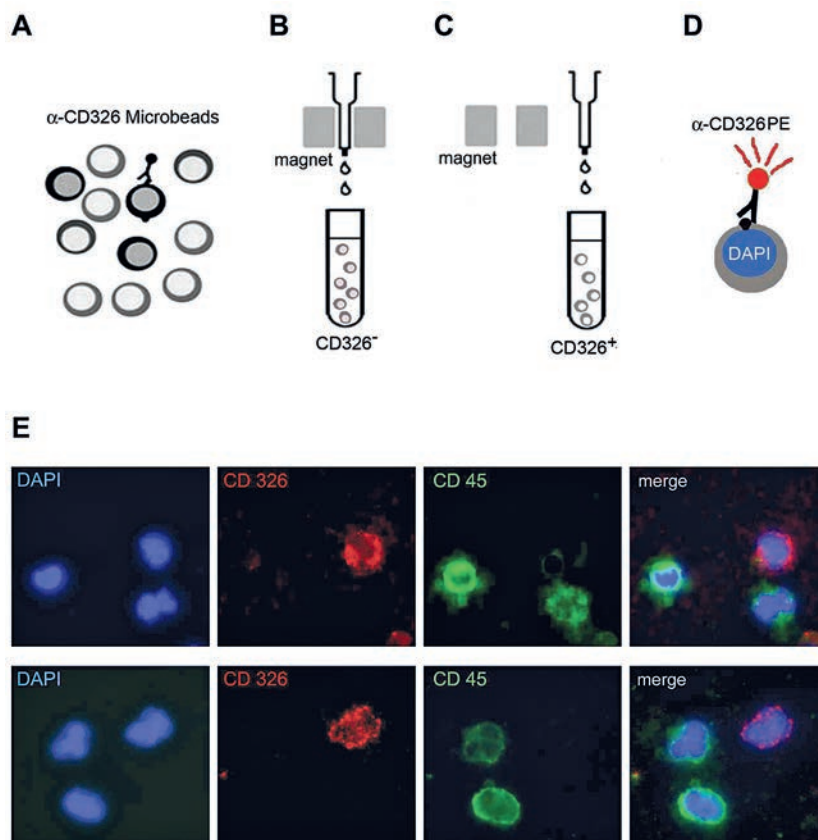


Fig. 1. A–D: Immuno-enrichment and immunofluorescence methods to detect circulating tumor cells from peripheral blood samples (see text). E: CTCs CD326+/DAPI+/CD45- (red/blue cells) surrounded by leukocytes CD45+/DAPI+/CD326- (green/blue cells) [15]

performed to analyze the effect of all variables on OS, and only those factors significantly associated at univariate analysis were included. For all comparisons, a P value <0.05 was considered statistically significant. Statistical analysis was performed using the SPSS PASW Statistics ver.19.0.

RESULTS

Seventy-five (M/F: 47/28; median age: 68, range 29-81 years) patients were enrolled in the study, including 54 patients (72%) with an early stage disease (I-III) and 21 (28%) with a metastatic disease (stage IV) (Table I). A total of 55 tumors (73.3%) were located in the colon, and 20 (26.7%) in the rectum. Forty-three (79.6%) patients with stages II-III received adjuvant chemotherapy (79% fluoropyrimidin plus oxaliplatin). None of the patients with stage IV disease underwent metastases resection before entering the chemotherapy protocol, and 52% received oxaliplatin-based chemotherapy. Overall, a positive baseline CTC (T0) count was detected in 21 (28%) patients. As shown in Table II, the prevalence of CTC positivity did not significantly differ according to age, gender, differentiation and site of primary tumor or recurrence. CTCs (T0) were strongly associated with either disease stage ($P = 0.001$) and ECOG PS ($P = 0.02$). At the follow-up time point (T1), 11 (22.4%) out of 49 patients had ≥ 2 CTCs. There was no difference in CTC (T1) count irrespective of type of chemotherapy received (regimens

Table I. Baseline clinical-pathological characteristics of patients.

Characteristic	Patients	
	Number	%
Total	75	
Median age, years	68 (29-81)	
Gender: F/M	28/47	37.3/62.7
UICC Stage		
I	2	2.7
II	23	30.6
III	29	38.7
IV	21	28
Differentiation		
Well to Moderate	35	50.7
Poor	34	49.3
Tumour Localization		
Colon	55	73.3
Rectum	20	26.7
ECOG Performance Status		
0	42	56
1	26	34.7
2	7	9.3
Oxaliplatin		
Yes	45	60.0
No	30	40.0
Recurrence		
No	42	58.3
Hepatic	23	31.9
Extrahepatic	7	9.7

with or without oxaliplatin), and site of tumor recurrence (hepatic vs. extrahepatic) (data not shown).

Cumulatively, the mean PFS and OS were 48.8 (CI: 41.8-55.8) and 55.5 (CI: 49.0-61.9) months, respectively. When patients were categorized according to the CTCs (T0), a positive status was associated with a reduction of both PFS

Table II. Association between baseline (T0) CTC detection and clinical-pathological parameters.

