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ORIGINAL ARTICLE

Prognostic significance of *CEACAM5*mRNA-positive circulating tumor cells in patients with metastatic colorectal cancer

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Abstract

Purpose To evaluate the clinical relevance of *CEACAM5*mRNA-positive circulating tumor cells (CTCs) in patients with metastatic colorectal cancer (mCRC).

Methods Peripheral blood was obtained from 436 patients with mCRC before the initiation of systemic therapy. A second sample was obtained on treatment assessment from 296 (67.9%) patients. The detection of *CEACAM5*mRNA-positive CTCs was performed using a real-time PCR assay.

Results The patients' median age was 67 years and PS (EGOG 0–1) 92%; *KRAS* exon 2 and $BRAF^{V600E}$ mutated primary tumors were identified in 31.9% and 6.4% of the tested patients, respectively, whereas metastasectomy was performed in 17.7% of the patients. Circulating *CEACAM5*mRNA-positive CTCs were detected in 125 (28.7%) and 85 (28.7%) patients at baseline and on treatment assessment, respectively. The detection of *CEACAM5*mRNA-positive cells was revealed, in multivariate analysis, as an independent prognostic factor associated with decreased PFS (HR 1.6; 95% CI 1.1–2.5; $p=0.026$) and OS (HR 2.2; 95% CI 1.3–3.2; $p<0.001$). The detection of *CEACAM5*mRNA-positive CTCs in patients with KRAS and $BRAF^{V600E}$ mutations was correlated with shorter PFS ($p=0.041$ and $p=0.022$, respectively). Moreover, OS was significantly shorter in patients with *CEACAM5*+/KRAS mutations compared to those with *CEACAM5*+/KRAS wt tumors ($p=0.023$).

Conclusions Detection of peripheral blood *CEACAM5*mRNA-positive CTCs is an adverse prognostic factor correlated with poor clinical outcome in patients with mCRC, especially in patients with *KRAS* and *BRAF* mutated tumors.

Keywords *CEACAM5* · mRNA · CRC · Metastatic · CTCs

Introduction

Colorectal cancer (CRC) is the second most common cause of mortality due to cancer [1, 2], and is a major health problem in the Western world representing approximately 10% of all cancer cases with 40–50% of all patients experiencing metastasis [2–4]. Unfortunately, the current routine clinical manifestations, radiologic evaluations and serum tumour markers do not provide enough information of the ongoing metastasis as early as possible or predicting the clinical outcome with high accuracy and reproducibility [5]. The high metastatic potential of the disease is due to the dissemination of tumor cells through the hematogenous and/or the lymphatic vasculature. The detection of tumor cells in the peripheral blood (circulating tumor cells; CTCs) and bone marrow aspirates (disseminated tumor cells; DTCs) has been described in cancer patients [6–12] and has been shown to be associated with

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shorter progression-free (PFS) and overall (OS) survival in various tumor types, including CRC [13–21].

In patients with early-stage CRC, the detection of malignant cells in the bone marrow, the peritoneal lavage, and the involvement of the regional lymph nodes are associated with poor survival [22, 23]. Quantitative real-time RT-PCR (RT-qPCR) has been shown to provide the sensitivity and the practicability that is necessary to detect rare CTCs in patients' blood [24]. In CRC, the most frequently analyzed marker is the carcinoembryonic antigen (CEA). The initial clinical studies have analyzed the usefulness of *CEACAM5*mRNA for the detection of CTCs in blood samples of patients with CRC, but none of them have quantified the PCR product [25–28]. Nevertheless, a low level of *CEACAM5*mRNA expression has been detected in normal subjects, suggesting an illegitimate expression of the *CEACAM5* gene in hematopoietic cells [29, 30] whereas other studies have failed to show significant differences of *CEACAM5*mRNA expression between cancer patients and healthy individuals [31]. These studies have been conducted using sets of primers that amplify a splice variant of *CEACAM1* expressed in hematopoietic cells (WBCs), in which an "intron" sequence replaces part of the exon 10 [29], thus explaining the findings of *CEACAM5*mRNA expression in normal blood samples [30], in patients with inflammatory bowel disease [31] and in cultured WBCs after induction with G-CSF [32]. On the basis of the aforementioned results, the overall usefulness of *CEACAM5* as a PCR-based tumor cell detection marker remained questionable.