Characteristic	Patients Number (%)		P value
	CTC (T0) 0-1	CTC (T0) ≥ 2	
Patients	54 (72)	21 (28)	
Age			
< 65 years	23 (79.3)	6 (20.7)	0.3
≥ 65 years	31 (67.4)	15 (32.6)	
Gender			
Female	19 (67.9)	9 (32.1)	0.5
Male	35 (74.5)	12 (25.5)	
UICC Stage			
I-II	21 (84.0)	4 (16.0)	0.05
III	22 (75.9)	7 (24.1)	
IV	11 (52.4)	10 (47.6)	
Differentiation			
Well to Moderate	26 (74.3)	9 (25.7)	0.8
Poor	26 (76.5)	8 (23.5)	
Tumour Localization			
Colon	39 (70.9)	16 (29.1)	0.7
Rectum	15 (75)	5 (25)	
ECOG Performance Status			
0	32 (76.2)	10 (23.8)	0.03
1	20 (76.9)	6 (23.1)	
2	2 (28.6)	5 (71.4)	
Recurrence			
No	33 (78.6)	9 (21.4)	0.18
Hepatic	13 (56.5)	10 (43.5)	
Extrahepatic	5 (71.4)	2 (28.6)	

(34.8 vs 53.6 months; $P=0.06$) and OS (36.2 vs. 61.6 months; $P = 0.002$) (Tables III, IV, Fig. 2).

At multivariate regression analysis, only the UICC stage was found to be an independent predictor of both PFS ($p<0.001$; HR: 19.41, 95% CI: 7.36-51.2) and OS ($p<0.001$; HR: 13.94, 95% CI: 5.62-34.62). However, a CTC positive count at T1 was found to be an independent predictive factor for reduced OS ($P = 0.03$; HR: 3.55, CI: 1.09-11.47), while CTC positive count at T0 showed a trend towards the significance ($P = 0.09$; HR: 1.97, CI: 0.89-4.37).

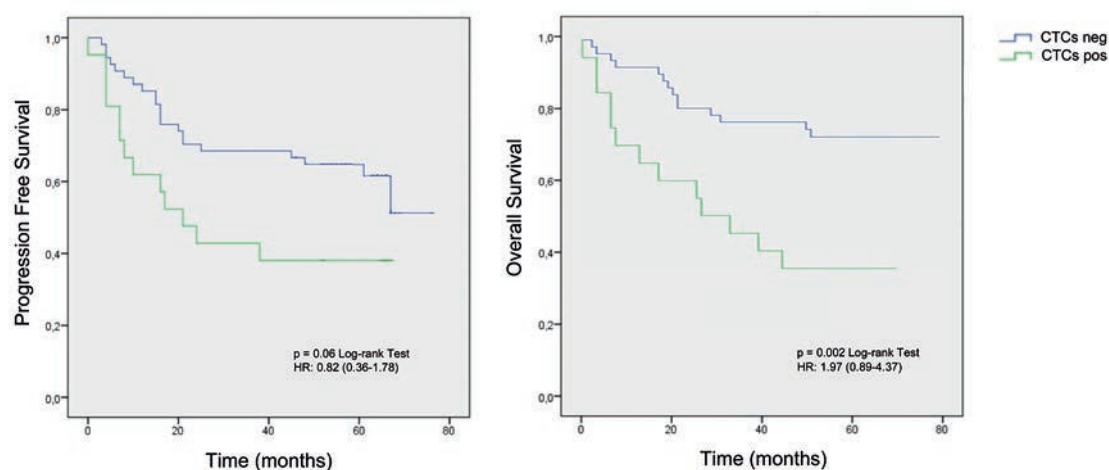
DISCUSSION

Presence of CTCs in the blood corresponds to the circulation step of cancer cells after the intravasation during the complex multistep process of cancer metastasis. CTCs may be

Table III. Progression Free Survival according to clinical characteristics.

Characteristic	Univariate analysis		Multivariate Cox regression analysis					
	Mean PFS (months)	Log-rank P-value	HR	CTC (T0)		CTC (T1)		
				95% CI	P-value	HR	95% CI	P-value
All patients	48.8							
Age								
< 65 years	49.7							
≥ 65 years	45.5	0.16	1.13	0.52-2.46	0.77	0.69	0.26-1.81	0.45
Gender								
Female	46.1	0.47	0.93	0.46-1.90	0.84	0.63	0.23-1.74	0.38
Male	48.1							
UICC Stage								
I-III	63.4							
IV	11.8	<0.001	19.41	7.36-51.2	<0.001	60.0	11.50-313.2	<0.001
ECOG PS								
0	48.3	0.49						
1-2	47.0							
Oxaliplatin								
Yes	51.9	0.35						
No	43.1							
CTC (T0)								
0-1	53.6							
≥ 2	34.8	0.06	0.82	0.38-1.78	0.61			
CTC (T1)								
0-1	51.0							
≥ 2	36.3	0.34				0.92	0.30 – 2.78	0.88

Abbreviations: CI=confidence interval; HR=Hazard ratio. HR for both CTC (T0) and CTC (T1) was estimated by Cox regression analysis, adjusted for age, gender and UICC stage.