Our group has previously reported the development of a reliable and reproducible RT-qPCR assay for the detection of *CEACAM5*mRNA CTCs in CRC. The detection of *CEACAM5*mRNA-positive cells in patients with operable (stages II–III) CRC has been correlated with poor clinical outcome [21]; in this study, we have analyzed 100 samples from patients with mCRC as validation set. We reported that *CEACAM5*mRNA-positive CTCs could be detected in 44% of patients with mCRC. The detection of *CEACAM5*mRNA-positive CTCs in patients with mCRC has been associated with liver metastases and with decreased progression-free and overall survival [21].

The aim of the present study was to prospectively investigate the clinical relevance of *CEACAM5*mRNA-positive CTCs in patients with mCRC providing, thus, a clinically useful new prognostic biomarker.

Patients and methods

Patients' population

Four hundred and thirty-six consecutive patients, with newly diagnosed and histologically documented mCRC treated at the Department of Medical Oncology, University Hospital of Heraklion (Greece), were enrolled in the study. There were no other exclusion criteria for enrolment. All patients were tested for the presence of circulating *CEACAM5*mRNA-positive cells before the initiation of any systemic front-line treatment; moreover, in 294 (67.4%) patients a second blood sample was obtained at the time of treatment assessment. Treatment administration as well as treatment assessment were coded without the knowledge of the CTC status. The study was approved by the Ethics Committee/Institutional Review Board of the University Hospital of Heraklion (Greece) (Number 7302/19-8-2009) and all patients signed written informed consent to participate in the study.

Peripheral blood (15 mL in EDTA) was obtained at the middle-of-vein puncture after the first 5 mL of blood was discarded to avoid contamination with epithelial cells from the skin. The evaluation of the analytical sensitivity and specificity of the method has been previously described [21].

Specimen characteristics and assay methods

Peripheral blood mononuclear cells (PBMCs) were isolated by gradient density centrifugation and RNA extraction was performed as previously described [33]. RNA concentration was determined using the NanoDrop (Thermo Scientific, USA) equipment. Amplification of the β-actin as internal reference gene was done to verify the RNA integrity. RNA prepared from the Lovo colorectal and ARH-77 leukemic cell lines was used as positive and negative controls, respectively. The reverse transcription and the qPCR conditions have been previously described [21]. Quantification of gene expression was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, USA). All experiments were performed in triplicates. Quantification was based on an external calibration curve that was obtained using external standard cDNAs [21]. The SDS 2.3 software was used for the analysis of the results. Finally, genomic DNA contamination was excluded, as no RNA transcripts could be detected in each analyzed sample in the absence of reverse transcriptase.

KRAS exon 2 and $BRAF^{V600E}$ mutational analysis were performed in the context of the standard clinical practice using Sanger sequencing after PCR amplification [34] and an allelic discrimination method based on real-time PCR [35], respectively. Since the study was initiated before the description of the predictive value of *NRAS* mutations for treatment efficacy [35], a retrospective mutation analysis of this gene was not performed.

Study design and statistics

The aim of this study was to explore the prognostic significance of *CEACAM5*mRNA-positive CTCs in patients

with mCRC and correlate their detection with pathological and clinical characteristics of the disease. Due to its observational nature no formal sample size calculation was performed. The cut-off value of ≥ 0.69 Lovo cells equivalents/5 µg of RNA has been previously suggested [21]. Based on this cut-off value and to better investigate the role of *CEACAM5*mRNA-positive CTCs in patient's outcome, we attempted to divide the patients into three groups according to the levels of *CEACAM5*mRNA-positive CTCs with the assumption of the higher risk associated with the higher levels of *CEACAM5*mRNA-positive CTCs: (1) low ≤ 0.69 (negative group), (2) middle 0.7–1.91 (intermediate group) and (3) high (positive group) ≥ 1.92 Lovo cells equivalents/5 µg of RNA. Summary tables (descriptive statistics and/or frequency tables) are provided for all baseline and efficacy variables, as appropriate. Continuous variables are summarized with descriptive statistics (*n*, median and range). Ninety-five percent confidence intervals (95% CI) are also presented, as appropriate. Progression-free survival (PFS) was defined as the time from first treatment cycle until clinical or radiological disease relapse or death from any cause. Overall survival (OS) was measured from the date of first treatment cycle until the date of death from any cause or the date of last follow-up. Qualitative factors were compared by Pearson's Chi-square test or Fisher's exact test whenever appropriate. Differences in continuous variables were assessed using the Kruskal–Wallis test. PFS and OS were estimated using the Kaplan–Meier analysis and the comparisons were computed with the log-rank test. Associations between prognostic factors and PFS or OS were examined using Cox proportional hazards regression models. All statistical tests were two sided, and p values < 0.05 were considered statistically significant. Data were analyzed using the SPSS statistical software, version 22.0 (SPSS Inc., Chicago, IL, USA).

Results

Patients' characteristics and clinico-pathological features

The patients' characteristics and the disease features were typical for mCRC (Table 1). The median patients' age was 67 years (range 20–88) and 39% of them were aged > 70 years, 62% were males and 92% had a PS (ECOG) of 0–1; the primary tumor was located in the rectum in 32.3% of the patients and in 39% of the cases was undifferentiated (high grade), while *KRAS* exon 2 and $BRAF^{V600E}$ mutations were detected in 31.9% and 6.4% of the analyzed tumors, respectively. All patients were treated with 5-FU-based first-line chemotherapy combined in 95% of them with oxaliplatin or irinotecan. In 178 (40.8%) patients, chemotherapy was combined with Bevacizumab, while an anti-EGFR-based combination was administered in 104 (23.9%) patients (Table 1). Seventy-seven (17.7%) patients underwent a metastasectomy before the initiation of front-line chemotherapy.

Detection of CEACAM5mRNA-positive CTCs before chemotherapy

According to the degree of *CEACAM5*mRNA positivity at baseline, 311 patients were considered as negative whereas 101 and 24 patients were considered to have an intermediate and a high positivity (Table 1). There was no correlation between the patients' clinico-pathologic characteristics and the degree of *CEACAM5*mRNA positivity, except for the patients who did not undergo surgery (Table 1). Among the 77 patients who underwent up front metastasectomy for liver or pulmonary metastases, 16 (20.8%) had detectable *CEACAM5*mRNA-positive CTCs (intermediate or high positivity) compared to 109 (30.4%) patients who received only chemotherapy ($p = 0.241$; Table 1).

Effect of chemotherapy on the detection of CEACAM5mRNA-positive CTCs

In 294 (67.4%) patients, a second blood sample was obtained at the time of treatment assessment. *CEACAM5*mRNA-positive CTCs could be detected in 84 (28.6%) patients. According to the CTC status before and after chemotherapy, 33 (11.2%) patients who were initially *CEACAM5*mRNA positive (high and/or intermediate) turned negative whereas 30 (10.2%) patients who were initially *CEACAM5*mRNA negative turned positive (high and/or intermediate; Suppl. Table 1). Similarly, 54 (18.4%) and 177 (60.2%) patients were classified as positive (high and/or intermediate) and negative, respectively, at both time points (Suppl. Table 1).