**Fig. 2.** Progression Free Survival and Overall survival according to baseline CTC count.

isolated from other cells by using immune-magnetic techniques that recognize specific surface markers such as EpCAM. We used the MACS® system, that is one of these accurate methods [13, 14]. The prevalence rate we detected in patients with CRC was consistent with data of previous studies, ranging from 29% to 36% [7, 8, 10]. To date, only few studies suggested the

potential role of CTCs in predicting survival in CRC patients [8-10]. In the present study we found that a CTC positive status before chemotherapy is a significant factor associated with patients' survival. In detail, the OS in patients with CTC (T0) positive was near half as compared to that of patients with negative CTCs, with a HR of 1.97. In addition, CTC monitoring

Table IV. Overall survival according to clinical characteristics.

Characteristic	Univariate analysis		Multivariate Cox regression analysis					
	Mean OS (months)	Log-rank P-value	CTC (T0)			CTC (T1)		
			HR	95% CI	P-value	HR	95% CI	P-value
All patients	55.5 (49.0-61.9)							
Age								
< 65 years	55.2 (47.6-62.8)							
≥ 65 years	51.5 (42.8-60.0)	0.2	1.32	0.55-3.13	0.53	0.81	0.26-2.52	0.71
Gender								
Female	51.7 (40.9-62.5)	0.3	0.95	0.42-2.17	0.9	1.01	0.32-3.20	0.98
Male	52.9 (45.8-60.0)							
UICC Stage								
I-III	68.9 (64.2-73.7)							
IV	20.1 (12.2-27.9)	< 0.001	13.94	5.62-34.62	<0.001	43.07	8.88-208.84	<0.001
ECOG PS								
0-1	52.9 (45.5-60.4)	0.35						
2	52.6 (42.7-62.5)							
Oxaliplatin								
Yes	57.1 (49.0-65.2)	0.53						
No	48.2 (39.0-57.4)							
CTC (T0)								
0-1	61.6 (54.9-68.3)							
≥ 2	36.2 (24.9-47.5)	0.002	1.97	0.89-4.37	0.09			
CTC (T1)								
0-1	59.7 (51.5-68.0)							
≥ 2	39.2 (24.0-54.5)	0.08				3.55	1.09-11.47	0.03

Abbreviations: CI=confidence interval; HR=Hazard ratio. HR for both CTC (T0) and CTC (T1) was estimated by Cox regression analysis, adjusted for age, gender and UICC stage.

at follow-up is useful to further predict a reduced survival in those patients with a positive status, suggesting a more strict follow-up and/or a more aggressive cancer treatment. In detail, we observed that survival was significantly reduced in patients with CTCs positivity detected after 1 month of chemotherapy. Our data are in keeping with the results of a recent meta-analysis showing that presence of CTCs in peripheral blood is associated with a lower OS as compared to those with a negative count (HR = 2.28, 95% CI: 1.55-3.38) [15]. In addition, it was found that CTC determination is more accurate than post-chemotherapy CEA levels assessment in predicting relapse in these patients [16].

Based on the results of our study, it would appear clinically relevant to further investigate the predictive value of CTCs in localized (Stage I-II) CRC patients in whom data are still scanty [17-19]. Indeed, CTCs may be detected in 15–22% of these patients. For instance, chemotherapy is not routinely advisable for low-risk stage II colorectal cancer patients [20, 21], despite the fact that a definite risk of disease recurrence has been demonstrated also in these patients. In such a scenario, CTC assessment, along with other traditional prognostic factors, could allow a more accurate selection of patients deserving enhanced follow-up and/or an adjuvant treatment.

CONCLUSION

Our data support the prognostic role for CTC count in CRC patients. However, further studies aimed at confirming these findings in the early stages of the disease are necessary.

Conflicts of interest: None to declare.

Authors' contribution: AR, SR: Conception and design of study, analysis of data, drafting of article and critical revision, final approval of manuscript. MR, AM: Acquisition of clinical data, analysis of data, critical revision and final approval of manuscript. RDR, IS, VB, CDA, VD, MF: Acquisition of clinical data, analysis of data, final approval of manuscript. LL, DR, FM: Acquisition of laboratory data, analysis of data, final approval of manuscript. AZ, EM: Analysis of data, critical revision and final approval of manuscript. MRT: Conception and design of study, critical revision, and final approval of manuscript. PM: Conception and design of study, critical revision, and final approval of manuscript.

Acknowledgments: This work was partially supported by grants from MIUR and from AIRC—Associazione Italiana per la Ricerca sul Cancro (IG 10272), Italy.

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