Clinical outcome according to the detection of CEACAM5mRNA-positive CTCs

Patients with a negative and an intermediate *CEACAM-5mRNA* status had a comparable PFS (8.8 vs 8.7 months, $p = 0.671$; Suppl. Table 2). Conversely, there was a statistically significant difference of PFS between the patients with negative *CEACAM5mRNA* and high positivity status (8.8 vs 7.0 months, $p = 0.013$) (Suppl. Table 2; Fig. 1a). The comparison of PFS between the *CEACAM5mRNA*-intermediate and *CEACAM5mRNA*-high patients' groups showed a marked but not statistically significant difference (8.7 vs 7.0 months, $p = 0.063$; Suppl. Table 2). Moreover, the median OS was significantly higher in the group of patients with a *CEACAM5mRNA*-negative status compared with that of patients with a *CEACAM5mRNA*-positive

Table 1 Patient characteristics according to different cut-offs

			*CEACAM5*mRNA status						
	Total ($n=436$)		Negative < 0.7 ($n=311$)		Intermediate 0.7–1.91 ($n=101$)		High ≥ 1.92 ($n=24$)		p value
	N	%	*N*	%	*N*	%	*N*	%	
Gender									
Male	270	62.0	189	60.8	68	67.3	13	54.2	0.361
Female	166	38.0	122	39.2	33	32.7	11	45.8	
Median age	66.5 (20–88)		62 (25–88)		67 (20–83)		70 (37–82)		0.249
≤ 70 years	266	61.0	191	61.4	63	62.4	12	50.0	0.516
> 70 years	170	39.0	120	38.6	38	37.6	12	50.0	
Tumor location									
Colon	295	67.7	210	67.5	72	71.3	13	54.2	0.272
Rectum	141	32.3	101	32.5	29	28.7	11	45.8	
Grade									
I–II	254	58.3	181	59.7	59	60.2	14	60.9	0.992
III	170	39.0	122	40.3	39	39.8	9	39.1	
UN	12	2.8	8	2.6	3	3.0	1	4.2	
Surgery									
Yes	353	81.0	54	17.4	19	18.8	10	41.7	0.014
No	83	19.0	257	82.6	82	81.2	14	58.3	
Metastasectomy									
Yes	77	17.7	61	19.6	13	12.9	3	12.5	0.241
No	359	82.3	250	80.4	88	87.1	21	87.5	
PS (ECOG)									
0–1	401	92.0	288	92.6	93	92.1	20	83.3	0.273
2	35	8.0	23	7.4	8	7.9	4	16.7	
KRAS exon 2 mutations									
Mutated	139	31.9	104	33.4	28	27.7	7	29.2	0.07
Wild type	213	48.9	150	48.2	47	46.5	16	66.7	
ND/UN	84	19.3	57	18.3	26	25.7	1	4.4	
$BRAF^{V600E}$ mutations									
Mutated	28	6.4	17	5.5	8	7.9	3	12.5	0.315
Wild type	387	88.8	276	88.7	91	90.1	20	83.3	
ND/UN	21	4.8	18	5.8	2	2.0	1	4.2	
Regimens									
CPT-11 based	197	45.2	143	46.0	49	48.5	5	20.8	0.009
LOHP based	151	34.6	108	34.7	27	26.7	16	66.7	
Both	80	18.3	56	18.0	22	21.8	2	8.3	
Other	8	1.8	4	1.3	3	3.0	1	4.2	

(high) status (23.4 vs 11.2 months, $p<0.001$); similarly, a significant difference in terms of median OS was observed between the patients with a *CEACAM5mRNA*-intermediate and *CEACAM5*mRNA-positive (high) status (23.7 vs 11.2 months, $p=0.001$; Fig. 1b). Finally, the detection of *CEACAM5*mRNA-positive CTCs did not demonstrate any significant association with the clinical outcome (both PFS and OS) of patients who underwent a metastasectomy (data not shown).

Patients' clinical outcome according to the tumors' molecular profile and the CEACAM5mRNA CTCs' status

Patients with KRAS mutant tumors and detectable *CEACAM5mRNA*-positive CTCs presented significantly lower PFS (7.6 months, 95% CI 5.1–10.2 months) compared to those with undetectable CTCs and KRAS mutant tumors (9.0 months, 95% CI 7.4–10.6 months, $p=0.041$) (Table 3; Suppl. Fig. 1a); moreover, these patients had a significantly

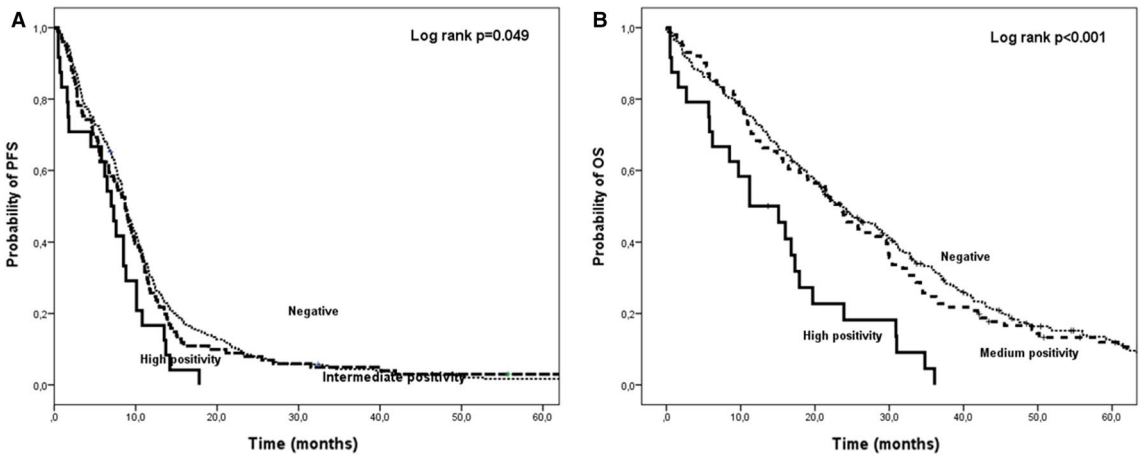

Fig. 1 Kaplan–Meier curves for progression-free survival (**a**) and overall survival (**b**) according to *CEACAM5*mRNA positivity at baseline

lower OS (21.2 months, 95% CI 10.9–31.5 months) compared to those with detectable CTCs and KRAS wt tumors (25.8 months, 95% CI 19.1–32.6 months, $p = 0.023$). In addition, patients with undetectable CTCs and KRAS mutant tumors had a lower median OS (22.4 months, 95% CI 18.9–26.0 months, $p = 0.028$) compared to those with undetectable CTCs and KRAS wt tumors (28.1 months, 95% CI 23.0–33.2 months, $p = 0.008$) (Table 3; Suppl. Fig. 1b). Similarly, patients with $BRAF^{V600E}$ wt tumors and undetectable *CEACAM5*mRNA-positive CTCs presented significantly higher PFS (9.0 months, 95% CI 8.3–9.7 months) compared to those with detectable *CEACAM5*mRNA-positive CTCs and $BRAF^{V600E}$ mutant tumors (2.5 months, 95% CI 0.1–10.6 months, $p = 0.022$; Table 3, Suppl. Fig. 1c). Finally, patients with $BRAF^{V600E}$wt tumors and undetectable *CEACAM5*mRNA-positive CTCs had significantly higher OS (24.5 months, 95% CI 20.5–28.5 months) compared to those with undetectable CTCs and $BRAF^{V600E}$ mutant tumors (11.1 months, 95% CI 5.8–16.4 months, $p = 0.036$; Table 3, Suppl. Fig. 1d).

Univariate and multivariate analyses

Univariate analysis revealed that PFS was significantly lower in patients with high-grade tumors (HR 1.4, 95% CI 1.2–1.8; $p < 0.001$), PS (ECOG) ≥ 2 (HR 3.4, 95% CI 2.3–4.8; $p < 0.001$), in patients who did not undergo metastasectomy (HR 2.2, 95% CI 1.7–2.9; $p < 0.001$) and high positivity levels of *CEACAM5*mRNA at baseline (HR 1.7, 95% CI 1.1–2.5; $p = 0.018$) (Table 2). Conversely, there was no significant association between PFS and age, gender, tumor location, $BRAF^{V600E}$ or *KRAS* mutations. Moreover, univariate analysis revealed that the detection of *CEACAM5*mRNA-positive CTCs at baseline was associated with significantly lower median OS (HR 2.3, 95% CI 1.5–3.6; $p < 0.001$) (Table 2), independently of the administered chemotherapy regimen. In addition, high-grade tumors and PS (ECOG) ≥ 2 were associated with an increased incidence of death (HR 1.4, 95% CI 1.2–1.8; $p < 0.001$ and HR 4.0, 95% CI 2.8–5.6; $p < 0.001$, respectively). An increased risk of death was also revealed for patients who did not undergo metastasectomy (HR 2.9, 95% CI 2.2–3.9; $p < 0.001$; Table 2). There was no significant association between the median OS and the gender, the age, the tumor location, or the mutation status of $BRAF^{V600E}$ and *KRAS*.

Multivariate analysis confirmed these results and revealed that the detection of *CEACAM5*mRNA-positive CTCs at baseline, the tumor's high grade, the PS (ECOG) ≥ 2 and the inability to perform a metastasectomy with curative intent were strongly associated with decreased patients' PFS and OS (Table 2).

Discussion

There are few validated prognostic factors in mCRC including clinico-pathological parameters such as the stage of the disease, the performance status, the Kohne prognostic index, and the tumor differentiation [36]. More recently, the $BRAF^{V600E}$ mutation status was added as an adverse prognostic and predictive biomarker for these patients [37, 38]. Our group has been previously described a reliable and reproducible assay for the detection of *CEACAM5*mRNA-positive CTCs in patients with operable CRC; using this assay, it was shown that the detection of *CEACAM5*mRNA-positive CTCs in the blood of patients is an independent prognostic factor for reduced DFS and OS [21]. In the study by Vardakis *et al.* [21] was included, for validation purposes, a group of patients with mCRC; the detection rate of *CEACAM5*mRNA-positive CTCs in these patients was 44%. Based on this initial observation, it was decided to extend this patients' cohort to better investigate the clinical

Table 2 Univariate and multivariate analyses for progression-free survival (PFS) and overall survival (OS)

Feature	Univariate				Multivariate			
	PFS		OS		PFS		OS	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Histologic grade								
III vs I–II	1.4 (1.2–1.8)	< 0.001	1.5 (1.2–1.8)	< 0.001	1.3 (1.1–1.6)	0.003	1.4 (1.1–1.7)	0.002
PS								
≥ 2 vs 0–1	3.4 (2.4–4.8)	< 0.001	4.0 (2.8–5.8)	< 0.001	3.0 (2.1–4.3)	< 0.001	3.4 (2.3–4.9)	< 0.001
Metastasectomy								
No vs yes	2.2 (1.7–2.9)	< 0.001	2.9 (2.2–3.9)	< 0.001	2.1 (1.6–2.7)	< 0.001	2.7 (2.0-3.7)	< 0.001
*CEA*mRNA CTCs at baseline								
High (≥ 1.92) vs all others (< 1.92)	1.7 (1.1–2.5)	0.018	2.3 (1.5–3.6)	< 0.001	1.6 (1.1–2.5)	0.026	2.1 (1.3–3.2)	0.001
KRAS								
Mutant vs WT	1.0 (0.8–1.2)	0.839	1.1 (0.9–1.4)	0.329	1.0 (0.8–1.3)	0.971	1.1 (0.9–1.4)	0.274
BRAF								
Mutant vs WT	1.4 (0.9–2.0)	0.119	1.1 (0.7–1.7)	0.557	1.3 (0.8–1.9)	0.280	1.1 (0.7–1.7)	0.717

Table 3 Progression-free survival (PFS) and overall survival according to CTC status and molecular profiling of patients

	PFS, median (months)	*p*
CEACAM5+/KRASmut vs *CEACAM5−/KRASmut*	7.6 vs 9.1	0.041
CEACAM5+/BRAFmut vs CEACAM5−/*BRAFwt*	2.5 vs 9.0	0.022
	OS, median (months)	*p*
CEACAM5+/KRASmut vs *CEACAM5−/KRASmut*	21.2 vs 22.4	0.028
CEACAM5+/KRASmut vs *CEACAM5+/KRASwt*	21.2 vs 25.8	0.023
CEACAM5+/*KRASmut* vs *CEACAM5−/KRASwt*	21.2 vs 28.3	0.008
CEACAM5−/BRAFmut vs *CEACAM5−/BRAFwt*	11.1 vs 24.5	0.036

relevance of *CEACAM5*mRNA-positive CTCs in chemotherapy-naïve mCRC patients.

The presented data revealed, practically, three groups of patients according to the *CEACAM5*mRNA positivity and the detection of high *CEACAM5*mRNA positivity before the initiation of any systemic treatment emerged as an independent factor associated with decreased PFS and OS (Fig. 1a, b). When analyzed in conjunction with other clinico-pathological features, the detection of high *CEACAM5*mRNA-positive status may provide additional information. The two other groups (intermediate and low *CEACAM5*mRNA positivity) were not associated with patients' clinical outcome. Similarly, the detection of *CEACAM5*mRNA-positive CTCs did not demonstrate any significant association with PFS and OS in the group of patients who underwent a metastasectomy, irrespectively of the degree of positivity. However, concerning patients with isolated colorectal liver metastases undergoing liver metastasectomy, little is known about the possible prognostic value for CTCs [39, 40]. Only recently Seeberg et al. published an article where the CellSearch platform was used for the detection of CTCs in 7.5 ml blood in patients with isolated colorectal liver metastases [41]. They reported that CTCs can predict non-resectability and impaired survival. CTC positivity was significantly higher in nonresectable (46%) than in resectable patients (11.7%), $p < 0.01$ [41]. However, the authors have also included patients who were not eligible for resection and it is not clear whether the patients who underwent liver surgery had extrahepatic disease [41]. On the contrary, in our study we demonstrated, for the first time, a significant association between the detection of *CEACAM5*mRNA-positive cells and the clinical outcome in patients with *KRAS* or $BRAF^{V600E}$ mutated tumors. More importantly, patients with detectable *CEACAM5*mRNA-positive cells and *KRAS* mutant tumors presented significantly shorter PFS and OS compared to the other patients' groups (Table 3, Suppl. Figs. 2a, b). Additionally, patients with $BRAF^{V600E}$ wt tumors and undetectable CTCs presented significantly higher median PFS compared with those with detectable and $BRAF^{V600E}$ mutant tumors (Table 3, Supplementary

Fig. 2c), whereas patients with *BRAF*^{V600E} wt tumors and undetectable CTCs presented significantly higher median OS compared with those with undetectable and *BRAF*^{V600E} mutant tumors (Table 3, Suppl. Fig. 2d). This strong correlation confirms the aggressive physical history of colon cancer with *BRAF*^{V600E} mutations, which is characterized by early dissemination and the frequent presence of multi-metastatic disease at the time of diagnosis [34, 42–44]. These results are in concordance with previous studies using either molecular- or cytology-based techniques for the detection of CTCs [21, 45]. Indeed, using the CellSearch platform, the CTC detection rate in patients with CRC was 36.2% and the quantification of CTCs could be a valuable prognostic factor [45]. In a prospective multicenter study, it has been demonstrated that the number of CTCs before and during treatment is an independent predictor for both PFS and OS in patients with mCRC whereas the presence of liver metastases was associated with the detection of CTCs [46]. However, it should be noted that *CEACAM5*, which is used as a marker for the detection of CTCs in the current study, represents a marker of epithelial cells. Cancer cell heterogeneity has been widely described and includes a wide range of differentiation states from epithelial-to-mesenchymal types, a process known as epithelial–mesenchymal transition (EMT) [47–49]. Whether *CEACAM5* is similarly modulated during EMT is still unknown. On the other hand, it has been proposed that the combination of epithelial and mesenchymal markers may capture CTCs undergoing EMT resulting, thus, in an increase of the sensitivity of the assays used for the detection of CTCs [50]. However, both the CellSearch assay and the molecular assay, which was used for the detection of CTCs in the current study, are based on epithelial markers, such as EpCAM and cytokeratins or *CEACAM5*mRNA expression, respectively. Despite the observed positive correlations between the detection of CTCs with any of these assays and the patients' clinical outcome, it is unclear whether these assays recognize the same subpopulation of CTCs. Therefore, only the direct comparison of these assays has the possibility to demonstrate which of them is the most appropriate for clinical use and decision-making. This comparison should not evaluate only their clinical relevance as a tumor biomarker but also their capacity to capture the vast majority of CTCs undergoing EMT, as well as their reproducibility, cost and subjectivity of the interpretation of images [51, 52].

Beyond its prognostic significance, the detection of CTCs may be used as surrogate marker for patients' outcome under specific treatments as already has been reported [27]. According to its design, the current study could not provide information concerning the predictive value of *CEACAM5*mRNA-positive CTCs according to the location of the primary tumor or the efficacy of the different chemotherapy regimens which are frequently used for the treatment of mCRC; a subsequent prospective study, focused on the tumor location and the specific molecular tumors' characteristics is required to more appropriately define the clinical relevance of *CEACAM5*mRNA-positive CTCs during treatment of patients with mCRC. In addition, the clinical relevance of *CEACAM5*mRNA-positive CTCs in homogeneously treated patients should be investigated in future studies, to clearly define whether they may be used as a surrogate marker for the efficacy of systemic treatment in patients with mCRC. Furthermore, the isolation and genetic–molecular characterization of CTCs may allow the non-invasive genotyping of CTCs and, thus, the continuous monitoring of the disease tailoring the therapeutic decisions. Indeed, it has been reported that using modern technologies it is possible to monitor the molecular/mutational profile in CTCs and in some cases this profile may be different from that of the primary tumor [20, 53–55] and the corresponding metastases [55]. In another study, our group investigated the detection of *KRAS* mutations in CTCs from patients with mCRC and compared their mutation status during treatment or disease progression with that of the corresponding primary tumors. It was revealed that although 29.2% of the patients had *KRAS* mutant primary tumors, *KRAS* mutations were revealed in 45% of them and in 16.7% of those with wild-type tumors [54]. In a more recent study, we investigated the *KRAS* exon 2 mutations in serial CTCs samples of patients with *RAS* wild-type mCRC captured by the isolation by size of epithelial tumor cells (ISET) system in an effort to evaluate the evolving genetic heterogeneity of these cells in patients receiving front line treatment [20]. The results demonstrated that *KRAS* exon 2 mutations could be detected in CTCs from patients with *RAS* wt primary tumors, either before the initiation of systemic treatment or during treatment and at the time of disease progression. An interesting observation was the fact that in three patients who received anti-EGFR-based chemotherapy, *KRAS* mutations were first detected only at the time of disease progression suggesting the presence of a resistance mechanism against anti-EGFR treatment. Moreover, despite the presence of *KRAS* mutations in serial samples of two patients, each sample presented a different mutation, possibly indicating the heterogeneity of the CTC population [20]. Therefore, it is reasonable to hypothesize that the discordance of the mutational status of *KRAS* gene between the primary tumor and the CTCs may reflect the heterogeneity of the tumor clones which may have potential predictive and/or prognostic relevance and could be emerged as a dynamic molecular marker representing more appropriately the real-time tumor heterogeneity and evolution of mCRC.

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Compliance with ethical standards

Conflict of interest No potential conflicts of interest were disclosed.

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